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Circadian control and environmental response

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Zusammenfassung

Das Wachstum von Pflanzen im Tagesverlauf ist ein komplizierter und vielschichtiger Prozess, der durch endogene regulatorische Systeme und Umwelteinflüsse gesteuert wird. In der Natur sind die verschiedenen Organe der Pflanze (z.B. Blatt und Wurzel) unterschiedlichen, im Tagesverlauf schwankenden Umgebungsfaktoren ausgesetzt. Diese Umweltimpulse und eigene endogene Kontrollsysteme, wie die innere (zirkadiane) Uhr, können spezifische, organabhängige Einflüsse auf die Wachstumsaktivität haben. Ziel dieser Dissertation ist es, die Bedeutung der zirkadianen Uhr und der Bodentemperatur auf die Wachstumstagesgänge verschiedener Organe der Pflanzen zu analysieren.

Das dynamische Blatt- und Wurzelwachstum von zwei zirkadianen Mutanten, der *CCA1*-Überexpressionsmutante (*CCA1ox*) und der Mutante *prr9-prr7-prr5* (*prr975*), wurde untersucht. Eine defekte Uhr verursacht in diesen Mutanten Veränderungen der täglichen Wachstumsraten. Beide Linien zeigten tagsüber im Vergleich zum Wildtyp (wt) eine verstärkte Wachstumsaktivität, die auf eine erhöhte Verteilung von Photo-Assimilaten zum Blattwachstum am Tag hinweisen. Korrelierend zu einem ineffizienten Stärkeabbau in den Blättern wurde das nächtliche Blattwachstum verringert und zwei Stunden vor Tagesanbruch vollständig inhibiert, wobei diese Reduzierung des Wachstums nicht durch einen Stärkemangel verursacht wurde. Insgesamt stimmen diese Ergebnisse mit der Theorie überein, dass eine defekte zirkadiane Uhr die zeitliche Kohlenstoff- und Energieverteilung stört, und dadurch die nächtliche Wachstumsreduktion verursacht. Des Weiteren war auch die Morphologie des Blattrosetten- und Wurzelsystems der Mutanten durch den Defekt in der inneren Uhr verändert. Beide Mutanten zeigten eine starke Wachstumsreduktion von sowohl primären als auch den sekundären Wurzeln, und ebenfalls die Unterdrückung von sekundärer Wurzelbildung. Die sekundären Wurzeln bildeten eine wellenartige Morphologie aus, als sie einem “Tag-Nacht“-Lichtzyklus ausgesetzt wurden und expandierten mehr in die Breite des Wurzelsystems. Daraus kann geschlossen werden, dass eine defekte zirkadiane Uhr das Wachstum der ganzen Pflanze beeinflusst, nicht nur in der zeitlichen Organisation sondern auch in der Kontrolle der Wurzel- und Rosettenmorphologie.

Die endogen kontrollierte Wachstumsdynamik ist auch abhängig von verschiedenen Umweltfaktoren und deren Interaktion. In dieser Dissertation wird der Fokus auf die Interaktion zwischen Wurzeltemperatur und Wachstum gelegt. Klimakammerexperimente zeigten, dass die Wurzeltemperatur den Metabolismus beeinflusst und dass eine niedrige Wurzeltemperatur die Kaltadaptierung stimuliert sowie das Wachstum verlangsamt. Der Einfluss von Temperaturverringerung im Gewächshaus oder im Feld wurde noch nicht untersucht. Im Gewächshausexperiment mit gekühltem Boden war die Anpassungen auf eine Absenkung der Wurzeltemperatur geringer. Während manche Wachstumsparameter, wie etwa Biomasseakkumulation, Blattexpansion und spezifische Blattbiomasse kleine Adaptionen aufwiesen, konnten keine

veränderten Werte des Kohlestoff- oder Flavonoidmetabolismus (Flavone und Anthocyane) bestimmt werden. Dies könnte dadurch zu erklären sein, dass in Experimenten unter natürlichem Licht sowie im Gewächshaus die allmähliche Veränderung der Lichtintensität am Morgen bzw. am Abend zusammen mit der maximalen Höhe der Lichtintensität zur Mittagszeit sich in anderer Art und Weise auf die Feinregulation des pflanzlichen Wachstums auswirkt.

Um gleichzeitig das Blatt- und Wurzelwachstum mit hoher zeitlicher Auflösung zu quantifizieren, wurde ein System entwickelt, das synchron Blatt- und Wurzelwachstumsraten auswerten kann, und bei dem die Temperatur an der Wurzel geändert werden kann. Erste Experimente wurden unter drei verschiedenen Bedingungen durchgeführt, wobei die Kontrollpflanzen unter 22°C Raumtemperatur kultiviert wurden. Veränderte Kultivierungsbedingungen waren zum einen das Kühlen des Wurzelsystems auf 10°C im Laufe des gesamten Tageszyklus, während der Spross 22°C Lufttemperatur ausgesetzt war. Zum anderen wurde das Wurzelsystem den selben Änderungen des Licht:Dunkel-Wechsels ausgesetzt wie der Spross. Die Wurzelwachstumsraten von *N. tabacum* waren bei 10°C um bis zu einem Drittel im Vergleich mit den Kontrollpflanzen reduziert, die bei 22°C kultiviert wurden. Außerdem änderte an den Wurzeln einfallen der Licht:Dunkel-Wechsel den Tageswachstumszyklus der Wurzeln. Jedoch konnten in beiden Experimenten keine Änderungen in der Blattexpansion gemessen werden.

Neben der zirkadianen Uhr und der Umwelt ist die Entwicklungsphase ein weiterer wichtiger Parameter, der das Wachstum beeinflusst. Der festgelegte Rhythmus des täglichen Wachstums von *N. tabacum* Keimlingen verlief deutlich abweichend von dem älterer Pflanzen, mit großen Unterschieden zwischen Tages- und Nachtwachstumsraten und minimalen graduellen Übergängen.

Der Tagesgang des Wachstums in Pflanzen ist das Gesamtergebnis eines präzisen Gleichgewichts zwischen der Physiologie der Pflanze, der molekularen Kontrolle (z.B. zirkadiane Uhr), der Umwelt und des Entwicklungsstadiums. Um den Tagesverlauf umfassend zu verstehen, muss man alle Parameter und deren Interaktionen einbeziehen.

Summary

Diel (24 h) growth is a complex and multi-layered process that is guided throughout day and night by endogenous regulatory systems and the environment. In nature, the different plant organs (e.g. root and shoot) are not exposed to the same diel fluctuating environmental stimuli. These environmental impulses as well as endogenous regulatory mechanisms might have specific organ-dependent effects on growth. In this frame, this study aimed to identify the importance of the circadian clock and soil temperature on the diel growth pattern of both shoot and root organs.

The dynamic growth phenotype of leaves and roots in two lines of *Arabidopsis thaliana* with a disrupted circadian clock: the *CCA1*-overexpressing line (*CCA1ox*) and the *prr9-prr7-prr5* (*prr975*) mutant were mapped. On a diel time scale, leaf growth defects observed due to a disrupted circadian clock. Both investigated lines showed enhanced leaf growth compared with the wild type during the diurnal period, suggesting increased partitioning of photosynthates for leaf growth at this time of the day. Nocturnal leaf growth was reduced and growth inhibition occurred by dawn, which can be explained by ineffective starch degradation in the leaves of the mutants. However, this growth inhibition was not caused by starch exhaustion. Overall, these results are consistent with the notion that the defective clock affects carbon and energy allocation, thereby reducing growth capacity during the night. Furthermore, rosette morphology and size as well as root architecture were strikingly altered by the defective clock control. Separate analysis of the primary root and lateral roots revealed strong suppression of lateral root formation in both *CCA1ox* and *prr975*, which was accompanied by peculiar changes in lateral root growth direction in light-dark cycles and increased lateral extension of the root system. It can be concluded that growth of the whole plant is severely affected by improper clock regulation in *A. thaliana*, resulting in not only altered timing and capacity of growth but also aberrant development of shoot and root architecture.

Growth responses depend besides endogenous regulatory mechanisms as the circadian clock on a series of environmental factors and their interplay. In this respect, this thesis focussed on the effects of root cooling on plant growth. In climate chamber experiments there is a consensus that soil temperatures have an effect on plant performance i.e. carbohydrate metabolism, phenylpropanoid biosynthesis, growth penalties and a general cold-acclimation response. However, not much is known about the effects of lowered soil temperature in greenhouse and field experiments. In greenhouse experiments with chilled soil, it was observed that most of the responses that were found in the climate chamber were nullified. While several growth parameters (biomass accumulation, leaf expansion and specific leaf weight) were affected, no up-regulation of the carbohydrate or flavonoid (flavonol and anthocyanin) metabolism was observed. From this result, it can be hypothesized that the field-like light regime in the greenhouse experiment (higher peak intensity and gradual increase/decrease in morning/evening) plays an important role in the amelioration of adverse effects

of root temperatures on shoot growth, carbohydrate and flavonoid metabolism. In order to quantify the growth rate of leaf and root simultaneously under root cooling in a high temporal scale, a system was developed that is capable of synchronous phenotyping of leaf and root growth. First experiments were carried out under three different conditions, a control condition where root and shoot were subjected to 22°C air temperature, a root cooling condition in which the root was cooled to 10°C throughout the diel cycle while the shoot was subjected to 22°C air temperature, and a third condition whereby the root was subjected to the same changes in the light-dark cycles as the shoot. Root growth rates in *N. tabacum* seedlings were reduced to one third when chilled (10°C vs. 22°C). Moreover, incident light:dark cycles affected the diel growth rhythm in roots. In either case, no response (e.g. lower amplitude or changed oscillation) was seen in leaf expansion.

The developmental stage might be another important factor that determines growth and the growth pattern. The leaf expansion pattern in *N. tabacum* seedlings showed marked differences as opposed to leaves that emerge later during development.

Diel growth patterns in plants are the result of fine balance between the plant's physiology, the molecular frameworks such as the circadian clock and the environment. In order to fully comprehend diel growth in all it facets it is necessary to understand each layer underlying growth.

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1 Introduction

1.1 Plant growth

Plant growth is one of the most intensively studied processes in plant biology. The growth process can be defined as the irreversible increase in plant size or biomass and is accomplished by two cellular processes, cell division and cell expansion. Growth in plants depends on the species, the developmental stage and the environment where the plant is growing in. Plants use a wide range of regulatory pathways to converge all signalling inputs and guide the growth process. This interplay between specific endogenous and exogenous signalling factors gives each individual plant its phenotype.

The question how leaf and root growth are controlled lies at the base of many attempts to generate more successful plants for various purposes and under a range of boundary conditions. In order to achieve this goal it is crucial to understand, amongst others, the control mechanisms of short-term growth responses on a scale that is relevant to important (and/or) recurring environmental variations. Functional plant modelling, vegetation analysis in the context of global climate change and modern plant breeding require an improved understanding of dynamic growth processes, including those observed under 24-h day-night (diel) fluctuations of environment.

While part of the same organism, leaves and roots are exposed to completely different environments; the atmosphere and the pedosphere, respectively. These environments differ from each other in chemical composition and physical properties and have distinct spatial and temporal heterogeneity. The atmosphere is characterized by strong and often predictable diel and seasonal variations in temperature, light-dark cycles and day length. Other atmospheric factors such as wind or air humidity can also affect plant growth (de Langre, 2008), yet these changes are less predictable and do not follow regular cycles. In contrast to the atmosphere, the pedosphere is mainly characterized by spatial heterogeneity. The physicochemical properties of the soil substrate determine the soil capacity for water and mineral retention. Temporal changes in temperature do occur in the pedosphere as well, but they are slower and dampened in amplitude compared to those in the atmosphere. For example, in a typical diel temperature cycle the atmospheric temperature varies by 16°C and reaches a maximum around 2 p.m., whereas soil temperature at 10 cm depth varies by merely 3°C and reaches a maximum two hours later at 4 p.m. (Walter *et al.*, 2009). These different growth environments seem to lead to different diel growth strategies (Ruts *et al.*, 2012b).

The most diverse group of land plants are the angiosperms or flowering plants. Traditionally the angiosperms have been divided into the monocotyledonous and dicotyledonous plants; plants with one cotyledon or two cotyledons, respectively. The diversion between monocots and dicots occurred early in the angiosperm evolution, approximately some 200 million years ago (Wolfe *et al.*, 1989).

Aside from the cotyledon number, there are several other characteristics that distinguish monocotyledonous from dicotyledonous plants (see Table 1 for more characteristics). However it must be pointed out that there are many exceptions to these characteristics in both groups.

Dicots	Monocots
Double cotyledon	Single cotyledon
Major veins are branched; system is reticulated	Major leaf veins are parallel
Root develops from radical	Adventitious roots
Vascular bundles in a ring	Vascular bundles scattered
Flowers are tetramerous or pentamerous	Flowers are trimerous
Pollen with three furrows or pores	Pollen with one furrow or pore
Shoot apical meristem exposed	Shoot apical meristem protected by older leaves
Secondary growth often present	Secondary growth is absent

Table 1: Differences between dicots and monocots; adapted from http://www.ucmp.berkeley.edu.

The anatomical differences between leaves of mono- and dicotyledonous plants, especially the position of the growth zone in which cell division and elongation takes place, predispose their leaf growth to a distinct perception and sensitivity to atmospheric environments (Fig. 1). In dicots the leaf growth zone is more directly exposed to environmental changes, whereas that of monocots is in a more protected microclimate shielded by sheaths of older leaves (Davidson and Milthorpe, 1966). In addition, growing leaf tissue is already engaged in photosynthesis in many dicot species, while growth zones of monocot leaves remain heterotrophic for a longer time. Similar to the monocot leaf, roots grow unidirectional and have well-defined, linearly organized growth zones. Unlike the monocot leaf however, the root growth zones are exposed more directly to the rhizosphere or pedosphere environment.

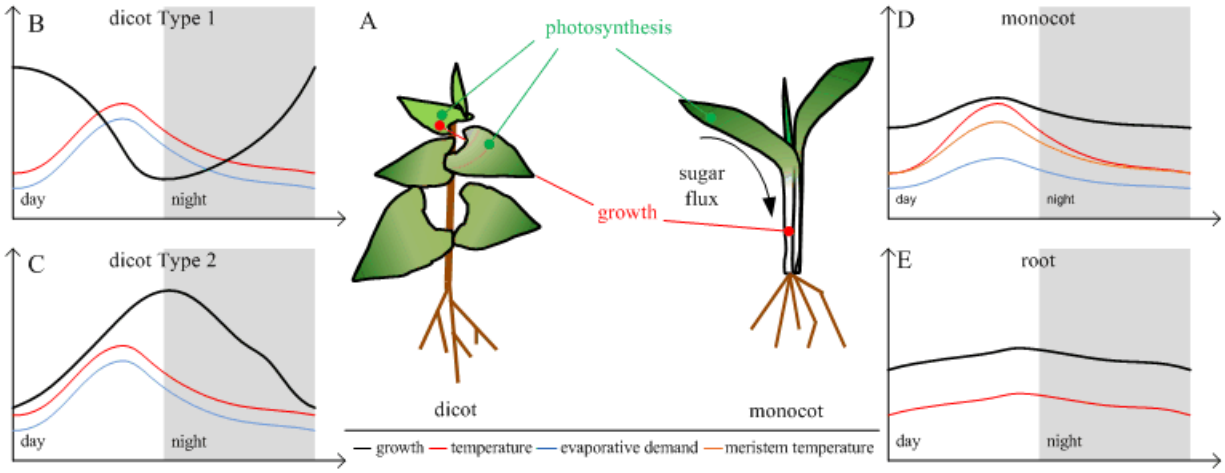


Figure 1: Plant architecture, prevailing diel variations of environmental factors and predominant diel leaf growth patterns. Schematic for a characteristic monocot and dicot with the growth zone (red) and photosynthesis zone

(green) marked (a). Schematic diel pattern of dicot Type 1 leaf growth (b), dicot Type 2 leaf growth (c), monocot leaf growth (d) and root growth (e) under changing temperature and evaporative demand. Adopted from (Ruts *et al.*, 2012b).

1.2 Root growth dynamics

The first diel measurements of root elongation rate (RER) go back to 1965 (Head, 1965). Time lapse movies with an interval of 4 hours were made for several days to study cherry (*Prunus avium*) root growth. The author reported a diel RER pattern with the highest growth rate at night and the lowest growth rate during the day. Unfortunately, no comments were made on the environmental conditions of the experiment (day length, temperature, soil properties or water availability), making it difficult to interpret these diel root growth patterns.

More recent studies with higher temporal resolutions (minutes instead of hours) revealed that root growth is highly responsive to temporal changes in environmental conditions. Root growth of *Zea mays* and *Nicotiana tabacum* quickly adjusted to new temperature regimes or to aboveground changes in light regimes within a few minutes (Walter *et al.*, 2002; Nagel *et al.*, 2006). Especially, root elongation growth seems to follow alterations in root temperature almost linearly within a physiological temperature range between 20°C and 30°C (Fig. 1e) (Walter *et al.*, 2002; Hummel *et al.*, 2007). The RER is furthermore sensitive to changes in nutrient availability (Walter *et al.*, 2003), soil water potential (Sharp *et al.*, 1988) and mechanical impedance of the soil (Bengough *et al.*, 2006) (for a review see: (Bengough *et al.*, 2011)). When environmental conditions were kept constant, diel root growth patterns were found to be constant in a number of species such as *Arabidopsis thaliana* (Chavarría-Krauser *et al.*, 2008), *Oryza sativa* (Iijima *et al.*, 1998), *Sorghum bicolor* (Iijima *et al.*, 1998), *Z. mays* (Walter *et al.*, 2002; Walter *et al.*, 2003) and *N. tabacum* (Walter and Schurr, 2005; Nagel *et al.*, 2006) (see Table 2). These results indicate that the environment plays a crucial role in determining root growth rates.

However, this notion is recently being contested. Marked oscillations of root tip growth rates have been reported in *A. thaliana* and *O. sativa* (Yazdanbakhsh and Fisahn, 2010; Iijima and Matsushita, 2011). For *A. thaliana*, under constant conditions; a growth maximum was recorded one hour after dawn followed by a steep decrease to reach a minimum at dusk and recuperation during the night (Yazdanbakhsh and Fisahn, 2010). Furthermore, in 2011 Iijima and Matsushita provided evidence for diel root rhythmicity in *O. sativa*; thereby contesting their results from 1998 (Iijima *et al.*, 1998; 2011).

The nature of the various rhythms observed in root growth is not known, however developmental stage and the environment may be crucial factor that can explain the different observations. For example, an important difference between the experimental conditions of Yazdanbakhsh and Fisahn (2010) and those of Walter *et al.* (2002, 2005) or Iijima *et al.* (1998, 2011) is the exposure of the entire root system, including the growing root tips, to light-dark cycles in the study by the former authors. As light is known to inhibit root growth (Pilet and Ney, 1978), the observed oscillation patterns in Arabidopsis root growth may be influenced by the inhibitory effect of root illumination

(Schmidt and Walter, 2009). Furthermore, complete enclosure of seedlings in a Petri dish - a widely used condition for root growth analysis - can affect growth processes through ethylene emission by leaves (Eliasson and Bollmark, 1988; Hummel *et al.*, 2009). Although there is a considerable amount of data on diel root elongation rates, there are also some contradictory observations. These can be partially attributed to differences between the species, developmental stages, environmental and growth conditions. An extended time-course analysis on several species may provide further information regarding the root elongation rhythms.

Species	Velocity (mm h^{-1})	Pattern	Temperature	Light intensity	Day length	Medium	Reference
Z. mays	0.8 - 1.3	constant	21°C	150 µE	12h/12h	-	Walter *et al.*, 2002
	1.9	constant	26°C	150 µE	12h/12h	-	Walter *et al.*, 2002
	2.6	constant	26°C	150 µE	12h/12h	water	Walter *et al.,* 2003
	1.8	constant	26°C	150 µE	12h/12h	nutrient solution	Walter *et al.,* 2003
N. tabacum	0.04 - 0.11	constant	-	-	-	-	Walter *et al.,* 2005
	0.05 - 0.06	constant	23°C	60 µE	12h/12h	Ingestad solution	Nagel *et al.*, 2006
	0.235 - 0.245	constant	23°C	300 µE	12h/12h	Ingestad solution	Nagel *et al.*, 2006
A. thaliana	0.055 - 0.120	oscillation	21°C	100 µE	12h/12h	½ Skoog	Yazdanbakhsh and Fisahn, 2010
	0.101	constant	23°C	60 µE	12h/12h	Ingestad solution	Krauser *et al.,* 2008
O. sativa	1.1 - 1.7	constant	25°C	380 µE	12h/12h	¼ Hoagland + loamysand	Iijima *et al.*, 1998
	1.03	oscillation	28°C	0 µE	DD	¼ Hoagland + loamysand	Iijima and Matsushita, 2011
	1.28	constant	33°C	0 µE	DD	¼ Hoagland + loamysand	Iijima and Matsushita, 2011
	1.48	oscillation	28°C	380 µE	12h/12h	¼ Hoagland + loamysand	Iijima and Matsushita, 2011
	2.02	oscillation	oscillation	380 µE	12h/12h	¼ Hoagland + loamysand	Iijima and Matsushita, 2011
S. bicolor	2.9 - 3.3	constant	25°C	380 µE	12h/12h	¼ Hoagland + loamysand	Iijima *et al.*, 1998

Table 2: Diel root elongation rates of several species; the parameters given are: velocity, pattern observed, temperature, light intensity at shoot, day length, medium and the reference. The roots were covered from the light in all experiments with the exception of Yazdanbakhsh and Fisahn, 2010. (Dashes signify that the information was not found in the reference).

1.3 Leaf growth dynamics

1.3.1 Dicots

In many dicot species, expanding cells of growing leaves are photosynthetically active (Stessman *et al.*, 2002). Because gas exchange and growth processes take place in one and the same tissue, pronounced diel fluctuations of carbohydrate and water availability accompany growth processes. Leaf veins of dicot plants are often arranged in a net-like structure and leaf lamina expand in both width and length with specific genetic control (Tsukaya, 2006). Moreover, considerable cell division still occurs in elongating parts (Beemster *et al.*, 2005). All of these make the growth process and their controlling mechanisms more complex for dicot leaves than for monocot leaves.

In growth a base-tip gradient is often observed with a maximum relative expansion rate at the base and the minimum at the tip region of the growing leaf (Schnyder and Nelson, 1988; Durand *et al.*, 1995; Tardieu *et al.*, 2000). This gradient is coordinated by earlier maturation of the tip part of the lamina compared to the basal part and it decreases with time (Schmundt *et al.*, 1998; Donnelly *et al.*, 1999; Walter and Schurr, 2005). In *N. tabacum*, for example, there is approximately a four-day delay in maturation over the gradient (Walter and Schurr, 1999). However, this base-tip growth gradient is not a general feature of all dicots; species such as *Glycine max*, *Populus deltoides* and *Theobroma cacao* show a homogeneous growth distribution over the entire leaf (Ainsworth *et al.*, 2005; Walter and Schurr, 2005; Matsubara *et al.*, 2006; Czech *et al.*, 2009).

Diel growth oscillation in dicots differs between species. In *Helianthus annuus* (Boyer, 1968) and *Acer pseudoplatanus* (Taylor and Davies, 1985), maximal leaf growth rates were reported at night, in *Phaseolus vulgaris* (Davies and Van Volkenburgh, 1983) and *Vitis vinifera* (Shackel *et al.*, 1987) maximal growth was reported at day, and in *Lycopersicon esculentum* highest growth rates were found at the day-night transition (Price *et al.*, 2001). In previous studies dicot leaf growth was mostly analysed by using linear variable displacement transducers that measure leaf elongation along the mid-vein, not taking expansion in width into account. Moreover, many earlier studies distinguished only between diurnal and nocturnal leaf growth by recording leaf dimensions at the beginning of day and night, respectively. When time-lapse imaging based methods became available, about a decade ago, it enabled the analysis of two dimensional expansion dynamics for different dicot plants under a range of environmental conditions (Schmundt *et al.*, 1998). For all species so far investigated, growing leaves exhibit repetitive diel leaf growth patterns without clear correlation to the diel atmospheric temperature regime (for a review of these analyses see Walter *et al.*, 2009). The observed diel leaf growth patterns can be categorized in two main types called Type 1 and Type 2 (Fig. 1b, c) (Walter *et al.*, 2009). External environmental parameters, such as temperature and light intensity, can influence the amplitude, but not the basic character, of the observed pattern (Poiré *et al.*, 2010b). Type 1 plants, such as *N. tabacum* (Walter and Schurr, 2005) and *A. thaliana* (Wiese *et al.*, 2007), show a

sinusoidal growth pattern with maximal growth rates around dawn and directly after onset of light (Wiese *et al.*, 2007). The diel growth pattern of Type 2 plants, such as *G. max* (Ainsworth *et al.*, 2005), *P. deltoides* (Matsubara *et al.*, 2006) and *T. cacao* (Czech *et al.*, 2009), is also sinusoidal but has a maximum at the end of the day. These three Type 2 plants have a homogenous growth distribution over the entire leaf, while *P. trichocarpa*, another Type 2 plant, shows a base-tip growth gradient across the lamina (S. Matsubara, unpublished data), indicating that formation of spatial and temporal (diel) growth patterns is not necessarily coupled with each other. Contribution of different growth processes (cell division and expansion) cannot explain variations in diel leaf growth patterns, either; the specific diel growth patterns, albeit with decreasing amplitude, are maintained over the base-tip gradient of Arabidopsis leaves (Wiese *et al.*, 2007) and during transition from predominantly cell division to cell expansion phase in growing leaves of *T. cacao* (Czech *et al.*, 2009).

The mechanisms regulating diel growth strategies of dicot plants are yet to be elucidated. Nevertheless, a study comparing the behaviour of several dicot and monocot species on a transfer from day-night conditions to continuous light conditions, has indicated that the circadian clock is an important driver of the observed diel growth patterns in leaves of dicot species (Poiré *et al.*, 2010b). Whereas a circa 24-h leaf growth rhythm continued in dicot plants after transfer to constant light and temperature regimes, the diel leaf growth rhythm in monocot species stopped when plants were transferred to constant light conditions. In addition to these findings, the presence of the Type 1 growth pattern in isolated leaf discs floating on nutrient solution without any contact to the sink-source system of the intact plant (Biskup *et al.*, 2009) indicates that the circadian clock within the growing leaf itself plays an important role in regulation of the diel dicot leaf growth pattern. For *A. thaliana*, the diel pattern of hypocotyl elongation growth is also shown to be influenced by the circadian clock and by carbohydrate availability (Nozue *et al.*, 2007; Stewart *et al.*, 2011); there, the observed Type 1 like growth pattern depends on the diel cycling of phytochrome-interacting factor 4 (PIF4) and PIF5 (see *1.6 Roles of the circadian clock and light in controlling diel dicot growth*).

1.3.2 Monocots

Leaf elongation in monocots occurs in defined growth zones at the basal part of the shoot (Fig. 1a). Growing cells in this zone undergo three phases: 1) exponential elongation, 2) stable elongation, and 3) progressive decrease of elongation until it reaches the final cell size (Parent *et al.*, 2009). Leaf elongation rates (LER) in several monocots, such as *Hordeum vulgare*, *Z. mays* and *S. bicolor*, have been shown to be stable and constant for a relatively long period (5 - 7 days) following the initial exponential growth phase (Bernstein *et al.*, 1993; Munns *et al.*, 2000; Sadok *et al.*, 2007). In *O. sativa* on the other hand, this stable elongation period is very short, if existing at all, and is followed by a long period of gradual elongation decrease (Parent *et al.*, 2009).

Close analysis of diel growth patterns in monocot leaves has revealed either constant elongation or slow decrease independent of time in the day/night cycle (Munns *et al.*, 2000; Parent *et al.*, 2009; Poiré *et al.*, 2010b). Increasing evidence indicates that there is a strong correlation between the short-term behaviour of monocot LER and changes in temperature or soil water status (Ben-Haj-Salah and Tardieu, 1995; Munns *et al.*, 2000; Sadok *et al.*, 2007; Poiré *et al.*, 2010b). Part *1.4 environmental factors affecting growth* elaborates further on the effects of temperature and water relations on LER in monocot plants.

1.3.3 Interaction between leaf and root growth

Roots and leaves live in completely different environments and have adapted to these conditions in unique ways. Diel growth patterns often differ between above- and belowground organs. However these organs are integral parts of a single plant system and they are highly dependent on each other for growth and survival. Optimal resource use efficiency and allocation demand highly coordinated fluxes of carbohydrates, water and mineral nutrients, all of which are acquired by one organ and delivered to the other. Hence, organ growth patterns that have evolved under certain environmental constraints can be considered to reflect optimal reaction patterns with which that certain plant organ can grow in its environmental context. The signalling between shoot and root growth is modulated, partially by phytohormones (Sharp and LeNoble, 2002; Ghanem *et al.*, 2011). In addition, the effects of carbohydrate translocation that modulate root growth upon alteration of light intensity (Nagel *et al.*, 2006), and other carbohydrate- as well as water-related effects of dynamic organ growth control are known to be linked with shoot-root signalling. The reduced transport of water by cooled roots, for example, has been shown to diminish diurnal but not nocturnal leaf growth (Poiré *et al.*, 2010a). A photosynthesis signal generated in the shoot, possibly sucrose or a derivative, has been proposed to influence the root circadian clock (James *et al.*, 2008). Furthermore, the diel and circadian expression of genes coding for transporters of water, ions, metabolic solutes such as sucrose, micronutrients and signalling molecules, including Ca^{2+}, might also contribute to the control of fluxes between root and shoot, thereby optimizing the overall plant performance (Haydon *et al.*, 2011).

1.4 Environmental factors affecting growth

Environmental factors that affect plant growth can be can be roughly categorized in 'temporal' factors which have a strong reoccurring diel or seasonal oscillation, such as temperature, the light:dark cycle, day length; and the more 'spatial' factors such as soil structure, and nutrient availability where the spatial distribution plays a important role.

Mineral nutrients such as nitrogen, phosphorus and potassium are mainly acquired in the soil in the form of inorganic ions. These minerals are essential in the synthesis of organic compounds, energy storage, structural integrity, signalling within the cell or involved in redox reactions (Taiz and Zeiger, 2007). An inadequate supply of an essential element often results in a nutritional disorder that is manifested by a characteristic deficiency phenotype (Piper, 1942; Vert *et al.*, 2002). Excess of minerals in the soil also restrict plant growth by limiting water availability and the accumulation of heavy metals becomes toxic at high concentrations (Li *et al.*, 2005; Wani *et al.*, 2007). Although there is continuous cycling of minerals in the ecosystem, mineral concentrations do not cycle within a diel time scale. However, seasonal oscillations in mineral content can be observed in arid regions and regions with strong seasonal rainfall (Taiz and Zeiger, 2007).

Soil structure, like mineral deposition is also mainly a spatial factor. In friable soil, roots can growth rapidly but the uptake of water and nutrient might be limited due to the loose contact with the soil (Bengough *et al.*, 2006). In hard soil, the high mechanical impedance inhibits root growth so that the foraging ability of the root system is poor which leads to inadequate water and nutrient acquisition (Bengough *et al.*, 2006). Soil structure not only affects root growth and resource acquisition, it also induces hormonal signalling which slows shoot growth even when the current resource uptake is adequate (Passioura, 1991).

In the atmosphere, gaseous compounds such as carbon dioxide, ozone and nitrous oxide have diel and seasonal oscillations (Christensen, 1983; Moriwaki and Kanda, 2004; Du *et al.*, 2006). For example, seasonal and diel variations in the CO_2 flux from soil to atmosphere have been reported and seem to follow the seasonal temperature trends (Parkin and Kaspar, 2003). Additionally, there is also the emission of volatile organic compounds (VOCs) such as ethylene by the canopy which show diel rhythmicity (Prévôt *et al.*, 2000; Na *et al.*, 2003; Ho *et al.*, 2004). However, very little is known on the effects of these rhythmic changes in gaseous compounds on diel plant growth.

Plant development and carbon acquisition strongly depends on light and its signalling pathways. As such, the transition of the seedling from skotomorphogenesis to photomorphogenesis is triggered by a short flash of light (Taiz and Zeiger, 2007). Flowering is in several plant species induced by increasing day length (Suarez-Lopez *et al.*, 2001; Hayama and Coupland, 2004). Furthermore, light intensity plays an important role in the functioning of the photosystem and the total carbon acquisition

over the day (Poorter and Nagel, 2000). With the spectrum of absorbed light (e.g. light quality) the plant can sense its position in the canopy and adjusts the organ growth accordingly to compete for photosynthetic active wavelengths (Morelli and Ruberti, 2000). This light signalling in plants occurs mostly over the phytochromes and cryptochromes. Depending on the processes studied wavelength, exposure time (e.g. day length), intensity or a combination of the before mentioned play a crucial role in the light signalling processes. Light has strong and reliable diel and seasonal rhythms and is therefore one of the most important factors that the plant uses to synchronize its endogenous rhythm to the environment. For more information on how light affects plant processes on the diel time-scale I refer to section *1.6 Roles of the circadian clock and light in controlling diel dicot growth*.

The influence of diel temperature and diel water status on plant growth is explained in more detail in the next sections due to their relevance for diel plant growth in the context of this thesis.

1.4.1 Temperature

Nocturnal LER in monocots (*Z. mays*, *O. sativa*) has been shown to follow temperature alterations linearly in a range between 10°C and 30°C (Ben-Haj-Salah and Tardieu, 1995; Pietruszka and Lewicka, 2007; Poiré *et al.*, 2010b). Variations of LER and temperature coincide almost perfectly throughout the diel cycle when evaporative demand is low (Poiré *et al.*, 2010b; Parent and Tardieu, 2012). Thus, the meristem temperature seems to be a primary factor in controlling the rate of leaf elongation in monocots (Ben-Haj-Salah and Tardieu, 1995). Indeed, at constant temperature and low evaporative demand, no diel pattern of LER is observed (Parent *et al.*, 2010; Poiré *et al.*, 2010b) although transient effects of light-dark transitions may appear (Sadok *et al.*, 2007). Given the strong correlation with temperature, nocturnal (or diel) monocot LER (at low evaporative demand) can be described on the basis of thermal time (Sadok *et al.*, 2007). The classical concept of 'thermal time' is based on the linearity between the elongation rate and temperature:

$$R = a\,(T - T_0) \qquad \text{Eqn. 1}$$

where R is the leaf elongation rate, T is the temperature, T_0 is the threshold temperature below which the elongation rate is considered to be zero, and a is constant (Granier and Tardieu, 1998a; Bonhomme, 2000). When a time component is added, the LER over a given time t can be described as:

$$R = a \int_0^t (T - T_0) dt \qquad \text{Eqn. 2}$$

In other words, the leaf elongation rate is a linear function of the thermal input the leaf receives over a certain time. It is important to notice that this formula can be used only when the relationship between elongation rate and temperature is *linear*, which holds true within a certain, species-specific temperature range (Bonhomme, 2000). For calculation of development rates in a temperature range extending to extremes, a recent equation (Parent *et al*,. 2010) based on a combination of molecular

reaction rates (Eyring equation; Eyring 1935) and the reversible inhibition of enzymes (Eyring, 1935; Johnson *et al.*, 1942; Parent *et al.*, 2010) with temperature changes can be considered. In contrast, thermal time cannot be directly used for modelling of diel leaf expansion rates in dicots since growth of dicot leaves correlates less strongly with temperature changes (Poiré *et al.*, 2010b).

1.4.2 Water relations

Beside the metabolic constraints, water availability is one of the dominant factors controlling leaf expansion. Without enough water the necessary turgor pressure cannot be built up in a cell, which is a prerequisite for leaf expansion. Reduced water availability can act on plant growth via reduced cell number, cell expansion and carbon accumulation. Of those three, cell expansion is the process most affected by water availability since it is governed by the mechanical properties of the cell wall as well as the plants hydraulics which is dependent on the balance between the soil water potential, water conductivity of the vessels/tissues and leaf transpiration (Spollen and Sharp, 1991; Tang and Boyer, 2002; Bouchabké *et al.*, 2006). Hydraulic control is therefore thought to be more restrictive during the day, when transpiration demand is potentially the highest, rather than during the night when stomata are closed and the leaf water potential recovers. Additionally during the course of leaf ontology there seems to be a switch from tight metabolic control to hydraulic control over leaf growth and water relations become more important in older leaves (Pantin *et al.*, 2011).

In sunflowers, water deficit does not affect the duration of cell division and expansion. Regardless of the time of the deficit there is a reduced relative expansion rate by approx. 36% and relative cell division rate by 39% in all positions within the leaf (Granier and Tardieu, 1998b). However, the extent of reduction in the final leaf area and cell number in a given zone of the leaf largely varied depending on the timing of deficit, with the maximum effect found for earliest deficits (Granier and Tardieu, 1998b).

1.4.3 Evaporative demand and water deficit on a diel scale

During the day period evaporation and transpiration of water can be a dominant factor in determining LER. The LER has been shown to decrease with increasing evaporative demand (Munns *et al.*, 2000; Reymond *et al.*, 2003). Moreover, diurnal changes in LER are closely related to the transpiration rate and correlate with changes in evaporative demand even in the absence of a soil water deficit (Acevedo *et al.*, 1979; Ben-Haj-Salah and Tardieu, 1996, 1997; Sadok *et al.*, 2007). Hence, leaf elongation rate can be described by (Sadok *et al.*, 2007):

$$R = a\,(1 - d J_w) \qquad \text{Eqn. 3}$$

Where J_w is the transpiration rate per unit leaf area.

Likewise, a decrease in the water potential in the growing tissue due to diminished root water conductivity and resulting increase in xylem tension can reduce LER during the afternoon (Tang and

Boyer, 2008). The effects of soil water deficit on LER (in the absence of evaporative demand) have been shown to be proportional to the predawn leaf water potential (Chenu *et al.*, 2008). Moreover, it has been proposed that abscisic acid (ABA), which plays an important role in regulation of water relations, has three main effects on growth by: (a) increasing the water conductance in the plant, (b) buffering the negative effect of evaporative demand and related day-night alteration of LER, and (c) a modest influence on non-hydraulic effects (Tardieu *et al.*, 2010).

1.5 Endogenous control of growth

Earth's rotation brings almost all organisms into changing but recurring environmental conditions. Therefore most living organisms adjust their physiology and behaviour with the help of internal oscillators called "circadian clocks" to anticipate these recurring events. For autotrophic plants there is the need to fine-tune their photosynthesis, carbohydrate metabolism and carbohydrate storage during the entire diel cycle of day and night (Lu *et al.*, 2005; Gibon *et al.*, 2009; Graf *et al.*, 2010). As described above, leaf growth of monocot and dicot plants as well as growth of other plant organs follows different diel rhythms, requiring an adapted rhythmic control which, for leaves, seems to be much stronger in dicots than in monocots. The dicot leaf seems to regulate its expansion by balancing the timing of many processes according to metabolic constraints and environmental cues (Green *et al.*, 2002; Dodd *et al.*, 2005; Covington and Harmer, 2007).

The diel control of growth occurs at many different levels of regulation. Changes in metabolite levels (glucose, starch) and the influence of the circadian clock are briefly explained in the next sections due to their relevance in the context of this thesis.

1.5.1 Carbohydrates

Carbohydrates are required as building blocks for growth (e.g. cell wall polymers) and as energy carriers to drive growth activities. To control the availability and quality of carbohydrates, plants and other organisms evolved a complex signalling system that allows the integration of carbohydrates as signalling molecules into the control of gene expression, metabolism, growth and development (Rolland *et al.*, 2006; Smeekens *et al.*, 2010). Carbohydrates are the product of photosynthesis during the day and a fraction of them is stored as starch. This fraction provides a nocturnal supply to source and sink cells and its degradation is adjusted to the expected length of the night period (Gibon *et al.*, 2004; Lu *et al.*, 2005; Graf *et al.*, 2010). Diurnal growth on the other hand is limited by the amount of carbohydrates that can be derived from photosynthesis and which are not used for storage (Sulpice *et al.*, 2009; Graf and Smith, 2011). Also starch degradation, but not starch synthesis, was shown to correlate strongly and positively with the relative growth rate (RGR) of Arabidopsis in varying day lengths (Gibon *et al.*, 2009). Therefore, carbohydrate metabolism plays a very important role in the control of leaf growth. Leaves and roots of Arabidopsis starch deficient mutants do not grow or grow very slowly during the night due to a lack of available carbohydrates (Gibon *et al.*, 2004; Wiese *et al.*, 2007; Biskup *et al.*, 2009; Yazdanbakhsh *et al.*, 2011). Instead, in such mutants a leaf growth increase at the end of the day correlates with an excess of soluble carbohydrates which, in wild-type (wt) plants, is converted to starch. The carbohydrate status apparently has a direct impact on the amplitude of leaf growth, as manifested by increased growth rates in the afternoon, while still retaining the general phasing of the leaf growth cycle comparable to that of wt plants (Wiese *et al.*, 2007). Overall the starch metabolism is supposed to act as an integrator of the metabolic response in a regulatory

network that balances growth with the carbon supply (Sulpice *et al.*, 2009; Graf *et al.*, 2010; Graf and Smith, 2011). Furthermore, many sugar-responsive genes show marked diel expression changes and low sugar levels have a profound impact on diel gene regulation (Bläsing *et al.*, 2005). The diel changes in carbohydrate status of dicot plants have a strong effect on the observed growth pattern of leaves (Gibon *et al.*, 2004; Nagel *et al.*, 2006; Wiese *et al.*, 2007). Similarly, carbohydrate availability is an important driving force in the basal zone of monocot leaf growth (Davidson and Milthorpe, 1966; Schnyder and Nelson, 1987). The completely shielded growth zone of the monocot leaf is heterotrophic and photosynthates are provided from the already fully differentiated tip part of the growing leaf (Allard and Nelson, 1991) as well as other surrounding fully expanded leaves (Brégard and Allard, 1999). Thus in monocot leaves, photosynthesis is spatially clearly separated from the growth zone. The diel export of carbohydrates from the distal, source part of a young maize leaf correlates positively with LER (Kalt-Torres and Huber, 1987). Yet, the diversity in monocot carbohydrate storage forms (fructans, sucrose, starch) and cellular compartmentalisation of storage carbohydrates leads to a very complex situation that is far from being elucidated. Also in roots, carbohydrate availability strongly regulates dynamic growth activity (Aguirrezabal *et al.*, 1994; Freixes *et al.*, 2002). A change of light intensity for the shoot can affect root growth via changes in carbohydrate availability within less than one hour (Nagel *et al.*, 2006), demonstrating the important role of carbohydrates in short-term whole-plant growth control.

1.5.2 Circadian clock

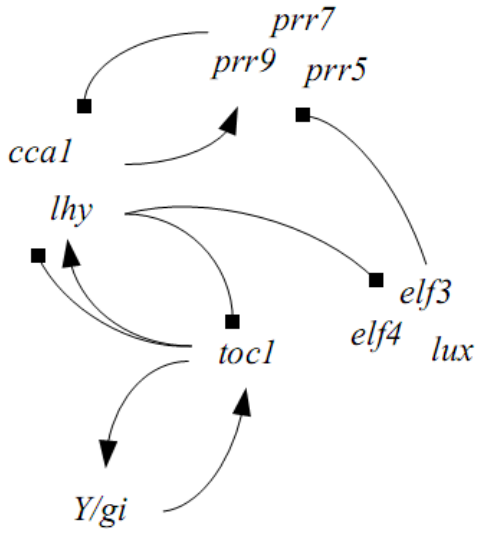
The circadian clock is a molecular network that keeps track of time and that enables organisms to respond to daily occurring changes in the environment and maintains a period of *circa* 24 hours. A match of the oscillation period of the clock with daily rhythms in environmental changes is therefore essential for their fitness, including plants (Green *et al.*, 2002; Michael *et al.*, 2003b; Dodd *et al.*, 2005; Yerushalmi *et al.*, 2011).

The clock is involved in multiple processes during the plant's life cycle, ranging from germination (Penfield and Hall, 2009), hypocotyl elongation (Dowson-Day and Millar, 1999; Nozue *et al.*, 2007), petiole and leaf growth dynamics (Wiese-Klinkenberg *et al.*, unpublished) to the photoperiod control of flowering time (Yanovsky and Kay, 2003; Imaizumi and Kay, 2006). Besides these processes the clock has been shown to control stomatal responses (Gorton *et al.*, 1993; Meidner and Willmer, 1993), phytochrome signalling (Covington and Harmer, 2007), cold tolerance (Nakamichi *et al.*, 2009), transitory starch degradation (Graf *et al.*, 2010) and plant defence mechanisms (Kerwin *et al.*, 2011).

1.5.2.1 Circadian components and their interaction

The circadian clock runs with a period close to 24 h even in the absence of environmental triggers, can be reset by environmental cues (e.g. light, temperature) and is temperature compensated. The

oscillator is truly endogenous as rhythmicity is observed in etiolated seedlings that never have been exposed to changing environmental conditions (Salomé *et al.*, 2008). The circadian clock is an endogenous control network consisting of various transcriptional-translational feedback loops (Fig. 2). Conventionally, the core of the central loop consists of three components CCA1 (CIRCADIAN CLOCK ASSCIATED 1), LHY (LATE ELONGATED HYPOCOTYL) and TOC1 (TIMING OF CAB EXPRESSION 1), also referred to as PSEUDO-RESPONSE REGULATOR 1, (PRR1) (Wang and Tobin, 1998; Alabadı́ *et al.*, 2001). *CCA1* and *LHY* are dawn-phased genes and code for partially redundant MYB transcription factors, which inhibit the expression of the evening-phased gene *TOC1* by binding directly to the evening element of the *TOC1* promoter (Alabadı́ *et al.*, 2001; Mizoguchi *et al.*, 2002). TOC1 in turn, reciprocally regulates the expression of *CCA1* and *LHY* transcripts, directly by binding the *CCA1* and *LHY* promoter (Gendron *et al.*, 2012) and indirectly via CHE, a TCP transcription factor that binds specifically to the *CCA1* promoter (Alabadı́ *et al.*, 2001; Makino *et al.*, 2002; Pruneda-Paz *et al.*, 2009). The expression of *CHE* is regulated by CCA1, thus adding a CCA1/CHE feedback loop to the clock model (Pruneda-Paz *et al.*, 2009). Recently, it has been demonstrated that TOC1 is a general repressor of oscillation through its rhythmic association to the promoters of the oscillator genes (Gendron *et al.*, 2012; Huang *et al.*, 2012). In addition to TOC1, there are several other evening genes that are in a similar feedback loop with CCA1/LHY. These include the putative MYB transcription factor LUX (LUX ARRYTHMO), also called PCL1 (PHYOCLOCK 1) (Hazen *et al.*, 2005; Onai and Ishiura, 2005), as well as BOA (BROTHER OF LUX ARRYTHMO), GI (GIGANTEA) (Lu *et al.*, 2012), ELF3 and ELF4 (EARLY FLOWERING 3, 4) (Schaffer *et al.*, 1998; Doyle *et al.*, 2002; Kikis *et al.*, 2005; Dai *et al.*, 2011; Lu *et al.*, 2012).



These components are, like TOC1, repressed by CCA1 and/or LHY and are necessary for the proper morning expression of *CCA1* and *LHY*. It has been demonstrated that the evening components ELF3, BOA and TOC1 can directly bind to the *CCA1* promoter region (Dai *et al.*, 2011; Gendron *et al.*, 2012; Huang *et al.*, 2012; Lu *et al.*, 2012). Furthermore, the evening components LUX, ELF3 and ELF4 can bind together and form a complex (Nusinow *et al.*, 2011). Additionally, it is shown that LUX can bind to its own promoter (Helfer *et al.*, 2011).

Figure 2: Simplified working model of the Arabidopsis circadian clock. See text for the abbreviations of the genes.

During the diurnal period there are consecutive peaks of transcripts of the PRR family; in the early morning, *PRR9* is expressed, followed by *PRR7*, *PRR5* and *PRR3* to end with *TOC1* around dusk (Nakamichi *et al.*, 2007). CCA1 and LHY positively act on the expression of *PPR9* and *PRR7*. A feedback loop is formed by the repressor activity of the PRR9, PRR7 and PRR5 on promoters of their activators *CCA1*and *LHY* (Farré *et al.*, 2005; Nakamichi *et al.*, 2010). There are also several other feedback loops between the PRRs and the evening components. The expression of the active morning PRRs are controlled by the evening components. So is the expression of *PRR9* directly regulated by LUX, ELF3 and ELF4, in which the latter two might function in an ELF3/ELF4 complex (Dixon *et al.*, 2011; Helfer *et al.*, 2011; Kolmos *et al.*, 2011; Herrero *et al.*, 2012). The expression of *PRR7* is regulated by ELF3 and ELF4 (Dixon *et al.*, 2011). This relationship changes as the day progresses and the expression of the evening genes comes under control of the PRRs. PRR5 together with PRR3 enhances TOC1 stability at dusk (Para *et al.*, 2007; Wang *et al.*, 2010). PRR5 binds to TOC1 and thereby enhances the nuclear import of TOC1 (Wang *et al.*, 2010). ZTL (ZEITLUPE), which is a cytosolic F-box protein, binds to TOC1 and PRR5, targeting them for proteasomal degradation (Mas *et al.*, 2003). However, when PRR3 binds to TOC1 it perturbs interaction of TOC1 with ZTL and thus results in the inhibition of the proteasome-dependent degradation of TOC1(Para *et al.*, 2007). Based on mathematical modelling and gene expression analyses, a negative transcriptional feedback loop between TOC1 and GI has been proposed (Locke *et al.*, 2005; Locke *et al.*, 2006).

Current evidence suggests that the plant circadian clock is tissue autonomous (Thain *et al.*, 2002; James *et al.*, 2008). Roots for instance, have a simplified version of the circadian clock consisting solely of a functional morning loop (James *et al.*, 2008). The evening genes, although expressed, do not oscillate and - in general - a smaller number of genes shows diel gene expression in darkened roots compared to the shoot (James *et al.*, 2008). Furthermore, in the shielded developing maize ears diel gene expression is strongly dampened compared to the photosynthetically active leaf, and the expression of all clock genes displays strongly reduced amplitudes (Hayes *et al.*, 2010). For detailed reviews on the circadian clock, see (Harmer, 2009; McClung and Gutierrez, 2010; Pruneda-Paz and Kay, 2010; Nakamichi, 2011).

1.5.2.2 Clock entrainment by the environment

Plants synchronize their clock by signal inputs from the environmental cycles, in particular by those of light and temperature.

Light

Several photoreceptors have been shown to mediate light signalling to the circadian clock (for recent reviews see (Jones, 2009; McWatters and Devlin, 2011)).The red and blue light photoreceptors, phytochromes (PHY) and cryptochromes (CRY), mediate part of the light signal input into the clock

(Somers *et al.*, 1998). The interactions between the PHY/CRY signalling pathways are synergistic and depend on light quality as well as fluence rate (Casal and Mazzella, 1998). Even though light signalling via PHY and CRY is important for normal clock function, these pathways are not essential for clock functioning and can be compensated by other input signals (Yanovsky *et al.*, 2000; Strasser *et al.*, 2010).

ZTL and two ZTL homologues, FLAVIN BINDING KELCH F-BOX 1 (FKF1) and LOV KELCH PROTEIN2 (LKP2), act as photoreceptors by a photochemically active LOV domain to regulate *TOC1* expression in the evening part of the oscillator (Mas *et al.*, 2003; Kim *et al.*, 2007; Sawa *et al.*, 2007; Baudry *et al.*, 2010).

Furthermore, LIGHT-REGULATED WD1/2 (LWD1/2) have recently been shown to act in light signalling into the clock although the exact mechanisms have not been elucidated (Wu *et al.*, 2008). LWD1 forms a positive feedback loop by directly targeting the promoter regions of *PRR9* and *PRR5*, enhancing their expression (Wang *et al.*, 2011). LWD1 also binds to the promoter region of *TOC1* (Wu *et al.*, 2008; Wang *et al.*, 2011). In addition *SRR1* (Staiger *et al.*, 2003), *LIP1* (Kevei *et al.*, 2007), *XCT* (Martin-Tryon and Harmer, 2008), *ELF3* (McWatters *et al.*, 2000) as well as *ELF4* (Kikis *et al.*, 2005) all affect light input into the oscillator, suggesting a complex regulation of input signalling by light.

Temperature

The circadian clock is temperature compensated; maintaining relatively constant periodicity over a broad range of physiological temperatures. Current results indicate that temperature compensation is established by a dynamic balancing of morning components (*LHY/CCA1*) versus evening components (*TOC1/GI*) (Gould *et al.*, 2006; Portoles and Mas, 2010).

Nevertheless, temperature cycles can entrain the circadian oscillator (Salome and McClung, 2005; Thines and Harmon, 2010). Temperature cycles alone are sufficient to drive at least half of all transcripts critical for synchronizing cellular processes, such as cell cycle and protein synthesis (Michael *et al.*, 2008). The differences in temperature sensitivity of circadian clock outputs suggest the presence of multiple oscillators with different temperature sensitivities (Michael *et al.*, 2003a). The genes involved in temperature compensation (*LHY*, *CCA1*, *TOC1*, *GI*), together with *ELF3*, *PRR9* and *PRR7*, have been proposed to be components of temperature input into the oscillator (Salome and McClung, 2005; Thines and Harmon, 2010).

The circadian oscillator takes inputs during the diel cycle to sense the environment and to regulate output accordingly (Sawa *et al.*, 2007). The impact of light and temperature via the clock on growth, in contrast to their direct effect on growth, depends on the importance of the circadian network that in

turn varies between plant species and the plant's organ/tissue. Hence, to derive the exact connection between circadian clock elements and the diel control of leaf growth processes, a precise understanding of the role of the above mentioned elements will be required. This may eventually contribute to clarification of differences between Type 1 and Type 2 species as well as to understanding the variable fine-tuning of diel leaf growth patterns in different species to environmental variations.

1.5.2.3 Different clocks - monocot vs dicot

Most of the work in unravelling the molecular components of the circadian clock and their function has been done in the dicot *A. thaliana*. However, homologues for most of the genes of the central oscillator are found in other species, both monocots (*Lemna gibba, Lemna paucicostata, O. sativa, Z. mays*) and dicots (*G. max, Ipomoea nil, Solanum lycopersicum, P. trichocarpa, P. deltoides*, *Castanea sativa*) (Izawa *et al.*, 2003; Miwa *et al.*, 2006; Hayama *et al.*, 2007; Murakami *et al.*, 2007; Facella *et al.*, 2008; Ibañez *et al.*, 2008; Serikawa *et al.*, 2008; Takata *et al.*, 2009; Hayes *et al.*, 2010; Hudson, 2010). Recently, *LATE BLOOMER1* (*LATE1*) and the *DIE NEUTRALIS* (*DNE*) genes were characterized in pea as homologues of the Arabidopsis clock genes *GI* and *ELF4*. Like Arabidopsis *gi* and *elf4* mutants, *late1* and *dne* mutants are both photoperiod insensitive and always flower late and early, respectively (Hecht *et al.*, 2007; Liew *et al.*, 2009). However, the clock does not appear to function the same way in all dicots. For example in *Pharbitis*, the circadian clock is reset at dusk, thereby distinguishing itself from Arabidopsis and Oryza in which the clock is mainly reset at dawn (Hayama *et al.*, 2007). Although there are some species-specific modifications in the clock, overall expression patterns of clock components appear to be conserved within dicots. Recent studies also have revealed the function of some clock genes in several monocot species (Miwa *et al.*, 2006; Murakami *et al.*, 2007). In both the *Oryza* genus and the *Lemna* genus homologues of Arabidopsis *CCA1, TOC1, LHY, GI* and *PRR*s have been identified (Murakami *et al.*, 2003; Miwa *et al.*, 2006). In some monocots like *L. giba*, clock components have another function in the clock. For example, down regulation of the GI homologue in *L. giba* abolishes circadian rhythms, a phenotype that is much more severe than observed in its Arabidopsis counterpart (Serikawa *et al.*, 2008). In summary, although homologues of clock genes have been identified in dicots and monocots, variations are found in terms of the amplitude, phase gene expression and role in the regulation of the oscillation (Miwa *et al.*, 2006; Hayama *et al.*, 2007; Serikawa *et al.*, 2008; Hayes *et al.*, 2010). Together, these results suggest that the roles of some clock genes have diverged during angiosperm evolution in both monocot and dicot clades.

1.5.2.4 Interaction of carbohydrates and the clock - a link between resource utilisation and integration of environmental changes

In *A. thaliana* roughly 30% and in maize 10% of the transcriptome is under circadian regulation (Covington *et al.*, 2008; Khan *et al.*, 2010). When different conditions are taken into account, the total sum of transcripts that can show diel rhythmicity is estimated to be close to 90% (Michael *et al.*, 2008; Filichkin *et al.*, 2011). Many clock-controlled genes are modulated in a diurnal/nocturnal regime concomitant with the changing carbohydrate metabolism (Bläsing *et al.*, 2005). Sucrose modulates the phase and amplitude of the expression of circadian-regulated genes in Arabidopsis shoot and root tissues (Dalchau *et al.*, 2011). It has been proposed that the observed differences of circadian gene expression in developing ears of *Z. mays* and in seeds of *G. max* (Hudson, 2010) when compared to leaves are caused by the sugar importing sink status of these organs (James *et al.*, 2008; Hayes *et al.*, 2010). A significant proportion of genes under circadian regulation are involved in metabolism and hormone signalling in plants (Michael *et al.*, 2008; Khan *et al.*, 2010). The circadian clock influences plant metabolism and hormone signalling, including auxin gating, tricarboxylic acid (TCA) cycle, carbohydrate metabolism and storage (Lu *et al.*, 2005; Covington and Harmer, 2007; Fukushima *et al.*, 2009; Graf *et al.*, 2010). Increased starch accumulation has been shown for mono- and dicotyledonous plants lacking the clock gene *GI* (Eimert *et al.*, 1995; Izawa *et al.*, 2011). By regulating the rate of starch degradation, the circadian clock ensures carbohydrate availability throughout the night (Graf *et al.*, 2010), exerting an indirect control of the nocturnal growth potential (Graf and Smith, 2011). On the other hand, another line of evidence is also accumulating to suggest that circadian signalling is not unidirectional but reciprocally obtains information from metabolic profiles (Kerwin *et al.*, 2011).

1.6 Roles of the circadian clock and light in controlling diel dicot growth

Hypocotyl elongation has long been recognised for its sensitivity to a very wide range of endogenous and environmental factors (Smalle *et al.*, 1997; Dowson-Day and Millar, 1999). Elongation of the hypocotyl appears to be controlled by a signalling cascade in which the response to any single factor depends critically upon the interactions among other regulators (Cary *et al.*, 1995; Chory and Li, 1997; Trewavas and Malho, 1997; Jensen *et al.*, 1998). Hypocotyl elongation in light-dark cycles includes a period of rapid growth at the end of the night with a peak elongation rate at dawn and a period of reduced elongation spanning the subsequent light period and early night (Nozue *et al.*, 2007). Upon transfer to continuous light this growth pattern is phase shifted by 8–12 h relative to plants in light-dark cycles, and the elongation peak is observed at subjective dusk (Dowson-Day and Millar, 1999; Nozue *et al.*, 2007). Hypocotyl elongation rates of several clock mutants deviate from this wt growth pattern. For example, the hypocotyl elongation rates of *CCA1ox* and *elf3* mutants increase as soon as the dark period starts, continue to increase throughout the nocturnal period and diminish rapidly at dawn (Nozue *et al.*, 2007). At the molecular level, these growth rhythms are regulated by the interaction of the phasing of transcription and the regulation of protein abundance by light (Nozue *et al.*, 2007).

1.6.1 Clock and light control on hypocotyl elongation

The circadian clock regulates the transcript levels of *PIF4* and *PIF5*, two positive growth regulators necessary for hypocotyl elongation at dawn, by three clock components ELF3, ELF4 and LUX (Nozue *et al.*, 2007; Nusinow *et al.*, 2011). ELF3, ELF4 and LUX are expressed at dusk and form a complex during the early night (Evening Complex, EC) that can bind to the promoter region of *PIF4* and *PIF5*, thereby inhibiting the expression of these transcription factors (Nusinow *et al.*, 2011). The loss of any Evening Complex components leads to early *PIF4* and *PIF5* expression and hence increased nocturnal growth (Nusinow *et al.*, 2011). The expression of ELF4, ELF3 and LUX is partially regulated by light (Nusinow *et al.*, 2011).

PIF4 and PIF5 proteins accumulate in the dark, but are rapidly turned over in response to exposure to normal to high red to far-red (R:FR) ratio of the light spectrum. This is due to the degradation of PIF4 and PIF5 upon interaction with the nuclear localised PHYs (Lorrain *et al.*, 2008). In non-shaded conditions the PIFs are actively promoting hypocotyl elongation in a small time window at dawn. The promotion of hypocotyl elongation is also dependent on the photoperiod (Niwa *et al.*, 2009). Under short-day conditions, internal (circadian) and external (photoperiod) time cues coincide in such a manner that *PIF4* and *PIF5* expression is induced (Niwa *et al.*, 2009). In long-day conditions, on the

other hand, there is an insufficient match between internal and external cues and the nighttime expression of *PIF4* and *PIF5* tends to disappear (Niwa *et al.*, 2009).

The coincidence of high transcript levels (by the clock) and protein accumulation (in the dark) of PIF4 and PIF5 allows maximum hypocotyl growth at dawn under diurnal non-shaded conditions (Nozue *et al.*, 2007; Lorrain *et al.*, 2008). Thus, these two genes integrate clock and light signalling as well as the coordinated regulation between them.

1.6.2 Shade avoidance syndrome

Plants need light in order to survive and are often in competition with each other for the photosynthetically active wavelengths of light (most of the visible light, with exception of some green light that is reflected or transmitted). As the light travels through the canopy photosynthetic pigments such as chlorophylls and carotenoids absorb most of light in blue and red wavelength regions. However, far-red light (725 and 735 nm) is poorly absorbed by the canopy and gets enriched relative to the other wavelengths. Plants have developed a suite of photoreceptors (e.g. PHYs, CRYs, phototropins) that enables them to detect light quality. Phytochromes (PHY A-E) are the dominant photoreceptors and light signal transducers in the red/far-red regions.

The R:FR ratio is a useful parameter that provides plants with information on the presence of competitors (shade effects). Normal daylight has a R:FR ratio of about 1.15 and this varies little under different weather conditions or in different times of the year (Smith, 1982). In contrast, the R:FR is typically between 0.005 and 0.7 underneath the canopy (Smith, 1982).

When the R:FR ratio is low, morphological changes to avoid shade (in shade-intolerant plants such as *A. thaliana*) are triggered in a process called the shade avoidance syndrome (SAS). One of the most dramatic shade avoidance responses is the stem elongation. This response is reversible and remarkably rapid, with a lag phase of a few minutes. Many plants react within 5 to 10 min of exposure to FR-rich light by accelerating stem elongation rates up to 3- or 4-fold (Morelli and Ruberti, 2000). The elongation response of stem and petiole are often linked with reduced leaf area development and underdeveloped storage organs (Devlin *et al.*, 1992; Reed *et al.*, 1993; Smith and Whitelam, 1997). If the plant fails to outgrow its competitors and stays shaded, it will also start to flower early (Halliday *et al.*, 1994).

Phytochromes perceive red and far-red light by switching between two convertible spectral forms (Pr and Pfr) (Schäfer *et al.*, 1972; Rockwell *et al.*, 2006). Phytochromes are synthesised in the Pr form (red-absorbing form) in the cytosol. Upon absorbing red light, the Pr form is converted to the Pfr form (far-red-absorbing form) and translocated to the nucleus where it can interact with a set of PHY interacting factors (PIFs) and PIF-like (PIL) proteins (Schäfer *et al.*, 1972; Kircher *et al.*, 1999; Oh *et*

al., 2004; Park *et al.*, 2004; Shen *et al.*, 2005). Several of the PIFs and PILs, such as PIF4 and PIF5, are known to be involved in SAS (de Lucas *et al.*, 2008; Lorrain *et al.*, 2008; Hornitschek *et al.*, 2009). In the shade (i.e. low R:FR ratio) PIF4 and PIF5 are stabilised, thereby promoting stem elongation (Lorrain *et al.*, 2008).

The shade-avoidance response is also gated by the circadian clock and this is most apparent at dusk (Salter *et al.*, 2003). In low R:FR there is a clear circadian gating of the shade-induced expression of *PIL1* transcript levels. The evening component TOC1 is necessary for the normal promotion of elongation by low R:FR (Salter *et al.*, 2003). PIL1 and TOC1 have been shown to interact with each other and are required for the accelerated growth associated with the shade-avoidance response (Makino *et al.*, 2002; Salter *et al.*, 2003).

1.7 Hypothesis

Monocots and dicots, as well as different plant organs, cope in a different way with their surrounding environment and this is reflected in the growth patterns. Differing organ and plant architectures conceivably contribute to the evolution of differing growth strategies. Through the course of evolution the leaves of dicots started adjusting their growth to a greater extent to endogenous regulatory systems to avoid growth at unfavourable times during the diel cycle as the growth zone is vulnerably exposed to strong fluxes in the environment. In contrast, the monocot leaf growth zone and root growth zones are less exposed to environmental fluctuations in the diel cycle and probably therefore do not require such a stringent diel control by the circadian clock.

2 Motivation & Objectives

In a diel cycle the plant experiences various changes in the environment associated with the earth's rotation. Temperature and light are two parameters that fluctuate consistently in a diel manner. The circadian clock allows the plant to anticipate these recurring cycles, giving a fitness advantage by timing critical processes to favourable periods of the day (Green *et al.*, 2002; Dodd *et al.*, 2005). As such, diel growth patterns in plants are thought to be a result of fine integration of endogenous regulatory mechanisms and the environment.

During the last decade much progress has been made in measuring and understanding how growth rates of plant organs change in a diel cycle. To date, we know much about the molecular mechanisms and components involved in the diel hypocotyl elongation rhythm. However, this knowledge cannot be directly transferred to shoot and root growth as different organs have a different loop composition in their circadian clock (James *et al.*, 2008). Moreover, in nature the root is not in the same manner exposed to diel fluctuating environmental stimuli as the shoot. Environmental impulses might have a different effect on growth and regulatory processes depending on the organ. Therefore, when looking at plant growth and its regulatory mechanisms it is important to take these organ-specific difference into account and preferably analyse both shoot and root. Therefore, the importance of the circadian clock for root and shoot growth was investigated in this study.

Of all environmental factors that have an effect on plant growth only a few show clear and reliable diel rhythmicity; thereby light and temperature are the most important ones. Light has been studied the most exhaustively. There is a bulk amount of data on the effects of light intensity, light quality and day length on growth mechanisms in several species (Poorter and Nagel, 2000; Maloof *et al.*, 2001; Nagel *et al.*, 2006; Walter *et al.*, 2007). Effects of temperature on diel growth were mainly studied in monocots and crop species were a lot of interesting work is published on the relation between temperature and temperature fluctuations on diel growth rates (Ben-Haj-Salah and Tardieu, 1995; Bonhomme, 2000; Tardieu and Parent, 2009). When referring to the effects of temperature on growth, soil temperature is often neglected. Yet, in studies that do expose shoot and root to different temperatures, a clear influence of soil temperature has been shown to affect leaf growth and metabolite partitioning (Watts, 1972; Davies & Van Volkenburgh 1983; Walter *et al.*, 2009; Poiré *et al.*, 2010). Therefore this study aimed to investigate the role of soil temperature in the growth patterns observed in roots and shoots.

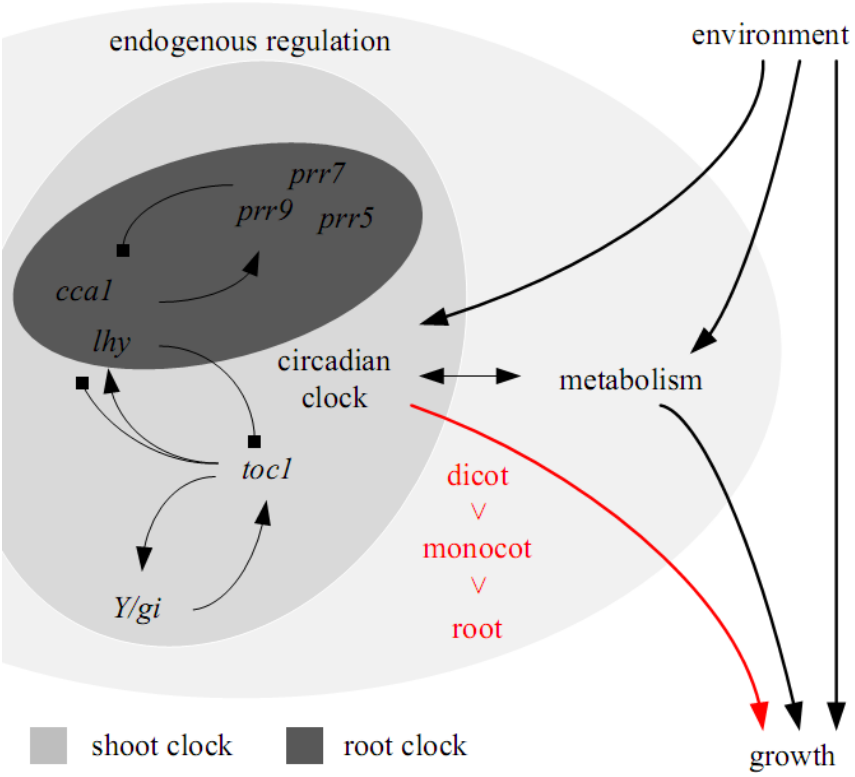


Figure 3: Schematic drawing of the endogenous regulation and environmental control on diel leaf and root growth patterns. The circadian clock comprises three feedback loops. The core oscillator consists of early morning genes, *CCA1* and *LHY*, which inhibit expression of *TOC1*, an evening gene. *TOC1* expression will lead to up regulated *CCA1* and *LHY* gene expression by the early morning. In the 'morning' loop the *PRR*s and *CCA1/LHY* form a negative feedback loop. The 'evening' loop consists of *TOC1*andan unknown component *Y* which reciprocally regulate each other. The root clock consists only of one actively oscillating morning loop. It is hypothesized that clock oscillation influences growth to a greater extent in the dicot leaf than in the monocot leaf and in roots. Adopted from (Ruts *et al.*, 2012b).

In order to investigate the role of the circadian clock in the regulation of diel leaf and root growth, I analysed shoot and root growth patterns of several arrhythmic clock mutants in *A. thaliana* and sought to explain the observed phenotypes by correlating them to the plant's carbohydrate status.

As for the soil temperature, diel growth responses were studied during cold soil temperatures and the cold-acclimation. Because many scientific studies have already been performed on root cooling in growth chambers, an attempt was made to bridge the knowledge gap between lab-based experiments and field trials. In semi-field conditions, an experimental setup was established to phenotype the acclimation response to soil cooling and several growth parameters, carbohydrate and polypropanoid metabolism were analysed in several different species.
Furthermore, diel leaf and root growth patterns of *N. tabacum* were investigated simultaneously under root cooling.

3 Results & Discussion

3.1 Circadian clock

3.1.1 Chapter I: Aberrant temporal growth pattern and morphology of root and shoot caused by a defective circadian clock in *A. thaliana*

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Designed research: T.R., S.M. & A.W; Performed research: T.R. & A.W.K.; Analysed data: T.R., S.M. & A.W.; Wrote the paper: T.R., S.M., A.W.K. & A.W.

3.1.1.1 Introduction

One of the most predictable changes in nature is the day-night cycle governed by the earth's rotation around its own axis. Correspondingly, almost all living organisms exhibit diel (24 h) changes in their behaviour and physiology to cope with and take advantage of this phenomenon. Also in plants many physiological events such as nastic leaf movements (Bunning, 1964; Hoshizaki and Hamner, 1964), flowering time (Yanovsky and Kay, 2003; Imaizumi and Kay, 2006) and hypocotyl growth (Dowson-Day and Millar, 1999; Nozue *et al.*, 2007; Nusinow *et al.*, 2011) are adapted to these day-night cycles. The circadian clock is an endogenously driven regulatory mechanism with an approximate period of 24 h that allows plants to anticipate recurring diel environmental changes, and thus confers a fitness advantage (Green *et al.*, 2002; Michael *et al.*, 2003b; Dodd *et al.*, 2005). The clock network is composed of multiple interlocking transcriptional/translational feedback loops that contribute to the robustness of the regulatory system.

Diel transcript turnover is strongly governed by the clock. The sum of transcripts that show diel rhythmicity in *Arabidopsis thaliana* is estimated to be close to 90% (Michael *et al.*, 2008) and roughly 30% of the Arabidopsis transcriptome is supposed to be under direct circadian regulation (Harmer *et al.*, 2000; Schaffer *et al.*, 2001; Covington *et al.*, 2008; Michael *et al.*, 2008). A significant proportion of these genes is involved in metabolism and hormone signalling (e.g. tricarboxylic acid cycle, carbohydrate metabolism and auxin gating (Lu *et al.*, 2005; Covington *et al.*, 2008; Fukushima *et al.*, 2009; Graf *et al.*, 2010)), suggesting a connection between the circadian clock and plant growth. Indeed, recent studies report that the hypocotyl elongates with diel rhythmicity and that this phenomenon is circadian regulated (Dowson-Day and Millar, 1999; Nozue *et al.*, 2007; Nusinow *et al.*, 2011). Hypocotyl elongation rates are repressed in the early night by the evening complex

(EC) that consists of three clock proteins: EARLY FLOWERING-4, EARLY FLOWERING-3 and LUX ARRHTYHMO (ELF4-ELF3-LUX) (Nusinow *et al.*, 2011). The EC binds to the promoter region of two positive hypocotyl growth regulators, PHYTOCHROME-INTERACTING FACTOR 4 (PIF4) and PIF5 (Nusinow *et al.*, 2011). The growth repression is removed by the end of the night, inducing a growth peak at dawn. Furthermore, by controlling starch degradation the clock ensures carbohydrate availability throughout the night (Graf *et al.*, 2010). The available carbon determines in turn the nocturnal growth potential, implying an indirect control of growth by the clock through carbohydrate metabolism.

Hypocotyl growth is highly dependent on the carbon reservoir, having little carbon and energy input from photosynthesis (Bewley and Black, 1994). It is likely that the extent of circadian regulation of growth changes during the development of plants, as sink-source relations and the dependency of growth processes on exogenous factors, such as light and water, change. Nonetheless, comparable to hypocotyl growth, leaf growth in several dicotyledonous species maintains diel growth rhythmicity in continuous light, suggesting growth regulation by the clock beyond the hypocotyl stage (Poiré *et al.*, 2010b). Interestingly, these diel growth patterns are organ-specific (Walter *et al.*, 2002; Nozue *et al.*, 2007; Wiese *et al.*, 2007; Ruts *et al.*, 2012b), which is somehow analogous to the tissue/organ-specificity of many clock components (Thain *et al.*, 2002; James *et al.*, 2008). While the relation between the circadian clock and growth is emerging for individual organs, the role of the circadian clock in regulating and coordinating growth of different organs, and thus controlling whole-plant performance, can only be understood by taking the whole plant into account (Walter *et al.*, 2009; Farré, 2012; Ruts *et al.*, 2012b).

In order to assess the impact of the circadian clock regulation on whole-plant growth performance, we investigated growth phenotypes of the two Arabidopsis lines, *CCA1ox* and *prr9-prr7-prr5* (hereafter *prr975*) having alterations in the circadian feedback loops that operate in both shoots and roots, on the short term (diel) and the long term (several days), respectively. Relative growth rate (RGR) was determined for individual leaves and rosette as well as the total root system and day-night temporal growth patterns were continuously monitored in expanding leaves and growing root tips. In addition, root growth and architecture was analysed for the primary root and lateral roots separately.

3.1.1.2 Results

Rosette and root growth

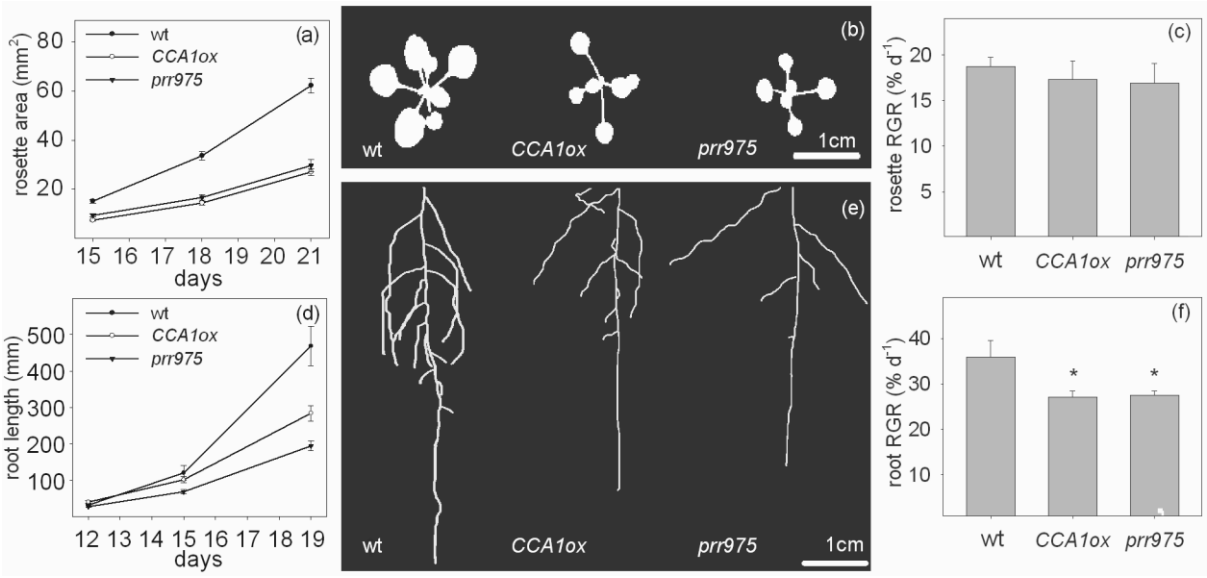


Figure 4: The rosette and root system growth of wt, *CCA1ox* and *prr975*. (a) Total projected leaf area in days after germination (d.a.g.). (b) Mask image of the total projected leaf area16 d.a.g. (c) Relative rosette growth rate per day on day 16. (d) Total length of the root system in d.a.g. (e) Image of the root system growing in Petri dishes.(f) Relative root growth rate per day 16d.a.g.. The replicate plants were n=40 for all three genotypes in (a) and (c). The replicate plants for the data in (d) and (f) were n=12 for wt, n=14 for *CCA1ox* and n=17 for *prr975*. Asterisks (*) indicate significant differences compared to the wt at $P < 0.005$. Error bars show S.E..

The rosette of *CCA1ox* and *prr975* is characterized by long petioles with small leaf lamina; the plants had only 50% soil coverage compared to the wt 16 days after germination (Fig. 4a, b). Despite the large differences in rosette area developing over time, there was no significant difference in the diel RGR of the total rosette between the wt and the mutants, reaching 16-18% per day under our experimental conditions (Fig. 4c). Yet, there was a clear tendency for rosette RGR of the clock mutants to be somewhat lower compared to the wt; during an exponential growth phase these small variations in RGR develop into a significant difference in the total rosette area over several days (Fig. 4a). For example, between day 14 and day 21 the rosette of the wt plants grew more compared to the rosette of *CCA1ox* and *prr975* (Fig. 5). Thus, the effects of short-term RGR (Fig. 4c) are cumulative and can result in significant growth differences in the long term (Fig. 4a, b).

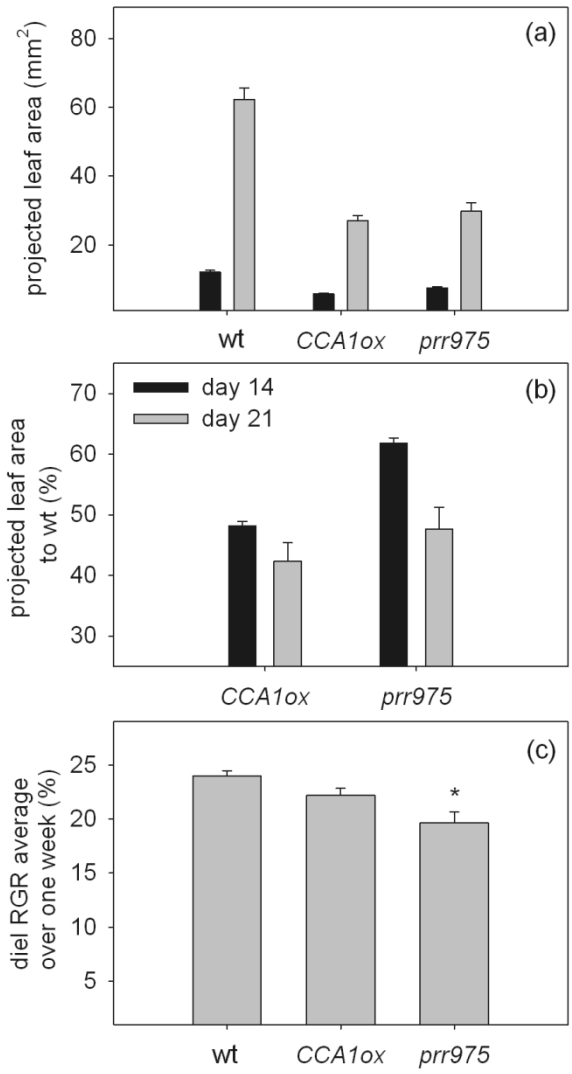


Figure 5: Decreased growth of *CCA1ox* and *prr975* compared to wt. (a) Projected leaf area at day 14 and at day 21. (b) Ratio of the projected leaf area of the mutant over the wt. n=40 for all plants; (c) Diel RGR average over a one week period, Error bars show S.E.

The root system growth was remarkably different in the wt and the two lines (Fig. 4d, e; 5). The total root length was significantly shorter for *CCA1ox* and *prr975* compared to the wt (Fig. 1d), which was accompanied by lower root growth rates (Fig. 4f).

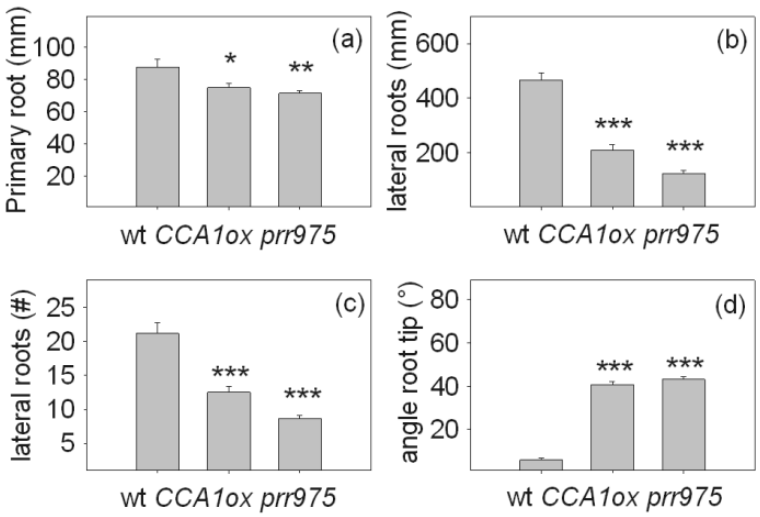


Figure 6: Root architecture of wt, *CCA1ox* and *prr975*. (a) Primary root length. (b) Total length of lateral roots. (c) Number of lateral roots. (d) Angle of the lateral root tips with respect to the gravity vector. wt (n=12), *CCA1ox* (n=14) and *prr975* (n=17). Asterisks indicate significant differences compared to the wt at $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***). Error bars show S.E..

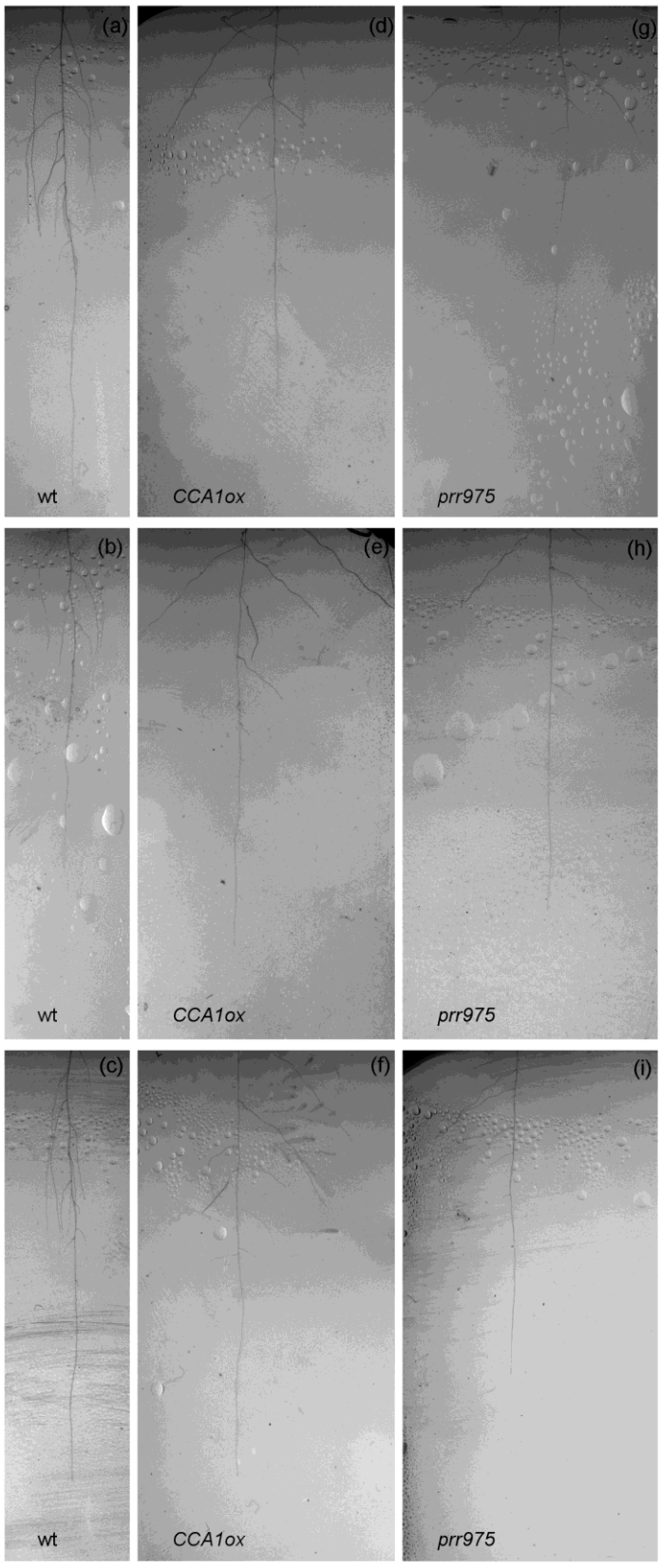


Figure 7: Raw images of the root system growing in Petri dishes under light-dark cycles 16 days after germination with a light intensity of around 100 µmol photons $m^{-2}s^{-1}$. (a)-(c), wt; (d)-(f), *CCA1ox*; (g)-(i), *prr975*.

Analysing the primary root and lateral roots separately, the growth repression in *CCA1ox* and *prr975* was found to be much more pronounced for lateral roots than the primary root (Fig. 6a, b). The 'dwarfed' lateral root development in the two lines seems to be a result of a combination of slower root growth and less lateral root formation (Fig. 6c).

Furthermore the distinct root architecture of *CCA1ox* and *prr975* included lateral root development in more horizontal directions, with the angle of lateral roots to the gravity vector being 40-45˚ in both *CCA1ox* and *prr975* compared to 7-9° in the wt (Fig. 6d). Notably, lateral roots of *CCA1ox* and *prr975* showed a wave-like pattern repeating in a diel manner during light-dark cycles, which however disappeared completely in continuous light (Fig. 8).

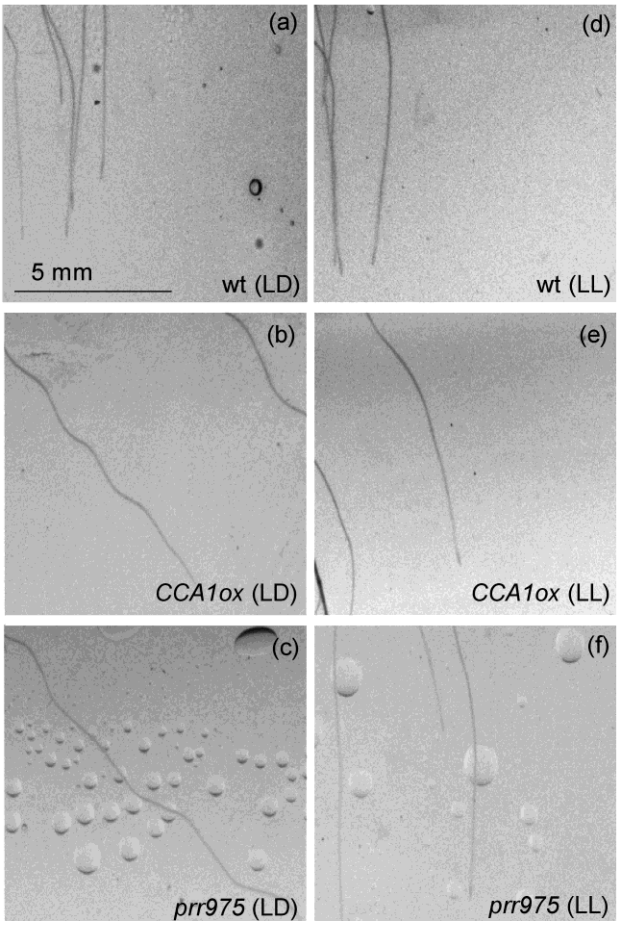


Figure 8: Images of the lateral roots growing under light-dark cycles (LD, a-c) or continuous light (LL, d-f). (a) and (d), wt; (b) and (e), *CCA1ox*; (c) and (f), *prr975*.

Diel patterns of leaf and root growth

Diel leaf growth pattern of the wt showed the maximum growth in the morning followed by a plateau in the afternoon (Fig. 9a). At night leaf RGR was reduced to reach a minimum of 1% h^{-1} towards the

end of the night. A similar phasing, but distinct from that of the wt, was observed in *CCA1ox* and *prr975*. Both lines showed strong up-regulation of leaf growth in the morning followed by a gradual increase towards the end of the day. At late night (from 03:00 a.m. onwards) leaf growth was drastically repressed in *CCA1ox* and *prr975*, suggesting nocturnal leaf growth inhibition. Interestingly, *CCA1ox* had higher leaf RGR compared to the wt throughout the entire diurnal period (Fig. 9a). The leaf growth rates of *prr975*, on the other hand, were comparable with the wt during the morning and exceeded wt RGR only in the afternoon. On average diurnal growth activity of leaves was higher in *CCA1ox* than in the wt and *prr975* (Fig. 9b). There was a marked decline in leaf growth rate from day to night in the two arrhythmic clock lines, whereas the wt maintained a similar average growth rate during the diel cycle.

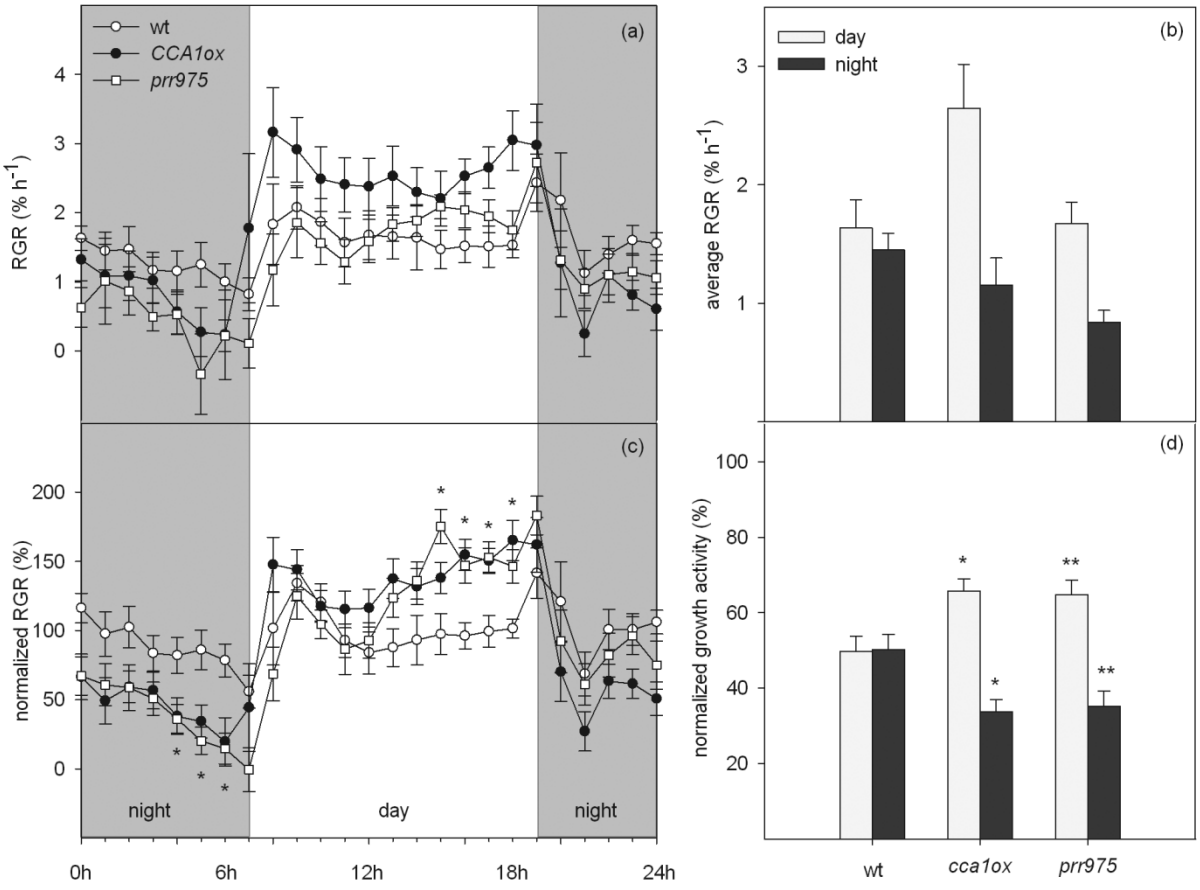


Figure 9: Diel leaf growth patterns and diurnal and nocturnal leaf growth activity. (a) Diel changes in leaf relative growth rates (% h^{-1}) of the wt (open circles), *CCA1ox* (closed circles) and *prr975* (open squares). (b) Average of the diurnal (open bars) and nocturnal (closed bars) leaf relative growth rates (% h^{-1}). (c) Leaf growth rates (%) normalized to the daily average (=100%). (d) Normalized integrated diurnal and nocturnal growth activity ($\sum_{\text{diurnal growth}} + \sum_{\text{nocturnal growth}}$=100%). The nocturnal growth activity of the wt is equal to the diurnal growth activity. In *CCA1ox* and *prr975*, the growth activity is shifted towards the day. wt (n=5), *CCA1ox* (n=7) and *prr975*(n=6). Asterisks indicate significant differences compared to the wt at $P < 0.05$ (*) or $P < 0.01$ (**). Error bars show S.E..

In order to compare the intrinsic growth patterns of different genotypes, we normalized the data to the average growth rate of the corresponding diel cycle (Fig. 9c). Strikingly uniform growth patterns were found in leaves of *CCA1ox* and *prr975*. The morning growth peak was followed by a steady increase during the afternoon, in a stark contrast to the stable RGR found in leaves of the wt. During the night leaf growth activity was reduced in all genotypes to reach the minimum at the end of the night, yet this nocturnal growth repression was much more pronounced in *CCA1ox* and *prr975*. When the day-night distribution of leaf growth activity was calculated (Fig. 9d), a nearly equal distribution was found for the wt, whereas it was shifted similarly and strongly towards the day in *CCA1ox* and *prr975*.

Root system growth was measured continuously and the average root RGR was calculated for the day and night period (Fig. 10).

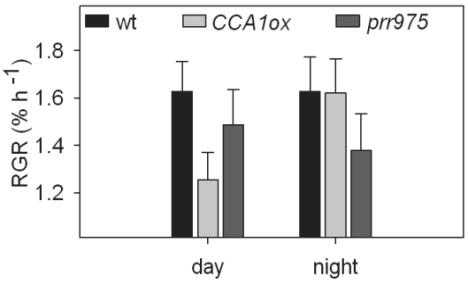
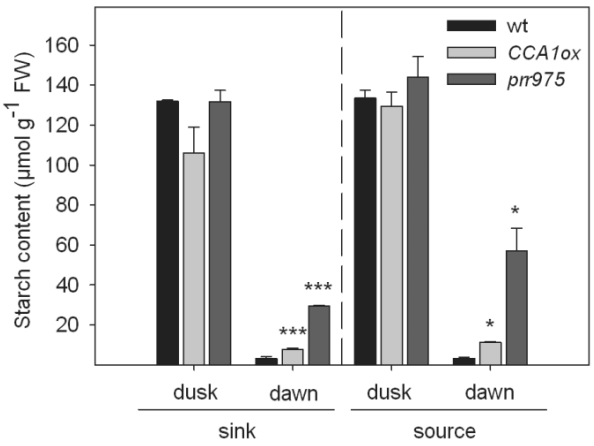


Figure 10: Average relative growth rate (% h^{-1}) of the entire root system during day and night (20 days after sowing). wt (n=24), *CCA1ox* (n=14) and *prr975* (n=13). Error bars show S.E..

Diurnal and nocturnal average growth rates were equal in wt roots. Both *CCA1ox* and *prr975* showed day-night variations in root RGR, albeit in the opposite directions. The nocturnal root growth rates of *CCA1ox* were higher than diurnal growth rates and comparable to growth rates in the wt, while root growth rates of *prr975* were slightly lower during the night than during the day. When the growth rates were averaged over a diel cycle, both *CCA1ox* and *prr975* had lower values than the wt.

Starch metabolism

Starch content was measured at the time points of the highest (dusk) and lowest (dawn) leaf growth rates in *CCA1ox* and *prr975* (Fig. 9). In both sink and source leaves *prr975*and the wt accumulated nearly equal amounts of starch at the end of the day (Fig. 11).



starch turnover (%)	wt	*CCA1ox*	*prr975*
sink	97.4 ± 1.0	91.8 ± 4.1	76.9 ± 2.7
source	97.5 ± 0.7	91.2 ± 1.6	59.0 ± 15.0

Figure 11: Starch concentration in sink and source leaves at dusk and dawn and the estimated starch turnover. For all data n=5 (± S.E., indicated by error bars for starch concentration). Asterisks indicate significant differences compared to the wt at $P < 0.05$ (*) or $P < 0.001$(***).

The source leaves of *CCA1ox* contained the same amount of starch as the source leaves of the wt and *prr975*; however, the growing sink leaves showed slightly lower starch accumulation. By the end of the night all plants had much reduced leaf starch contents, with the lowest levels found in the wt having its starch reserves nearly exhausted during the night. This is also reflected in high starch turnover of approximately 97% in both sink and source leaves of the wt (Fig. 11). Leaves of *CCA1ox* and *prr975*, regardless of sink or source, retained significantly more starch than the wt at the end of the night, leading to lower starch turnover especially in the source leaves of *prr975*. The larger starch reservoir remaining in leaves of *CCA1ox* and *prr975* at the end of the night, together with the similar (or lower for sink leaves of *CCA1ox*) leaf starch contents at the end of the day, implies that less carbohydrate is stored in these plants in the form of starch during the day, presumably due to increased carbon and energy supply needed to support the enhanced diurnal leaf growth (Fig. 9). With increasing maturity of the plants, leaves of *prr975* accumulated more and more starch (Fig. 12) while the starch levels in *CCA1ox* and the wt did not change substantially.

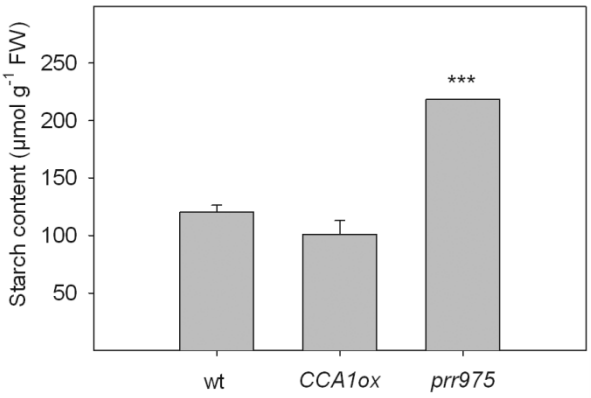


Figure 12: Starch content of source leaves (leaf 6) of 5-weeks-old plants. wt (n=6), *CCA1ox* (n=6) and *prr975* (n=6). Asterisks (***) indicate significant differences compared to the wt at $P < 0.001$. Error bars show S.E..

3.1.1.3 Discussion

The circadian clock enhances plant fitness by enabling anticipation of recurring changes in the environment (Green *et al.*, 2002; Michael *et al.*, 2003b; Dodd *et al.*, 2005). So far it has been shown that the circadian clock is an important regulator of diel hypocotyl growth activity (Nozue *et al.*, 2007; Nusinow *et al.*, 2011). Here we show by phenotyping both shoot and root growth and morphology of the two arrhythmic lines *CCA1ox* and *prr975* in the short and long term that growth of the whole plant is under clock control in the model dicotyledonous plant *A. thaliana*.

In long-term development, growth of both root system and rosette area is vastly hampered by a disrupted clock (Fig. 4). The small root system of the clock mutants is consistent with their significantly lower diel root RGR (Fig. 4f). For the rosette, the decrease of RGR was statistically not significant (Fig. 4c). However, these small differences in RGR add up in the long term to a significantly reduced rosette size of *CCA1ox* and *prr975* compared to the wt (Fig. 5), highlighting the cumulative effect of the recurring failure in diel growth regulation (Fig. 9). In addition, slower

germination of the clock mutants might also contribute to their reduced rosette area, as the circadian clock is known to influence germination (Penfield and Hall, 2009).

Clock control of diel leaf and root growth: a matter of allocation?

Diel leaf growth activity exhibited a conspicuous shift towards the diurnal period in both *CCA1ox* and *prr975* (Fig. 9). This indicates increased carbon and energy partitioning to leaf growth in the light period, which may partly compete with diurnal starch accumulation in leaves (Fig. 11). The fact that *CCA1ox* and *prr975* share further growth characteristics, e.g. rosette morphology (Fig. 4) and root architecture (Fig. 7; 8), is in correspondence with the direct *in vivo* interaction between PRR9, PRR7 and PRR5 with the promoter of *CCA1* (Nakamichi *et al.*, 2010). Knock-out of these *prr* genes results in elevated *CCA1* transcript levels (Farré *et al.*, 2005; Nakamichi *et al.*, 2005), thus phenocopying *CCA1ox*. However, only in *CCA1ox* the temporal growth shift in leaves was accompanied by higher diurnal leaf RGR compared to the wt (Fig. 9a, c). Yet, these higher values of diurnal (and also diel) leaf RGR found in *CCA1ox* did not lead to increased rosette expansion and larger rosette size in the long term. Early growth deceleration and leaf maturation during development could be a possible reason for this. Furthermore, the influence of the clock, especially on the diel growth patterns, may change as the main factor controlling growth in *A. thaliana* shifts from metabolic to hydraulic governance during leaf expansion (Pantin *et al.*, 2011).

Concomitant with the increased diurnal leaf growth, root growth of *CCA1ox* was reduced during the day (Fig. 10); as the leaf RGR declined at night, root growth recovered to the wt level. In contrast, *prr975* showed parallel growth down-regulation in leaves and roots during the dark period, together with low starch turnover (Fig. 11). The different allocation patterns found in the two lines may be attributable to variations in CCA1 concentration, more specific up-regulation of *CCA1* expression in *prr975* compared to the over-expression in *CCA1ox* using the 35S-promoter, or the lack of PRR9, PRR7 and/or PRR5 having additional functions beside the *CCA1* down-regulation (Farré *et al.*, 2005; Ito *et al.*, 2008; Wang *et al.*, 2011).

Taken together, these results provide further evidence to clock control of diel carbon allocation, and further, show clearly the distinct timing of temporal growth anomaly in leaves and roots of *A. thaliana* due to a disrupted clock.

Multiple pathways linking the clock and diel growth

The total amount of starch accumulated at the end of the day is a good proxy for the nocturnal growth capacity, as plants degrade their starch reservoir in a linear fashion over the expected night period to result in a nearly complete depletion of the starch pool at the end of the night (Smith and Stitt, 2007; Gibon *et al.*, 2009; Graf *et al.*, 2010). The importance of having a sufficient carbohydrate reservoir for

nocturnal growth is best exemplified by rapid and strong leaf growth inhibition found in the starch-free mutant *stf1* during the dark period (Wiese *et al.*, 2007). Furthermore, inappropriate rapid starch degradation due to a fast-running clock (as seen in *cca1/lhy*; Graf *et al.*, 2010) can cause starvation at the end of the night and might be responsible for the inhibited root growth (Yazdanbakhsh *et al.*, 2011), underpinning a tight connection between growth activity and carbohydrate availability.

In the present study, leaves of *CCA1ox* and *prr975* showed severe growth depression at the end of the night (Fig. 9a, b), which corresponds well to the end-of-the-night starvation observed in a mutant with a fast-running clock (*cca1/lhy*; Graf *et al.*, 2010). Contrary to previous studies, in neither of the two lines was the starch pool completely exhausted at dawn (Fig. 11), suggesting that it was not starvation that caused leaf growth inhibition in these plants. Instead, carbon and energy allocation between growth and carbohydrate reservoir and between leaf growth and root growth can explain the leaf growth inhibition in *CCA1ox* and *prr975*. Starch degradation is a pathway through which the clock can influence the allocation between nocturnal growth and carbohydrate reserves. How the clock interacts with the allocation between diurnal growth and starch accumulation, or that between leaf growth and root growth, is yet to be clarified.

Importantly, our results indicate that there is a direct nocturnal growth penalty attached to a defective clock, irrespective of whether it leads to early exhaustion of the starch pool as in the fast-running clock mutant *cca1/lhy* (Graf *et al.*, 2010) or to incomplete starch degradation as in the arrhythmic lines *CCA1ox* and *prr975* (especially in the latter). Avoiding growth depression during the nocturnal cycle thus requires proper functioning of the clock to ensure well-regulated degradation and allocation of stored carbohydrate.

Clock and root architecture

Our analysis is revealed profound differences in root architecture of *CCA1ox* and *prr975* compared to the wt. Formation of lateral roots was significantly decreased in both lines and lateral roots continued to grow in an angle of approximately 45° relative to the gravity vector, increasing the width extension of root systems (Fig. 6c, d). On top of this, the angle of the lateral root growth changed during the light-dark cycles, giving rise to the wave-like lateral root morphology (Fig. 8, 10); lateral roots grew more horizontally in the dark and less horizontally in the light. The wave pattern vanished under continuous light and lateral roots of both *CCA1ox* and *prr975* grew more or less parallel to the gravity vector as observed in the wt.

Aberrant directional growth of roots has been shown in several gravitropic mutants having altered auxin levels and/or transport (Rahman *et al.*, 2010; Mei *et al.*, 2011). Auxin is also involved in lateral root formation at several different stages (De Smet *et al.*, 2010; Jones and Ljung, 2012). In addition to auxin, formation of lateral root primordia requires periodically oscillating gene expression in *A.*

thaliana (Moreno-Risueno *et al.*, 2010). Lateral root primordia tend to be located in the positions of primary root bending that occurs at an average interval of 5-6 h at different temperatures and that persists in continuous light (Moreno-Risueno *et al.*, 2010). The periodically oscillating gene expression and the persistence in continuous light, together with our root system data, strongly suggest involvement of an endogenous clock in controlling the primary root bending and formation of lateral root primordia. The defective clock control may perturb these processes, resulting in the significantly reduced number of lateral roots in *CCA1ox* and *prr975* (Fig. 6c).

Unlike the clock-regulated formation of lateral root primordia in the primary root, the direction of lateral root growth in the arrhythmic lines is sensitive to light-dark changes. The functional clock in the wt seems to counteract the expression of light-dark waves by redirecting the lateral root growth downwards, especially during dark periods when the lateral roots of *CCA1ox* and *prr975* grow almost horizontally. The regularity in the root growth angles during the light periods and the dark periods implies that not the gravity sensing but the output of gravitropic and perhaps skototropic response may be affected in lateral roots of these plants. The role of the circadian clock in timing of growth responses to external cues has been established for hypocotyl elongation (Nozue *et al.*, 2007). The peculiar morphology of lateral roots observed in *CCA1ox* and *prr975* in the light-dark cycles illustrates the importance of the clock regulation in adjusting the growth responses to external cues (e.g. gravity, light) also for roots.

The strong root phenotypes in the clock mutants indicates that the circadian clock is a major factor contributing to root system development (i.e. root RGR, lateral root formation, lateral root angle).

Concluding remarks

The circadian clock is involved in the regulation of diel whole-plant growth activities in *A. thaliana* and its influence can be seen in both short and long term. When the circadian control is lacking, temporal coordination of leaf growth and root growth is disturbed. In both investigated lines with defective clock regulation, leaf growth activity is more confined to the daytime and starch turnover is reduced. The circadian clock also affects root architecture by timing the preparation of lateral root formation and by adjusting the direction of lateral root growth in the light-dark cycles.

3.1.1.4 Material and methods

Plant material

prr975 (prr9-1 prr7-3 prr5-1) (generated by E.M. Farré) and *CCA1ox#34* (Wang and Tobin, 1998) are in the *Arabidopsis thaliana* Col-0 background and were kindly provided by E.M. Farré (Department of Plant Biology, Michigan State University, East-Lansing).

Well-watered plants were grown for three to four weeks on soil (ED73, Einheitserde, Schopfheim, Germany) in growth chambers with 12h:12h day-night cycles at an air temperature of 23°C during the day and 18°C at night and at a constant relative humidity of 60%. The light intensity was 80 - 90 µmol photons $m^{-2}s^{-1}$.

Rosette growth analysis

Rosette growth of soil-grown Arabidopsis plants was measured from day 16 to day 20 after sowing with GROWSCREEN_FLUORO as described in Jansen *et al.* (2009).

Analysis of single leaf growth dynamics

For diel leaf growth assessment of Arabidopsis, the target leaf (leaf 7 - 9, ± 1cm in length) was mechanically fixed in a stationary position according to Wiese *et al.*(2007). Fixing the leaves with appropriate weights does not disturb growth of Arabidopsis leaves. Grey-value images were taken every 90s and were saved in a multi-Tiff format. The image sequences were analysed based on a structure-tensor approach (Schmundt *et al.*, 1998). For more details, see Schmundt *et al.* (1998) and Walter *et al.* (2002).

Carbohydrate analysis

Sink leaves were selected according to the same criteria as for the diel leaf growth assessment (leaf 9); leaf 6 of the same plant was taken as source leaf. The leaves were weighed, frozen in liquid nitrogen and stored at -80^0C until extraction. Starch concentrations were measured by a coupled enzymatic assay (Jones *et al.*, 1977) using a micro plate spectrophotometer (Synergy 2 Multi-Mode Microplate Reader, Biotek, USA) according to Walter *et al.*(2002).

Root growth analysis

Root growth was analysed in square Petri dishes (234mm x 234mm x 17mm, Nalgene Nunc International, Rochester, U.S.) filled up completely with 1% agarose (w/v) containing ⅓ Hoagland solution (Hoagland & Arnon (1941)). Seeds were placed on top of the agarose through three holes made in the Petri dish (for details see Nagel *et al.* (2009a)). The Petri dishes were placed in a climate chamber with 12h:12h day-night cycles at a constant air temperature and relative humidity of 22°C and 60%, respectively, and a light intensity of 100 µmol photons $m^{-2}s^{-1}$. The analysis of primary root length, secondary root length and number of lateral roots was conducted with the software GROWSCREEN_ROOT, as described by Mühlich *et al.*(2008). Angles of the lateral root tips were analysed with ImageJ 1.43u (Abramoff *et al.*, 2004).

Statistical analyses

Statistical significance of the differences in RGR, starch content, primary root length, lateral root length, number of lateral roots and lateral root angle was tested with a Bonferroni t-test. The Dunn's method was used to test the statistical significance of the differences in rosette area, rosette expansion rate, root system expansion and the starch content in source leaves at dawn. All data were statistically tested by comparing *CCA1ox* or *prr975* with the wt. Differences were considered significant when $P < 0.05$.

We would like to thank E. M. Farré (Department of Plant Biology, Michigan State University, East-Lansing) for kindly providing the seeds of *CCA1ox* and *prr975* as well as for critical reading and comments on an earlier version of the manuscript. T.R. acknowledges the support of his PhD thesis by the iGRAD-plant, the Heinrich-Heine University Düsseldorf and Michigan State University. This study was supported by a grant of the Deutsche Forschungsgemeinschaft (DFG) through a graduate program (IRTG 1525; iGRAD-Plant).

3.1.1.5 Supplementary information excluded from publication

Concomitant with the afternoon growth we found an increased stomatal opening in both mutants (Fig. 13, 14). It is well known that stomatal aperture exhibits diel rhythmicity (Stalfelt, 1963; Pallas *et al.*, 1974) and that these rhythms are partially controlled by the circadian clock, as demonstrated by the preserved rhythmicity in continuous light (Gorton *et al.*, 1989) and continuous darkness (Holmes and Klein, 1986). A higher stomatal conductance during the diurnal period has been reported for *CCA1ox* (Dodd *et al.*, 2005).

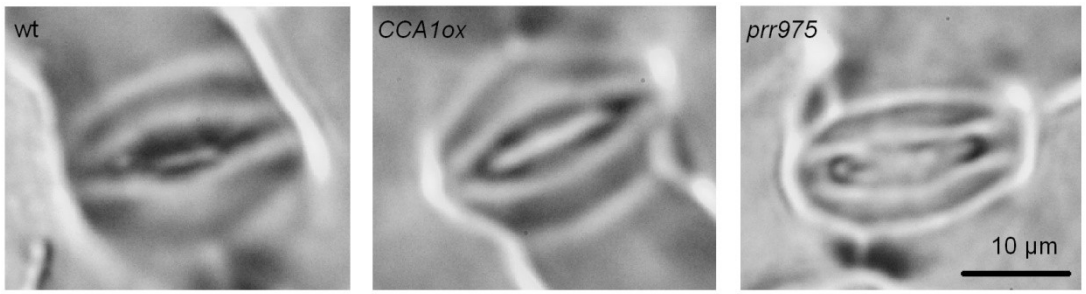


Figure 13: Stomatal opening in the wt and the circadian clock mutants in the afternoon.

Even though a higher stomatal conductance can potentially lead to increased carbon fixation, Dodd *et al.* (2005) found reduced rates of carbon fixation in *CCA1ox* plants, thus associated with a growth disadvantage in their study. In our (low-light) conditions starch levels at the end of the day were equally high in source leaves of the wt, *CCA1ox* and *prr975* while sink leaves showed slightly lower starch accumulation in *CCA1ox* compared to the wt or *prr975* (Fig. 9).

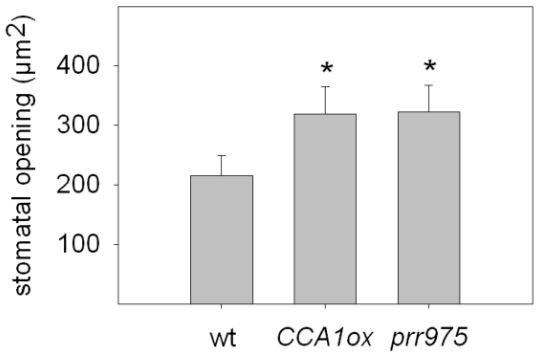


Figure 14: The area of stomatal opening. wt (n=6), *CCA1ox* (n=4) and *prr9-prr7-prr5* (n=6). Asterisks (*) indicate significant differences compared to the wt at $P < 0.05$. Error bars show S.E..

In low-light conditions light rather than CO_2 is the limiting factor for photosynthesis and growth (Perchorowicz *et al.*, 1981; Dietz and Heber, 1986). This, together with the increased afternoon growth observed in the two mutants, suggests that carbon fixation was not substantially impaired in *CCA1ox* and *prr975* plants in our experimental conditions. Furthermore, the average leaf RGR of *CCA1ox* over the entire diurnal period indicated, a growth advantage, rather than disadvantage. It might be that in our conditions the wider stomatal opening can result in a slight growth advantage; however more experiments are necessary to test this.

3.2 Environmental responses

3.2.1 Chapter II: The effect of root temperature on four greenhouse-grown species

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Designed research: T.R., H.B. & A.W; Performed research: T.R., H.B., E.A.P.T., M.S.; Analysed data: T.R., H.B. & A.W.; Wrote the paper: T.R. & A.W.

3.2.1.1 Introduction

In nature, light and temperature cycles persist with certain diel and seasonal fluctuations. Soil temperature, similar to air temperature has a sinusoidal oscillation. However, in the soil these oscillations are delayed and lowered in amplitude depending on the depth, and maximal values are reached later in the season. Soil temperature has a direct impact on root elongation, water- or nutrient uptake and root distribution (Nordin, 1977; Clarkson and Warner, 1979; Markhart *et al.*, 1979). When the plant is subjected to low soil temperature, water uptake is reduced and root hydraulic conductivity is curtailed (Markhart *et al.*, 1979; Melkonian *et al.*, 2004). This reduced conductivity is probably aquaporin-mediated (Aroca *et al.*, 2005; Murai-Hatano *et al.*, 2008). Some species can acclimate their hydraulic conductivity within hours upon exposure to low temperatures, thus partially compensating for prolonged cooling effects (Fennell and Markhart, 1998). Since the root system is a major regulator for water transport through the entire plant, a decrease in root hydraulic conductivity does not only affect the root system, but the entire plant. Indeed, over the past decades many studies reported growth underperformance of the shoot under root cooling (Watts, 1972; Reddell *et al.*, 1985; Malone, 1993; Pardossi *et al.*, 1994; Gavito *et al.*, 2001). In general, the shoot biomass is positively correlated to increased soil temperature until an optimum is reached (Al-Ani and Hay, 1983; Clarkson *et al.*, 1986; DeLucia *et al.*, 1992; Kaspar and Bland, 1992). Moreover, the temperature gradient in the soil seems to be important as well (Füllner *et al.*, 2012). Root cooling affects various processes in the shoot essential for proper growth such as photosynthesis, carbon partitioning and hormone signalling (Kudoyarova *et al.*, 1998; He *et al.*, 2009; Poiré *et al.*, 2010a).

Furthermore, exposure to low temperatures triggers the cold-acclimation signalling cascade in the plant involving the synthesis of secondary metabolites via the phenylpropanoid pathway (Christie *et al.*, 1994; Chalker-Scott, 1999). The phenylpropanoid biosynthesis is induced by a number of environmental factors including visible and UVB radiation, cold temperatures and water stress (Chalker-Scott, 1999). The subsequent production of phenylpropanoids may allow the plant to develop resistance to a number of environmental stresses. Low- and moderate low temperatures are known to induce the biosynthesis of flavonoids in several species (Christie *et al.*, 1994; Shichijo *et al.*, 1996; Terrence, 1998; Rivero *et al.*, 2001). In some cases, even cooled irrigation can lead to an up

regulation of the anthocyanin biosynthesis (Iglesias *et al.*, 2002). Under low temperature, anthocyanins and flavonols ensure that chlorophyll is not over-excited (Hannah *et al.*, 2006; Korn *et al.*, 2008). A large number of the stress-induced phenylpropanoids are derived from the C15 flavonoid skeleton, and are synthesized by chalcone synthase via the condensation of p-coumaroyl-coenzyme A (COA) and three malonyl-COA molecules. Flavonoid synthesis is not only critical for the plant's protection and acclimation to stress resistance, but it is also an important indicator for product quality at harvest (Ryder, 2010). Flavonoids are anti-oxidative substances and the consumption of fruits and vegetables rich in flavonoids has been associated with health promoting effects and lower occurrence of cardiovascular disease and certain forms of cancer (Hertog *et al.*, 1993; Crozier *et al.*, 1997; Ferreres *et al.*, 1997; Hollman, 2001; Llorach *et al.*, 2008).

Artificial growth conditions often seen in growth chamber and greenhouse setups differ in many aspects to the natural growth conditions plants have evolved to function in. In most greenhouse and growth chamber experiments plants are cultivated in relatively small, black pots, which can lead to a lot of adverse plant reactions (Passioura, 2006; Poorter *et al.*, 2012). The confinement of the root system does not only affect root expansion growth, it also indirectly affects the photosynthetic capacity (Thomas and Strain, 1991). Moreover, the root in these black pots is often subjected to uncommon temperature regimes due to radiative heating from the black pots when the lights are on (Poiré *et al.*, 2010a). Depending on the light conditions in the chamber, soil temperature can rise to values even higher then air temperature, having significant metabolic changes and effects on growth in the shoot and root as a consequence (Poiré *et al.*, 2010a).

In the present study we wanted to test the hypothesis that lower root temperature affects whole plant growth and metabolism (decreased shoot growth, increased carbohydrate concentrations and up regulation of the pigment composition) in a range of functionally differing species in the greenhouse. Therefore, we chose a typical monocotyledonous cereal crop (*Hordeum vulgare*), a dicot model crop often used for growth analysis (*Nicotiana tabacum*) and two horticultural crops cultivated partially for their anti-oxidative properties in greenhouses and in the field (*Lactuca sativa* and *Cucumis sativus*).

3.2.1.2 Material and methods

Plant material

Hordeum vulgare (cv. Barke), *Nicotiana tabacum* (cv. Samsun), *Lactuca sativa* (cv. Bughatti) and *Cucumis sativus* (cv. Dominicia) seeds were germinated and cultivated for five days in prefertilized soil (Einheitserde Typ VM Vermehrungserde, Einheitserde Werkverbande.V., Germany). After 10 days plants were transferred to pots (1L) filled with white sand and further cultivated in the temperature controlled greenhouse compartment. The treatment started after the plants had been in the root temperature controlled greenhouse for 10 days. The greenhouse experiments were done during April and early May 2010 in a closed greenhouse system in Jülich, Germany.

Experimental conditions

Figure 15: Setup of the greenhouse for root cooling.

Controlled root temperatures in the greenhouse compartment were obtained by a closed liquid (1:1 glycerol:water) circuit system (Fig. 15). A network of tubes (mats consisting of parallel tubes) was used to circulate the liquid through the greenhouse compartment, and were connected to a refrigerating/heating circulator (Julabo FP50, Julabo Labortechnik GmbH, Germany). The pots were bottomless to provide better temperature conduction between the mat and the substrate and were isolated on the outside with a white painted rubber insulator to avoid radiative heating. The substrate was kept in the pot by fine gauze. The pots were placed on top of the mats when the plants were transferred to the greenhouse compartments. Two compartments, each capable of holding 40 pots, were equipped with this root cooling system configuration. This allowed an even species distribution in the compartments to avoid placement biases (e.g. border effects) and furthermore allowed to cool roots of half of the plant population in each compartment, avoiding a treatment bias. At the start of the treatment the temperature circulator was set to 5°C for the treated plants and to 18°C for the control population, that resulted in a temperature gradient in the pot between 11-15°C (bottom to top) and 18-20°C respectively, with a diel fluctuation of 2°C. The air temperature and humidity in the greenhouse compartment was kept constant at 22°C and 60%, respectively. An automated watering system added 30 mL ⅓ Hoagland solution each hour. The average peak of light intensity each day was around 650 µmol photons $m^{-2}s^{-1}$, when the light intensity was lower than 370 µmol photons $m^{-2}s^{-1}$ between 6.00 a.m. and 10.00 p.m..

Growth analysis

Relative daily growth rates were calculated by using the equation: $RGR = [\ln(A_2) - \ln(A_1)]/[T_2-T_1]$, where A is the total leaf area and T is time. Total leaf area was acquired by ruler where leaf length, leaf width and specific leaf factor (*N. tabacum* = 0.7929 and *C. sativus* = 0.6603) were experimentally determined. At the end of the treatment plants were harvested and the fresh weight and dry weight of shoot and root were determined. Specific leaf weight (SLW) was determined by weighing a leaf disc (diameter: 4mm).

Carbohydrate analysis

Tissue sections of the third leaf from the top were taken from four individual plants every three hours for a period of 24 hours at day 5. Two discs per leaf (one tip and one basal disc, diameter: 4mm) were punched out, weighted, frozen in liquid nitrogen and stored at -80°C for further analysis. Soluble carbohydrates were extracted from the frozen leaf discs (Arnon, 1949) and glucose, fructose, sucrose and starch concentrations were measured in a coupled enzyme assay (Jones *et al.*, 1977) using a micro plate spectrophotometer (Synergy 2, BioTek Instruments, U.S.) according to Walter *et al.* (2002).

Secondary metabolite analysis

At the end of the experiment samples of the measured leaf were probed and analysed for their chlorophyll, flavonol and anthocyanin content in a biochemical assay to verify the results of the non-destructive optical measurements. Pigments and flavonoids were extracted with Ethanol 95%. Chlorophyll content was calculated according to Lichtenthaler and Buschmann (2001), whereas total flavonoid content was determined according to the Aluminum Chloride colorimetric method using quercetin as a calibration standard (Lin and Tang, 2007). Anthocyanins were extracted with acidified methanol according to Rodriguez-Saona and Wrolstad (2001) and quantified using the pH differential method as described by Giustiand Wrolstad (2001), using cyanidin-3-glucoside as reference. Samples were read in an Enspire 2300 Microplate reader (PerkinElmer Inc., US).

Secondary metabolites were measured in a non-destructive way at day 6 of the treatment with the Multiplex® 3 device (FORCE-A, France, (Cerovic *et al.*, 2002; Ghozlen *et al.*, 2010)). Chlorophyll content was estimated by the simple fluorescence ratio after red excitation, which is the quotient of the far-red fluorescence (740nm) over the red fluorescence (690nm, far-red fluorescence ^{redexc}/red fluorescence ^{redexc}). Compounds absorbing at 375nm, which are mainly flavonols, were approximately quantified by calculation of the logarithm of far red fluorescence after red excitation (625 nm) over far red fluorescence after UV excitation [375nm, log(far-red fluorescence^{redexc}/far-red fluorescence^{UVexc})]. Anthocyanin content was estimated by the logarithm of the quotient of far red fluorescence after red excitation and far red fluorescence after green excitation [516nm, log(far-red

fluorescence^{redexc}/far-red fluorescence^{greenexc})]. This method mainly assesses anthocyanins and flavonols accumulated in the epidermal layer or bound to the cuticle and upper cell wall (Grammatikopoulos *et al.*, 1999).

Climate chamber conditions

N. tabacum was grown in growth chambers with 12h:12h day-night cycles with an air temperature of 23°C during the day and 18°C at night and a constant relative humidity of 60%. The light intensity was 80 - 90 µmol photons m^{-2} s^{-1}.

Statistical analysis

Data were tested by one-way ANOVA using SPSS software (17.0, SPSS Inc., Chicago, USA).

3.2.1.3 Results

Growth of four root cooled species in a greenhouse

The effect of root cooling on growth was assessed in multiple ways. A global overview of the species growth was generated by harvesting shoot and root material. Secondly, we focused on the effects of root cooling on new leaf growth by examining the specific leaf weight and relative leaf growth rates. No significant differences in fresh or dry biomass in either shoot or root were found after eight days of treatment (Table 3). However, the shoot:root ratio of all cultivars was slightly decreased upon root cooling (Fig. 16a).

Species	Treatment	Shoot (g FW)	Shoot (g DW)	Root (g FW)	Root (g DW)
H. vulgare	control	16,90 ± 1,73	1,75 ± 0,13	12,71 ± 0,12	0,72 ± 0,04
	cooling	14,37 ± 1,09	1,30 ± 0,14	13,03 ± 0,85	0,84 ± 0,14
N. tabacum	control	60,80 ± 11,46	4,19 ± 0,76	15,06 ± 2,63	1,24 ± 0,35
	cooling	68,53 ± 6,08	4,48 ± 0,25	19,05 ± 3,04	1,13 ± 0,04
L. sativa	control	42,83 ± 7,67	1,90 ± 0,40	7,87 ± 1,33	0,45 ± 0,10
	cooling	38,58 ± 4,10	2,39 ± 0,08	6,74 ± 0,40	0,2775 ± 0,02
C. sativus	control	94,55 ± 15, 18	8,01 ± 1,58	52,33 ± 6,26	3,44 ± 0,59
	cooling	84,03 ± 11,93	7,34 ± 0,88	52,50 ± 1,64	3,53 ± 0,20

Table 3: Fresh and dry weight of shoot and root.

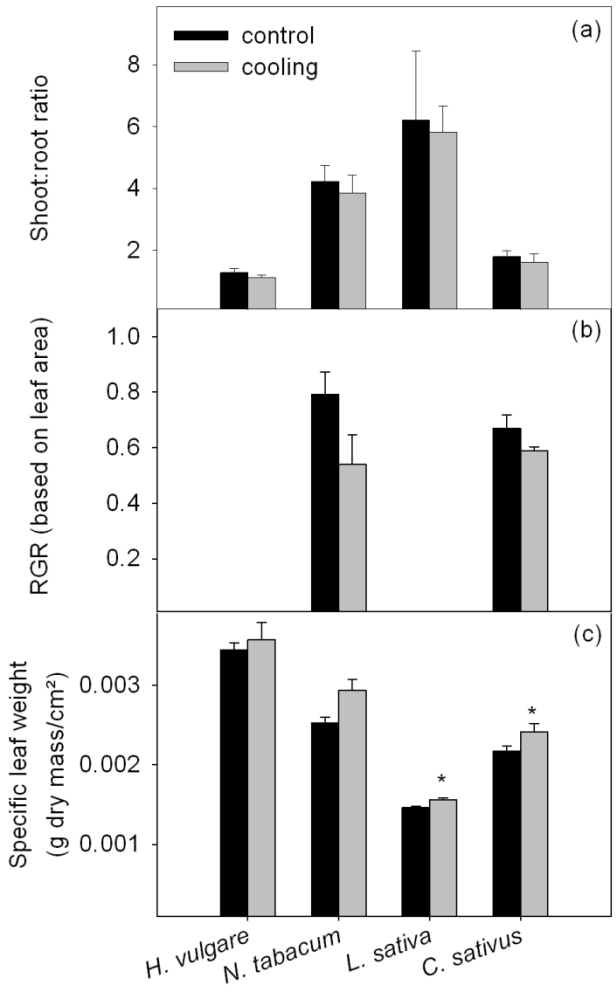


Figure 16: Growth of the four species in control and root cooling conditions. (a) shoot:root ratio. (b) Specific leaf weight. (c) Relative growth rate per day based on leaf area. n=4-6 for shoot:root ratio, n=10-12 for RGR and specific leaf weight; error bars are S.E.. Statistical significance is indicated by an asterisk.

Leaves subjected to chilling are known to increase their leaf thickness as protection. In order to quantify the increase in leaf thickness we measured the specific leaf weight in new growing tissue. The specific leaf weight was significantly induced by root cooling in *L. sativa* and *C. sativus*, and a similar trend of induction was seen in *H. vulgare* and *N. tabacum* (Fig. 16b). Relative growth rate per day (RGR d^{-1}) were measured for *N. tabacum* and *C. sativus* six days after the treatment started. Both species showed a slight decrease in their diel growth rates (Fig. 16b).

Carbohydrate metabolism

The four carbohydrates glucose, fructose, sucrose and starch were measured over the diel cycle. However, for none of the four carbohydrates there was a specific change in content due to the treatment over the diel cycle. Therefore, we report the carbohydrate data for one time point (12.00 a.m., Fig. 17). Of all the carbohydrates, glucose content was most variable between the species (Fig. 17a). Despite the strong species variability, no treatment dependent differences could be found. The fructose content showed less variability. However in *L. sativa*, fructose levels were two times higher compared to the other species (Fig. 17b). For fructose there was no statistically significant difference between the treatments. In *H. vulgare*, sucrose levels were approximately three times higher compared to the other investigated species (Fig. 17c). But also for the sucrose content no treatment-specific difference could be found. Of all species *C. sativus* had the lowest amount of soluble carbohydrates, but showed to have the highest starch content of the four species (Fig. 17d). *N. tabacum* had intermediate levels and *H. vulgare* and *L. sativa* showed low starch levels. Starch levels did not change significantly depending on the treatment. Although there are some remarkable

interspecies differences in glucose, fructose, sucrose and starch levels; neither of the carbohydrates showed significant increase or decrease dependent on the treatment (Fig. 17). Similar to the carbohydrate data at 12.00 a.m. we found no significant treatment differences at the seven other sampling time points.

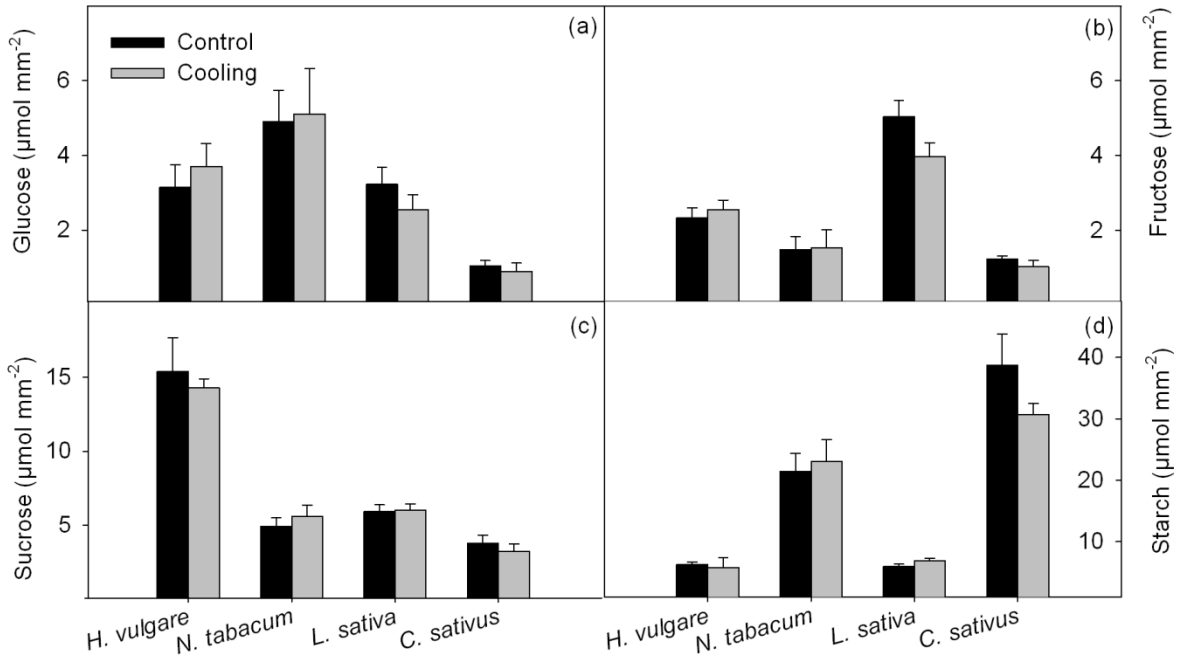
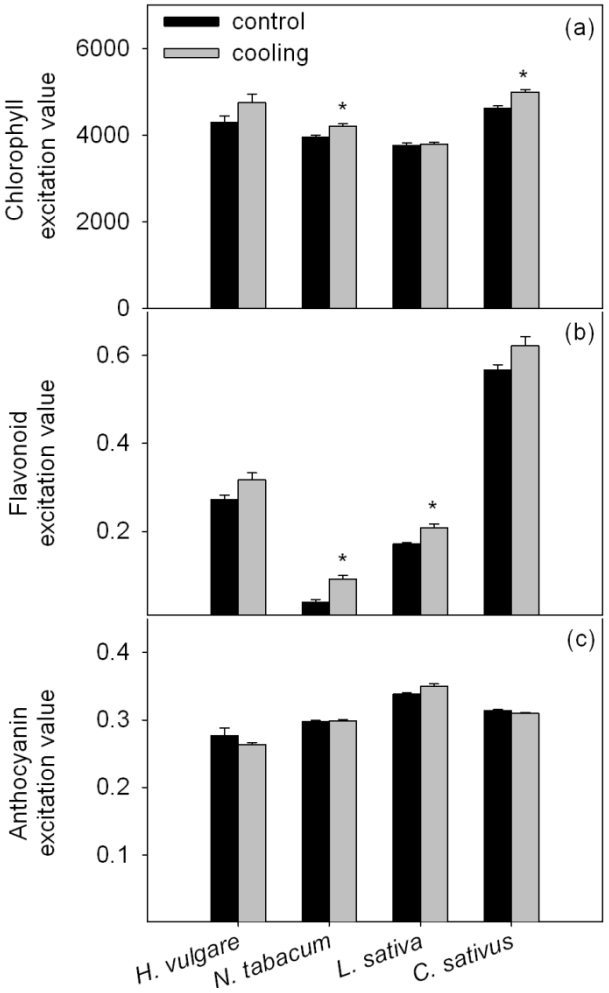


Figure 17: Carbohydrate levels for the four species in control and root cooling conditions. (a) Glucose levels at noon (b) Fructose levels at noon. (c) Sucrose levels at noon.(d)Starch levels of barley, tobacco, lettuce and cucumber at noon. n=4, error bars represent S.E..

Secondary metabolism – flavonols, anthocyanins and chlorophyll

Polyphenols such as flavonol and anthocyanin content were measured together with the chlorophyll content in a non-destructive way to phenotype the acclimation response to root cooling. Chlorophyll fluorescence was measured at day 4, 5 and 8 after the start of the treatment. In contrary to our hypothesis (i.e. a strong acclamatory response to root cooling) we found no fast initial response to the root cooling treatment. Only at the end of the treatment we found significant up regulation of the chlorophyll content in *N. tabacum* and *C. sativus*, and a similar tendency in *H. vulgare* and *L. sativa* (Fig. 18a). A similar increase upon root cooling was seen in the estimate of the flavonol content in all species, and significant results were found for *N. tabacum* and *L. sativa* (Fig. 18b). However, root cooling had no effect on the anthocyanin levels measured with the optical device (Fig. 18c). With a destructive biochemical assay we wanted to verify the results of the non-destructive optical measurements. The chlorophyll content did not differ significantly between the two treatments for all species. For *H. vulgare* we found no significant changes in the chlorophyll content with 2355 µg g^{-1} ± 159 µg g^{-1} DW and 2487 µg g^{-1} ± 180 µg g^{-1} DW for control and cooling respectively (Fig. 19a). Also



for the flavonoid content no significant difference was found in *H. vulgare*, the flavonoid content was around 2345 µg g^{-1} ± 342 µg g^{-1} DW and 2294 µg g^{-1} ± 413 µg g^{-1} DW for control and cooling respectively (Fig. 19b). Similar tendencies were made for all other species (Fig. 19a, b). Interestingly, in *L. sativa* the chlorophyll and flavonoid content found was three times higher than in the other species.

Figure 18: Chlorophyll, flavonol and anthocyanin excitation values of the four species in control and root cooling conditions in measured by the multiplex device. (a) Chlorophyll content. (b) Flavonol content. (c) Anthocyanin content. n=12 for *H. vulgare* and *N. tabacum*, n=8 for *L. sativa*, *C. sativus*; Error bars are S.E.. Statistical significance is indicated by an asterisk.

Neither for chlorophyll or flavonoid content there were significant differences between the control and the cooling treatment group (Fig. 19a, b).

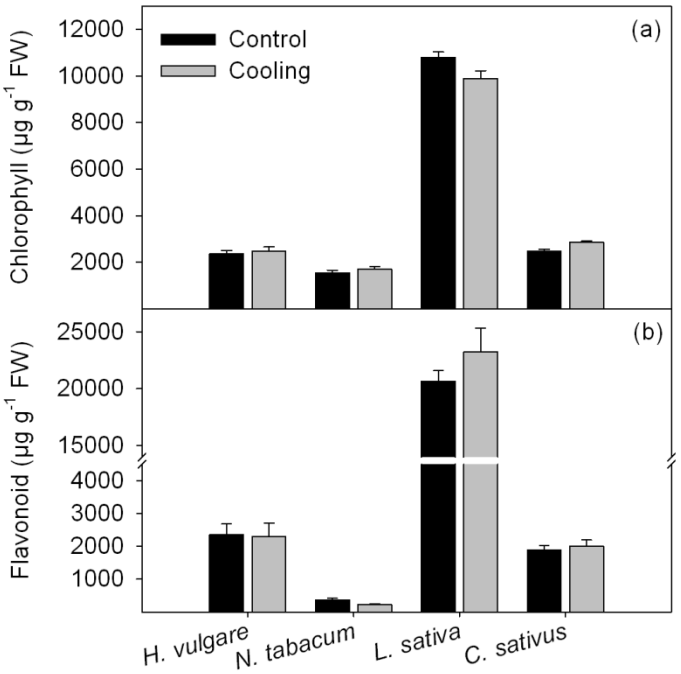


Figure 19: Chlorophyll and flavonoid content of the 4 species for control and root cooling conditions biochemically measured. (a) Chlorophyll content. (b) Flavonoid content. n=10, Error bars are S.E..

Growth of root cooled *N. tabacum* in a climate chamber

In comparison to the results of an earlier study from our group on *R. communis* (Poiré *et al.*, 2010a), the effects of the root cooling observed in the greenhouse are marginal for the four species studied. We hypothesized that either the effects of root cooling are species specific or that the growth conditions (e.g. the difference between greenhouse grown plants and climate chamber plants) are a dominant factor. Therefore we grew *N. tabacum* in a growth chamber and analysed the carbohydrate status of root cooled plants and control plant. Similarly to observations in *R. communis*, the carbohydrates (glucose, fructose and sucrose) of *N. tabacum* leaves increased significantly upon root cooling (Fig. 20).

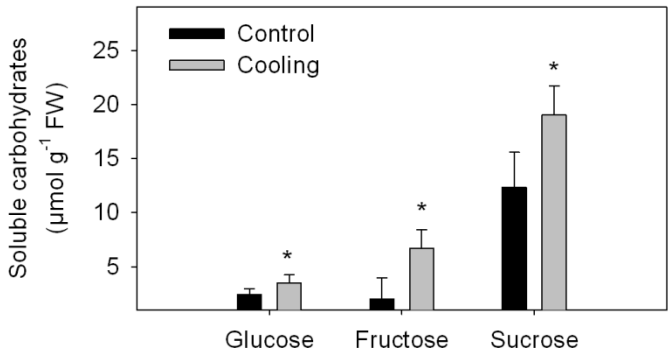


Figure 20: Glucose, fructose and Sucrose content of *N. tabacum* under root cooling in climate chamber conditions. (Light intensity 100 µmol photons $m^{-2}s^{-1}$) n=3, Error bars are S.E..

3.2.1.4 Discussion

Roots are often subjected to temperature cycles in growth chamber and greenhouse experiments with potted plants, which can differ strongly from temperatures experienced in nature. Several studies show profound effects of root cooling when the plants are growing in climate chambers. Growth, photosynthesis, carbon partitioning, hormone content are all affected by root cooling (Malone, 1993; He *et al.*, 2009; Poiré *et al.*, 2010a). Additionally in lowered temperature conditions, cold acclimation and stress responses are exhibited in the up regulation of the flavonoid pathway (Christie *et al.*, 1994; Catalá *et al.*, 2011). Here, we examined the growth response and change in metabolism (carbohydrate and pigment composition) upon root cooling in the greenhouse for a range of functionally differing species. Contrary to our hypothesis, a decrease in root temperature for plants grown in the greenhouse did not affect growth, carbohydrate metabolism and secondary metabolism in a profound manner.

In response to the root cooling a very mild response of growth to cold acclimation was observed. Shoot and root mass did not decrease significantly, although a slight tendency to decreased biomass accumulation was observed (Table 3). In general both shoot and root biomasses were marginally affected by the change in root temperature. This is partially due to the initial size of shoot and root mass at the onset of the experiment and to the experimental run time (5 days). Interestingly, in all four species a trend towards a lower shoot:root ratio is observed, indicating that the root cooling had a

more profound effect on shoot growth than on root growth (Fig. 16a). In young leaves the daily growth rates dropped and in all species a significant increase in specific leaf weight was observed (Fig. 16b, c). This is a typical response in cold acclimation whereby the cell wall is thickened as a form of cold protection, leading to thicker leaf and higher specific leaf weights (Huner *et al.*, 1981; Griffith *et al.*, 1985; Stefanowska *et al.*, 1999).

In climate chamber experiments with *R. communis* it was shown that upon root cooling there is an accumulation of carbohydrates such as glucose, fructose, sucrose and starch in roots and leaves (Poiré *et al.*, 2010a). In our greenhouse experiments no changes in carbohydrate accumulation were found in the leaves (Fig. 16). This could be either due to species specific reactions or due to effects of environmental factors that differed between growth chamber and greenhouse cultivation. When *N. tabacum* was subjected to root cooling whilst growing in a growth chamber, there was similar response as observed in *R. communis* (Fig. 20; Poiré *et al.*, 2010). The response is therefore unlikely to be species specific. One of the biggest differences between a greenhouse setup compared to a climate chamber setup is the light regime. In most cases, the light intensity experienced by the plants in climate chambers is much lower than it is in the greenhouse, where the continuous, gradual transition between no light at nighttime and peak daytime light intensity as well as the light spectrum might play a vital role. We hypothesize that the adverse effects of root cooling in climate chambers are partially compensated in the greenhouse by the more natural light climate.

Concomitant with the carbohydrate results there was no clear increase found in secondary metabolite levels, when invasive and non-invasive quantification methods were considered. Some significant changes were found by using the non-invasive method (e.g. the approximate chlorophyll content in *N. tabacum* and *C. sativus* as well as the approximate flavonoid levels in *N. tabacum* and *L. sativa*; Fig. 18). However, when the total flavonoid content was measured using invasive methods, very small but insignificant differences in flavonoid metabolism could be observed (Fig. 19). The different results between the invasive and non-invasive analyses might be attributed to the nature of the measurement. The non-invasive measurement for flavonols is based on the optical absorbance of 375nm which the vast majority of flavonols absorb, but not all; while the extraction quantifies all flavonoids. Furthermore, the non-invasive method is limited to the secondary metabolites located in the epidermis, cuticle or upper cell wall; whereas with the extraction all cell layers are pooled and analysed. Nonetheless, the treatment effect is also for secondary metabolites not-existent or very small, even with some significant difference found in the non-invasive method. The reason for this might be similar to the status quo found in the carbohydrate content under the different conditions, where the increased light intensity of climate chamber experiments versus greenhouse experiments might compensate the effects of root cooling in the greenhouse.

On the basis of these results we conclude that the natural transition of light intensity and quality in the diel cycle (the major fluctuating variable between climate chamber and greenhouse) might be an important factor to provide a balanced growth and metabolism. Where a difference by 6°C in root temperature was able to significantly changes growth and the metabolism balance in climate chambers (Fig. 20; Poiré *et al.*, 2010), it did not affect the balance in a pronounced way in greenhouse experiments (Fig. 17, 18, 19). Besides the differing light conditions, the diel oscillation in air (and soil) temperature together with absolute temperature differences (i.e. surpassing threshold values) between the two conditions might play an additional role in this shifted growth balance.

In contrast to growth chamber experiments light and temperature are not stable over the diel cycle in the greenhouse. The natural oscillation in light and temperature, together with the absolute differences temperature and light between greenhouse and climate chamber experiments might explain the weak response to root temperature in our experiments. The use of root cooling as an enhancer of potential health-promoting secondary metabolites in plants is only likely under constant and fairly low light conditions.

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3.2.2 Chapter III: Synchronic high temporal phenotyping of leaf and root growth in *N. tabacum*

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Designed research: T.R. & A.W; Performed research: T.R.; Analysed data: T.R .& A.W.; Wrote the paper: T.R. & A.W.

3.2.2.1 Introduction

Since time-lapse imaging based methods became available for measuring plant growth about a decade ago, several studies have shown that the diel growth rhythms in plants depend on the environment as well as on their intrinsic regulatory mechanisms such as the circadian clock (Schmundt *et al.*, 1998; Walter *et al.*, 2002; Walter *et al.*, 2009; Poiré *et al.*, 2010a; Poiré *et al.*, 2010b; Ruts *et al.*, 2012a). These diel growth patterns vary from species to species and are different in root and leaf (Walter *et al.*, 2002; Ainsworth *et al.*, 2005; Matsubara *et al.*, 2006; Wiese *et al.*, 2007).

Root growth, similar to monocot leaf expansion, is highly responsive to temporal changes in environmental conditions and the growth rate is adjusted consequently (Walter *et al.*, 2002; Poiré *et al.*, 2010b). For example, the root elongation rates (RER) of *Zea mays* and *Nicotiana tabacum* quickly adjust to new temperature regimes or to aboveground changes in the light regime within a few minutes (Walter *et al.*, 2002; Nagel *et al.*, 2006). The RER is sensitive to various environmental parameters including light (Nagel *et al.*, 2006), temperature (Walter *et al.*, 2002), nutrient availability (Walter *et al.*, 2003), soil water potential (Sharp *et al.*, 1988) and mechanical impedance of the soil (Bengough *et al.*, 2006). However, when environmental conditions are kept constant, no change in diel root growth can be found in a number of species such as *Arabidopsis thaliana* (Chavarría-Krauser *et al.*, 2008), *Oryza sativa* (Iijima *et al.*, 1998), *Sorghum bicolor* (Iijima *et al.*, 1998), *Z. mays* (Walter *et al.*, 2002; Walter *et al.*, 2003) and *N. tabacum* (Walter and Schurr, 2005; Nagel *et al.*, 2006). These results are consistent with the strong effects of environment on root growth which have been demonstrated under changing conditions as summarized above. Interestingly, marked diel oscillations of root tip growth have been reported recently in *A. thaliana* under constant conditions (Yazdanbakhsh and Fisahn, 2010); a growth maximum was recorded one hour after dawn followed by a steep decrease to reach a minimum at dusk and recuperation during the night. In 2011, Iijima and Matsushita provided evidence for diel root rhythmicity in *O. sativa*; thereby contesting their results from 1998 (Iijima *et al.*, 1998; 2011). The reason for the existence of various rhythms is not known. The developmental stage and the environment may be associated with the variation in the rhythms. For example, an important difference between the experimental conditions of Yazdanbakhsh and Fisahn (2010) and those of Walter *et al.* (2002, 2005) or Iijima *et al.* (1998, 2011) is the exposure of the entire root system, including the growing root tips, to light-dark cycles in the study by the former. As light is known to inhibit root growth (Pilet and Ney, 1978), the observed oscillation patterns in the

Arabidopsis root growth may be influenced by the inhibitory effect of root illumination (Schmidt and Walter, 2009). Furthermore, complete enclosure of seedlings in a Petri dish - a widely used condition for root growth analysis - can affect growth processes through ethylene emission by leaves (Eliasson and Bollmark, 1988; Hummel *et al.*, 2009). Although there is a considerable amount of data on diel root elongation rates, there is also a number of contradictory observations that can be partially attributed to differences between the species, developmental stages, environmental and growth conditions. An extended time-course analysis on several species may provide further information regarding the root elongation rhythms.

Contrary to root elongation and monocot leaf elongation, the rates of leaf expansion in dicot plants do not follow temperature and other environmental variations in the same manner (Walter and Schurr, 2005). The observed diel leaf growth patterns in dicots can be categorized in two main types: Type 1 and Type 2 (Walter *et al.*, 2009). External environmental parameters, such as temperature and light intensity, can influence the amplitude, but not the basic character of the observed diel pattern (Poiré *et al.*, 2010b). Type 1 plants, such as *N. tabacum*, show a sinusoidal growth pattern with maximal growth rates around dawn and directly after the onset of daytime illumination (Walter and Schurr, 2005; Wiese *et al.*, 2007). The origin of the different diel growth strategies of dicot plants is yet to be elucidated. Nevertheless, a study comparing the behaviour of several dicot and monocot species following a transfer from day-night conditions to continuous light conditions has indicated that the circadian clock is an important regulator of the observed, repetitive diel growth patterns in leaves of dicot species (Poiré *et al.*, 2010b; Ruts *et al.*, 2012a). In continuous light the growth rhythm, although dampened, is maintained (Poiré *et al.*, 2010b). Furthermore, leaf growth in circadian clock mutants is shown to be disturbed (Ruts *et al.*, 2012a).

Until now it was impossible to study the growth of different organs of the same plant at the same time and with high temporal resolution. Here we present a new technique that enables high-resolution temporal growth phenotyping in leaf and root synchronously. This technique combines the existing methods, leaf-DISP and root-DISP that allow capturing changes in leaf expansion and rooting elongation, to observe growth activities of the whole seedling on a diel scale. This allows us to gain a better insight into how growth rates change in leaves and roots over time and how their growth rates are balanced within a plant. Furthermore, it would be possible to quantify the growth response to experimental treatments applied to leaves or roots, both in the treated organ and the non-treated organ.

Here we subjected the root system of tobacco seedlings to three different conditions: A control condition were root and shoot were subjected to 22°C air temperature, a root cooling condition in which the root was cooled to (10°C throughout the diel cycle while the shoot was subjected to 22°C air temperature, and a third condition were in the root was subjected to the same changes in the light-dark cycles as the shoot. We asked how and to what extent changes in the environmental conditions

would impact the growth rates of the treated organ, in this case the root, and the non-treated organ, the leaf.

3.2.2.2 Method: cultivation and hardware setup

Seeds of *N. tabacum* (cv. Samsun) were surface sterilized in a sodium chloride solution and sown on sterile agar (1% $w.w^{-1}$). The medium contained a ⅓ modified Hoagland solution (Stock solution: 1M KNO_3, 1M $Ca(NO_3)_2$, 1M $MgSO_4$, 1M $KHPO_4$, trace elements; Hoagland & Arnon (1941)). Petri dishes (234mm x 234mm x 17mm, Nalgene Nunc International, Rochester, U.S.) were completely filled with the medium and sealed with fabric tape (Micropore, 3M Health Care, Neuss, Germany). Seeds were placed on top of the agarose through holes made in the Petri dish (three holes per Petri dish, one seed per hole). Laboratory film (Parafilm, Pechiney Plastic Packaging, Chicago, U.S.) was applied to cover the holes in order to keep the seeds moist. After germination the laboratory film was removed. For more details concerning this cultivation method, see Nagel *et al.*(2009b). The Petri dishes were placed in a climate chamber with 12 h-12h day-night cycles at a constant air temperature and relative humidity of 22°C and 60%, respectively, and a light intensity of 100 µmol photons $m^{-2}s^{-1}$.

A custom metal framework was constructed that included a holder for a Petri dish and an X-, Y-, Z-axis moving stage with a CCD camera (Scorpion SCOR-20SO, Point Grey Research, Vancouver, Canada) attached, allowing fine movements for tracking the growing root. The camera was equipped with a standard objective lens (25mm, Pentax, Tokyo, Japan) and an infrared filter (880nm, Edmund Optics, Karlsruhe, Germany). Constant illumination throughout day and night was provided by 800 nm infra-red LED bars (CSS, Kyoto, Japan).The Petri dishes were tightly fastened in the holder by several screws, locking them in the focal plane of the camera. A mirror foil is placed at the back of the plate for optimal contrast (Optimont – Spiegelfolie S1SK, Bleher, Ditzingen, Germany). To prevent incident light to reach the root a box was constructed around the Petri dish holder. Temperature regulation of the Petri dish was provided by attaching a copper plate connected to a Refrigerated/Heating Circulator (F25-ED, Julabo GmbH, Seelbach, Germany) to the Petri dish holder.

For the diel leaf growth assessment in *N. tabacum* (2-weeks-old seedlings), the target leaf (leaf 1 - 2, ± 1cm in length) was mechanically fixed in a stationary position according to Wiese *et al.* (2007). Images of the leaves were acquired with a CCD camera (Scorpion SCOR-20SO, Point Grey Research, Vancouver, Canada) positioned above the plant with a standard objective lens (25mm, Cosmicar/Pentax, Pentax, Tokyo, Japan) and an infrared filter (880nm, Edmund Optics, Karlsruhe, Germany). Constant illumination throughout day and night was provided by a cluster of six infrared diodes (880nm, Conrad Electronics, Hirschau, Germany).

Grey-value images were taken every 90s (for leaves) and every 30s (for roots) and saved in a multi-Tiff format. The image sequences were analysed based on a structure-tensor approach (optical flow via the brightness constancy constraint equation(BCCE)) (Schmundt *et al.*, 1998) that calculates the velocities from all moving visible structures at the leaf surface within the image sequence. Relative growth rates (RGR) were calculated as the divergence of the estimated velocity field by selecting an area of interest (AOI) within the image and tracking the structure within this AOI over time. For more details, see Schmundt *et al.*(1998), Walter *et al.* (2002), Matsubara *et al.* (2006).

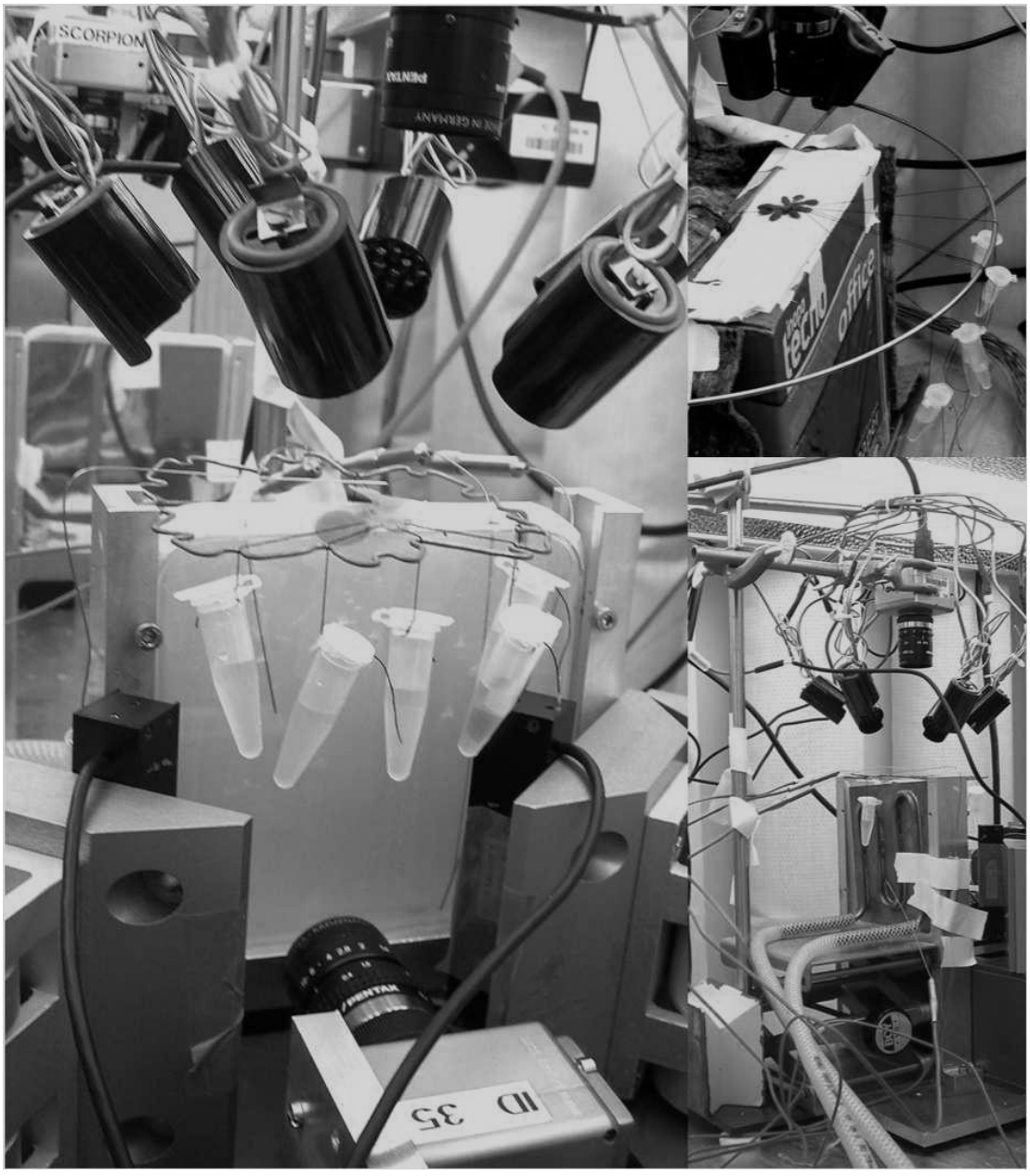

Figure 21: Dual-DISP setup. Left panel –synchronous monitoring of a tobacco leaf and root. Upper right panel – *Arabidopsis thaliana* in the dual DISP setup, a box protects the root system from incident light. Lower right panel – temperature regulation of the Petri dish with thermocouples for monitoring the temperature.

3.2.2.3 Results

In the seedlings with covered roots but no root zone cooling, root growth was mostly stable throughout the diel period (Fig. 22b). A small decrease in growth rates could be noticed at the start of the diurnal period. Leaf growth of these seedlings was more confined to the nocturnal period; during the nocturnal period leaf growth rates were stable and were approximately three times higher than during the diurnal period (Fig. 22a). Leaf growth at dawn was marked by a steep decrease in growth rates, after which the growth rates rapidly increased to the average nocturnal level (ca. 3% h^{-1}) followed by a slow decrease over the next two hours to reach a steady state (1 - 1.5% h^{-1}) for the remaining diurnal period.

When the roots of the *N. tabacum* seedlings were subjected to 10°C, root growth rates were found to be reduced by approximately 50% compared to the root growth rates with no cooling (± 22°C) (Fig. 22c). At these low rates root growth remained stable over the entire diel period. Leaf growth in these seedlings was not affected substantially at any point over the diel period compared to the wt (Fig. 22d).

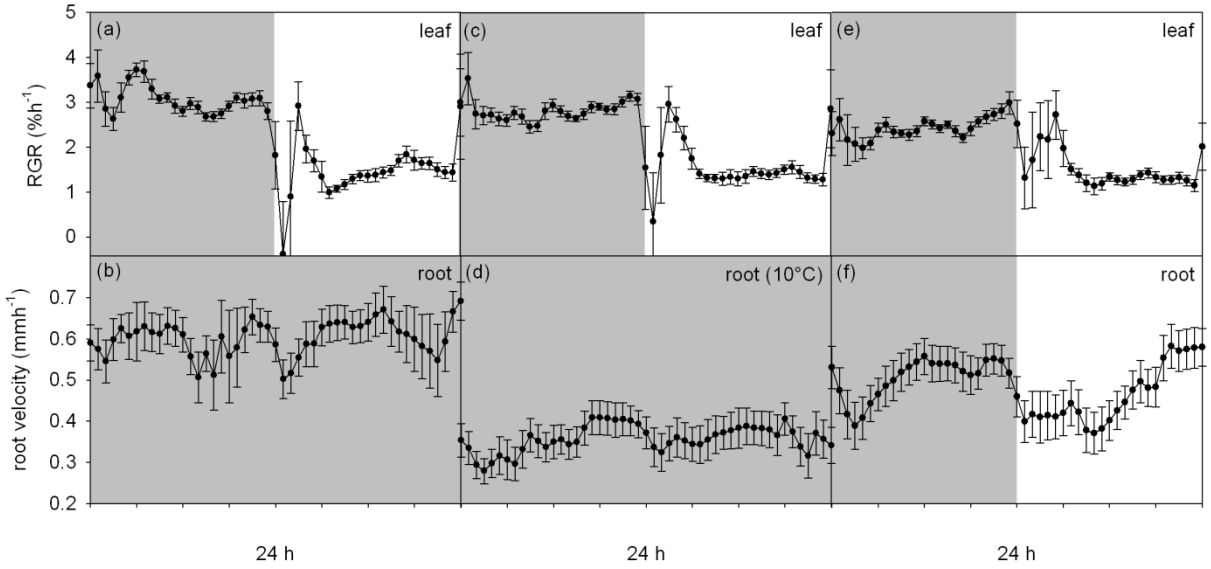

Figure 22: Leaf and root growth patterns of *N. tabacum* seedlings. (a) Leaf growth when the roots are growing in the dark. (b) Root growth in the dark. (c) Leaf growth during 10°C root cooling in the dark. (d) Root growth at 10°C in the dark. (e) Leaf growth when the whole plant is subjected to diel light-dark cycles. (f) Root growth in diel light-dark cycles. n = 10 for all treatments, root and shoot; Error bars are S.E..

Roots subjected to light-dark cycles (12h:12h) showed diel growth rhythmicity (Fig. 22f). Root growth rates were the highest towards the end of either the diurnal or nocturnal period. In the morning root growth rates were low but recovered slightly over the diurnal period to reach the rates similar to those seen at the end of the night or in the roots of the control seedlings in constant darkness (Fig. 22b). Similar observations are made at the beginning of the nocturnal period. However, the recovery of root growth rates during the nocturnal period started earlier. Leaf growth rates were overall very

similar to the rates found in the control seedlings (Fig. 22a, e). Interestingly, the prompt decrease in leaf growth rates at the beginning of the day was not that pronounced.

3.2.2.4 Discussion

The increasing computing power and advancement of image analysis software in the last decade made it possible to use high temporal time-lapse movies for phenotyping of growth patterns in leaves and roots (Walter and Schurr, 1999; Walter *et al.*, 2002).

Studies of leaf expansion in several dicotyledonous species showed that leaf expansion has a diel rhythmicity (Ainsworth *et al.*, 2005; Matsubara *et al.*, 2006; Wiese *et al.*, 2007; Poiré *et al.*, 2010b). Moreover this rhythmicity is maintained under continuous light, indicating underlying circadian control mechanisms (Poiré *et al.*, 2010b). Most diel growth patterns observed in leaves, including *N. tabacum*, are sinusoidal in nature and the growth rates reach the maximum amplitude around dawn or dusk (Ruts *et al.*, 2012b). In the *N. tabacum* seedlings studied here, however, the leaf growth pattern does not exhibit a sinusoidal oscillation (Fig. 22). The diel leaf growth pattern in these seedlings can be described as a step chart with two steps: high stable growth rates at night and low stable growth rates during the day. This pattern differs from the type 1 diel pattern typically found in leaves of *N. tabacum* at a later developmental stage (Walter and Schurr, 2005; Poiré et al., 2010b). In leaf 4 of *N. tabacum*, also relatively low RGR at day are measured and shallow amplitude is observed (Walter, 2009). Furthermore, Walter *et al.* (2005) showed that poplar leaves had more shallow diel leaf growth amplitudes early in the season (early developmental stage of the plant) compared to a time point later in the season, indicating that there is clear developmental gradient in rhythmic leaf expansion. Seedlings that have just exited the hypocotyl stage and have formed the first one or two primary leaves are in a transition phase. During the hypocotyl phase they were relying on the stored resources. As those resources deplete, seedlings become more and more dependent on their environment for resources; for light and CO_2 for photosynthesis they depend on the leaf and for minerals and water uptake they depended on root. The observed diel leaf growth pattern may be specific to the early seedling stage during development. The high fluctuations in growth rates at dawn can be partially attributed to a change in water potential due to large and abrupt changes in evaporative demand caused by the sudden increase in light intensity, leading to low cell turgor pressure and hence lower growth rates.

Root growth is highly responsive to temporal changes in environmental conditions (Walter *et al.*, 2002; Nagel *et al.*, 2006). However, when environmental conditions are kept constant, no change in diel root growth pattern was found in a number of species, including *N. tabacum* (Iijima *et al.*, 1998; Walter *et al.*, 2002; Walter *et al.*, 2003; Walter and Schurr, 2005; Nagel *et al.*, 2006). Also in this experiment, root growth was constant and stable in the *N. tabacum* seedlings over the entire diel period when the root system remained in the dark (Fig. 22b, d). A clear decrease in root growth was

seen when the temperature was lowered to 10°C. Low temperatures at the root reduce water uptake and root hydraulic conductivity (Markhart *et al.*, 1979; Wan *et al.*, 2001). Furthermore low temperatures are known to restrict apical root elongation (Stone and Taylor, 1982; Al-Ani and Hay, 1983).

Low root temperatures can have an effect on shoot growth by reducing water, nutrient or hormone supply (Ali *et al.*, 1997; Ali *et al.*, 1998; Ye *et al.*, 2003). Even at a constant air temperature it has been demonstrated that low root-zone temperature can cause a decrease of shoot biomass production (Nagel *et al.*, 2009b). Interestingly, we did not observe a reduction in leaf growth rates for *N. tabacum* seedlings. Davies & Van Volkenburgh (1983) showed that leaf growth is inhibited primarily during the day but not at night when the root is exposed to a constant low root temperature throughout the diel cycle. Since enhanced leaf growth of *N. tabacum* seedlings always occurred during the night (Fig. 22a, c, e), the negative effect of low root temperature on leaf growth may not be that pronounced during the day.

In the experiment with root-exposure to light-dark cycles, roots grew with clear diel rhythmicity (Fig. 22f). The diurnal root growth repression is probably caused by the light as light is known to have an inhibitory effect on root growth (Pilet and Ney, 1978; Schmidt and Walter, 2009). Consistent with this, marked diel oscillations of root tip growth have been reported recently in hypocotyls of *A. thaliana* (Yazdanbakhsh and Fisahn, 2010; Yazdanbakhsh *et al.*, 2011); in a measurement setup were entire root system was subjected to direct light exposure during the diurnal period. Interestingly, the circadian mutant *elf3* did not show any oscillation in root growth under the same condition (Yazdanbakhsh *et al.*, 2011). The peculiar root growth of *elf3* and altered sensitivity of the root system growth to gravity and light exposure in several other clock mutants (Ruts *et al.*, 2012a), might hint towards a regulatory role of the circadian clock in the observed root growth patterns in response to light.

In conclusion, this new technique allows detailed phenotyping of growth rates in leaf and root of the same plant simultaneously. Synchronous measurements in these organs enable detection of direct and indirect growth responses to various above- and belowground stress treatments, such as high-light stress or temperature stress. Furthermore, this technique can be used to quantify gene mutational effects on growth patterns and regulation, taking both roots and leaves into account. Use of this technique in transgenic plants having inducible promoters or tissue specific promoters could be especially powerful to investigate the impact of the induced effects in the induced and non-induced organs with high spatiotemporal resolution. Finally, the sensitivity of diel growth patterns to the environment revealed in the roots of *N. tabacum* seedlings underpins the importance to carefully control the growth conditions for root growth analysis in order to avoid or/and minimize complications.

4 Conclusion

Growth is the net result of multi-layered and complex processing that occurs in the plant. Here I show that the complexity of plant growth is determined by the environment, circadian clock and developmental stage.

4.1 Environment

Throughout their life cycle plants continuously sense their environment and adjust growth accordingly. The leaves of monocotyledonous plants and roots in general respond rapidly to environmental changes, resulting in highly variable diel growth rates mostly depending on the environment. When the conditions are kept constant, root growth and monocot leaf growth are stable over the diel cycle. In the experiments to study the root growth of *N. tabacum* seedlings over the diel cycle, I found little change in elongation rates when the roots were subjected to a continuous dark regime. When the root temperature was lowered (10°C), a decrease by two thirds was measured for the root elongation rate, while the constant diel growth pattern remained unchanged. However, when the roots were subjected to a light-dark regime, the root elongation rate oscillated. These results show that the root of *N. tabacum* dynamically adjust its elongation to rates to the environment. However, no significant cross-talk from root to shoot was found. Leaf growth rates of *N. tabacum* stayed stable when root temperature changed or when the root was subjected to a diel light-regime. The environmental changes in the pedosphere do mostly not underlie diel rhythmicity or in the case of temperature a rather weak rhythmicity. Therefore, an environmental cross-talk from root to shoot might only be necessary when the root experiences limiting or stress conditions such as a low soil water potential.

Many studies have been conducted to investigate the effects of root cooling under controlled conditions in growth chambers. There is, however, a substantial knowledge gap between the lab-based experiments and the field trials. In our greenhouse experiment with “semi-controlled” conditions, cool soil temperatures were imposed on the roots of four species; *H. vulgare*, *N. tabacum*, *L. sativa* and *C. sativus*. The root cooling treatment led to a decrease in leaf expansion and a slight reduction of shoot biomass. Furthermore the specific leaf weight increased in plants with the root cooling, which suggests a cold acclimation response. Contrary to the cooling experiments in climate chambers, no significant increase was found in carbohydrate or flavonoid content for cooled plants in the greenhouse conditions. It was hypothesized that the highly variable field-like light regime in the greenhouse experiment (higher peak intensity and gradual increase/decrease in morning/evening) may play an important role in the amelioration of adverse effects of reduced root temperature on shoot growth, carbohydrate and flavonoid metabolism. Also in these greenhouse experiments the environmental cross-talk from root to shoot seems to be very small. Only after a week of treatment

small phenotypic differences start to appear which suggests a slow acclimation response to a changing non-stressful environment instead of clear, fast signalling from root to shoot.

In mutants lacking a functional circadian clock, a clear dependence of the growth phenotype on the environment was observed. It was hypothesized that the growth rates and the phenotype became more dependent on the environment when clock functions were lacking (as example: the angle of the lateral root tip was determined by the light regime). This phenomenon is often observed in monocot leaves in roots were it is assumed that the clock had less influence on the growth pattern (Walter *et al.*, 2009; Poiré *et al.*, 2010; Ruts *et al.*, 2012).

4.2 Circadian regulation

Diel growth in dicots does not show as much plasticity as in monocots in response to environmental changes. Although the environment still determines the overall daily growth amplitude of dicots, it cannot explain the stringent pattern in diel growth rhythmicity of dicot leaves. We hypothesized that the diel growth pattern of dicot leaves is partially endogenously regulated. Indeed, circadian mutants of *A. thaliana* have profound changes in their diel leaf growth patterns. The arrhythmic clock mutants *CCA1ox* and *prr975* showed enhanced leaf growth compared to the wild-type during the diurnal period, suggesting increased partitioning of photosynthates for leaf growth. Nocturnal leaf growth was reduced and growth inhibition occurred by dawn, coinciding with ineffective starch degradation in their leaves. While nocturnal growth was reduced in both leaves and roots in *prr975* which retained substantial amounts of starch during the night, leaf and root growth rates exhibited inverse diel patterns in *CCA1ox*, i.e. high diurnal and low nocturnal growth for leaves and *vice versa* for roots. These results are consistent with the notion that the defective clock affects carbon and energy allocation between leaf growth, root growth and carbohydrate reservoir, leading as a whole to a reduced growth capacity. Furthermore, rosette morphology and size as well as root architecture were strikingly altered by the defective clock control. Separate analysis of the primary root and lateral roots revealed strong suppression of lateral root formation in both *CCA1ox* and *prr975*, which was accompanied by peculiar light-dark changes in lateral root growth direction. Thus, growth of the whole plant is severely affected by improper clock regulation, resulting in not only altered timing and capacity of growth but also aberrant development of shoot and root architecture.

4.3 Developmental stage

The developmental stage of the plant also seems to play a role in shaping the diel leaf growth pattern. In *N. tabacum* seedlings, leaf growth rates were three times higher at night than during the day giving rise to a step-chart pattern. This pattern has not been seen in leaf expansion of *N. tabacum* at later developmental stages (Walter *et al.*, 2005; Walter, 2009). After the seedling stage the diel leaf growth of *N. tabacum* exhibits a sinusoidal growth pattern with a maximum a few hours after dawn and a minimum at dusk (Type 1). At the hypocotyl stage, the plant is partially dependent on the stored

resource in the seed. It was hypothesized that the different diel leaf growth patterns may arise from changing equilibrium between the influence of the endogenous and the environmental control mechanisms at different developmental stages of plants.

4.4 The clock and the environment – a fine balance that determines growth

The astonishing phenotypes of the clock mutants in both shoot and root show how deep the clock network is enrooted in the endogenous regulatory mechanism of *A. thaliana*. The clock functions as a regulator that provides the plant with a daily modus operandi to cope with the expectable diel and seasonal environmental changes. When clock regulation in the dicot *A. thaliana* was lacking, the plant´s organs seem to become more susceptible to environmental change such as the light-dark cycle and illumination of the root. The increased susceptibility of growth to the environment is a trait that is often seen in monocots where it is thought that the control of the clock on growth isn´t that stringent (Poiré *et al.*, 2010; Ruts *et al.*, 2012). This daily script provided by the clock to assist growth is focussed on expected, mostly reoccurring changes. The environment and the unpredictability of it, however, remains the determining factor in plant growth. While the clock influences the diel growth rhythm (in dicots), the environment determines the amplitude of the diel growth pattern and thereby the overall growth velocity. Every unexpected environmental change or an unnatural environment has a noticeable impact on the growth pattern for which the clock cannot anticipate. Unexpected or unnatural conditions are probably intercepted by flexibility of the plant and by stress signalling pathways.

5 Outlook

There is a huge interest in understanding and influencing plant growth, which is driven by a socio-demographic and economic demand for more agricultural output to sustain a stable food and energy supply. Characterising plant growth in different environmental conditions and understanding the underlying mechanisms is essential to advance our knowledge of plant growth processes.

In the last years, the research community interested in plant circadian clocks has witnessed the achievement of several fundamental discoveries with respect to understanding the basic components of the circadian clock and to how the clock can influence hypocotyl elongation in *A. thaliana*. Further unravelling of the molecular mechanisms and functioning of the circadian clock in different tissues, as well as determining the input/output pathways to/from the clock will be important to elucidate the overall function and impact of the circadian clock on the plant's phenotype and growth capacity under various environmental conditions. An interesting but often neglected topic is the role and function of the circadian clock in root growth. Aberrant root growth rates and root architecture have been shown in several clock mutants as part of this thesis (Ruts *et al.*, 2012) and in previous studies (Yazdanbakhsh *et al.*, 2011). The molecular mechanics behind these phenotypes are yet to be elucidated. While it is clear that the components of which the clock is build up differs in shoot and root (James *et al.*, 2008), there is unfortunately no proper mapping of the molecular framework of the clock in the root. Furthermore, no direct clock output mechanisms are known that affect root system development. Observing signalling pathways that are known to influence root growth and architecture such as the auxin flow in the roots of clock mutants, together with the study of the circadian rhythm in mutants disturbed in important signalling pathways for root growth might enlighten the role of the clock in the root system.

With the emerging technologies for plant phenomics, we can acquire high-dimensional phenotypic data on different temporal and spatial scales. It is possible to exhaustively screen *genotype x environment* interactions in high throughput. Phenotyping combined with genetic screens can be a powerful tool in the search for genes and pathways that regulate growth or growth-related processes. Moreover, to get systems biology understanding of plant growth it will be crucial to observe the entire plant instead of only organs or cell layers. Changes in regulatory mechanisms due to environment, treatment or gene modification have direct and indirect effects on all plant organs. With the new phenotyping technique demonstrated in this thesis it is possible to determine the treatment effects on growth of treated and non-treated organs in high temporal and spatial resolution. Environmental effects on growth rates, such as high-light stress, can be assessed for the shoot and the root on a diel scale. Furthermore, the study of mutants which are affected in signalling pathways and/or transport between shoot and root might give us new insights in plant signalling. Moreover, the use of tissue-

specific over-expression or knock-outs to determine the direct and indirect effects on diel growth might be of great interest. For example, the screening of different circadian mutants with shoot and root growth phenotypes, which show abolished normal clock function in only one organ, might be of interest to learn more about the synchronisation of the circadian clock and its relevance in shoot and root growth.

The management and curation of all these genotypic and phenotypic data for different regulatory and organisational levels as well as their interaction with the environment is one of the biggest challenges in plant sciences for the next decades.

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7 Publications in this dissertation

7.1 Manuscript 1(Basis of the introduction)

Tom Ruts, Shizue Matsubara, Anika Wiese-Klinkenberg, and Achim Walter
Diel patterns of leaf and root growth: endogenous rhythmicity or environmental response?
J Exp Bot 2012 May;63(9):3339-51
Journal of Experimental Botany
Impact factor: 5.364 (Ranking 2011)

7.2 Manuscript 2 (Chapter I)

Tom Ruts, Shizue Matsubara, Anika Wiese-Klinkenberg, & Achim Walter
Aberrant temporal growth pattern and morphology of root and shoot caused by a defective circadian clock in *A. thaliana*
Plant J. 2012 June, DOI: 10.1111/.j1365-313X.2012.05073.x.
The Plant Journal
Impact factor: 6.16 (Ranking 2011)

7.3 Manuscript 3 (Chapter II)

Tom Ruts, Helen Behn, Eduardo Alejandro Pérez Torres, Marieke Scheffen & Achim Walter
The effect of root temperature on four greenhouse-grown species
In preparation

7.4 Manuscript 4 (Chapter III)

Tom Ruts & Achim Walter

In preparation

8 List of abbreviations

°C	degree Celcius
%	percent
µ	micro
ABA	abscisic acid
a.m.	ante meridiem
ANOVA	analysis of variance
AOI	area of interest
BCCE	brightness constancy constrain equation
BOA	BROTHER OF LUX ARRHYTHMO
CAB2	CHLOROPHYLL A/B-BINDING PROTEIN 2
CAT3	CATALASE 3
CCA1	CIRACADIAN CLOCK ASSOCIATED 1
CCD	charge-coupled device
CHE	CCA1 HIKING EXPEDITION
cm	centimetre
COA	co-enzyme A
col-0	Columbia-0
CRY	cryptochrome
cv.	cultivar
d	day
d.a.g.	days after germination
d.a.s.	days after sowing
DFG	Deutsche Forschungsgemeinschaft
DISP	dual image sequence processing
DNE	DIE NEUTRALIS
DW	dry weight
E	Einstein
EC	evening complex
e.g.	exempli gratia
ELF	EARLY FLOWERING
et al.	et alii (and others)
ETH	Eidgenössische Technische Hochschule
exc.	excitation
Fig	figure
FKF1	FLAVIN BINDING KELCH F-BOX 1
FW	fresh weight
g	gram
GI	GIGANTEA
h	hour
IBG	Institüt für Bio- und Geowissenschaften
i.e.	id est (that is)
L	litre
IRTG	international research training group
LATE1	LATE BLOOMER 1

LD	light-dark
LED	light emitting diode
LER	leaf elongation rate
LHY	LATE ELONGATED HYPOCOTYL
LIP1	LIGHT INSENSITIVE PERIOD 1
LKP2	LOV KELCH PROTEIN2
LL	continuous light
LOV	light, oxygen and voltage
LUX	LUX ARRHYTHMO
LVDT	linear variable differential transformer
LWD	LIGHT-REGULATED WD
m	metre
mm	millimetre
n	number of biological replicates
nm	nanometre
MYB	myeloblast
p	probability
p.m.	post meridiem
Pfr	far-red absorbing form of the phytochrome
PHY	phytochrome
PIF	PHYTOCHROME-INTERACTING FACTOR
PIL	PIF-like
Pr	red absorbing form of the pytochrome
PRR	PSEUDO-RESPONSE REGULATOR
R:FR	red to far-red ratio
RER	root elongation rate
RGR	relative growth rate
s	second
S.E.	standard error
SAS	shade avoidance syndrome
SLR	single lens reflex
SLW	specific leaf weight
SRR	SENSITIVITY TO RED LIGHT REDUCED
STF1	STARCH FREE 1
TCA	tricarboxylic acid
TIFF	tagged image file format
TOC1	TIMING OF CAB 1
UV	Ultraviolet
v	volume
VOC	volatile organic compound
w	weight
wt	wild-type
XCT	XAP5 CIRCADIAN TIMEKEEPER
ZTL	ZEITLUPE

9 List of tables and figures

Figures

Tables

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