A CRITICAL ROLE FOR THE ARABIDOPSIS CIRCADIAN CLOCK AT HIGH TEMPERATURE

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

by

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September 2010

ABSTRACT

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The circadian clock is an internal mechanism found in most organisms generating a 24h rhythm, evolved to anticipate predictable environmental changes thus making best use of resources. Intensely studied in the model plant *Arabidopsis thaliana* (L.) Heynh, the circadian clock was found to control a large number of physiological traits and the expression of more than a third of the plant genes. Clocks therefore play a central role in the life of organisms, in fact plants lacking clock functionality lose their ability to anticipate the dawn showing a reduced fitness. In a perspective of raising global temperatures, impact on crops production of plants with a non optimised clock could be of major importance.

The present study therefore investigated the importance for *Arabidopsis* plants of having a functional clock at high temperatures. Arrhythmic plants over-expressing the *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) gene were compared to wild-type Col-0 plants: while at 17°C wild-type plants showed more leaf area, biomass and chlorophyll content than CCA1ox, at 27°C the difference was even greater. This increased difference at high temperature was also confirmed in successive transcript and metabolite profiling of the two lines.

Not only a functional but also an accurate clock was previously found to be important for a good plant performance. For this reason investigation on the impact of high temperature was extended to plants with an internal clock period not matched to the external light/dark cycle. Period mutant lines *ztl* (30h) and *toc1* (20h) along with their wild-types (24h) were grown in 12LD, 15LD or 10LD cycles. *ztl* line showed no difference from Col-0 at 17°C but a marked difference at 27°C, where the lines with a period matching the external light/dark cycle performed better. Similarly the *toc1-1* line performed better at high temperature when its clock period matched the environment.

Another key feature of circadian clocks is temperature compensation, a mechanism able to maintain an accurate and robust rhythm with a period close to 24h over a broad range of temperatures. To quantify the importance of temperature compensation at high temperatures, the mutant line *gi-11*, defective for the temperature compensation mechanism, was used. Little differences were found at both 17°C and 27°C between the *gi-11*line and its wild-type WS. Finally, a screen was performed to identify new components of the clock specifically required for function at high temperatures. At the end of the screen pipeline, four putative circadian mutants were identified, which will need to be further characterised to confirm their altered rhythmicity and eventual position in the current clock model.

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ACKNOWLEDGMENTS

I would like to thank Dr. Anthony Hall who guided me through this PhD project, all the Sensible team led by Dr. Meriel Jones for offering me the great opportunity to work at Liverpool University as a Marie Curie fellow. It has been a very important time for my professional and personal growth.

But it would have been only work, messy without Jean the lab angel and boring without the multicultural bunch of friends in Lab G. Thanks to Nicolas and Peter for guiding my first steps in the lab, Charlie for sharing my Italian blood and passion for good food, Sean for guiding the whole lab in the Liverpool nightlife. Thanks to all the Sensible Fellows, for sharing this three year exploration of a new city and country. Thanks to Roland for sharing some true lab craziness between dry-ice clouds and popping eppendorfs, Maurycy for his fancy-dress international parties. Thanks to Angel for Mediterranean dinners, Jelena for the fantastic Lithuanian sweets, both for sharing Arabidopsis work, sunbathing in the growth chambers while drinking a good pint of AraBeer. Thanks to Magdalena and Michal for sharing their family life and the joy of being travelling parents, thanks to Jana and Tom, a true rambling couple.

I would have been lost without the welcoming community of the Chaplaincy: Father Ian, Jennifer, Rhea, Sandro, Basia... thanks for sharing Taize singing, trips, and every Sunday mass. Thanks to Seel Street Sisters for keeping me down to earth, for never letting down any man in need. Ema, thanking will never be enough: I simply would not be me without you. Without the support of both families I would never have gotten where I am, and never taken the following big choices that you little smiling man-cub primed.

CHAPTER 1 - Introduction

1.1 Circadian Rhythms

1.1.1 Biological Rhythms

Day and night, tides, heartbeat, migrations: rhythms are everywhere in the life of living organisms. But what are rhythms? They can be described as sequential events repeating in time, with the same interval and in the same order; if they come from within an organism they are called biological rhythms. Rhythms can have a period longer than 24 hours (Infradian) as bird migration, flowering and leaf fall, or shorter (Ultradian) as heartbeat, blinking and the activity of organisms depending on tidal movements (crabs and diatoms). When the rhythm is daily (approx. 24h) is called Circadian (from the Latin "Circa-Die", about a day): examples are the sleep-wake cycle and plant growth.

The first observation of a circadian rhythm was in plants, by a French physicist (de Mairan, 1729): he noted that *Mimosa pudica* daily leaf movements continued even in constant darkness, proving their endogenous origin. Circadian rhythms in animals were described only much later, with the measure of rats daily activity (Richter, 1922). Proving these rhythms are endogenous however leaves the question of what drove their evolution in such a wide array of living organisms, from cyanobacteria to all eukaryotes species. The answer is in the profound cyclical changes occurring in the environment, generated by the daily rotation and the yearly progression of the earth around the sun. These changes were a strong selective pressure to evolve a system which could predict them, leading to several advantages.

Clocks allow bees and birds to navigate and orientate with the sun (Paranjpe and Sharma, 2005), control the sleep and wakefulness in mammals helping them escaping predators and determining the season for mating (Oster *et al.*, 2002). Photosynthetic organisms however, are the one who gain the greatest advantages from the ability to keep the time, as they rely on the sun's energy for growing. Predicting the onset of dawn allows making the most of the available daylight, preparing the photosynthetic apparatus for

using the first glimpse of light. In plants knowing when dawn is approaching avoids starvation during the night, allowing production of the exact amount of starch required for respiration and no energy waste (Graf *et al.*, 2010). Reactions negatively affected by sunlight or oxygen can be temporally programmed during the night (Pittendrigh, 1993), such as the temporal segregation of nitrogen fixation in cyanobacteria (Johnson, 2001). Finally, tracking the duration of the day, light quantity and quality along with temperature variations tells the plants when is best to flower (Davis, 2002, Jackson, 2009, Amasino, 2010). Giving so many advantages, it's not surprising that the clock has such a central role in many organisms and that its malfunction generates severe syndromes and reduced growth. Circadian desynchrony can lead to obesity (Scott *et al.*, 2009) and metabolic dysfunction in humans (Ramsey and Bass, 2009), while choosing the right time can even help preventing and curing cancer (Hede, 2009).

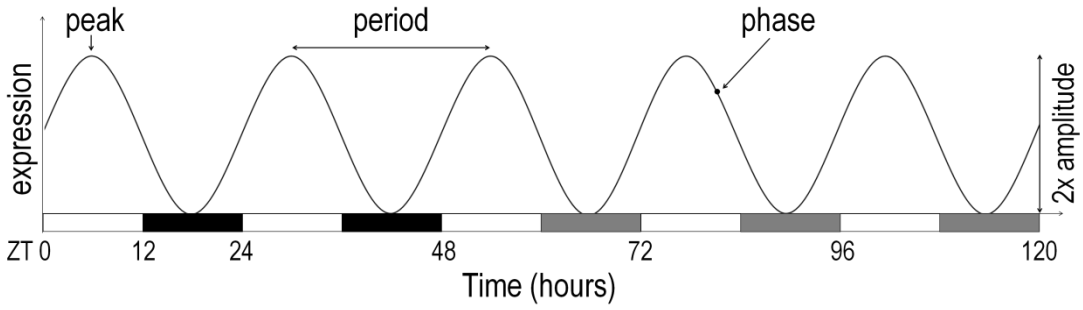


Figure 1-1. Hypothetical circadian clock output showing a 24h period in light/dark cycle (white/black boxes). In constant light the rhythm persists with a period close to 24h (grey boxes indicate subjective night). Circadian properties of peak, period, phase and amplitude are shown, see text below for details.

1.1.2 Properties of Circadian Rhythms

Circadian clocks have been found and studied in several different organisms, allowing researchers to compare their features across kingdoms of life. Some components were found to be common in distant organisms, others shared the same mechanism but with a different molecular basis (Dunlap, 1999; Young and Kay, 2001). These comparisons clearly indicated there has been a convergent evolution towards a circadian system with similar properties. Before outlining them however is useful introducing some of the basic terminology used in circadian research. Figure 1-1 represents an output of the circadian clock: it could be the expression of a gene which in normal conditions peaks every 24h, in the middle of the day. The distance between two peaks is called period and is the time necessary to complete a cycle, while half the height of the peak is the amplitude. Phase is the time of the day an event occurs and is usually measured in Zeitgeber time (ZT). Zeitgeber is the German word for "time giver", indicating any environmental input that can "give time" or entrain the circadian clock. As the most powerful signal is light, ZT is conventionally set as 0 for the beginning of the light time (dawn).

The first defining property of circadian rhythms is their ability to continue to oscillate under constants conditions with a rhythm close to 24h. Constant conditions (free run) signifies the absence of environmental time cues: constant temperature and either constant light (LL) or constant dark (DD). This is represented in Figure 1-1, where there is an entraining light/dark environment (white/black boxes) switching to constant light after 48h: grey boxes indicate subjective night, that is dark periods predicted by the endogenous rhythm but absent in the environment. The described feature is what differentiates a circadian from a diurnal rhythm: both are present in light/dark cycles, but only the circadian rhythm persists in free run conditions.

A second feature of circadian rhythms is that can be entrained by environmental cues. Entrainment is defined as the synchronization of the internal clock to the environment and could be explained as the ability of environmental signals (Zeitgeber) to alter the phase of the clock. The endogenous clock therefore, can be reset to the current environmental time (review in Devlin and Kay, 2001). The best studied and most common signal is light, but other signals as temperature, social cues and food can be

effective in resetting the clock (Devlin, 2002). Common life examples are the recovery from jet lag, daylight saving time, and the tracking of seasons by plants and mammals. Environmental signals however do not have the same capacity to entrain the clock throughout the day. This characteristic has been well studied in plants for light and named "gating". Gating allows the central oscillator to have a greater sensitivity to light pulses around dawn and dusk, reduce its responsiveness during subjective night, and have a sort of blindness to very short light pulses. (Jones, 2009).

The third property of circadian clocks is all about reliability. We expect our mechanical clock to work in all daily life circumstances, but what about our body clock, is it reliable? In fact it is, but it is not obvious at all. Biological clocks are based on biochemical processes, and normally chemical reaction rate doubles every 10°C increase in temperature. Q_{10} is the measure of this and for a doubling of reaction rate Q_{10} would equal 2. Circadian clocks have a Q_{10} close to 1, which means their period is minimally affected by temperature: they are temperature compensated. Temperature compensation was firstly measured in *Phaseolus multiflorus* leaf movement (Q_{10}=1.2, Bünning, 1931) then more precisely in other organisms. By increasing the temperature from 16°C to 26°C, Pittendrigh discovered that the Q_{10} for eclosion rhythm period in *Drosophila pseudoobscura* was only 1.02 (Pittendrigh, 1954). Mutations in core clock genes confirmed the first experiments: arrhythmic mutants for the *FREQUENCY* (*FRQ*) gene of the fungus *Neurospora crassa* lost their temperature compensation. At high temperature *frq* mutants showed conidial bands closer together (lower period) and vice versa at low temperature: in contrast wild-type rhythm was constant (Loros *et al.*, 1986; cited in Hall, 1997). Likely, in *Arabidopsis* the *gi-11* mutant showed a leaf movement period significantly shorter than wild-type above and below 17°C (Gould *et al.*, 2006). Phenotypes of the mutations just described exemplify another common feature of biological circadian systems: they are under genetic control. Circadian rhythms can be altered constitutively by mutations: period can be shorter, longer or even completely abolished leading the clock to arrhythmia.

1.2 Arabidopsis Circadian Clock

1.2.1 Inputs and Outputs

The circadian clock, as said earlier, is an internal mechanism found in most organisms that generates a constant 24h rhythm in all environmental conditions and with defined properties. The Circadian system that generates this rhythm can be described as consisting of three parts: input pathways that receive and relay environmental cues entraining the oscillator; a central oscillator that generates rhythmicity; and output components that generate detectable rhythms (Figure 1-2). This model is however oversimplified, as clock input components such as photoreceptors can also be clock outputs (Bognar *et al.*, 1999), and clock outputs can feed back on the clock itself, affecting the pace at which the central oscillator runs. In other words, the central oscillator is able to regulate the sensitivity of input pathways to environmental stimuli, depending on when these stimuli arrived, a feature known as gating (Hotta *et al.*, 2007). Furthermore, accumulating evidence is moving the current opinion away from a single one-way path, to an idea of regulatory network. A reciprocal regulation is being unveiled between the clock and the major environmental signaling pathways of light and temperature signaling, hormone and stress responses, cytosolic calcium and nutrient availability (Pruneda-Paz and Kay, 2010).

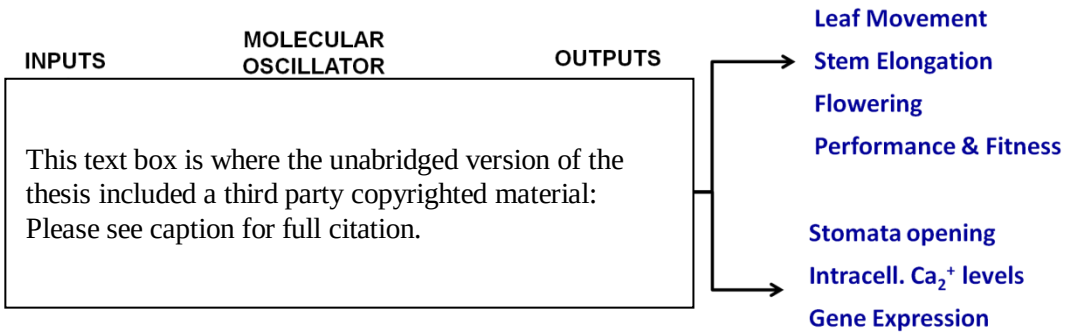


Figure 1-2. Basic model of a plant circadian system. Inputs from the environment entrain a central oscillator, which generates a rhythm traduced in visible physiological outputs. Adapted from http://millar.bio.ed.ac.uk/andrewM/Jo%20Selwood%20site/cogs_of_clock.htm

In higher plants, there is evidence for the presence of multiple independent oscillators that are distributed throughout the entire organism: these oscillators are independent from a central oscillator and can be entrained to free run with different phases in different organs (Thain *et al.*, 2000). Moreover, a recent paper proved that not only phase can vary in different organs, but even the clock composition, depending on environmental conditions. In whole plants, when only shoots but not roots are exposed to light, only a part of the clock genes are expressed rhythmically in roots (James *et al.*, 2008). In contrast, in the mammalian system, almost every cell of the body holds all the components necessary to build a functional biological oscillator but all these peripheral clocks are driven by the master circadian pacemaker in the Suprachiasmatic Nucleus (SCN) of the hypothalamus (Oster *et al.*, 2002).

Transcriptomic studies carried out on the model plant *Arabidopsis* identified that from 16 to 36% of the transcriptome is clock regulated (Michael and McClung, 2003; Edwards *et al.*, 2006), no need to say that such a big part of the transcriptome controls many physiological traits. Leaf movement was the first output ever monitored, leading in fact to the discovery of the existence of the circadian clock itself by de Mairan in 1729. Nowadays advanced monitoring techniques of the same leaf movements (Edwards and Millar, 2007) allowed for example study of natural variation in circadian clock function (Edwards *et al.*, 2005).

Other outputs measured to be clock controlled are hypocotyls growth, flowering, photosynthesis, stomatal opening, cytosolic free calcium (Webb, 2003), ethylene emissions (Thain *et al.*, 2004) and many others (reviewed in Yakir *et al.*, 2007a). Combined approaches has been used too, integrating metabolite with transcript and enzyme profiling (Gibon *et al.*, 2006), but with any doubt the most used technique to date has been luciferase bioluminescence assay. The firefly luciferase gene (*LUC*) is fused to the promoter of a gene of interest: the promoter's activity is then quantified by measure of the light emitted by luciferase protein (full details in Hall and Brown, 2007). With this method the very first *Arabidopsis* clock mutant was discovered (*TOC1*, Millar *et al.*, 1995) and successively even larger screens has been setup (Onai *et al.*, 2004). The disadvantage of bioluminescence assays however is the need to build and transform a construct into the plant: this is easy in *Arabidopsis* but may be impossible in other species hard to regenerate.

Recently however a new method has been added to the plant biologist's toolbox: delayed fluorescence (DF). Right after illumination, a luminescence is produced from chlorophyll A that can be easily measured; most importantly, this luminescence is under circadian control. DF represents a high-throughput technique not requiring any transformation, potentially applicable to all photosynthetic organisms (Gould *et al.*, 2009).

1.2.2 Central Oscillator

The heart of the central oscillator is a delayed negative feedback loop that integrates elements of transcription and translation. Oscillations are created by transcription factors activating the expression of clock genes that then negatively regulate their own transcription (Figure 1-3). This negative feedback loop occurs at least once in every biological clock (Dunlap, 1999). The molecular machinery that underpins these oscillations is still not completely understood, but using existing data a mathematical model incorporating three loops has been designed, validated by experimental results and successively extended (Locke *et al.*, 2006).

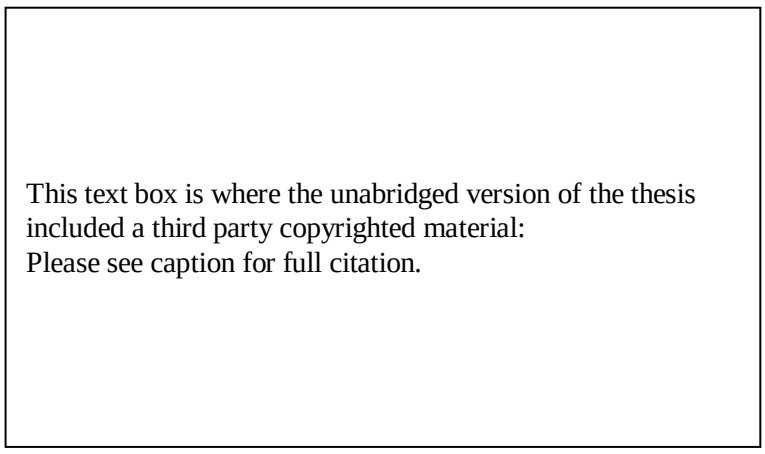


Figure 1-3. Example of a circadian clock negative feedback loop. Adapted from Dunlap, 1999.

The first model of the clock in *Arabidopsis* was based on a negative feedback loop where in one hand *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*), two MYB related transcription factors, repress the transcription of *TIMING OF CAB* (*TOC1*) expression, a pseudoresponse regulator. In turn *TOC1* activates a putative gene *X*, which then activates the expression of *CCA1* and *LHY* (Alabadi *et al.*, 2001; Figure 1-4, outer path). Although this first model of the clock was able to explain the basic mechanism of the oscillator, it was incomplete because it could not explain all experimental data. Most importantly *lhy/cca1* double-null mutants (Alabadi *et al.*, 2002) as well as *toc1* mutant plants (Mas *et al.*, 2003b) are not completely arrhythmic, but a short-period rhythm persists for several cycles.

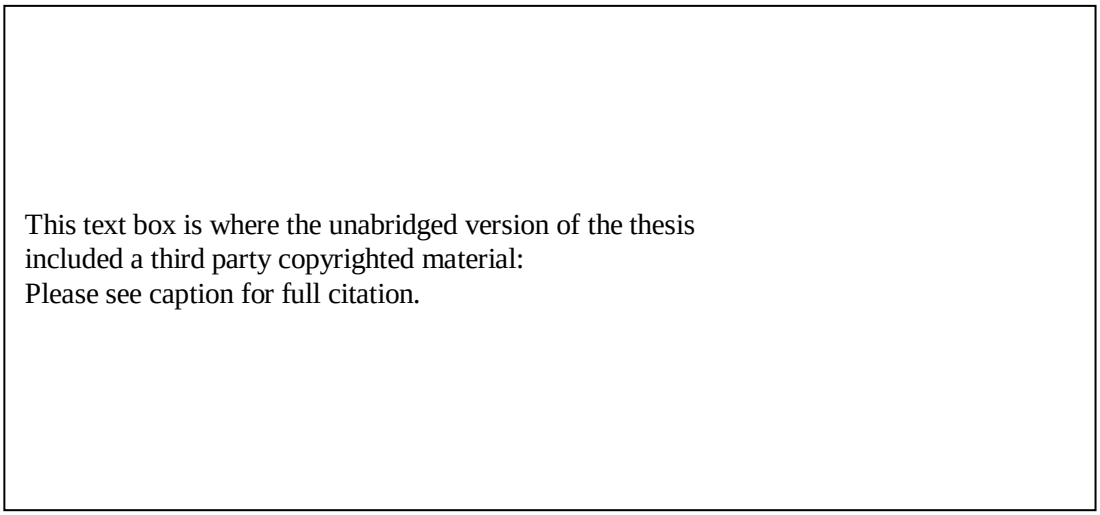


Figure 1-4. Overview of the three-loop clock model, showing only genes (boxed), light inputs (suns), positive (solid arrows) and negative (dashed bars) regulatory interactions. Genes in the "morning" and "evening" oscillator are white and dark grey, respectively. The *LHY/CCA1-TOC1-X* loop is shown in the outer path. Adapted from Locke *et al.*, 2006.

A second model comprising another feedback loop was therefore proposed with an unknown component Y, activating *TOC1* transcription and TOC1 repressing *Y* (Figure 1-4, right side). Y expression is also activated by light but inhibited too by *LHY* and *CCA1* (Locke *et al.*, 2005a). The *GIGANTEA* (*GI*) gene, expressed in the evening (12h after dawn, Fowler *et al.*, 1999) acts as part of the evening loop, explaining at least a part of the unknown Y component. The second loop is able to explain the residual rhythms of the *lhy/cca1* mutants but not of *toc1* mutant plants.

Finally a third loop was included with *CCA1* and *LHY* acting as positive factors inducing the expression of two morning expressed genes *PSEUDO RESPONSE REGULATOR 7* (*PRR7*) and *PSEUDO RESPONSE REGULATOR 9* (*PRR9*). *PRR7* and *PRR9* and have been proposed to repress the expression of *CCA1* and *LHY* forming a third loop (Nakamichi *et al.*, 2010; Figure 1-4, left side). These Pseudo-Response regulators are involved in the transmission of both light and temperature signals to the clock, gaining therefore an important role in the central oscillator (Farre *et al.*, 2005; Salome and McClung, 2005). To sum up, the three loops model suggests the presence of a morning oscillator (*PRR7/9-LHY/CCA1* loop) and an evening oscillator (*TOC1-GI* loop) coupled together by the first loop (*LHY/CCA1-TOC1-X*) (Locke *et al.*, 2006).

The three loop model just described is in constant evolution: every year new components or functions are unveiled, extending the complexity and our understanding of it. In fact, recently a new transcription factor called CCA1 HIKING EXPEDITION (CHE) was found to bind the *CCA1* promoter (Pruneda-Paz *et al.*, 2009). This binding takes place on the same region with TOC1 protein to repress CCA1 expression, which in turn represses CHE. By the end of the night, TOC1 seems to antagonize CHE, to eliminate the repression on CCA1 promoter. An updated model of plants' circadian network with further details of regulatory mechanisms can be found in Pruneda-Paz and Kay, 2010.

Classic transcriptional modulation however, is not the only system controlling circadian clock function (Mas, 2008). Chromatin remodelling for instance is involved in the rhythmic regulation of *TOC1* gene: activation of repressive chromatin structures enhances the repressing action of LHY/CCA1 transcription factors (Perales and Mas,

2007). Regulation continues at the post-transcriptional stage, where light was found regulating CCA1 transcript stability: CCA1 mRNA is stable in the dark but its half-life is reduced in blue and red light (Yakir *et al.*, 2007b). Messenger RNA is also involved in alternative splicing: in *Arabidopsis* the AtGRP7 RNA binding protein seems to autoregulate its abundance by binding to its own pre-mRNA, creating a post-transcriptional negative feedback mechanism (Staiger *et al.*, 2003). Similarly, low temperature can activate an alternative splicing in *Drosophila* PER Gene 3-UTR (Majercak *et al.*, 2004). Moving on to proteins it was found ZTL targets to degradation in the 26S proteasome complex the central clock component TOC1 (Mas *et al.*, 2003a) and its homolog PRR5 (Kiba *et al.*, 2007). Aside protein turnover, also phosphorylation acts at the protein level. In fact CASEINKINASE2 (CK2) can modulate CCA1 activity in vivo (Daniel *et al.*, 2004) and proteinkinase WNK1 regulates the phosphorylation of PRR3 (Murakami-Kojima *et al.*, 2002).

1.3 Plants temperature perception

Temperature, with light and nutrients, is a key component of plants' growth, necessary for good development and reproduction. It influences the whole plant function, from the rate of biochemical reactions to membrane fluidity. Plants therefore need to be able to measure accurately temperature changes, tracing the slow seasonal temperature changes but also reacting fast to sudden heat or frost. In a perspective of environmental changes with global raising temperatures, plants need to be able to adapt and we need to understand how temperature is perceived by plants to be able to face the threatened food shortages.

Plants' growth is strongly influenced by temperature, and there are evidences this growth is not just due to a mere increase of metabolic rate, but is mediated by endogenous hormones. Plants lacking gibberellin (GA) signalling are unable to increase their growth rate with the raise of temperature (Tonkinson *et al.*, 1997). In addition giving exogenous GA to *Pisum sativum* plants seems to saturate the signalling pathway, blocking stem elongation as a result of thermoperiodic growth (Grindal *et al.*, 1998). Thermoperiodic growth is the growth caused by a treatment with high differential temperatures between day and night. In *Arabidopsis* GA seems also to be involved in low-temperature dependent seed germination (Yamauchi *et al.*, 2004), explaining at least part of the germination mechanism, requiring different temperature treatments in different plant species to be able to occur. But GA is not the only way temperature interacts with the plant growth, as also auxin translates temperature inputs into a physiologic effect. In fact auxin (IAA) levels are increased by high temperature, leading to cell elongation in *Arabidopsis* hypocotyls (Gray *et al.*, 1998)

Hormones regulate also acclimation to cold and acquired thermotolerance. Abscisic acid (ABA)-insensitive and -deficient plants for instance cannot tolerate freezing after a cold acclimatization (Gilmour and Thomashow, 1991). On the other side of the temperature scale ABA is necessary also for acquired (but not basal) thermotolerance: in fact ABA mutants lack the ability to withstand very high temperatures of 45° for 3 hours (Larkindale *et al.*, 2005). Acclimatization to extreme temperatures involves, among other

things, maintaining membrane fluidity constant: plants accomplish this by altering the membrane's fatty acid composition. When temperature is low, plants increase the content of polyunsaturated fatty acids, while at high temperatures synthesis of saturated and monounsaturated fatty acids is increased (Falcone *et al.*, 2004). Temperature regulation of fatty acid synthesis is believed to be a direct consequence of fatty acid desaturase stability, controlled by their protein turnover mechanism. Several experiments were conducted to better understand this temperature regulation. Artificially altering plant fatty acid composition reduced plant tolerance to temperature extremes, while using chemicals that modify cell membranes fluidity it was possible to modify expression of temperature-regulated genes. This led to the controversial hypothesis that membrane fluidity itself could be a temperature sensing mechanism (Reviewed in Penfield, 2008).

Temperature effect however is not just limited to plants' germination, growth and membrane fluidity. Its global effect is mediated by the circadian clock, regulating itself several physiological traits. Temperature is perceived by the clock as an entraining cue, fluctuating with day and night. Removing the light cyclic signal (constant light), it was possible to quantify in 4°C the minimum temperature differential between subjective day and night effective for *Arabidopsis* clock entrainment (McClung *et al.*, 2002). A remarkably smaller differential of 0.5°C is sufficient to entrain *Kalanchoë* clock-controlled CO_2 assimilation rate (Rensing and Ruoff, 2002). Moreover in *Arabidopsis*, temperature cycles were proven to be enough to entrain the rhythmic expression of core clock components CCA1, LHY and TOC1. Expression of the same genes was altered in *ppr7-ppr9* double mutants, but only when entrained solely with temperature cycles, identifying the two related proteins PRR7 and PRR9 as critical for temperature entrainment (Salome and McClung, 2005).

An important developmental transition such as flowering is regulated too by temperature (together with light), as expression of seasonal changes. Rise of temperature is able to shorten the vegetative phase, increasing growth rate and promoting flowering. In *Arabidopsis* this switch to reproductive phase is even stronger if the high temperature is given during the night, rather than in the day (Thingnaes *et al.*, 2003). This however seems to happen independently from GA (an important promoter of flowering), converging directly on the floral promoter FT, while GA promotes flowering through the LEAFY

gene (Penfield, 2008). Balasubramanian *et al.*, 2006 show evidence that the mediators between temperature and the floral promoter FT are FLOWERING LOCUS C (FLC) and his homologue FLOWERING LOCUS M (FLM).

In conclusion, temperature affects plants in many ways, as via hormone-mediated growth, germination, membrane fluidity, flowering and circadian timing. Proper temperature sensors however have not been identified yet. Interestingly, some of the temperature response pathways were found sharing signalling components. Cytosolic calcium oscillations for example have been suggested to integrate temperature and circadian signalling, keeping a memory of past environmental temperatures (Hotta *et al.*, 2007). Moreover, phytochromes could integrate light with temperature. Both far-red light and high temperature result in early flowering response, reduced chlorophyll and petiole elongation. High far-red light, triggering the shade avoidance response, is mainly perceived by the phyB photoreceptor. Halliday *et al.*, 2003 found that the early flowering phenotype of *phyB* mutants is abolished at temperatures below 16°C, suggesting that phytochromes could have a role in both light and temperature signalling pathways.

1.4 Arabidopsis thaliana as a model organism

Arabidopsis is a small dicotyledonous flowering plant of the Brassicaceae family, commonly known as "thale cress". Native to Europe, Asia, and northern Africa (GRIN, Germplasm Resources Information Network) it now has a widespread habitat: over 750 natural accessions have been collected from around the world and are available from seed stock centers (ABRC, American; NASC, European) All these accessions show a remarkable variability in terms of form, development and physiology, and are used by researchers to understand the genetics behind this great variability in plant response to different environments (Figure 1-5).

Figure 1-5. Geographical distribution of ecotypes of *Arabidopsis thaliana* (L.) Heynh. Copyright Jonothan Clarke, Kingston University London (1993). Based on original map by George Redei (1969). Available at www.arabidopsis.org/images/geo_distribution.png

Unlike other members of its family such as cabbage, rapeseed, turnip, mustard, broccoli or cauliflower (NCBI Taxonomy Browser, Brassicaceae), it has no economic or agronomic importance. Despite this, it has been chosen by the scientific community as the principal model organism for plant laboratory research: for over 60 years it has been used for research in plant physiology, genetics, biochemistry and molecular biology. Several traits, firstly described by Langridge (Meyerowitz, 2001), made this small plant a very useful research tool.

Arabidopsis being a small plant (about 25 cm tall) makes it easy and economical to grow in high densities and restricted space, such as petri plates or multicell trays. As a spring ephemeral it has a short (fast) life cycle that can be entirely completed in six weeks, speeding up experiments. The flowers naturally self-pollinate producing fruits (siliques) containing ~30 seeds each for a total of ~10000 seeds per plant; dry seeds can then be stored at ambient temperature for many years, allowing the storage of entire mutagenised collections in a very limited space. Immature seeds are easily transformed with the efficient "floral dip" protocol (Clough and Bent, 1998; Davis *et al.*, 2009), which makes use of *Agrobacterium tumefaciens* to transfer DNA to the plant genome. Finally, *Arabidopsis* plant tissues (whole young seedlings and roots) are relatively translucent, well suited for light microscopy analysis: this allows non-invasive live cell imaging using both fluorescence and confocal laser scanning microscopy, including the possibility of performing time-lapse measurements (Moreno *et al.*, 2006).

The genome of *Arabidopsis* is one of the smallest among plants and the first to be fully sequenced: it has about 125 Mb over 5 chromosomes, 19 times smaller than maize and 128 times less than wheat. Despite its size, has a complement of most higher plant genes (about 30000) making easier the transfer the accumulated wealth of knowledge to higher crops, forestry, ecology and more recently bio energy production. Research is greatly helped by the large international research community and the information by this generated, such as high-resolution genetic and physical maps of chromosomes at The *Arabidopsis* Information Resource (Gene Structural Annotation; Map Viewer; SequenceViewer, TAIR), databases of annotated genes and proteins, seeds stocks and collections of mutants, cloned genes, gene expression data.

An example of how basic research performed in *Arabidopsis* can be transferred to crops is the work done by David Laurie group at the John Innes Centre (Turner *et al.*, 2005). In short, they identified Ppd-H1 gene as responsible for photoperiodic adaptation of barley and located it as a member of the Pseudo-Response-Regulator (PRR) family. This explained the molecular basis of what breeders did by selecting winter and spring barley varieties with a strong or weak response to long days. They were able to do so thanks to all the previous research done in rice and *Arabidopsis* (Laurie *et al.*, 2004): full genome sequences and dissection of the flowering pathways. This work increased the overall understanding of the control of flowering time in cereals, critical for improved grain yield (reviewed in Cockram *et al.*, 2007).

1.5 Aims of PhD

One of the key features of the circadian clock is the ability to maintain an accurate and robust rhythm with a period close to 24 hours over a broad range of temperatures, this feature is believed to be critical for the plant performance and growth. An initial experiment to test this assumption has been done with transgenic plants over-expressing the gene *CCA1* (CCA1ox). The over-expression of the single gene *CCA1* causes a complete arrhythmic phenotype (Wang and Tobin, 1998). At 17°C three week old plants with no functional clock (CCA1ox-o38 line) have similar phenotype to the wild-type (*WT*) plants; however, at 27°C these plants are not performing well and at 33°C they almost do not survive (Figure 1-6). These results indicated how critical a functional clock could be in *Arabidopsis* for optimal performance at higher temperatures, and led to a thorough investigation, performed with this thesis.

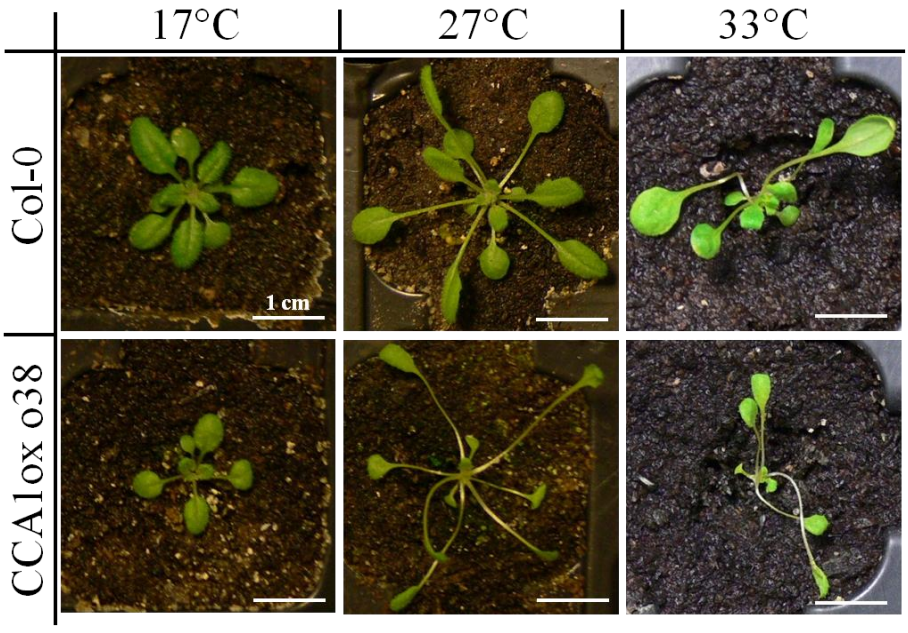


Figure 1-6. Transgenic plants over-expressing CCA1 (CCA1ox) have no functional clock and grow poorly at higher temperatures.

The main aims of the project were therefore to:

- Quantify the importance of circadian resonance and temperature compensation in the enhancement of plant growth at high temperatures.
- Identify new components of the clock specifically required for function at high temperatures.

From these main aims three questions were formulated.

Firstly is a clock important for optimized performance at high temperature, secondly is a temperature compensated clock important?

The first step of the project was the quantification of the performance of plants with either no functional clock, using the line *CCA1ox*, or altered clock function, using the mutant lines *gi11*, *zeitlupe* (*ztl*) and *toc1* (see 2.1.1 Performance experiments Lines). Plants were grown in standard conditions (21°C and 12L:12D), then transplanted in a randomized grid and transferred to 17 or 27°C in 12h Light/12h Dark cycles. After two weeks, various measurements were taken, indicative of growth efficiency and performance. These included measurements of chlorophyll content, wet and dry aerial weight, visible leaf area.

Is circadian resonance important at high temperature?

The second step of the project was to determine how important an accurate clock is as the temperature rises. Seeds of long (*ztl*) and short (*toc1*) period mutants were germinated in standard conditions (21°C and 12L:12D) and then transferred to either 17°C or 27°C in either 10h L/10h D, 12h L/12h D or 15h L/15h D cycles. The same assays done in the first set of experiment were performed.

Identify new components of the clock specifically required for function at high temperatures.

In parallel to the described experiments, I performed a screen using a collection of activation tagged *Arabidopsis* lines (see 2.1.2 Screen Lines), looking for plants that have an altered phenotype at 27°C, with the aim of identifying novel clock genes potentially

required for the maintenance of rhythmicity at high temperature in *Arabidopsis*. Putative mutant plants identified with the screening were transplanted, recovered and the seeds produced harvested. The seeds were screened again at 17°C and 27°C to confirm the poorly performing phenotype at high temperature. On the putative mutant lines which confirmed to have a high-temperature dependent phenotype, a leaf movement experiment is carried out to look at their circadian phenotype. If the plants have an altered clock phenotype, they can be regarded as real clock mutant lines and analyzed at molecular level, to identify the up regulated gene responsible for the phenotype. Once the gene is identified, it can be positioned in the current model of the circadian clock.

CHAPTER 2 - Materials & Methods

2.1 Plant Material

2.1.1 Performance experiments Lines

The over-expressed CCA1ox-o38 and CCA1ox-o34 *Arabidopsis* lines with no functional clock were obtained by transformation of Columbia (Col-0) WT plants with a 35S:CCA1 construct (Wang and Tobin, 1998). The period mutant *toc1-1* (20h, short period) was identified among a population of EMS mutagenized lines in a screening for altered expression of the *CAB2:LUC* transgene (Millar *et al.*, 1995). The *ztl-3* mutant (28-32h, long period) is a knock-out mutant containing a T-DNA insertion at amino acid 440 (Jarillo *et al.*, 2001; Somers *et al.*, 2004). ZTL is a protein involved in the light-dependent degradation of clock components such as TOC1 (targets for degradation by the ubiquitine/26s Proteasome). The temperature compensation mutant *gi-11* was isolated in a screen of T-DNA insertion lines (Richardson *et al.*, 1998; Fowler *et al.*, 1999). The ecotypes used in the experiments as background were Columbia 0 for *ztl-3*, CCA1ox-o38 and CCA1ox-o34; C24 2CAC for *toc1-1* (NASC ID: N3755), Wassilewskija for *gi-11* (Wang *et al.*, 1986).

2.1.2 Screen Lines

The collections of activation tagged *Arabidopsis* lines used for the screening were obtained from the European *Arabidopsis* Stock Centre (NASC). Scheible and Somerville lines (N31100) are a complete set of activation tagged lines obtained by transformation of wild-type *Arabidopsis* ecotype Columbia plants (Col-2, N907) with the pSKI15 vector from D. Weigel (Weigel *et al.*, 2000). The T-DNA vector contains multimerized transcriptional enhancers from the cauliflower mosaic virus (*CaMV*) *35S* gene which leads primarily to an enhancement of the endogenous expression of a gene close to the T-DNA insertion. Yokoi,Koiwa,Bressan lines (N31400, N31402, N31404) are a complete set of activation tagged lines obtained by vacuum infiltration transformation of C24 plants (already transformed with *RD29A-LUC* reporter gene and carry Kan resistance) with the pSKI15 vector from D. Weigel into which a luciferase-stress expression construct was inserted (http://arabidopsis.info/CollectionInfo?id=61).

2.2 Surface seed sterilization

A maximum of 100µl *Arabidopsis* seeds were sterilized in a 1.5ml eppendorf tube, under a sterile hood. The seeds were briefly (~1min) washed with 1ml 70% ethanol (EtOH), carefully mixed and the ethanol removed by decanting the seeds. One ml of diluted bleach solution was added to the tube (6% w/v sodium hyperchloride + 0.01% Tween 20), gently mixed for about 8min, but no longer than 15min, and centrifuged at 13000rpm for 10sec to pellet the seeds; the bleach was removed using a P1000 automatic pipette. The seeds were then washed in 1ml of sterile distilled water, centrifuged as before and the water was removed using a P1000 pipette. The seeds were finally suspended in 500µl 0.15% agar and stored at 4°C for up to one week.

An alternative method for sterilizing seeds involves the use of chlorine gas permitting sterilization of large amount of seeds at the same time. Under an aerated hood, two bleach tablets (Clo-Tabs 3mg/tablet, Arrow Solutions) were dissolved in 500ml RO water and the solution poured into a glass vacuum desiccator jar. Up to 100 open 1.5ml tubes with 100µl *Arabidopsis* seeds were positioned in a square eppendorf storage box and the box placed in the jar. Five ml of concentrated HCl were added to the water to release the chlorine gas and the lid quickly closed. After 3h the box containing the sterile seeds was moved under a sterile hood, aerated for 1h and the seeds suspended in 500µl 0.15% agar.

2.3 *Arabidopsis* multiplication

Arabidopsis sterilized seeds suspended in 0.15% agar were plated, under a sterile hood, on agar media (1.5% agar, 3% sucrose, Murashige & Skoog 4.3g/l) in 120x120x17mm square Petri plates (Greiner Bio-One GmbH, Germany) sealed with 3M micropore tape and stored at 4°C for 2 days. The plates were moved to 22°C and low light intensity for one week, to allow germination of the plant. Light was provided by a mixture of Philips TLD 36w/89 and General Electric Warm White 2850 Lm F36w/29 tubes adjusted to photon flux density of 100 μmol/m^2/s at plate level. Adequate ventilation was provided to avoid condensation inside the plate. Well established plants were transplanted to Araflats (Arasystem, Betatech bvba, Gent, Belgium) filled with a 3:1 mixture of compost and perlite (John Innes compost No.2, KS Horticultural Products, Seaview Nurseries, Egremont, Cumbria) saturated with water containing 0.2g/l insecticide Intercept 70WS (The Scotts Company Ltd, UK). To plants with a fully matured rosette and the flowering stem about 10cm high was applied the ARACON base + tube system. Plants were regularly watered from below, leaving 1cm of water in the Aratray (Figure 2-1).

Completely dry *Arabidopsis* plants were cut just above the rosette and the Aracon system (tube+base) removed. Wearing exam gloves the whole plant was crushed over two sheets of A4 white paper, the seeds were separated from the rest of the plant with a sieve and stored in a 1.5ml eppendorf tube. All the process was made on a 60 cm x 45 cm raised grid, sitting on an A2 paper, in a seed harvesting tray (Garland Products, Prod. No. G47). Particular attention was paid to avoid cross-contamination of seeds of different plant lines, cleaning throughfully the sieve and the tray with EtOH after each plant. A piece of 1cm^2 micropore tape was placed over the open eppendorf tubes to allow complete essication of the seeds for about 2 weeks.

Figure 2-1. Mature *Arabidopsis* plant in the Aracon System.

Figure 2-2. Germinating seeds, 6 days after sowing.

2.4 Performance Experiments

Plants from total of 6mg seeds (~300 seeds) were grown in two half trays for each genotype used in the experiments. Plants were germinated and grown in half trays of 230x176x55mm (Desch-Plantpak) filled with compost (John Innes compost No.2, KS Horticultural Products, Seaview Nurseries, Egremont, Cumbria) and then saturated with water containing 0.2g/l insecticide Intercept 70WS (The Scotts Company Ltd, UK). Seeds were weighed, mixed with 500μl of sterile silver sand and sprinkled on one half tray. Two half trays could fit in one gravel tray, which was covered with cling film: the name tags were reclined and used to keep the film separated from the soil (Figure 2-2 above).

Sown seeds were placed for 72 hours in a 4°C dark cold room, to allow a subsequent synchronised germination. Trays were then moved to a 21°C growth room with 12L:12D light cycles. After three days several holes were made in the cling film to avoid condensation; the film was removed on day 7. Plants were watered from the bottom of the trays as needed. Light was provided by a mixture of Philips Aquarelle TLD 36w/89 and General Electric Warm White F36w/29 tubes and were adjusted to photon flux density of 200 $\mu mol/m^2/s$ at soil level.

The 15th day 160 plants for each genotype were transplanted (one plant per hole) to 20 hole multicell half trays (MC20, Desch-Plantpak) containing the same soil and left at 21°C to recover from the transplant stress. On day 18 all the trays were moved to test conditions, created ad hoc in two environment controlled plant growth Cabinets (MLR-351 SANYO Electric Co., Japan). Every other day the gravel trays (containing two multicell trays each) were randomly shuffled to avoid differential growth due to positional effect and a photo of one tray per genotype was taken to measure the visible leaf area. Before the plants bolted (about 28 days after) each plant was cut at soil level, individually weighed, wrapped in aluminium foil and frozen in liquid nitrogen, then stored at -80°C.

2.5 Performance Assays

2.5.1 Visible Leaf Area

To follow plants' growth, visible leaf area was measured taking sequential photos (every other day) of two pots (40 plants) per genotype per temperature used in the experiment. Each photo was taken with a DMC-FZ30 digital camera fixed to a Kaiser RS1 camera stand (height=75cm) using the following settings: F3.2, 1/15sec, Iso100, focal length 70 (35mm equiv.), manual focus, "Daylight" white balance (Figure 2-3). To normalize the results in each photo was included a stripe of black & white graph paper and 1cm^2 square of glossy green paper (Dulux Woodland Fern 3, 30GY 27/514). With the MetaMorph image-analysis software (Molecular Devices Corp., USA) a green colour threshold was applied to each image (Colour model: HSI Hue:40-80, Saturation:30-255, Intensity: 60-255), 21 region boxes were drawn to include each plant plus the normalization square and finally the thresholded areas were exported to a Microsoft Excel worksheet (Figure 2-4). In Excel the data were normalized and statistical analysis was performed.

2.5.2 Dry weight

Plant samples to be assayed were quickly moved from the -80°C freezer into the freeze dryer (Edwards Modulyo K4) and dried at -60°C for 36 hours. Dried samples were then weighed on a Fisherbrand PS-60 fine balance and stored in 1.5ml eppendorf tubes for following analysis.

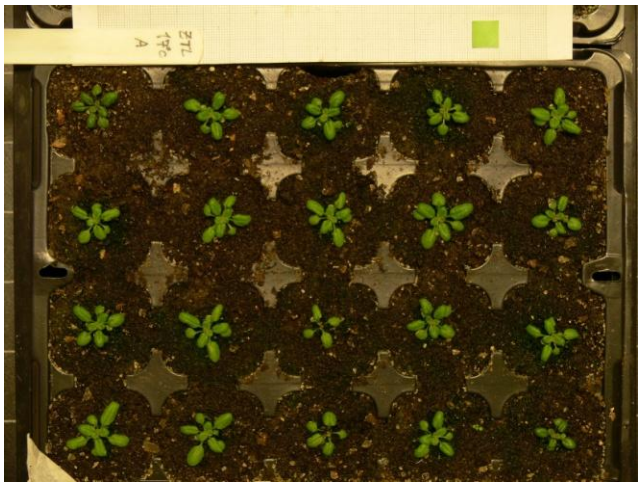


Figure 2-3. 20 hole multicell half tray containing *ztl* plants 25 day after sowing.

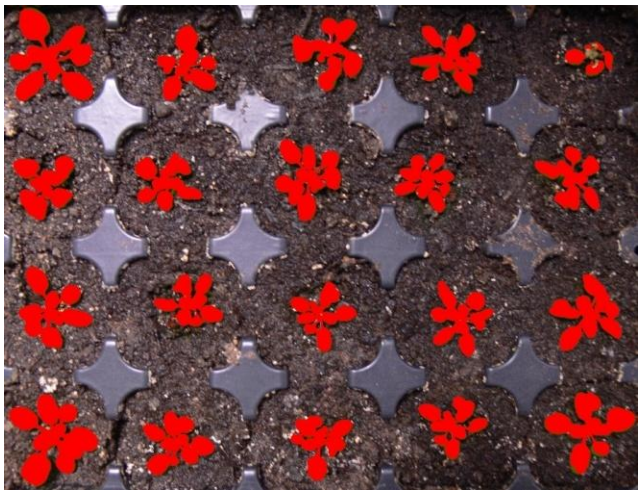


Figure 2-4. Thresholded image of *Arabidopsis* plants, the red area is recognised by the MetaMorph software as "visible leaf area"

2.5.3 Chlorophyll content

Each measurement was done using a minimum of 3-5 biological replicates. A frozen sample of about 100mg (10mg if freeze dried) was placed in a 2ml eppendorf tube, containing 500µl EtOH and one stainless steel bead (5 mm Ø, QIAGEN Cat. No. 69989). The tissue was homogenised using a bead mill (QIAGEN TissueLyser II, Cat. No. 85300) with a 2 x 24 TissueLyser Adapter Set (QIAGEN Cat. No. 69982). After centrifugation at 13000rpm for 2 minutes the supernatant was removed and placed in a new test tube. The extraction was repeated with 500µl EtOH, and after vortexing and a 2 min centrifugation at 13000rpm the supernatant was added to the first. The supernatant was then diluted 2.5 to 5 times, according to the initial amount of tissue. Absorbance was read at 649nm and 665nm using a SpectraMax 340 (Molecular Devices Corp., USA) microplate reader (200µl). The blank was 200µl EtOH. Total chlorophyll was calculated using the equations from Ritchie (Holden, 1976; Ritchie, 2006):

Chl a (µg/ml) = – 5.2007 x A_{649} + 13.5275 x A_{665} (± 0.03125 µg/ml)
Chl b (µg/ml) = 22.4327 x A_{649} – 7.0741 x A_{665} (± 0.02623 µg/ml)
Chl tot (µg/ml) = 17.232 x A_{649} + 6.4534 x A_{665}

Final chlorophyll concentration was expressed as µg/mg of tissue and the amount corrected for the dilution factor applied to the initial extract. The SpectraMax 340 microwell reader was chosen to increase greatly the output and quality of the experimental results. This instrument is able to read a 96 wells plate in less than 30 seconds, export the results to a PC and uses 200µl of sample per well, allowing to increase the number of technical replicates to 5 per biological sample (hence reducing experimental error). Ritchie equations however, are based on 1cm path length standard cuvettes: the equations were therefore corrected with a calibration curve between the two systems.

2.5.4 Data analysis

To obtain robust results, performance experiments were repeated two or more times. When comparing results among repeated experiments, the overall trend of the results was found to be quite uniform, the magnitude of them however was more variable. This is likely to be caused by the initial seed cold stratification and germination stages: these steps allowed a uniform germination of the plants used for the same experiment but even following a rigid protocol, the same could not be achieved completely for different experiments. A good statistical analysis of the results was therefore required.

First of all data was inserted in a Microsoft Access Database (Microsoft Corp., Redmond, WA, USA), creating one table for each performance assay. Each result was inserted as a "record", and for each record several descriptive fields were created: Light cycle, performance experiment number, assay type, temperature, genotype and finally the numeric value of the result. This approach can be lengthy at the stage of inputting the results, but allows extreme flexibility in retrieving the data when needed. With a simple "query" for example it is possible to get all the results of the same temperature, assay, light cycle, genotype or any combination of them, and use the results to draw a graph or perform statistical analysis.

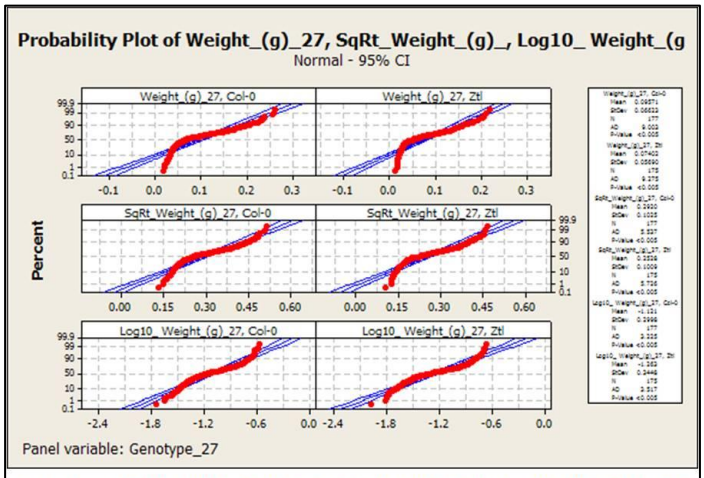
In the Compact Disc attached to this thesis it is possible to find the aforementioned database, containing three data tables and a number of queries. To allow a meaningful sorting, queries were prefixed with AA (fresh weight), AB (dry weight), BA (chlorophyll on fresh weight), BB (chlorophyll on dry weight), LA (Leaf Area) and GR for the data used to produce thesis figures with SPSS (IBM Corporation, Route 100 Somers, NY 10589). For users who don't have the Microsoft Access software, data tables were exported in more common formats: Microsoft Excel, tab delimited text (.txt) and comma separated values (.csv).

Through an ODBC connection, data was imported from the Access Database into the Minitab software (Minitab Inc., State College, PA, USA) and two different transformations were applied: Log10 and SquareRoot. The rationale behind transforming data is to obtain a set of values with a normal distribution (checked with a probability plot,

Figure 2-5) and equal variances of groups being compared (checked with descriptive statistics, Figure 2-5). A normal distribution and equal variances are a prerequisite for the following Analysis of Variance (ANOVA). In most cases, log10 transformation was used.

Performing an analysis of variance requires first of all knowing the structure of data. Completely randomised block design is the experimental design used for all performance experiments. In detail the fixed effects were: performance experiments (the blocks), genotypes, light cycle and temperature (the treatments). Within each experiment there were also random effects: 2 pots and 20 plants per pot. As the number of plants survived was different for each experiment, data resulted unbalanced: this required, to perform the analysis of variance, the use of the General Linear Model (GLM) tool in Minitab. ANOVA tells whether samples are statistically different from each other, but not which (and how much) is different from which: to obtain this information too, a following Tukey test (with 95% confidence intervals) was performed taking into account the "Center value" as shown in Figure 2-6.

At the end of each results subchapter, a table summarises the descriptive statistics of all experiments performed. For every assay and genotype, the number of independent experimental replicates used for the ANOVA is indicated. For each replicate (both at 17°C and 27°C) is then reported the number of biological samples (N), the Mean, Standard Error of the Mean (SEM), Standard Deviation and Variance (i.e. Table 5, page 48).



Descriptive Statistics: Weight_(g)_27, SqRt_Weight_(g)_27, Log10_ Weight_(g)_27

Variable	Genotype_27	N	N*	Mean	SE Mean	StDev	Variance
Weight_(g)_27	Col-0	177	0	0.09571	0.00498	0.06623	0.00439
	Ztl	175	0	0.07402	0.00430	0.05690	0.00324
SqRt_Weight_(g)_27	Col-0	177	0	0.29200	0.00770	0.10251	0.01051
	Ztl	175	0	0.25280	0.00762	0.10086	0.01017
Log10_ Weight_(g)_27	Col-0	177	0	-1.1211	0.0225	0.2998	0.0899
	Ztl	175	0	-1.2625	0.0261	0.3448	0.1189

Figure 2-5. Example of Probability plot (Above) and Descriptive statistics (Below) calculation in Minitab.

General Linear Model: Log10_ Weight_(g versus Genotype_27, Perf_Exp_27

Factor	Type	Levels	Values
Genotype_27	fixed	2	Col-0, Ztl
Perf_Exp_27	fixed	3	P1, P4, P7

Analysis of Variance for Log10_ Weight_(g)_27, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Genotype_27	1	1.7598	1.9080	1.9080	76.04	0.000
Perf_Exp_27	2	27.7763	27.7763	13.8881	553.47	0.000
Error	348	8.7323	8.7323	0.0251		
Total	351	38.2684				

S = 0.158408 R-Sq = 77.18% R-Sq(adj) = 76.98%

Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Log10_ Weight_(g)_27
All Pairwise Comparisons among Levels of Genotype_27
Genotype_27 = Col-0 subtracted from:

Genotype_27	Lower	Center	Upper	------+---------+---------+---------+
Ztl	-0.1805	-0.1473	-0.1140	(------*-----)
				------+---------+---------+---------+
				-0.150 -0.100 -0.050 -0.000

Tukey Simultaneous Tests
Response Variable Log10_ Weight_(g)_27
All Pairwise Comparisons among Levels of Genotype_27
Genotype_27 = Col-0 subtracted from:

Genotype_27	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
Ztl	-0.1473	0.01689	-8.720	0.0000

Figure 2-6. Example of ANOVA (Above) and Tukey 95% test (Below) outputs in Minitab.

2.6 Screen Experiments

For the screening experiments were used the complete set of activation tagged lines from Scheible and Somerville (N31100) and from Yokoi, Koiwa, Bressan (N31400, N31402, N31404), all obtained from the European *Arabidopsis* Stock Centre (NASC). The relevant wild-type background (Col-2 for Scheible and Somerville and C24 for Yokoi, Koiwa, Bressan) were grown along with the plant lines screened. 3mg of seeds (~150 seeds) from each tube provided by NASC were germinated and grown in the same way as done for the performance experiments up to day 10 (see 2.4 Performance Experiments): sown seeds were placed for 72 hours in a 4°C dark cold room, then moved to a 21°C growth room in 12L:12D light cycles.

After 11 days the growth chamber temperature was raised to 27°C and started the observation of the plants' phenotype. All plants were continuously monitored for unusual morphological features, such as hypocotyl and leaves elongation, leaf colour and distortion, petioles length, early flowering. When a plant showed a phenotype different from the phenotype of the wild-type plants, a toothpick was planted next to it and a number was assigned: the plant was periodically observed to spot any change in its phenotype, which was inserted in an Excel database. The plants which showed the most interesting phenotypes were transplanted to an Araflat and grown for seed as explained in the *Arabidopsis* multiplication section. The experiment was terminated about 27 days after, when all the plants flowered.

2.7 Re-Screen Experiments

The putative mutant lines selected in the screening experiments were transplanted, grown to set seed and the seeds harvested. These seeds were used for the re-screening experiment, whose aim is to determine if the phenotype spotted in the screen is expressed only at high temperatures. For this purpose were prepared 32 half trays filled with compost (John Innes compost No.2) and then saturated with water containing 0.2g/l insecticide Intercept 70WS. Each tray was divided in four sections and in three of them were sown 1mg of seeds (~50 seeds) of a different putative mutant, in the fourth 1mg of seeds of the wild-type Col-2 as control (Figure 2-7). The same three lines plus WT were sown again in an identical pot; in 32 pots were therefore sown 48 different putative mutants in two replicates. The sown seeds were stratified for 48 hours in a 4°C dark cold room, trays were then moved to two environment controlled plant growth Cabinets (MLR-351 SANYO Electric Co., Japan) set at the temperatures of 17 and 27°C with 12L:12D light cycles. Water was provided from the bottom of the trays as needed. Plants were constantly monitored for altered morphological features and flowering time and compared both to the wild-type sown in the same pot and the plants of the same genotype sown in the other cabinet. The genotypes which showed an altered phenotype only at 27°C were selected for the leaf movement experiments to look at their circadian phenotype.

Figure 2-7. Pot of plants during a re-screen experiment.

2.8 Leaf Movement

The aim of the leaf movement experiment is to measure the circadian phenotype of the putative mutant plant lines selected from re-screening experiments. About 50 *Arabidopsis* seeds were sterilized, under a sterile hood, in a 1.5ml eppendorf tube and suspended in 500μl 0.15% agar. The seeds were plated singularly, distant 1cm from each other, on agar media (1.5% agar, 3% sucrose, Murashige&Skoog 4.3g/l) in 120x120x17mm square Petri plates (Greiner Bio-One GmbH, Germany) sealed with 3M micropore tape and stored at 4°C for 2 days. A total of 8 different lines were plated, one line per plate, two plates per line. The plates were moved to 22°C and 12h light/12h dark cycles, to allow germination of the plant. Light was provided by a mixture of Philips TLD 36w/89 and General Electric Warm White 2850 Lm F36w/29 tubes adjusted to photon flux density of 100 μmol/m^2/s at plate level. Adequate ventilation was provided to avoid condensation inside the plate. After ten days the plates were opened, always in sterile conditions, and the plants transferred to a 100mm square Petri dish with 25 compartments (Cat. No.103, Sterilin, Barloworld Scientific). Each plant was removed from the original dish cutting a 1cm^2 square of agar around the plant with an ethanol flamed scalpel, then placed in one compartment with the two cotyledons parallel to the line of sight. Plants were placed only in the three central rows (2,3 and 4) and in the 4 angles 150μl of water were pipetted (Figure 2-8B). The plate was sealed with Parafilm M (Pechiney Plastic Packaging Company) and, always kept in vertical position, moved back to the original growth room. A total of 15 plants were moved to each plate, and two plates per genotype were prepared. The following day each plate was placed in vertical position in front of a Sony Exwave HAD Black & White video security camera, fitted in an environment controlled plant growth Cabinet (MLR-351 SANYO Electric Co., Japan) set at the experimental conditions with constant light (Figure 2-8A). The 16 cameras (8 cameras per cabinet) were controlled by a Nortek RC616 controller connected to a computer: with the MetaMorph software (Molecular Devices Corp., USA) 1 picture every 20 min was taken for 10 days.

With the MetaMorph software, a threshold was applied to each image of the series, isolating the plant silhouette from the background; a region was then drawn around each leaf and the coordinates of a centroid were positioned in the centre of the leaf. Through MetaMorph the X (horizontal) and Y (vertical) position values of the centroid were logged and exported to a Microsoft Excel worksheet. A 80 hours window of data was chosen to be analysed, always excluding the first 24h, to eliminate any adverse effect that adaptation to the new experimental conditions may have had on the seedlings. Data analysis by fast Fourier transform nonlinear least-squares (Plautz *et al.*, 1997) was performed with the Biological Rhythm Analysis Software System (BRASS) package, available from **www.amillar.org** (Edwards *et al.*, 2005). For each leaf were generated a circadian period estimate and a real amplitude error (RAE). The period estimate is a weighed mean, while RAE is a measure of rhythm robustness varying from 0 (a perfect fit to the cosine wave) to 1 (not statistically significant, rhythm not robust).

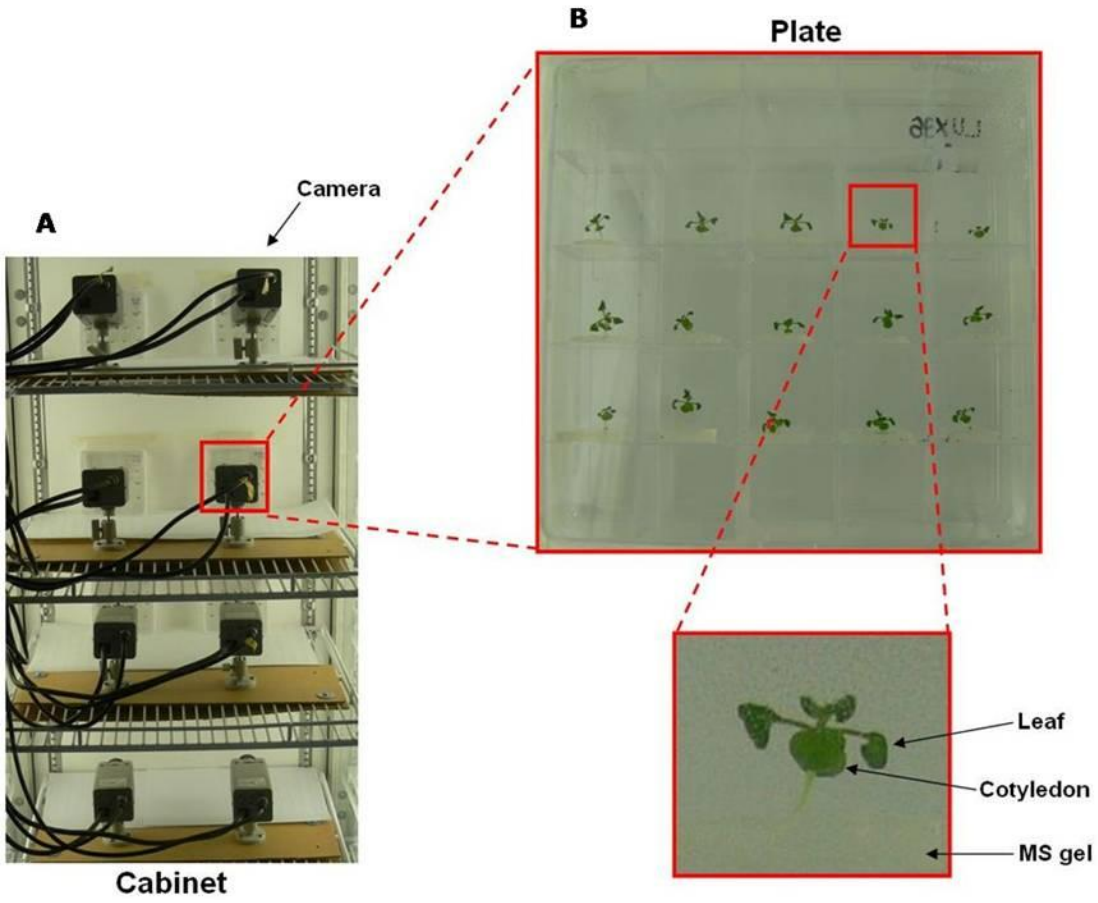


Figure 2-8. Leaf Movement System.

2.9 Quantitative PCR analysis

Arabidopsis seeds were sterilized (Surface seed sterilization, page 21), under a sterile hood, in a 1.5ml eppendorf tube and suspended in 500μl 0.15% agar. Seeds were plated in six groups of about 100 on agar media (2% agar, no sucrose, Murashige&Skoog 2.15g/l) in 120x120x17mm square Petri plates (Greiner Bio-One GmbH, Germany) sealed with 3M micropore tape and stored at 4°C for 2 days for stratification. Plates were moved to 21°C and 12h light /12h dark cycles, to allow germination of the plant. Light was provided by a mixture of Philips TLD 36w/89 and General Electric Warm White 2850 Lm F36w/29 tubes adjusted to photon flux density of 100 μmol/m^2/s at plate level. Adequate ventilation was provided to avoid condensation inside the plate. After six days the plates were moved to test conditions (12LD light cycles, 17°C or 27°C), created ad hoc in two environment controlled plant growth Cabinets (MLR-351 SANYO Electric Co., Japan).

After three days of entrainment aerial part of seedlings was harvested intact into liquid nitrogen in six time points: ZT 1, 6, 11, 13, 18, 23. To each time point frozen sample were added 450μl of RLT buffer, then a stainless steel bead (5 mm Ø, QIAGEN Cat. No. 69989). The tissue was homogenised using a bead mill (QIAGEN TissueLyser II, Cat. No. 85300) with a 2 x 24 TissueLyser Adapter Set (QIAGEN Cat. No. 69982). Lysed tissue was placed with its tube in the QiaCube and total RNA was extracted using RNeasy plant mini kit consumables and program (Qiagen). RNA quality was assayed with a denaturing formaldehyde gel and its purity with an absorbance reading (NanoDrop ND-1000, Thermo Scientific). 0.5 μg of template RNA was reverse transcribed using Qiagen QuantiTect Reverse Transcription Kit with random hexamer primers following the manufacturer's instructions.

Abundance of the target transcript was quantified relative to the housekeeping gene *UBQ10* with quantitative real-time PCR in a 7500 Fast Real-Time PCR System (Applied Biosystems). Each RNA sample was assayed in triplicate. Quantification data was acquired with Applied Biosystems 7500 Software v2.01. Amplification efficiency values for each set of primers were determined measuring transcript abundance from a five-points cDNA dilution series. Primers efficiency and normalization calculations were performed with the

aid of the Q-gene software (Muller *et al.*, 2002). Details on the PCR program, reaction mix and the primers used for transcript quantification are listed in Table 1, Table 2 and Table 3 respectively.

In particular primers for endogenous *CCA1* gene quantification were designed to amplify a sequence of 150-300bp spanning across the start of translation: from the 5'UTR to the start of the *CCA1* gene. This allows to amplify only mRNA produced from genomic DNA and not from the over-expressing construct transformed into the plant. This has been already accomplished by Wang and Tobin, 1998: they designed a probe for RNA gel blot analysis, starting from -68bp before the beginning of 5'UTR to +186 after. Analysing the position where they designed the probes, two sets of primers were designed with the Primer3 program available online (http://frodo.wi.mit.edu/primer3/input.htm).

Temperature (°C)	Time (sec)	Cycles
95	600	1
95	3	40
60	30	
95	15	1
60	60	
95	15	
60	15	

Table 1. qPCR program used for transcript quantification.

Component	Amount (µl)	Final Conc (µM)
SYBR Green Mix	5	1x
Primer FW	0.5	0.5
Primer RW	0.5	0.5
Water	3	
cDNA	1	
Total Volume	10	

Table 2. qPCR reaction mix used for transcript quantification.

Primer Name	Sequence (5' - 3')
CCA1 FW	TCTGTGTCTGACGAGGGTCG
CCA1 RW	ACTTTGCGGCAATACCTCTCTGG
CCA1_Endo FW	TCGAATCCAAGCTGATTTTG
CCA1_Endo RW	GTCCACCTTTCACGTTGCTT
LHY FW	ACGAAACAGGTAAGTGGCGACATT
LHY RW	TGGGAACATCTTGAACCGCGTT
LUC FW	GATGCACATATCGAGGTGGA
LUC RW	ATAAATAACGCGCCCAACAC
UBQ FW	CACACTCCACTTGGTCTTGCGT
UBQ RW	TGGTCTTTCCGGTGAGAGTCTTCA

Table 3. List of primers used for transcript quantification.

2.10 Microarray experiment

Seeds from the over-expressed CCA1ox-o38 *Arabidopsis* line with no functional clock and the wild-type Columbia-0 were sown and grown as in the performance experiments up to day 17. At day 18 plants were then transferred to 12L:12D cycles (160 μmol/m^2/s) and temperatures of 17ºC or 27ºC. Plants were grown for a further 7 days at these two temperatures, until six to eight leaves had developed (developmental stage 1,6-1,8). At this point none of the plants had flowered. Plant material was collected one hour before subjective dawn and one hour after subjective dusk. A green safety light was used to harvest plants prior to dawn cutting at soil level; each sample was wrapped in aluminium foil and frozen in liquid nitrogen, then stored at -80°C. A composite sample of 10 plants was collected for each treatment combination, in total eight samples: 2 genotypes x 2 temperatures x 2 sample times.

Total RNA was extracted from the samples, using Qiagen RNAeasy Plant Mini Kit and sent to NASC for Array hybridisation using an Affymetrix ATH1 chip, ca. 22,800 genes (NASC Transcriptomics Service, http://affymetrix.arabidopsis.info/). Results data obtained from NASC (NASCArrays-436,) was normalized using the GC RMA file pre-processor normalization in the GeneSpring program (Agilent Technologies) and analysed with MapMan for the functional analysis of gene responses (Thimm *et al.*, 2004).

CHAPTER 3 - A functional clock (CCA1ox line)

3.1 Introduction

One of the main aims of this project was to test the importance for an *Arabidopsis* plant of having a functional clock at high temperatures. Recent research proved that at 20°C a functional clock can increase plants' fitness (Dodd *et al.*, 2005), however, nothing is known about the interactions between high temperatures and the clock in regulating plants' performance. In a perspective of raising global temperatures, the impact on crop production with a non optimised clock could be of major importance. An image to better explain this concept is the limping runner. While walking, performance of person with a lame leg could be only slightly impaired, if not comparable, to that of a healthy man. If the two start running however, the differences would become evident. In the same way a plant could withstand a defect in clock functionality, but consequences could become dramatic in a stressful environment.

To test the outlined hypothesis, the CCA1ox line was chosen, together with the wild-type Col-0. The CCA1ox o38 line was obtained by transformation of Columbia WT plants with a 35S:CCA1 construct (Wang and Tobin, 1998). In CCA1ox plants, the over-expression of the single gene *CCA1* generates a complete arrhythmic phenotype both in leaf movement and *CAB* (*Lhcb*) gene expression. This is caused by a complete blockage of the circadian core oscillator, with suppression of two of its core components, *CCA1* and *LHY*. In addition to the clock phenotype the growth and development of the plant is profoundly affected, with very long and weak hypocotyl, elongated stems and late flowering (Wang and Tobin, 1998). Phenotypic alterations were then associated with a reduced fitness at 23°C, when Col-0 plants are grown in comparison to those over-expressing *CCA1*. In fact, CCA1ox plants were found to have lost the ability to anticipate the arrival of daylight and were less viable under very short-day conditions (Green *et al.*, 2002). Later on Dodd *et al.*, 2005 quantified these alterations at 20°C, finding CCA1ox-o38 plants fixed less carbon, produced less chlorophyll, aerial biomass and leaf area than the wild-type Col-0.

Over-expression of the single *CCA1* gene is able to generate the observed phenotypes due to its central role in the clock oscillator. *CCA1* was one of the first circadian clock genes to be discovered in plant circadian research as a myb-related transcription factor, binding to the promoter of the CAB gene and expressed with a diurnal rhythm peaking around one hour after dawn (Wang *et al.*, 1997; Wang and Tobin, 1998). Partially redundant to the *LHY* gene, *CCA1* binds with it to the pseudoresponse regulator *TOC1* promoter, creating the core negative feedback loop generating circadian rhythmicity (Alabadi *et al.*, 2001). *CCA1* expression is then directly repressed by the *CHE* transcription factor and indirectly activated by *TOC1*, which antagonizes *CHE* eliminating the repression (Pruneda-Paz *et al.*, 2009). At the post-transcriptional level, *CCA1* mRNA stability is controlled by light (Yakir *et al.*, 2007b); as a protein CCA1 is able to form homodimers, and with LHY even heterodimers (Lu *et al.*, 2009; Yakir *et al.*, 2009). Finally *CCA1,* together with *TOC1 LHY* and *GI* genes, was found to be a component of the circadian temperature compensation mechanism. *cca1-11* mutants are unable to compensate for temperature changes and *CCA1* role becomes critical to maintain a constant rhythm at lower temperatures (Gould *et al.*, 2006).

Being able to stop completely the plants' circadian mechanism, *CCA1* over-expressing lines were therefore chosen to test the importance of circadian functionality at high temperature. Initially performance of CCA1ox and Col-0 plants was quantified and compared. Experiments were performed in standard light conditions (12h Light/12h Dark) and at the two temperatures of 17ºC and 27ºC. Each experimental condition was repeated up to four times for Col-0 and CCA1ox-o38 lines, and up to two times for the CCA1ox-o34 line. Col-0 plants were found to be better performers than CCA1ox-o38, and this difference always increased with temperature (3.2.1, page 41). This was confirmed by transcript (3.2.3, page 59) and metabolic profiling (3.2.4, page 64). Finally monitoring the expression of *CCA1* and other genes driven by the 35S promoter (3.2.2.2, page 56), highlighted the tendency of the promoter to be increasingly expressed at high temperature, raising questions about the interpretation of the performance results.

3.2 Results

3.2.1 - Col-0 performs better than CCA1ox in 12h L/12h D

Across the experiments done the performance of the arrhythmic line CCA1ox o38 was found to be lower than Col-0: at 27ºC this difference was even greater than at 17ºC, showing a definite temperature effect. These performance differences were clearly visible even from the initial visual inspection of the seedlings (Figure 3-1): at 17ºC all plants are green, small and compact. At 27ºC wild-type plants remain healthy and green, with elongated petioles; CCA1ox o38 plants however show a much stressed phenotype, with very long etiolated petioles, a long hypocotyl and small leaves.

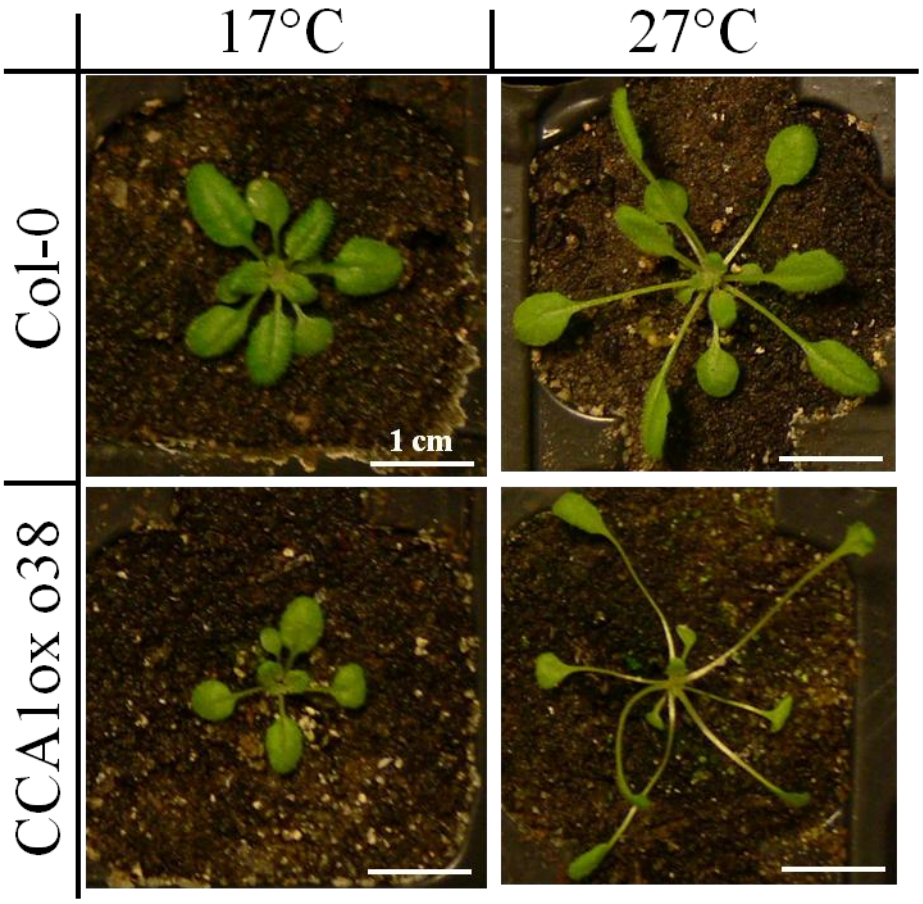


Figure 3-1. Col-0 and CCA1ox o38 plants, grown at 17ºC and 27ºC. The photo was taken 25 day after sowing, white bars equal to 1cm.

To quantify the observed phenotypes visible leaf area was measured (Figure 3-2). Over the two temperatures CCA1ox o38 plants had the same leaf area (P=0.435) while Col-0 had a greater leaf area at higher temperature (**P<0.001). Balancing wild-type against the mutant line within each temperature, at 17°C shows CCA1ox o38 plants with a reduced performance when compared to Col-0 (**P<0.001); difference that is even greater at 27°C (**P<0.001) where Col-0 plants have more than twice the leaf area of CCA1ox o38 plants.

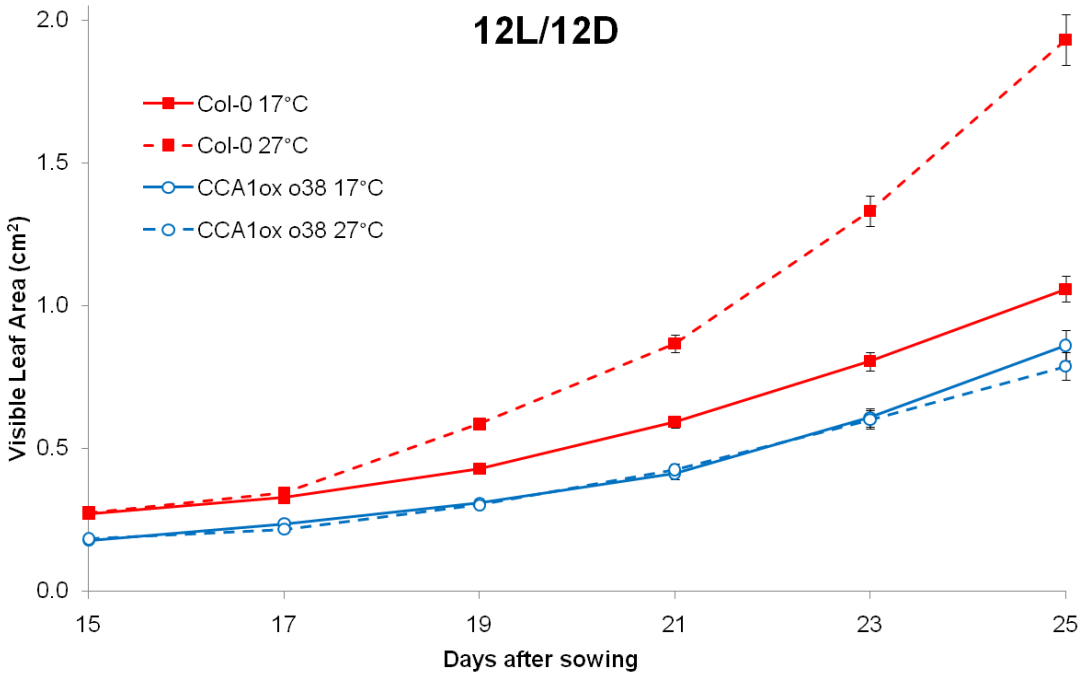


Figure 3-2. Visible leaf area of Col-0 and CCA1ox o38 plant lines 15 to 25 day after sowing, grown at 12h L/12h D light cycle and 17ºC or 27ºC. Data is mean ±1SEM. At day 25 in ANOVA test comparing CCA1ox to Col-0, **P<0.01 at 17°C and 27°C. Figure representative of four independent replicates.

To exclude the possibility that the reduced performance measured in CCA1ox o38 line is merely due to the insertion site of the transgene in the plant genome, the CCA1ox 034 plant line was also used. This arrhythmic line was obtained in the same way CCA1ox o38 was. The performance of the CCA1ox 034 line follow the same trends of CCA1ox o38 (Figure 3-3 to Figure 3-6), confirming that the over-expression of the *CCA1* gene is the cause of the measured phenotype.

Fresh weight results (Figure 3-3) follow the same trends just seen in visible leaf area, especially regarding the differences among genotypes. Between 17 and 27°C all the three genotypes tested gained weight with the raise of growth temperature, giving very significant results (**P<0.001 for all in ANOVA). A comparison within each temperature remarks the better performance of Col-0 over CCA1ox o38/o34 lines, having always greater weight values, at both temperatures (**P<0.001 for all in ANOVA). In Figure 3-3 for example, CCA1ox had about 55% less fresh weight than Col-0 at 17°C and 65% less at 27°C. The magnitude of these differences however increases against CCA1ox o38 but decreases against CCA1ox o34.

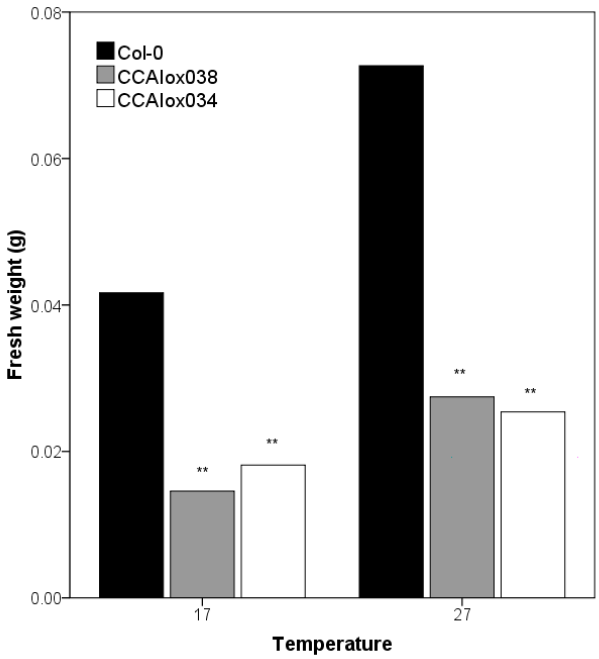


Figure 3-3. Fresh weight of Col-0 and CCA1ox o38/o34 plant lines 25 day after sowing, grown at 12h L/12h D light cycle and 17ºC or 27ºC. Data is mean, **P<0.01 in ANOVA test comparing CCA1ox to Col-0. Figure representative of four independent replicates for CCA1ox o38 and two for CCA1ox 034.

Differences among genotypes are once again confirmed by dry weight results (Figure 3-4). Matching Col-0 against the two CCA1ox lines showed Col-0 plants heavier both at 17°C and at 27°C, all with significant ANOVA P-Values of less than 0.001. In Figure 3-4 for example CCA1ox had about 50% less fresh weight than Col-0 at 17°C and 65% less at 27°C, with differences increasing with temperature. Comparing 17°C to 27°C however revealed quite diversified results: the wild-type had a greater weight at high temperature (**P<0.001, ANOVA), CCA1ox o38 had a greater weight at 17°C (*P=0.028, ANOVA) and CCA1ox o34 did not show any difference between the two temperatures (P=0.646, T-test).

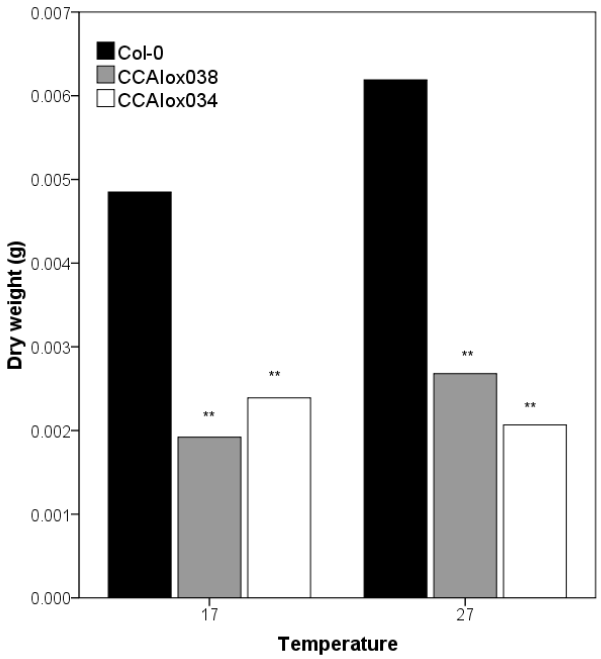


Figure 3-4. Dry weight of Col-0 and CCA1ox o38/o34 plant lines 25 day after sowing, grown at 12h L/12h D light cycle and 17ºC or 27ºC. Data is mean, **P<0.01 in ANOVA test comparing CCA1ox to Col-0. Figure representative of four independent replicates for CCA1ox o38 and one for CCA1ox 034 (T-Test).

Total chlorophyll content on fresh weight (Figure 3-5) confirms leaf area results only at high temperature. In fact, juxtaposing the genotypes within each temperature, shows at 17°C an equal amount of chlorophyll for all three lines: probability values were of P=0.133 (ANOVA) for Col versus CCA1ox o38 and P=0.261 (T-test) for Col versus CCA1ox o34. As said at 27°C things are different: Col-0 has greater chlorophyll content than CCA1ox lines, with significant P-values of **P<0.001 for both. In Figure 3-5 for example, CCA1ox had about 30% less chlorophyll than Col-0. Comparing 17°C to 27°C, all three lines showed an increased amount of chlorophyll at 17°C, with **P= 0.006 for Col-0 and ** P<0.001 for CCA1ox o38/o34.

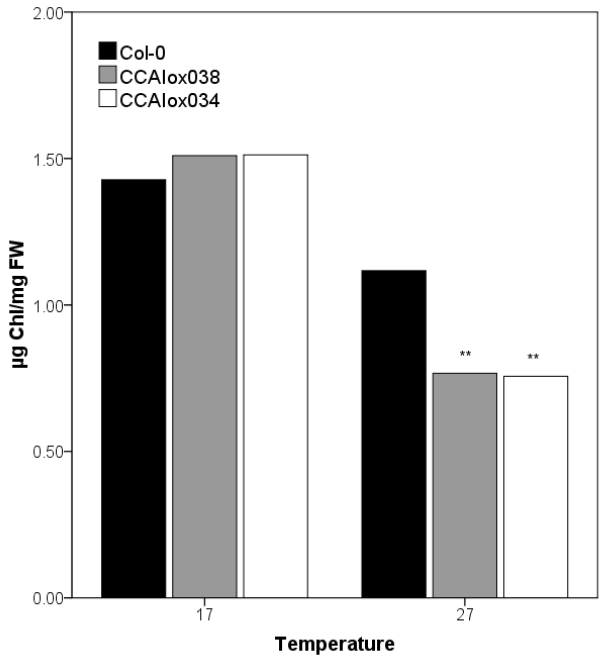


Figure 3-5. Total chlorophyll content (on fresh weight) of Col-0 and CCA1ox o38/o34 plant lines 25 day after sowing, grown at 12h L/12h D light cycle and 17ºC or 27ºC. Data is mean, **P<0.01 in ANOVA test comparing CCA1ox to Col-0. Figure representative of four independent replicates for CCA1ox o38 and one for CCA1ox 034 (T-Test).

Results from quantification of total chlorophyll on a dry weight basis (Figure 3-6) matched leaf area only for CCA1ox o38, and were quite different for CCA1ox o34. Analysing 17°C against 27°C resulted in Col-0 having a greater concentration at 27°C (**P<0.001) and in CCA1ox o38/o34 lines showing no temperature related change in concentration (P= 0.103 for o38 and P=0.127 for o34) Comparing the lines within the same temperature shows Col-0 greater than CCA1ox o38 at both temperatures, with ANOVA values of *P= 0.028 at 17°C and **P<0.001 at 27°C. On the other side Col-0 was greater than CCA1ox o34 only at 17°C (*P=0.032 in T-test), but not at 27°C (P=0.102 in T-test).

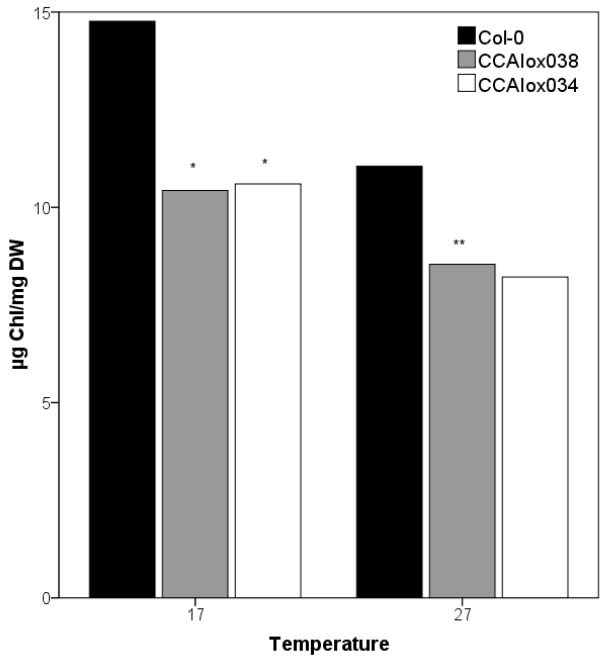


Figure 3-6. Total chlorophyll content (on dry weight) of Col-0 and CCA1ox o38/o34 plant lines 25 day after sowing, grown at 12h L/12h D light cycle and 17ºC or 27ºC. Data is mean, **P<0.01 and *P<0.05 in ANOVA test comparing CCA1ox to Col-0. Figure representative of four independent replicates for CCA1ox o38 and one for CCA1ox 034 (T-Test).

Summary of Results

Col-0 and CCA1ox o38 in 12hL/12hD light cycles

Leaf Area - 25 days				12LD
Different?	**How?**	**P value**	**Diff.**	
Yes **	Col-0 > CC38	<0.001	0.231	**17°C**
Yes **	Col-0 > CC38	<0.001	0.498	**27°C**
Yes				> with T°
Yes **	27 > 17	<0.001	0.507	**Col-0**
No	-	0.435	-	**CCA1ox-o38**

Fresh Weight				12LD	Dry Weight			
Different?	**How?**	**P value**	**Diff.**		**Different?**	**How?**	**P value**	**Diff.**
Yes **	Col-0 > CC38	<0.001	0.259	**17°C**	Yes **	Col-0 > CC38	<0.001	0.236
Yes **	Col-0 > CC38	<0.001	0.373	**27°C**	Yes **	Col-0 > CC38	<0.001	0.493
Yes				> with T°	Yes			
Yes **	27 > 17	<0.001	0.323	**Col-0**	Yes **	27 > 17	<0.001	0.202
Yes **	27 > 17	<0.001	0.144	**CCA1ox-o38**	Yes *	17 > 27	0.028	0.061

Chl FW				12LD	Chl DW			
Different?	**How?**	**P value**	**Diff.**		**Different?**	**How?**	**P value**	**Diff.**
No	-	0.133	-	**17°C**	Yes *	Col-0 > CC38	0.028	0.068
Yes **	Col-0 > CC38	<0.001	0.210	**27°C**	Yes **	Col-0 > CC38	<0.001	0.169
Yes				> with T°	Yes			
Yes **	17 > 27	0.006	0.051	**Col-0**	Yes **	27 > 17	<0.001	0.116
Yes **	17 > 27	<0.001	0.289	**CCA1ox-o38**	No	-	0.103	-

Table 4. Summary of results from the assays done on Col-0 and CCA1ox o38 plant lines, grown in 12h L/12h D light cycles and 17ºC or 27ºC. "P value" is probability from Analysis of Variance and "Diff." is the center value from Tukey test, both done as described in 2.5.4 Data analysis. ">with T°" indicates whether the difference between genotypes is increasing with the rise of temperature. *=P<0.05, **=P<0.01

Descriptive Statistics of all replicates

Col-0 and CCA1ox o38 in 12hL/12hD light cycles

Leaf Area 25d		N		Mean		SE Mean		St Dev		Variance	
	Replicate	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C
Col-0	1	20	20	1.4925	4.1947	0.1324	0.2827	0.5923	1.2643	0.3508	1.5984
	2	39	40	1.5574	2.9969	0.0627	0.1725	0.3915	1.0911	0.1533	1.1905
	3	39	36	0.8835	1.5396	0.0550	0.1197	0.3435	0.7185	0.1180	0.5162
	4	40	40	1.0583	1.9310	0.0454	0.0881	0.2869	0.5571	0.0823	0.3104
CC 38	1	20	20	0.6546	0.9851	0.0496	0.0566	0.2218	0.2533	0.0492	0.0642
	2	40	39	0.6706	0.7550	0.0387	0.0445	0.2451	0.2779	0.0601	0.0772
	3	40	38	0.6277	0.5482	0.0396	0.0349	0.2507	0.2151	0.0629	0.0463
	4	40	40	0.8615	0.7882	0.0538	0.0478	0.3403	0.3024	0.1158	0.0914

Fresh Weight		N		Mean		SE Mean		St Dev		Variance	
	Replicate	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C
Col-0	1	79	78	0.0633	0.1570	0.0019	0.0061	0.0172	0.0536	0.0003	0.0029
	2	40	40	0.0417	0.0727	0.0020	0.0040	0.0128	0.0255	0.0002	0.0007
	3	38	38	0.0219	0.0311	0.0013	0.0021	0.0079	0.0127	0.0001	0.0002
	4	40	40	0.0272	0.0419	0.0011	0.0021	0.0072	0.0131	0.0001	0.0002
CC 38	1	80	62	0.0366	0.0463	0.0014	0.0021	0.0128	0.0164	0.0002	0.0003
	2	40	40	0.0146	0.0275	0.0008	0.0019	0.0052	0.0118	0.0000	0.0001
	3	40	39	0.0143	0.0189	0.0009	0.0013	0.0059	0.0078	0.0000	0.0001
	4	38	40	0.0210	0.0279	0.0012	0.0016	0.0076	0.0099	0.0001	0.0001

Dry Weight		N		Mean		SE Mean		St Dev		Variance	
	Replicate	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C
Col-0	1	48	65	0.0071	0.0137	0.0003	0.0006	0.0019	0.0046	0.0000	0.0000
	2	10	10	0.0049	0.0062	0.0005	0.0007	0.0015	0.0023	0.0000	0.0000
	3	10	10	0.0034	0.0033	0.0003	0.0003	0.0008	0.0010	0.0000	0.0000
	4	10	10	0.0036	0.0042	0.0002	0.0004	0.0007	0.0014	0.0000	0.0000
CC 38	1	54	43	0.0043	0.0035	0.0002	0.0002	0.0014	0.0015	0.0000	0.0000
	2	10	10	0.0019	0.0027	0.0002	0.0003	0.0007	0.0009	0.0000	0.0000
	3	10	9	0.0022	0.0023	0.0002	0.0003	0.0007	0.0008	0.0000	0.0000
	4	9	10	0.0026	0.0020	0.0002	0.0002	0.0007	0.0005	0.0000	0.0000

Chl FW		N		Mean		SE Mean		St Dev		Variance	
	Replicate	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C
Col-0	1	6	6	0.9933	0.9902	0.0335	0.0560	0.0820	0.1371	0.0067	0.0188
	2	5	5	1.4273	1.1169	0.0557	0.0483	0.1246	0.1079	0.0155	0.0116
	3	5	5	1.1150	1.1376	0.0548	0.0297	0.1226	0.0664	0.0150	0.0044
	4	5	5	1.7847	1.5018	0.0702	0.0232	0.1569	0.0518	0.0246	0.0027
CC 38	1	4	4	1.1205	0.5231	0.0802	0.0241	0.1603	0.0483	0.0257	0.0023
	2	4	5	1.5097	0.7668	0.0940	0.0218	0.1881	0.0488	0.0354	0.0024
	3	5	5	1.4196	0.7826	0.0730	0.0297	0.1632	0.0664	0.0266	0.0044
	4	4	5	1.7674	0.8598	0.3153	0.0300	0.6307	0.0670	0.3978	0.0045

Chl DW		N		Mean		SE Mean		St Dev		Variance	
	Replicate	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C
Col-0	1	5	24	59.3600	82.1388	6.1897	2.0895	13.8406	10.2364	191.5630	104.7840
	2	5	5	14.7643	11.0546	1.3992	0.9624	3.1287	2.1520	9.7890	4.6310
	3	5	5	9.0023	10.1962	0.8451	1.0007	1.8897	2.2377	3.5710	5.0070
	4	5	5	11.5483	13.2915	0.3697	0.6711	0.8267	1.5006	0.6830	2.2520
CC 38	1	5	4	62.5785	64.2328	3.9394	6.4476	8.8087	12.8953	77.5930	166.2880
	2	5	5	10.4306	8.5413	1.3230	0.8691	2.9583	1.9433	8.7510	3.7770
	3	5	5	8.7200	6.8821	0.8972	1.7297	2.0062	3.8676	4.0250	14.9590
	4	4	5	8.3805	7.7720	0.2286	0.7351	0.4572	1.6436	0.2090	2.7020

Table 5. Descriptive Statistics of all the independent experimental replicates of the assays performed on Col-0 and CCA1ox o38 lines. Plants were grown in 12h L/12h D light cycles and 17ºC or 27ºC.

Summary of Results

Col-0 and CCA1ox o34 in 12hL/12hD light cycles

Leaf Area - 25 days				12LD
Different?	**How?**	**P value**	**Diff.**	
Yes **	Col-0 > CC34	<0.001	0.200	**17°C**
Yes **	Col-0 > CC34	<0.001	0.431	**27°C**
Yes				> with T°
Yes **	27 > 17	<0.001	0.507	**Col-0**
No	-	0.258	-	**CCA1ox-o34**

Fresh Weight				12LD	Dry Weight (T-Test)			
Different?	**How?**	**P value**	**Diff.**		**Different?**	**How?**	**P value**	**Diff.**
Yes **	Col-0 > CC34	<0.001	0.235	**17°C**	Yes **	Col-0 > CC34	0.003	0.318
Yes **	Col-0 > CC34	<0.001	0.209	**27°C**	Yes **	Col-0 > CC34	<0.001	0.466
No				> with T°	Yes			
Yes **	27 > 17	<0.001	0.323	**Col-0**	Yes **	27 > 17	<0.001	0.202
Yes **	27 > 17	<0.001	0.233	**CCA1ox-o34**	No	-	0.646	-

Chl FW (T-Test)				12LD	Chl DW (T-Test)			
Different?	**How?**	**P value**	**Diff.**		**Different?**	**How?**	**P value**	**Diff.**
No	-	0.261	-	**17°C**	Yes *	Col-0 > CC34	0.032	0.143
Yes **	Col-0 > CC34	<0.001	0.168	**27°C**	No	-	0.102	-
Yes				> with T°	No			
Yes **	17 > 27	0.006	0.051	**Col-0**	Yes **	27 > 17	<0.001	0.116
Yes **	17 > 27	<0.001	0.301	**CCA1ox-o34**	No	-	0.127	-

Table 6. Summary of results from the assays done on Col-0 and CCA1ox o34 plant lines, grown in 12h L/12h D light cycles and 17ºC or 27ºC. "P value" is probability from Analysis of Variance and "Diff." is the center value from Tukey test, both done as described in 2.5.4 Data analysis. ">with Tº" indicates whether the difference between genotypes is increasing with the rise of temperature. *=P<0.05, **=P<0.01

Descriptive Statistics of all replicates

Col-0 and CCA1ox o34 in 12hL/12hD light cycles

Leaf Area 25d		N		Mean		SE Mean		St Dev		Variance	
	Replicate	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C
Col-0	1	39	40	1.5574	2.9969	0.0627	0.1725	0.3915	1.0911	0.1533	1.1905
	2	40	40	1.0583	1.9310	0.0454	0.0881	0.2869	0.5571	0.0823	0.3104
CC 34	1	40	39	0.7324	0.6783	0.0422	0.0387	0.2667	0.2419	0.0711	0.0585
	2	40	40	0.9678	1.1301	0.0532	0.0401	0.3363	0.2537	0.1131	0.0644

Fresh Weight		N		Mean		SE Mean		St Dev		Variance	
	Replicate	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C
Col-0	1	40	40	0.0417	0.0727	0.0020	0.0040	0.0128	0.0255	0.0002	0.0007
	2	40	40	0.0272	0.0419	0.0011	0.0021	0.0072	0.0131	0.0001	0.0002
CC 34	1	40	39	0.0181	0.0254	0.0011	0.0015	0.0067	0.0091	0.0000	0.0001
	2	40	40	0.0239	0.0447	0.0016	0.0017	0.0100	0.0104	0.0001	0.0001

Dry Weight		N		Mean		SE Mean		St Dev		Variance	
	Replicate	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C
Col-0	1	10	10	0.0049	0.0062	0.0005	0.0007	0.0015	0.0023	0.0000	0.0000
CC 34	1	10	9	0.0024	0.0021	0.0004	0.0002	0.0012	0.0006	0.0000	0.0000

Chl FW		N		Mean		SE Mean		St Dev		Variance	
	Replicate	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C
Col-0	1	5	5	1.4273	0.7568	0.0557	0.0158	0.1246	0.0353	0.0155	0.0012
CC 34	1	5	5	1.5125	8.2162	0.0377	0.9101	0.0843	2.0350	0.0071	4.1412

Chl DW		N		Mean		SE Mean		St Dev		Variance	
	Replicate	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C
Col-0	1	5	5	14.7643	1.1169	1.3992	0.0483	3.1287	0.1079	9.7888	0.0116
CC 34	1	5	5	10.5977	11.0546	0.8626	0.9624	1.9289	2.1520	3.7206	4.6310

Table 7. Descriptive Statistics of all the independent experimental replicates of the assays performed on Col-0 and CCA1ox o34 lines. Plants were grown in 12h L/12h D light cycles and 17ºC or 27ºC.

3.2.2 - Differential expression of the CCA1 gene at high temperature

3.2.2.1 - CCA1 expression timecourse

In the previous subchapter it was clearly shown that *CCA1* over-expressing plants perform poorly when compared to their wild-type Col-0, which is consistent with the work of Dodd *et al.*, 2005. We have extended the Dodd study to demonstrate this difference increases at higher temperature. To explore further the reasons for CCA1ox-o38 reduced performance, expression profile of the *CCA1* gene was monitored over time and at the two temperatures of 17°C and 27°C and in 12hL/12hD light cycles. The two lines CCA1ox-o38 and Col-0 were grown for nine days, then sampled in six time points over the 24h at ZT 1, 6, 11, 13, 18, 23. Two sets of primers for quantitative PCR (qPCR) were used, with the aim of monitoring selectively the expression of the *CCA1* gene or the *CCA1* gene from both plant and transformed construct. See materials for details of how endogenous primers were designed (Quantitative PCR analysis, page 35).

CCA1 gene expression has been previously monitored in wild-type plants in several publications, but never comparing the two temperatures in the CCA1ox-o38 line. In wild-type plants *CCA1* mRNA was found to have a rhythmic expression: mRNA abundance starts to rise before dawn, to peak a couple of hours after and drop again right before dusk. This happens in constant light (Wang and Tobin, 1998; Gould *et al.*, 2006) and in 16hL/8hD or 8hL/16hD (Green *et al.*, 2002). Moreover, Tobin's research group monitored endogenous CCA1 expression, but only at 23°C.

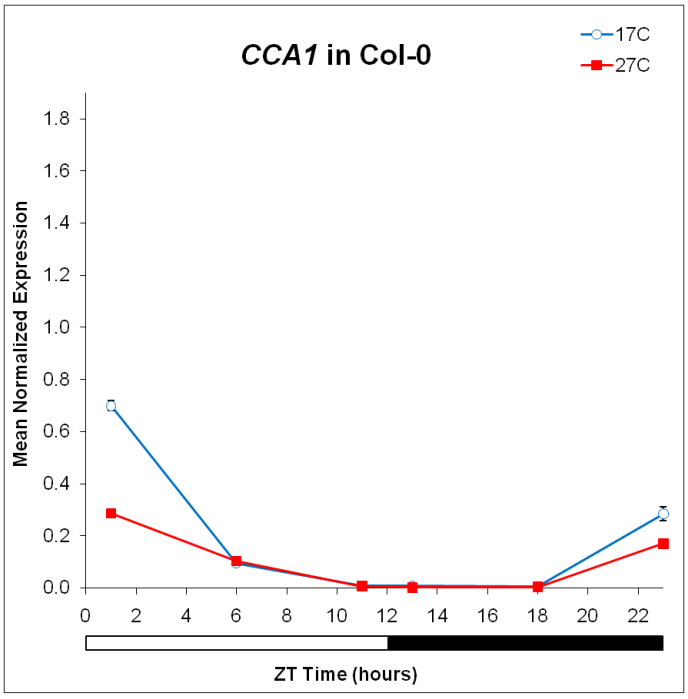


Figure 3-7. *CCA1* gene expression in Col-0 plants, normalized to internal *UBQ* control. Plants were grown in 17°C or 27°C and 12h light/12h dark cycle (white/black bar). Data is mean ±1SEM, n=3 technical repeats.

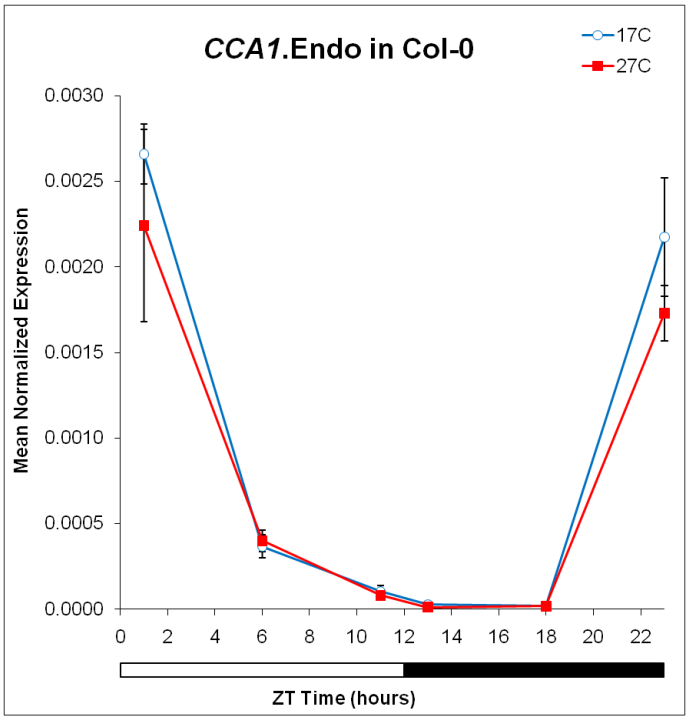


Figure 3-8. *CCA1* endogenous gene expression in Col-0 plants, normalized to internal *UBQ* control. Plants were grown in 17°C or 27°C and 12h light/12h dark cycle (white/black bar). Data is mean ±1SEM, n=3 technical repeats.

Expression of CCA1 and the endogenous CCA1 genes in the wild-type was measured with quantitative PCR (Figure 3-7 and Figure 3-8, respectively). The same mRNA abundance was detected, with both sets of primers. As expected, the same trend was found: expression starts before dawn, to reach its maximum right after it. During this peak expression at 17°C seems greater than at 27°C: with endogenous primers however, standard error is quite high and reduces this difference to zero. The trends found match those already published by Wang and Tobin, 1998 who targeted explicitly the endogenous expression (Their Fig.6B: measured in constant light and ~23°C) and those by Green *et al.*, 2002 (Their Fig.3A , 23°C and either 16L:8D or 8D:16L). Furthermore, Gould *et al.*, 2006 (Their Fig.3C, WS ecotype) monitored *CCA1* expression at 17°C and 27°C in constant light, finding equal expression, but one time point during the peak where 27°C was greater than 17°C.

In CCA1ox-o38 plants, a different response was observed. *CCA1* gene expression (Figure 3-9) was found to be far higher at 27°C than at 17°C, except for one time point one hour after dawn, where there was a higher expression at 17°C. At both temperatures there is an expression peak opposite to the one seen in wild-type, with its maximum at ZT12, right in the middle of the light hours. These results are similar to the trend found by Green *et al.*, 2002. Measuring total *CCA1* expression in CCA1ox plants (16L:8D or 8D:16L, at 23°C) they found a peak of expression later on the day than wild-type. This behaviour suggests the expression was induced by light, therefore not predicting the onset of dawn.

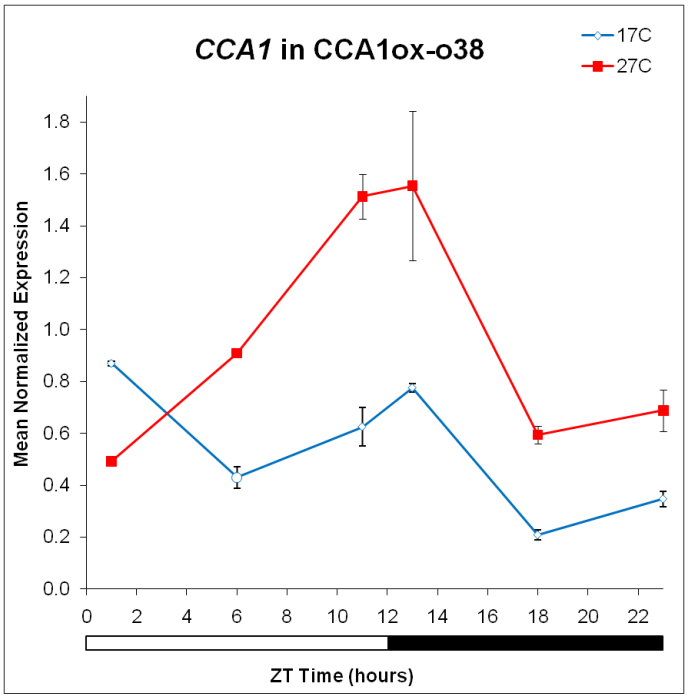


Figure 3-9. *CCA1* gene expression in CCA1ox-o38 plants, normalized to internal *UBQ* control. Plants were grown in 17°C or 27°C and 12h light/12h dark cycle (white/black bar). Data is mean ±1SEM, n=3 technical repeats.

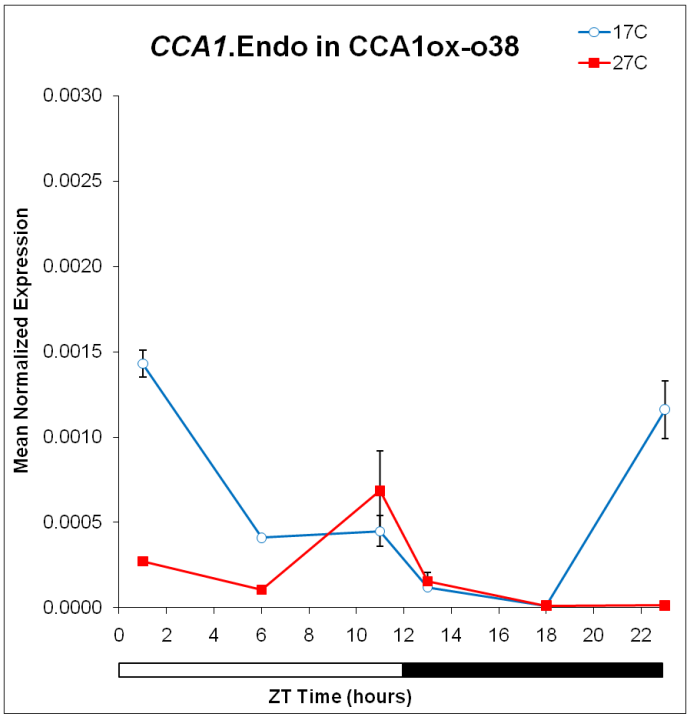


Figure 3-10. *CCA1* endogenous gene expression in CCA1ox-o38 plants, normalized to internal *UBQ* control. Plants were grown in 17°C or 27°C and 12h light/12h dark cycle (white/black bar). Data is mean ±1SEM, n=3 technical repeats.

The measured expression of the endogenous *CCA1* gene in CCA1ox-o38 plants is unexpected if compared to published results. Wang and Tobin, 1998 found no rhythm in constant light and ~23°C (Their Fig.6B). I here measured at 17°C a rhythm similar to wild-type (although lower in magnitude) and at 27°C no rhythm, with a single peak at ZT11 in the middle of the daylight (Figure 3-10). This different expression pattern was however measured in 12hL/12hD light cycles and not in constant light as in Wang and Tobin, 1998. The presented results could be interpreted as being due to a temperature dependant *CCA1* gene expression in the over-expressing construct of CCA1ox-o38 plants. If this is true, at 27°C the exogenous *CCA1* gene, highly expressed, would completely suppress the endogenous *CCA1* gene: its expression is very low in magnitude and produces just a little peak at ZT11, probably induced by the onset of light. On the other hand at 17°C the exogenous *CCA1*, expressed at a lower level, allows a greater expression of the endogenous *CCA1*; endogenous CCA1 shows a peak at dawn, as if could still predict the onset of it.

3.2.2.2 - 35S promoter enhanced at high temperature

One hypothesis is that 35S promoter itself is differentially expressed at higher temperature. A second experiment was therefore performed, to compare the expression of different constructs driven by the 35S promoter at 17°C and 27°C. All constructs have a 35S promoter, but constitutively express different genes and are very likely inserted in different parts of the plants' genome. The plant lines used were four: CCA1ox-o38 and CCA1ox-o34 both over-expressing *CCA1* gene, LHYox over-expressing *LHY* gene and 35S:LUC expressing the luciferase (LUC) gene. Plants were grown for nine days in 12hL/12hD light cycles and then sampled once in the dark, one hour before dawn (ZT 23). Different sets of primers for quantitative PCR were used, to monitor the expression of the following genes: *CCA1* and endogenous *CCA1* in CCA1ox-o38 and CCA1ox-o34 lines; *LHY* in LHYox line, *LUC* in 35S:LUC line. See materials for details of primers (Quantitative PCR analysis, page 35).

Figure 3-11 and Figure 3-12 represent qPCR results for *CCA1* and endogenous *CCA1* primers in CCA1ox-o38/-o34 lines at two temperatures. In both lines total *CCA1* gene expression is always higher at 27°C than at 17°C. Things however are inverted when measuring endogenous *CCA1* expression, in both lines 17°C is significantly greater than 27°C, which is almost not expressed. These results of course reflect those already described in the time course experiments (Figure 3-9 and Figure 3-10). Moreover, the fact that CCA1ox-o34 line gave similar results to CCA1ox-o38 line, highlights that the differential expression of CCA1 gene at high temperature is not due to the different insertion site of the transgene in the genome.

For the LHYox lines, the trend was found to be very similar to that of CCA1. Quantification of *LHY* gene shows at 27°C a greater expression than at 17°C (Figure 3-13). Similarly, *LUC* expression in 35:LUC plant lines is greater at higher temperature (Figure 3-14). *LUC* primers efficiency however was found to be very high compared to UBQ (150 vs 70, ideal is 100), possibly undermining the validity of these last results; a future repeat with newly synthesized primers could confirm results. Nonetheless, the result is consistent with the observation that 35S expression increases with temperature.

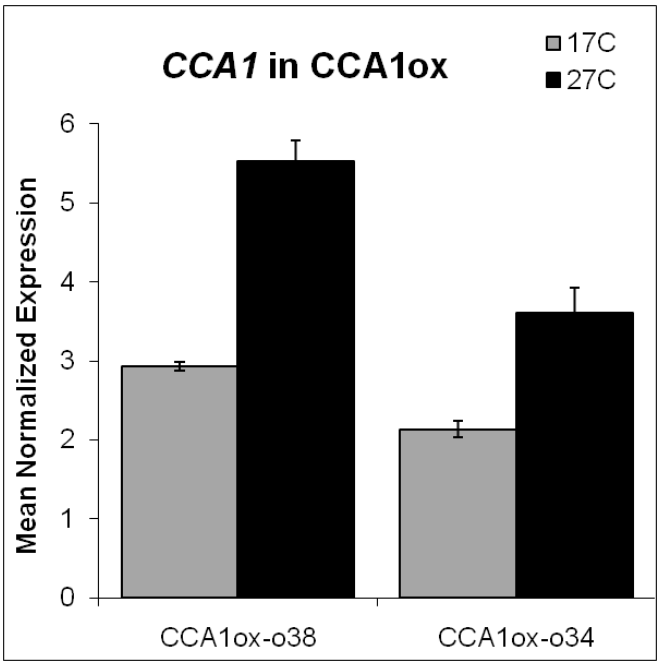


Figure 3-11. *CCA1* gene expression in CCA1ox-o38/-o34 plants, normalized to internal *UBQ* control. Plants were grown in 17°C or 27°C and 12h light/12h dark cycle, then harvested at ZT23. Data is mean ±1SEM, n=3 technical repeats. Figure representative of two independent replicates.

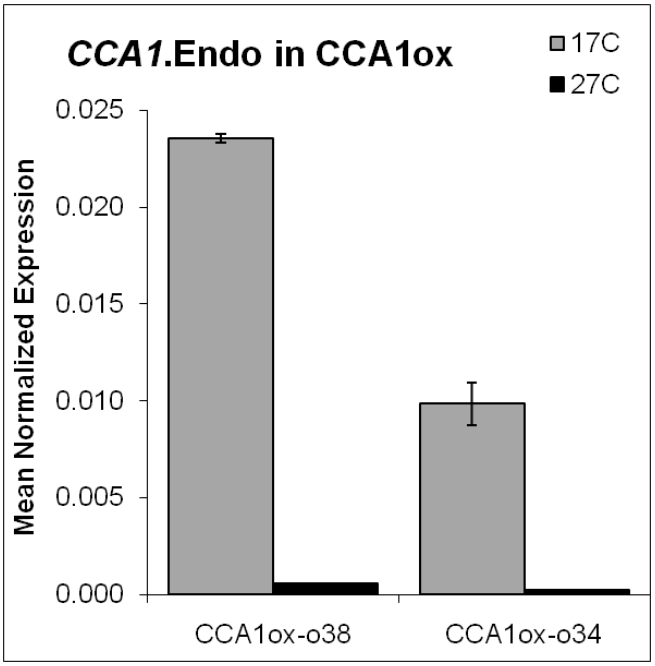


Figure 3-12. *CCA1* endogenous gene expression in CCA1ox-o38/-o34 plants, normalized to internal *UBQ* control. Plants were grown in 17°C or 27°C and 12h light/12h dark cycle, then harvested at ZT23. Data is mean ±1SEM, n=3 technical repeats. Figure representative of two independent replicates.

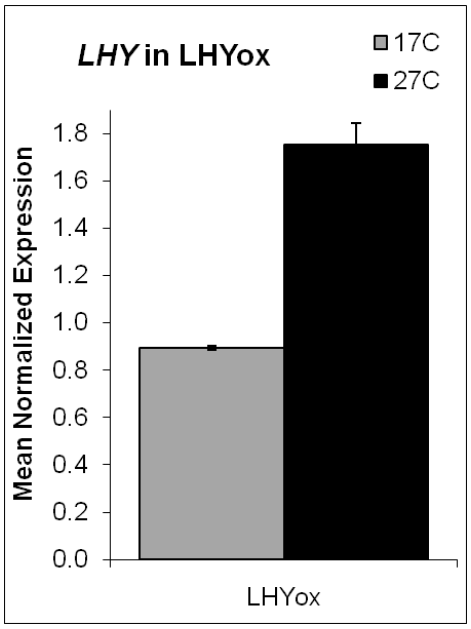


Figure 3-13. *LHY* gene expression in LHYox plants, normalized to internal *UBQ* control. Plants were grown in 17°C or 27°C and 12h light/12h dark cycle, then harvested at ZT23. Data is mean ±1SEM, n=3 technical repeats. Figure representative of two independent replicates.

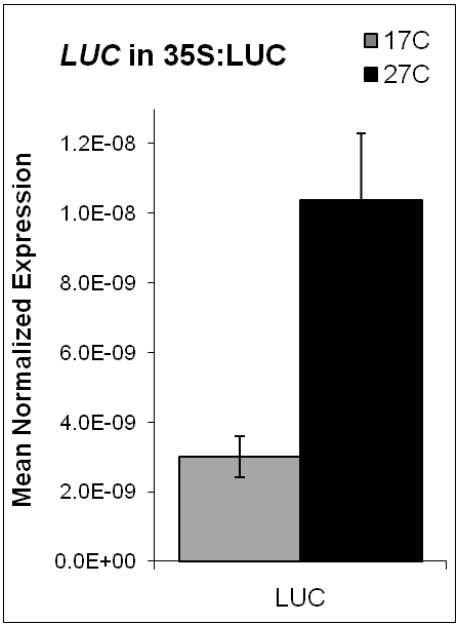


Figure 3-14. *LUC* gene expression in 35S:LUC plants, normalized to internal *UBQ* control. Plants were grown in 17°C or 27°C and 12h light/12h dark cycle, then harvested at ZT23. Data is mean ±1SEM, n=3 technical repeats. Figure representative of two independent replicates.

3.2.3 - Microarray experiment

In the experiments described above (3.2.1, page 41) performance of CCA1ox-o38 plants at high temperatures (27ºC) was measured and shown to be significantly reduced compared to WT plants Col-0. This effect at 17ºC while observable was not as pronounced. To further investigate the importance of circadian regulation with the increase of temperature a microarray experiment was conducted on plant lines CCA1ox-o38 (arrhythmic clock) and Col-0 (wild type). Measuring global gene expression differences could allow a better understanding of which pathways or key genes are causing the observed phenotypes. The experiment was designed and performed in collaboration with a visiting six months Marie Curie Fellow, Dr. Victor Resco de Dios (Universidad de Castilla-La Mancha, Toledo, Spain), who analyzed the results received from NASC Transcriptomics Service (methods in 2.10 Microarray experiment, page 38).

In *Arabidopis* research, microarray technology has been successfully used several times, allowing to build a vast database of expression data from several different experiments conducted under diverse environmental conditions (http://affymetrix.arabidopsis.info/). Recently, comparing multiple circadian microarray experiments it was possible to estimate in about one third the number of genes regulated by the circadian clock (Covington *et al.*, 2008). Many pathways have been found to be regulated, from plant hormones to multiple stress response: furthermore, more than half of the heat responsive genes were found to be circadian regulated.

Transcript profiling was performed on Col-0 and CCA1ox-o38, after four days of entrainment at 12h L/12h D cycles at 17ºC or at 27ºC. Samples were collected one hour before subjective dawn (ZT=23), and one hour after dusk (ZT=13). As we wanted to detect the greatest differences between the two lines, we choose our time points near the two daily transitions of light, the strongest entraining stimuli. We expect wild-type to predict the onset of dawn and dusk, modulating in advance its transcriptome as a consequence. On the other side CCA1ox, lacking a functional clock, is expected to change its transcript levels only after the light/dark transitions.

Comparing Col-0 vs CCA1ox responses

Differences in gene expression between the wild-type and CCA1ox-o38 were moderate at 17ºC, but widespread at 27ºC (Figure 3-15). There were larger differences between Col-0 and CCA1ox-o38 in samples collected at ZT13 than at ZT23 when plants were grown at 17ºC. However, at 27ºC, changes in expression level between the two genotypes were smaller when collected at ZT13.

Effect of temperature on gene expression

To explore further the larger differences between Col-0 vs CCA1ox-o38 transcripts at high temperature, the response of WT at 17ºC was compared with that at 27ºC and the response of CCA1ox-o38 at 17ºC with that at 27ºC. For a given collection time, transcript differences arising from different growth temperatures were more pronounced than those of the WT vs CCA1ox-o38 comparison. Most notably, differences between the WT at 17ºC against 27ºC were smaller than those between CCA1ox-o38 at 17ºC vs 27ºC, particularly at ZT23 (Figure 3-16).

Why CCA1ox plants show an impoverished phenotype?

From the functional analysis of gene responses the poor phenotype in CCA1ox-o38, particularly at high temperatures, seems to be associated with a wide range of transcripts differentially expressed between the mutant and the wild type. Carbon assimilation transcripts (linked to chlorophyll biosynthesis) were reduced, along with low transcript levels of "growth genes" (associated with PIF4 and growth hormones) and low transcript levels of isoprenoids and flavonoids. Furthermore, CCA1ox-o38 showed altered levels of transcripts leading to the inability to respond accurately to abiotic stresses and to an inappropriate red-ox regulation.

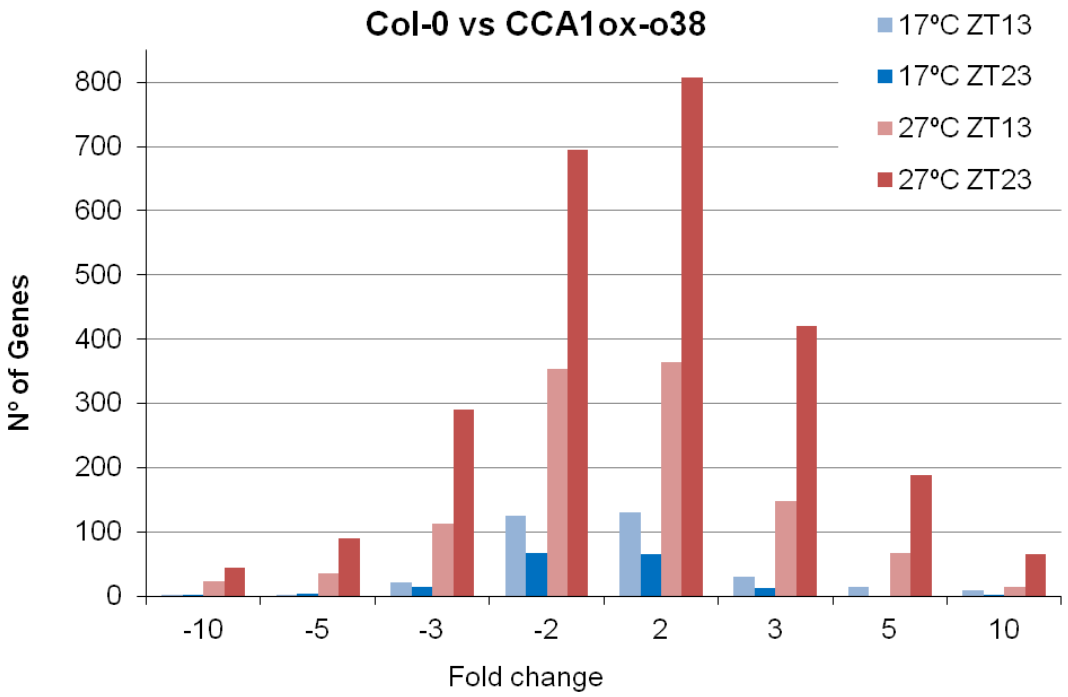


Figure 3-15. Differences in microarray gene expression between Col-0 and CCA1ox-o38.

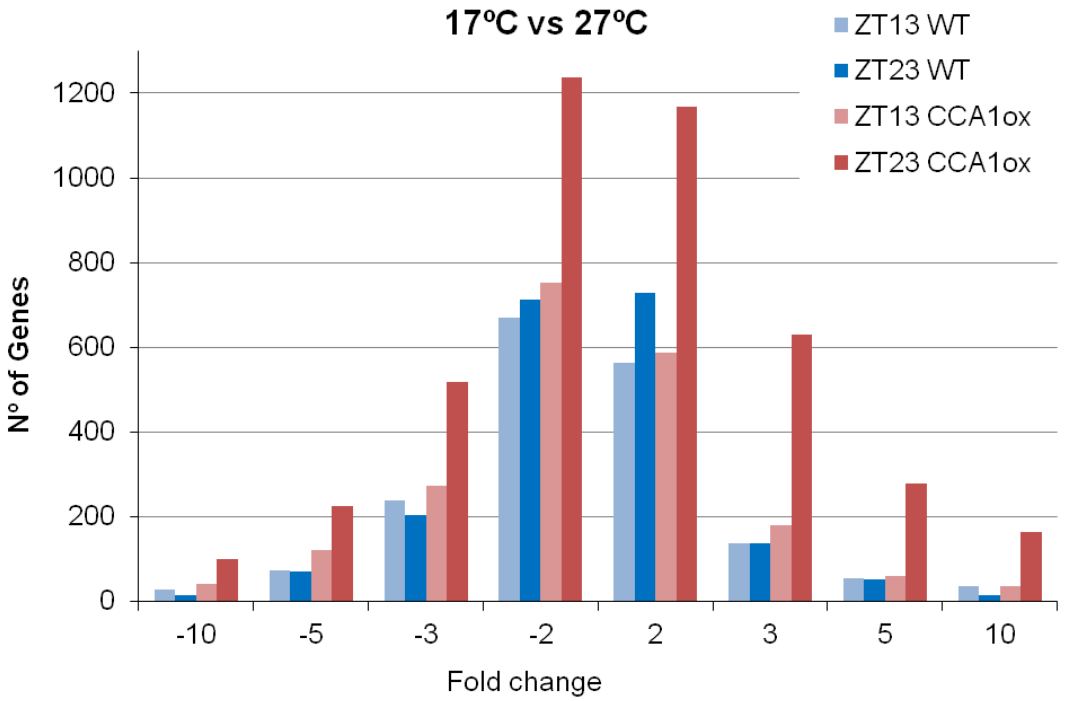


Figure 3-16. Differences in microarray gene expression between 17°C and 27°C.

3.2.3.1 - CCA1 expression levels

While measuring the expression levels of several core clock genes, the pattern of expression of *CCA1* gene was found to be altered in the CCA1ox-o38 plants, as would be expected. In wild type plants a detectable *CCA1* expression was measured only in pre-dawn samples (ZT23), with a negligible difference between the expression at 17ºC and 27ºC (Figure 3-17). These observations are consistent with the known *CCA1* expression profile and the quantitative PCR results previously described (Figure 3-7, page 52), confirming the little temperature variation in *CCA1* gene expression in a wild-type plant.

In arrhythmic plants CCA1ox-o38 however *CCA1* gene expression is very different. The gene was found to be expressed not only in pre-dawn (ZT23), but also in plants harvested at dusk (ZT13). In all cases expression levels are higher than in the wild-type, but appear particularly high at 27ºC (Figure 3-18). A comparison with quantitative PCR results (Figure 3-9, page 54) finds matching values within each time point, but not within the single temperature: in fact, both experiments show a higher expression level at 27°C than at 17°C. At 17°C however, while microarray indicates ZT23 greater than ZT13, qPCR results show vice versa ZT13 greater than ZT23. The very same trend is present in 27°C data, as there was still a rhythm, with gene expression values increasing near dawn.

Despite the differences with qPCR data, these microarray results seem to suggest that the exogenous over-expressed *CCA1* gene is transcribed even when in the wild type is not (ZT13), and with a greater abundance. This observation leads to the question whether the CCA1ox-o38 phenotype measured in the performance experiments is due to the impairment of the clock (the initial hypothesis) or it is due to this measured abnormal expression of CCA1 mRNA at high temperature.

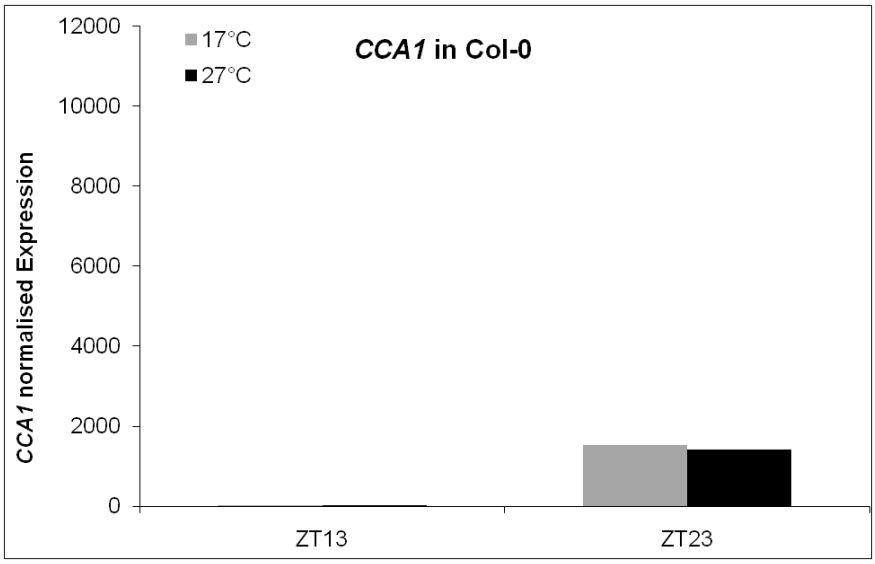


Figure 3-17. Microarray expression levels of the CCA1 gene in Col-0 plants.

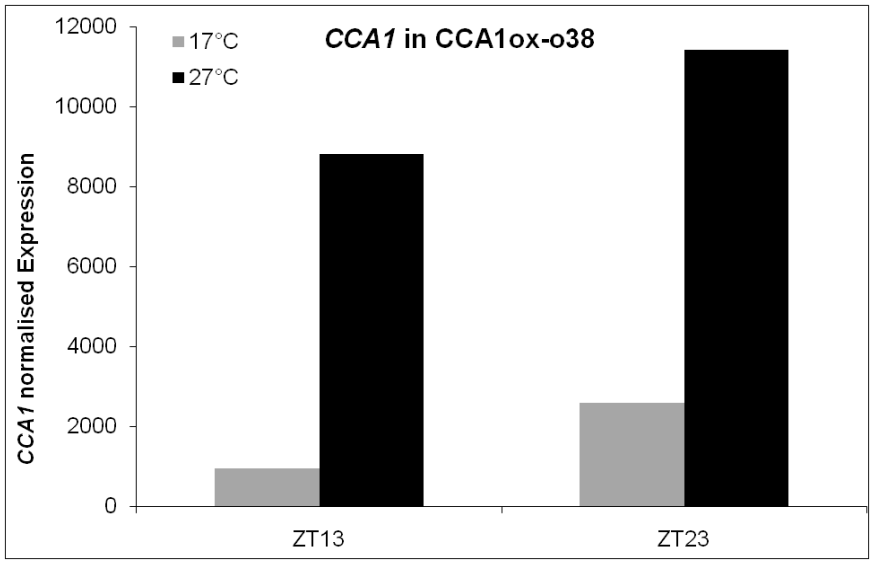


Figure 3-18. Microarray expression levels of the CCA1 gene in CCA1ox-o38 plants.

3.2.4 - Metabolic profiling

Investigation on the importance of circadian regulation with the increase of temperature included up to now quantification of plants' performance and transcript profiling. To have a more complete view of the differences between genotypes, metabolic profiling of the two lines was conducted. Experiments were designed and performed in collaboration with Dr. Simon Thain (Environmental Research Institute, Thurso) who gave his expertise in environmental metabolomics for this project.

Following the same protocol used for transcript profiling (page 38), CCA1ox-o38 and Col-0 plants were grown in a 12h Light/12h Dark light cycle and at two temperatures: 17 and 27ºC. Samples were harvested into liquid nitrogen at 6 different time points around dawn and dusk: ZT0, 1, 11, 12, 13, and 23. Freeze dried rosettes were milled and the metabolites extracted in a 70:30 methanol/water solution. The extract was then infused directly in a Time of Flight Mass Spectrometer. Finally the mass data was analysed via principle component analysis (PCA). PCA separates the principal components responsible for the total variability found in the data: this gives a good idea the overall structure of the data and the information contained in the quantities of each metabolite mass measured. Results from the second and third principal components, explaining about 35% of the variability have been reported here, as they were found to be the most informative in highlighting the differences between the two genotypes.

A comparison between the two genotypes at 17°C, regardless of time, shows little differences: data points are rather mixed (Figure 3-19). This is true also when data points are relabelled with their time of harvesting (Figure 3-20): only ZT12 data points are more clustered than the others. At 27°C however differences are more visible: Col-0 (WT) and CCA1ox-o38 (M) data points separate out into two distinct classes, indicating that temperature increased their overall difference (Figure 3-21). Renaming the data labels (Figure 3-22) shows a more distinct separation among them, especially between dawn and dusk time points.

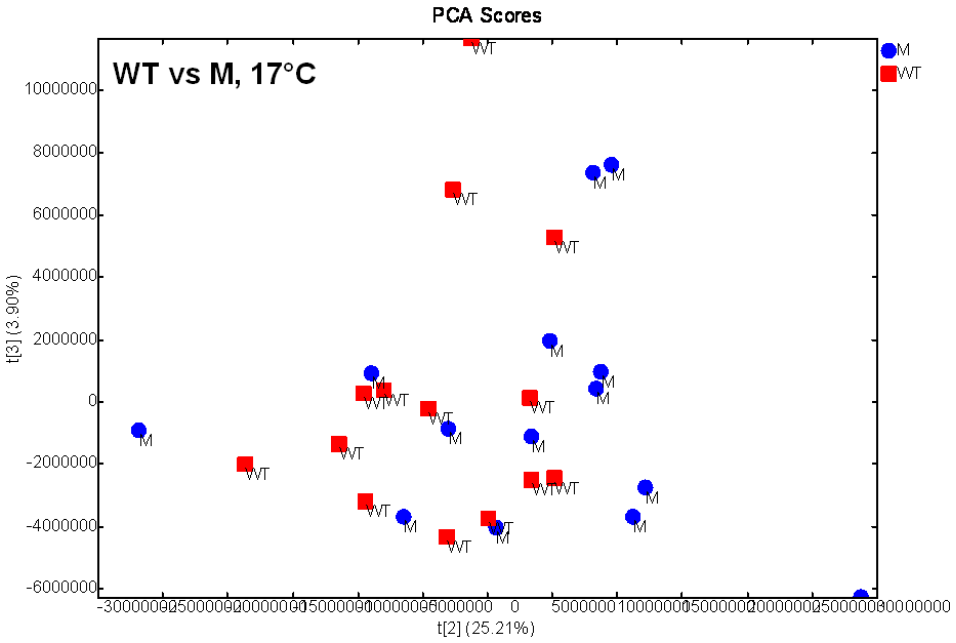


Figure 3-19. PCA plot comparing Col-0 and CCA1ox-o38 metabolite profile at 17°C, grouped by genotype.

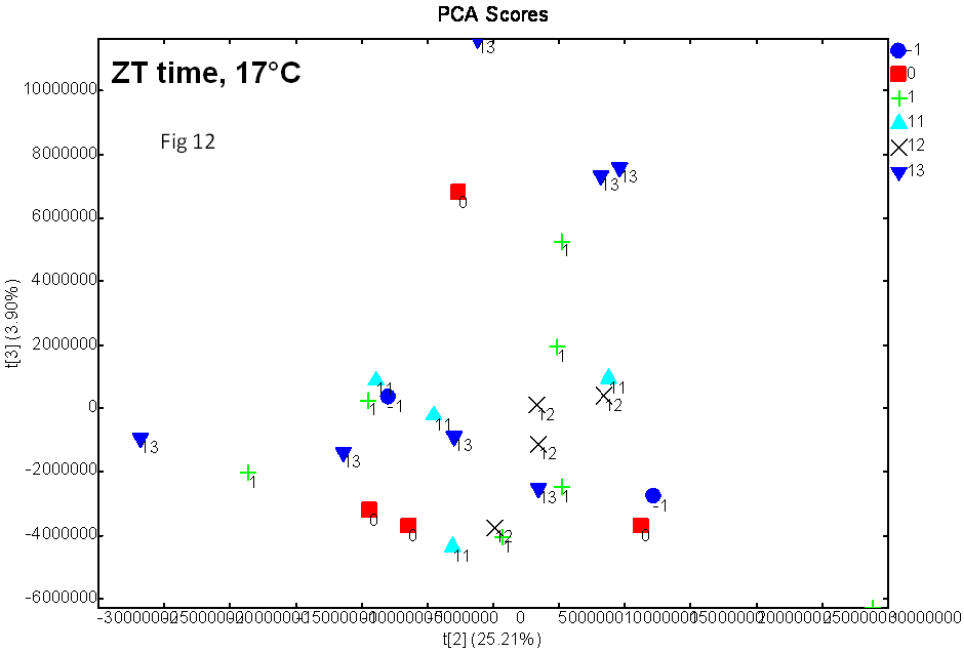


Figure 3-20. PCA plot comparing Col-0 and CCA1ox-o38 metabolite profile at 17°C, grouped by ZT time.

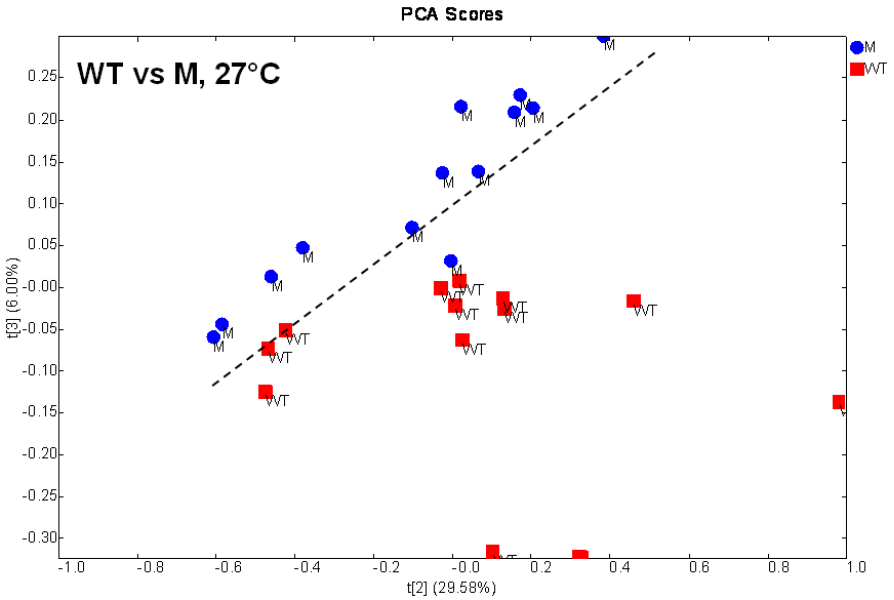


Figure 3-21. PCA plot comparing Col-0 and CCA1ox-o38 metabolite profile at 27°C, grouped by genotype.

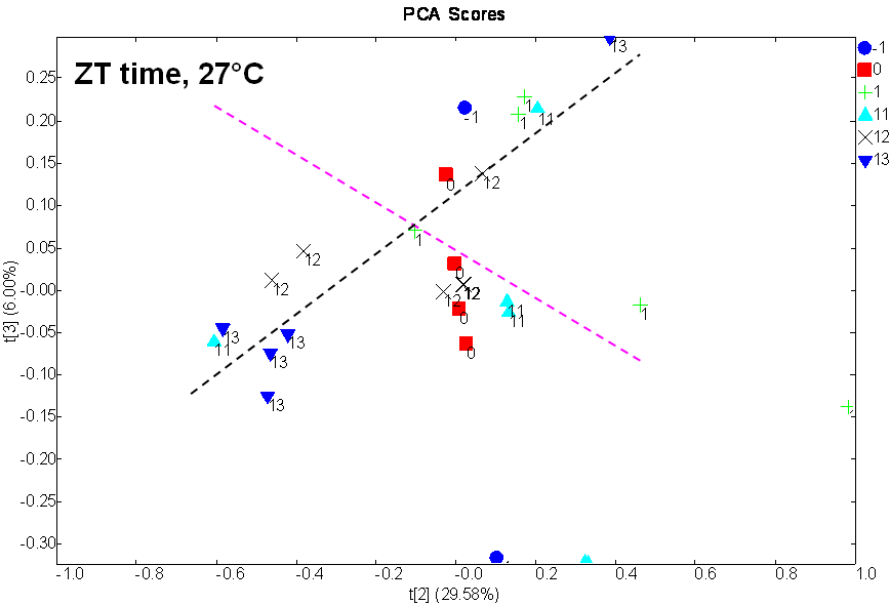


Figure 3-22. PCA plot comparing Col-0 and CCA1ox-o38 metabolite profile at 27°C, grouped by ZT time.

There are indications that responsible for some of the differences found could be secondary metabolites, as highlighted from transcript profiling results. The solution used for the initial extraction (70:30 methanol/water) is effective in extracting many secondary metabolites along with some primary metabolites. Transcript profiling too detected several changes in part of the secondary metabolism: isoprenoid and flavonoid genes showed at 17ºC small modifications in their expression levels, which increased at 27ºC where Col-0 genes had higher levels of expression than in CCA1ox-o38.

A closer look at the masses detected by the mass spectrometer indicates heavier masses as the most important: most differences between treatments occurred above 750Da, including therefore lipids, complex carbohydrates and some proteins. Due to unforeseen circumstances, Dr. Thain was unable to finish the data analysis of these and other samples assayed, identifying specific masses and structures within the time constrains of this project. However, the trends seem quite clear: the overall metabolic profile of the two genotypes is different, and this difference is greater at higher temperature, confirming the results seen in the performance experiments.

Identifying from mass information which specific metabolites are responsible for the differences seen would certainly clarify the links between metabolism and plant performance at high temperature. A future development of this research would be to extend the metabolite profiling to other metabolites, using a different the extraction method and compare the mass information with transcript profiling.

3.3 Discussion

Wang and Tobin, 1998 showed for the first time that over-expressing the *CCA1* gene affected multiple circadian regulated processes, from longer hypocotyls to delayed flowering. Successively this alteration was quantified as a whole plant poor performance: CCA1ox-o38 plants were less viable, fixed less carbon, produced less chlorophyll, aerial biomass and leaf area than the wild-type Col-0 (Green *et al.*, 2002; Dodd *et al.*, 2005). In this chapter I extended the research proving for the first time that CCA1ox-o38 performance is even poorer at higher temperature. In fact CCA1ox-o38 plants at 27°C showed 65% less biomass, 30% less chlorophyll and a minor leaf area than Col-0 (Paragraph 3.2.1, page 41). These observations were confirmed by transcript and metabolic profiling. Differences in gene expression between Col-0 and CCA1ox-o38 were moderate at 17°C, but widespread at 27°C (Figure 3-15, page 61). Most interestingly the bigger difference was found at 27°C one hour before dawn (ZT23): exactly when the wild type, predicting the onset of dawn, activates all the necessary genes to get the most of daylight. CCA1ox-o38 on the contrary seems unable to predict dawn. As a matter of fact, expression of the *CCA1* gene at 27°C doesn't start before dawn as in the wild type (Figure 3-8, page 52) but peaks later in the day, as if its expression was induced by light (Figure 3-9, page 54). At last, a further confirmation comes from a metabolomics experiment: the overall metabolic profile of the two genotypes was found to be different, and this difference was greater exactly at higher temperature (Figure 3-21, page 66).

Higher expression at 27°C of transgenic *CCA1* gene however (Figure 3-9, page 54) could be due to 35S promoter itself. To test this possibility, expression of different genes in different lines has been tested, all sharing 35S as their promoter in the construct (Paragraph 3.2.2.2, page 56). All genes tested, *CCA1*, *LHY* and even *LUC* were found to be more expressed at 27°C than at 17°C, making a strong argument for the 35S hypothesis. Moreover, the described results agree with some recent literature on the subject. Boyko *et al.*, 2010 (Their Figure 3C, page 72) growing *Nicotiana tabacum* plants at 50°C for 2 hours measured a 1.4 fold increase in 35S-driven *LUC* and actin genes, and a 10% statistically insignificant increase after a two weeks exposure to 28°C. Previously the same authors measured a significant increase in *Arabidopsis* plants grown at 32°C (Boyko *et*

al., 2005). A possible explanation of this unexpected behaviour is given by Pauli *et al.*, 2004, who suggested that the 35S promoter might contain potential heat shock motif. The viral nature of the promoter could be the founding cause of the measured instability, leading to the need of reconsidering expectations on the expression stability of genes driven by the 35S promoter. Harsh environmental conditions such as low temperatures, UVA and UVB radiations could alter significantly transgene expression (Boyko *et al.*, 2010).

35S-driven increased expression of the *CCA1* transgene requires therefore to reconsider all the performance results presented in this chapter. The reduced performance of CCA1ox-o38 plants at 27°C might be due to abnormal *CCA1* expression, which being a transcription factor could have altered the expression of many other clock controlled genes. Interpretation of microarray results would certainly deserve a similar attention. A future experiment that could solve this problem is to repeat the experiments with another *Arabidopsis* arrhythmic mutant, this time lacking the temperature-unstable 35S promoter in the genome.

CHAPTER 4 - An accurate clock (*ztl* line)

4.1 Introduction

Experimental results presented in Chapter 3, involving the arrhythmic plant line CCA1ox-o38 and its wild-type Col-0, proved that having a functional clock is important for the good performance of the plant at high temperatures. The validity of those results however is undermined by an abnormal *CCA1* gene expression at high temperature, likely caused by the 35S promoter used in the over-expressing construct. In this chapter the investigation goes further, showing the increased importance for a plant to have an accurate clock at high temperatures. This time however, the risk of having a temperature dependent gene expression driven by the 35S promoter is avoided, as the *ztl* line used for the experiments is a mutant and not a 35S driven overexpressor.

Clock accuracy is better defined as "circadian resonance": a plant is in circadian resonance with the environment when the period of its internal clock is matched to the external light/dark cycle, therefore accurately predicting light changes. A resonant clock was found to increase not only growth, but conferring a competitive advantage both in cyanobacteria and higher plants. In cyanobacterium *Synechococcus elongatus*, strains with a circadian period similar to the external light/dark cycle grew more if placed in competition with non resonant strains (Ouyang *et al.*, 1998). *S. elongatus* reproductive fitness is therefore improved, but this has been proved to happen only in cyclic environments (Woelfle *et al.*, 2004). As early as in the 1950's experiments were conducted in tomato plants (*Lycopersicon esculentum*) which identified that they were sensitive to changes in environmental light/dark cycle. Plants grew smaller, with yellow necrotic leaves when the external period did not match the 24h internal one (Highkin and Hanson, 1954). More recently, experiments similar to those done in cyanobacteria were performed in *Arabidopsis*. The period mutants *toc1-1* (21h, short period), *ztl-1* (28-32h, long period) and the wild-type Col-0 (24h, normal period) were grown in resonant and non resonant light/dark cycles. Plants grown in a light cycle matching the internal one showed enhanced fitness traits, such as photosynthesis, biomass and competitive advantage (Dodd *et al.*, 2005).

Hotta *et al.*, 2007 proposed five mechanisms through which the circadian clock is likely to increase plants' fitness, including temporal compartmentation of metabolic processes; anticipation of daily environmental changes; optimization of the turnover rate of proteins; anticipation of seasonal environmental changes; gating of environmental signals.

To test the importance of circadian resonance at higher temperatures the long period *ztl-3* mutant line was used, along with its wild-type Col-0. *ztl-3* is a knock-out mutant containing a T-DNA insertion at amino acid 440, lacking any detectable ZTL mRNA (Jarillo *et al.*, 2001). It has a late flowering phenotype, and a long period of 28-32h when measuring *CAB:LUC* expression or leaf movement respectively. (Somers *et al.*, 2004). Moreover, *ztl* long period phenotype seems to be independent from temperature (Nicolas Ugarte, personal communication).

Period length depends on fluence rate under red or blue light and, unlike central clock components, *ZEITLUPE* (*ZTL*) gene expression is independent of light/dark cycles and the circadian clock (Somers *et al.*, 2000). ZTL protein levels however oscillate (Kim *et al.*, 2003), due to the stabilization in blue light by the GIGANTEA (GI) protein (Kim *et al.*, 2007). Moreover, it was found ZTL targets to degradation in the 26S proteasome complex the central clock component TOC1 (Mas *et al.*, 2003a) and its homolog PRR5 (Kiba *et al.*, 2007). Gathering all the information together enables the positioning of *ZTL* in the evening loop of the circadian clock mechanism. *GI* (part of the unknown function *Y*) is activated by light, and activates *TOC1* transcription which in turn represses *Y*(*GI*). GI also confers a post-translational rhythm to *ZTL,* stabilizing its protein which targets TOC1 protein to degradation.

In the experiments presented here, *ztl* and Col-0 plants were germinated in standard conditions (21°C and 12L:12D) then transferred to either 17°C or 27°C in 12h L/12h D, 15h L/15h D or 10h L/10h D cycles. The two temperatures were chosen to compare plants grown in a normal and in a stressful environmental condition. To determine what meant "normal" for the Col-0 ecotype, historical weather data were searched for the area where Col-0 was originally collected by F. Laibach, near Frankfurt am Main in Germany (50°N and 8°E, Edwards *et al.*, 2005). The German Meteorological Service

(http://www.dwd.de/**,** 1961-1990 average) reports for the area a July temperature of Min.13°C, Avg.18.5°C, Max.24°C. As the cabinet temperature was kept constant, 17°C was thought to be a good estimate for "normal" temperature. Furthermore, recently the temperature compensation mechanism was found to become important moving away from 17°C. In Gould *et al.*, 2006 the leaf movement period of *gi-11* plants, mutants for the temperature compensation mechanism, diverged from the wild-type above and below 17°C. This finding could indicate that at 17°C temperature buffering mechanisms are dispensable and it's not felt as a stressful growing temperature. The stressful temperature (27°C) was chosen to be significantly higher than the ecotype's daily average, allowing the mutant to show its deficiencies, but not too extreme to overcome the wild-type's temperature buffering strategies. 33°C for example was proved to be too much, even for Col-0 (see Figure 4-19, panels A, B and D). Circadian rhythm in fact, was measured to be strongly temperature compensated only below 30°C in *Neurospora crassa* (Lakin-Thomas *et al.*, 1990) and at 32°C had already a Q_{10}=1.1 in C24 *Arabidopsis* plants (Somers *et al.*, 1998).

In 12LD cycles, little differences were found at 17°C, but moving at 27°C the resonant line Col-0 performed much better that the mutant *ztl*. The scenario was almost reversed in 15LD cycles: the resonant line *ztl* already performed better than Col-0 at 17°C, and the difference increased significantly at 27°C. Finally, in 10LD the two plants behaved in a similar way as at 12LD. This therefore indicates the importance of circadian resonance in *Arabidopsis* plants and for the first time this effect has been quantified to be even more important at higher temperature.

4.2 Results

4.2.1 - 12h L/12h D – Col-0 outperforms ztl

The first set of experiments was conducted in 12h L/12h D cycles, comparing the performance of the *ztl* mutant against the wild-type Col-0. Across the assays, the performance of the *ztl* line was found to be the same as Col-0 when grown at 17ºC, but became significantly lower at high temperature. At 27°C Col-0, having a period that matches the external light/dark cycle, performed much better than *ztl*. In Figure 4-1, photo of the seedlings just before harvesting, is already possible to spot some differences in the two lines. At 17ºC all plants are small and compact; at 27ºC while Col-0 plants have grown in size and elongated their petioles, *ztl* plants showed a phenotype extremely similar to 17ºC, indicating an almost complete halt in growth.

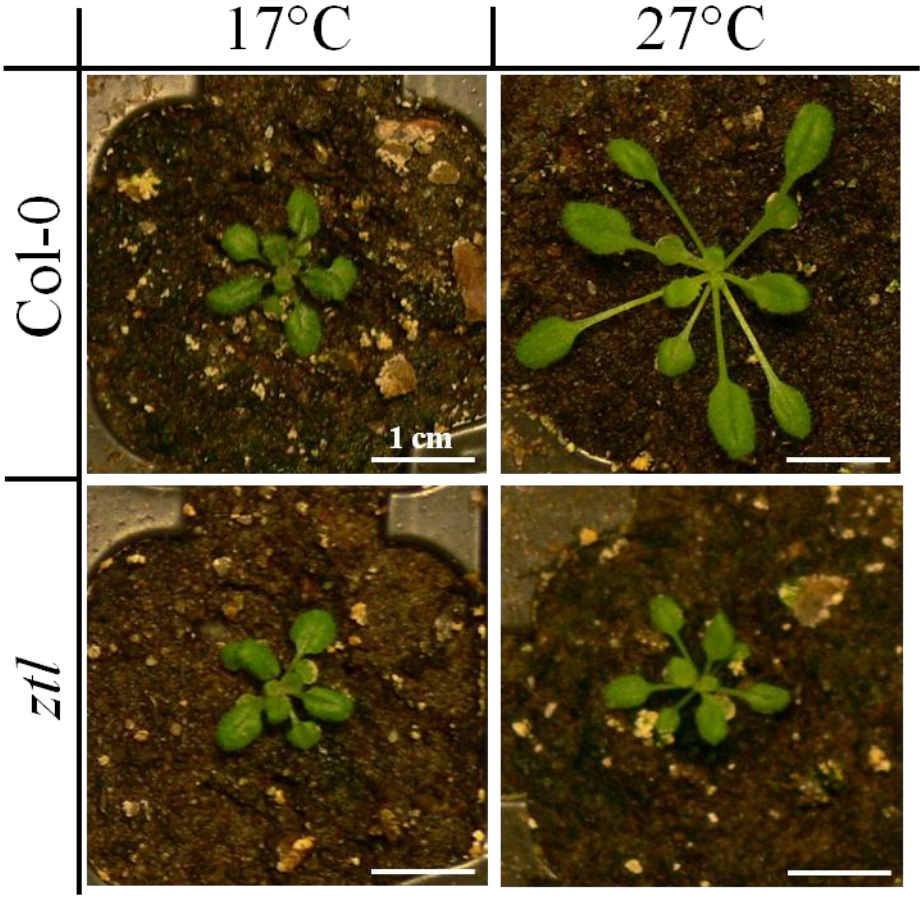


Figure 4-1. Col-0 and *ztl* plants, grown at 17ºC and 27ºC, in 12h L/12h D light cycles. The photo was taken 25 day after sowing, white bars equal to 1cm.

This visual phenotype is confirmed by quantitative results in visible leaf area (Figure 4-2). When comparing 17°C to 27°C, both Col-0 and *ztl* plants had a greater visible leaf area at high temperature, with P-values in the ANOVA test (at day 25) of **P<0.001 in both cases. In particular leaf area at 27°C was found to be about 1.5 times bigger for *ztl* at 17°C and more than 2.5 times for Col-0. Comparing within each temperature the two lines tested, results in no significant leaf area difference at 17°C (P= 0.121) but at 27°C in Col-0 the area is more than 1.5 times greater than *ztl* (**P<0.001). It is clear that the temperature increase has a profound effect on the differential performance of these two lines.

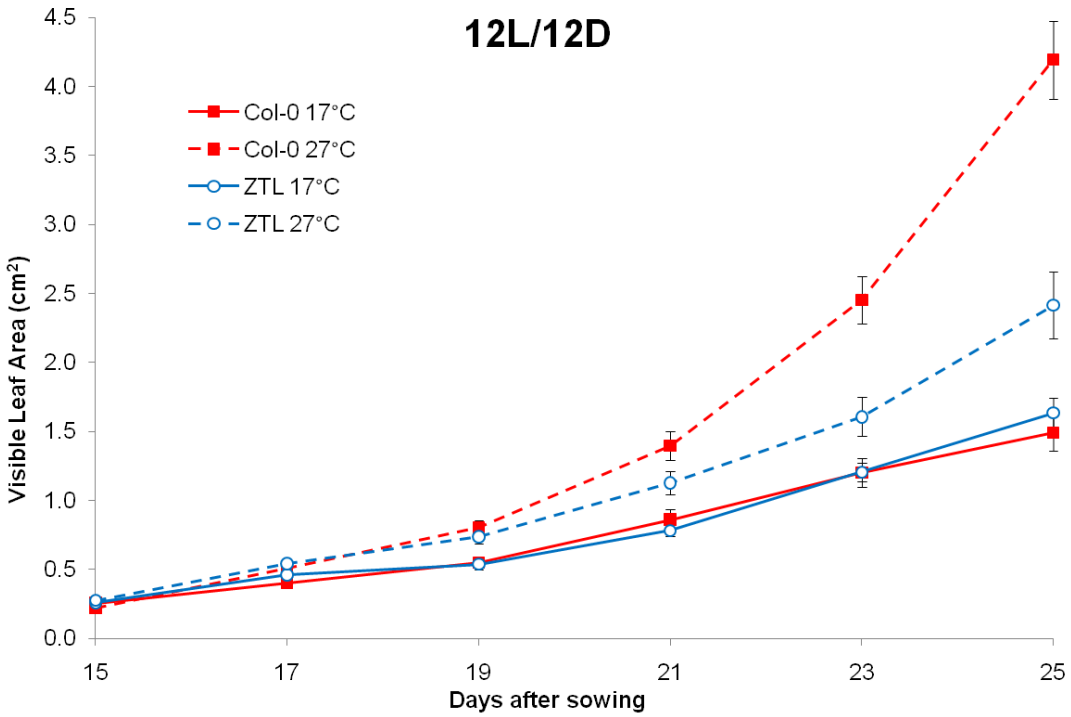


Figure 4-2. Visible leaf area of Col-0 and *ztl* plant lines 15 to 25 day after sowing, grown at 12h L/12h D light cycle and 17ºC or 27ºC. Data is mean ±1SEM. At day 25 in ANOVA test comparing *ztl* to Col-0, P>0.12 at 17°C and **P<0.01 at 27°C. Figure representative of three independent replicates.

Fresh weight results (Figure 4-3) show an identical trend to visible leaf area. Over the two growing temperatures both Col-0 and *ztl* mutant plants had a greater fresh weight at 27°C, with ANOVA test reporting significant P-values of less than 0.001. Balancing wild-type fresh weight against the mutant within each temperature, indicates no difference between the two lines at 17°C (P= 0.224), but at 27°C Col-0 values were always greater than *ztl* (**P<0.001). As a result, the differences between the two lines increased with temperature.

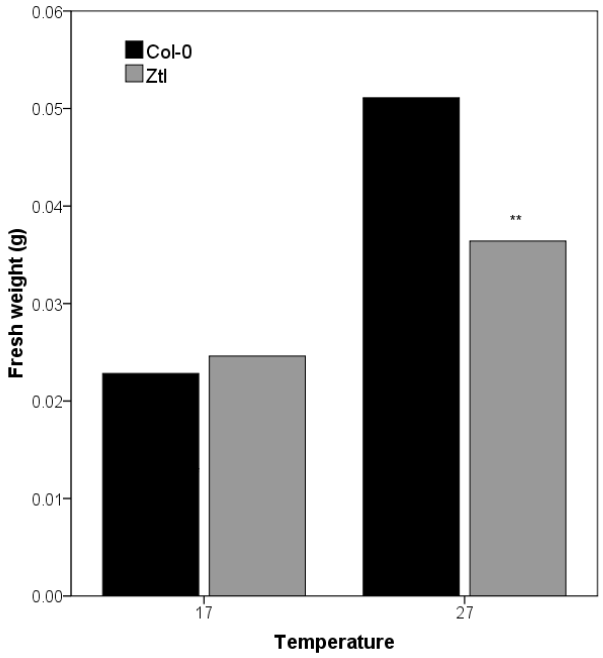


Figure 4-3. Fresh weight of Col-0 and *ztl* plant lines 25 day after sowing, grown at 12h L/12h D light cycle and 17ºC or 27ºC. Data is mean, in ANOVA test comparing *ztl* to Col-0 P>0.22 at 17°C and **P<0.01 at 27°C. Figure representative of three independent replicates.

Trends described up to now are confirmed by dry weight figures, described in Figure 4-4. Comparing 17°C to 27°C, both Col-0 and *ztl* plants had a greater dry weight at high temperature, with ANOVA P-values of **P<0.001 for Col-0 and **P=0.002 for *ztl*. Matching the wild-type against the mutant within each temperature highlights no difference at 17°C (P= 0.170) but at 27°C a greater weight for Col-0, with a very significant P-value of less than 0.001. It is clear the differences highlighted rose with the increase of temperature.

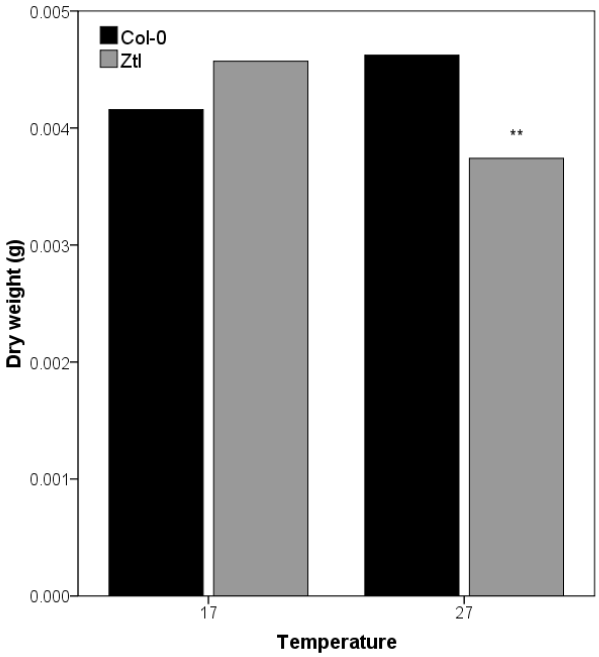


Figure 4-4. Dry weight of Col-0 and *ztl* plant lines 25 day after sowing, grown at 12h L/12h D light cycle and 17ºC or 27ºC. Data is mean, in ANOVA test comparing *ztl* to Col-0 P>0.17 at 17°C and **P<0.01 at 27°C. Figure representative of three independent replicates.

At first glance, the results obtained from total chlorophyll quantification on a fresh weight basis (Figure 4-5) look quite different from the previous data that has been presented. Comparing 17 and 27°C both lines had a greater chlorophyll concentration at lower temperature, with significant P-values in the ANOVA test: **P=0.006 for Col-0 and *P=0.013 for *ztl*. Comparing within each temperature the two lines tested, results in Col-0 were greater than *ztl* both at 17°C (**P=0.001) and at 27°C (**P=0.005). Moreover, these differences increased with temperature. This unexpected data may have been caused by the high variability found in the three distinct performance experiments done. In particular, variances were found to be 10 fold different between the two genotypes groups, and this could have impaired the validity of the statistical analysis.

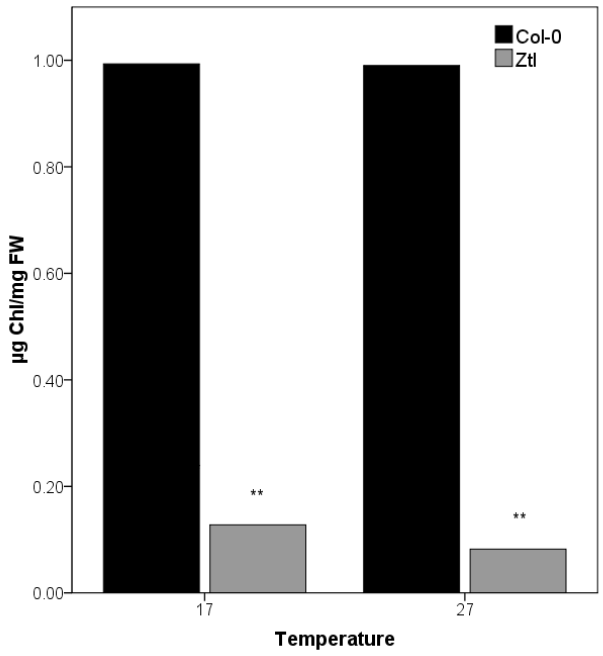


Figure 4-5. Total chlorophyll content (on fresh weight) of Col-0 and *ztl* plant lines 25 day after sowing, grown at 12h L/12h D light cycle and 17ºC or 27ºC. Data is mean, in ANOVA test comparing *ztl* to Col-0 **P<0.01 at 17°C and 27°C. Figure representative of three independent replicates.

Measurements of total chlorophyll on a dry weight basis (Figure 4-6) also led to partially different results. Analysing 17 against 27°C data within each genotype reveals that both Col-0 and *ztl* plants had a greater chlorophyll concentration at high temperature, with ANOVA P-values of **P<0.001 in either genotypes. On the other hand, juxtaposing the two genotypes inside each temperature, shows at 17°C no difference (P=0.796) and at 27°C *ztl* plants with more chlorophyll than Col-0 (*P=0.024). The difference between the two genotypes therefore have increased with temperature, but in the opposite way to what has been seen up to now.

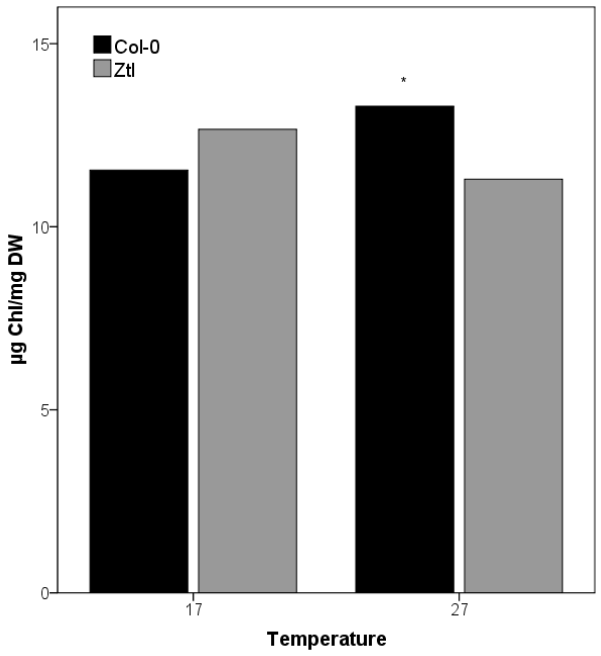


Figure 4-6. Total chlorophyll content (on dry weight) of Col-0 and *ztl* plant lines 25 day after sowing, grown at 12h L/12h D light cycle and 17ºC or 27ºC. Data is mean, in ANOVA test comparing *ztl* to Col-0 P>0.79 at 17°C and *P<0.05 at 27°C. Figure representative of two independent replicates.

Summary of Results

Col-0 and *ztl* in 12hL/12hD light cycles

Leaf Area - 25 days				12LD
Different?	**How?**	**P value**	**Diff.**	
No	-	0.121	-	**17°C**
Yes **	Col-0 > *ztl*	<0.001	0.295	**27°C**
Yes				> with T°
Yes **	27 > 17	<0.001	0.507	**Col-0**
Yes **	27 > 17	<0.001	0.173	***ztl***

Fresh Weight				12LD	Dry Weight			
Different?	**How?**	**P value**	**Diff.**		**Different?**	**How?**	**P value**	**Diff.**
No	-	0.224	-	**17°C**	No	-	0.170	-
Yes **	Col-0 > *ztl*	<0.001	0.147	**27°C**	Yes **	Col-0 > *ztl*	<0.001	0.099
Yes				> with T°	Yes			
Yes **	27 > 17	<0.001	0.323	**Col-0**	Yes **	27 > 17	<0.001	0.202
Yes **	27 > 17	<0.001	0.192	***ztl***	Yes **	27 > 17	0.002	0.072

Chl FW				12LD	Chl DW			
Different?	**How?**	**P value**	**Diff.**		**Different?**	**How?**	**P value**	**Diff.**
Yes **	Col-0 > *ztl*	0.001	0.318	**17°C**	No	-	0.796	-
Yes **	Col-0 > *ztl*	0.005	0.338	**27°C**	Yes *	*ztl* > Col-0	0.024	0.040
Yes				> with T°	Yes			
Yes **	17 > 27	0.006	0.051	**Col-0**	Yes **	27 > 17	<0.001	0.116
Yes *	17 > 27	0.013	0.071	***ztl***	Yes **	27 > 17	<0.001	0.141

Table 8. Summary of results from the assays done on Col-0 and *ztl* plant lines, grown in 12h L/12h D light cycles and 17ºC or 27ºC. "P value" is probability from Analysis of Variance and "Diff." is the center value from Tukey test, both done as described in 2.5.4 Data analysis. ">with T°" indicates whether the difference between genotypes is increasing with the rise of temperature. *=P<0.05, **=P<0.01

Descriptive Statistics of all replicates

Col-0 and *ztl* in 12hL/12hD light cycles

Leaf Area 25d		N		Mean		SE Mean		St Dev		Variance	
	Replicate	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C
Col-0	1	20	20	1.4925	4.1947	0.1324	0.2827	0.5923	1.2643	0.3508	1.5984
	2	20	20	1.0039	2.2081	0.0575	0.1520	0.2569	0.6799	0.0660	0.4622
	3	40	40	1.0583	1.9310	0.0454	0.0881	0.2869	0.5571	0.0823	0.3104
ztl	1	20	20	1.6351	2.4167	0.1084	0.2420	0.4848	1.0824	0.2351	1.1716
	2	20	20	1.0365	1.5000	0.0357	0.1124	0.1599	0.5026	0.0256	0.2526
	3	40	40	0.7551	1.0255	0.0344	0.0550	0.2176	0.3478	0.0473	0.1210

Fresh Weight		N		Mean		SE Mean		St Dev		Variance	
	Replicate	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C
Col-0	1	79	78	0.0633	0.1570	0.0019	0.0061	0.0172	0.0536	0.0003	0.0029
	2	57	59	0.0228	0.0511	0.0006	0.0023	0.0049	0.0175	0.0000	0.0003
	3	40	40	0.0272	0.0419	0.0011	0.0021	0.0072	0.0131	0.0001	0.0002
ztl	1	80	79	0.0671	0.1256	0.0020	0.0052	0.0180	0.0460	0.0003	0.0021
	2	59	57	0.0246	0.0364	0.0008	0.0017	0.0060	0.0130	0.0000	0.0002
	3	40	39	0.0186	0.0245	0.0009	0.0013	0.0054	0.0080	0.0000	0.0001

Dry Weight		N		Mean		SE Mean		St Dev		Variance	
	Replicate	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C
Col-0	1	48	65	0.0071	0.0137	0.0003	0.0006	0.0019	0.0046	0.0000	0.0000
	2	14	27	0.0042	0.0046	0.0005	0.0003	0.0018	0.0015	0.0000	0.0000
	3	10	10	0.0036	0.0042	0.0002	0.0004	0.0007	0.0014	0.0000	0.0000
ztl	1	52	64	0.0084	0.0114	0.0003	0.0005	0.0019	0.0037	0.0000	0.0000
	2	14	22	0.0046	0.0037	0.0010	0.0004	0.0036	0.0017	0.0000	0.0000
	3	10	10	0.0026	0.0026	0.0002	0.0002	0.0007	0.0006	0.0000	0.0000

Chl FW		N		Mean		SE Mean		St Dev		Variance	
	Replicate	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C
Col-0	1	6	6	0.9933	0.9902	0.0335	0.0560	0.0820	0.1371	0.0067	0.0188
	2	5	5	1.0055	0.8280	0.0538	0.0502	0.1203	0.1123	0.0145	0.0126
	3	5	5	1.7847	1.5018	0.0702	0.0232	0.1569	0.0518	0.0246	0.0027
ztl	1	6	6	0.1277	0.0821	0.0069	0.0024	0.0169	0.0059	0.0003	0.0000
	2	5	5	1.0592	1.3098	0.0425	0.0625	0.0949	0.1398	0.0090	0.0195
	3	5	5	1.9070	1.5574	0.0446	0.0768	0.0996	0.1718	0.0099	0.0295

Chl DW		N		Mean		SE Mean		St Dev		Variance	
	Replicate	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C
Col-0	1	5	24	59.3600	82.1388	6.1897	2.0895	13.8406	10.2364	191.5630	104.7840
	2	5	5	11.5483	13.2915	0.3697	0.6711	0.8267	1.5006	0.6830	2.2520
ztl	1	8	21	58.5746	95.4881	5.3031	2.1677	14.9994	9.9334	224.9810	98.6730
	2	5	5	12.6598	11.3001	0.8608	1.1315	1.9247	2.5301	3.7040	6.4020

Table 9. Descriptive Statistics of all the independent experimental replicates of the assays performed on Col-0 and *ztl* lines. Plants were grown in 12h L/12h D light cycles and 17ºC or 27ºC.

4.2.2 - 15h L/15h D – ztl outperforms Col-0

The second set of experiments was conducted in 15h L/15h D cycles, comparing the performance of the *ztl* mutant against the wild-type Col-0. Both at 17°C and at 27°C the *ztl* line proved to perform better than the wild-type. At 27°C however the differences measured were significantly greater than at 17°C. In these conditions *ztl* has an internal clock in sync with the external light/dark cycle, and this gives a net performance advantage to the plant. In the pre-harvesting photo (Figure 4-7) the differences between the two lines are already very clear. At 17°C all plants look approximately the same, but increasing the temperature reveals the differences: *ztl* plants remain healthy and green, with elongated petioles while Col-0 plants show a stressed phenotype, with very long petioles and small leaves (remarkably similar to CCA1ox o38 plants in the same conditions, Figure 3-1).

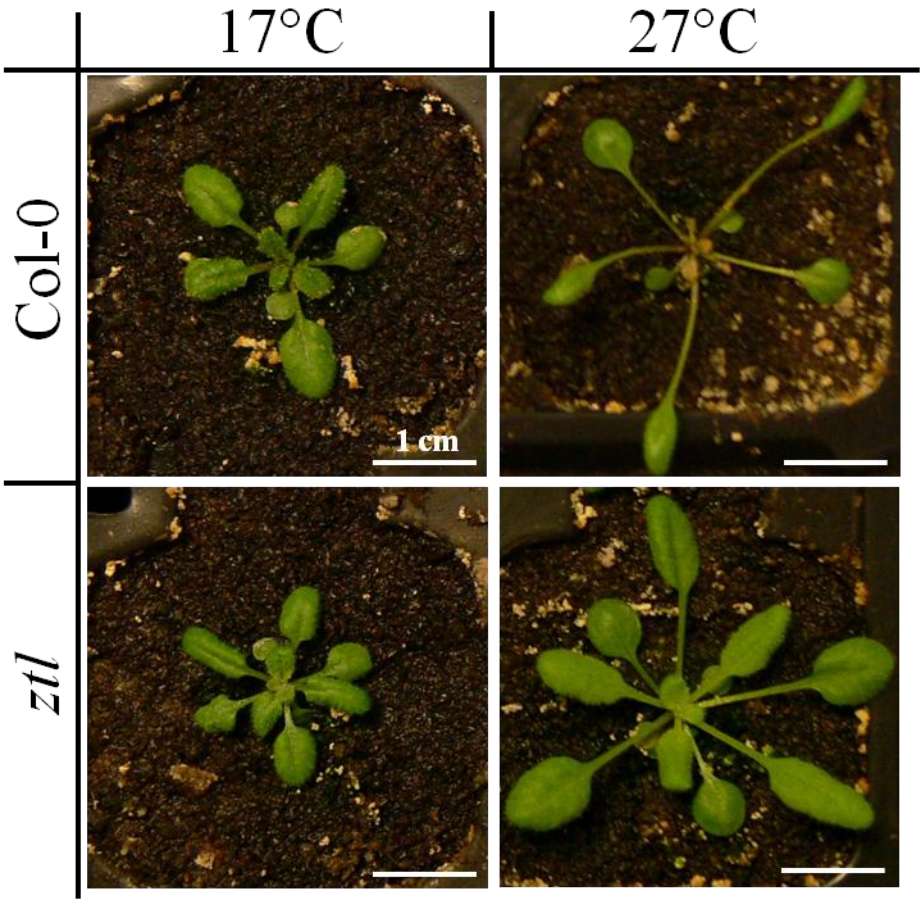


Figure 4-7. Col-0 and *ztl* plants, grown at 17ºC and 27ºC, in 15h L/15h D light cycles. The photo was taken 25 day after sowing, white bars equal to 1cm.

What was seen by eye was confirmed by results from visible leaf area measurements (Figure 4-8). A comparison of the two genotypes shows that *ztl* plants grown at 17°C are already performing better than their wild-type (**P= 0.005), and this difference is further increased by temperature. Actually, at 27°C *ztl* performs even better, showing almost three-fold the visible leaf area of Col-0 (**P<0.001). As a consequence, the difference between ztl and Col-0 increases with temperature. Finally, comparing 17°C to 27°C, both Col-0 and *ztl* plants had a greater leaf area at high temperature, with ANOVA P-values of **P=0.001 for the wild-type and **P<0.001 for the mutant.

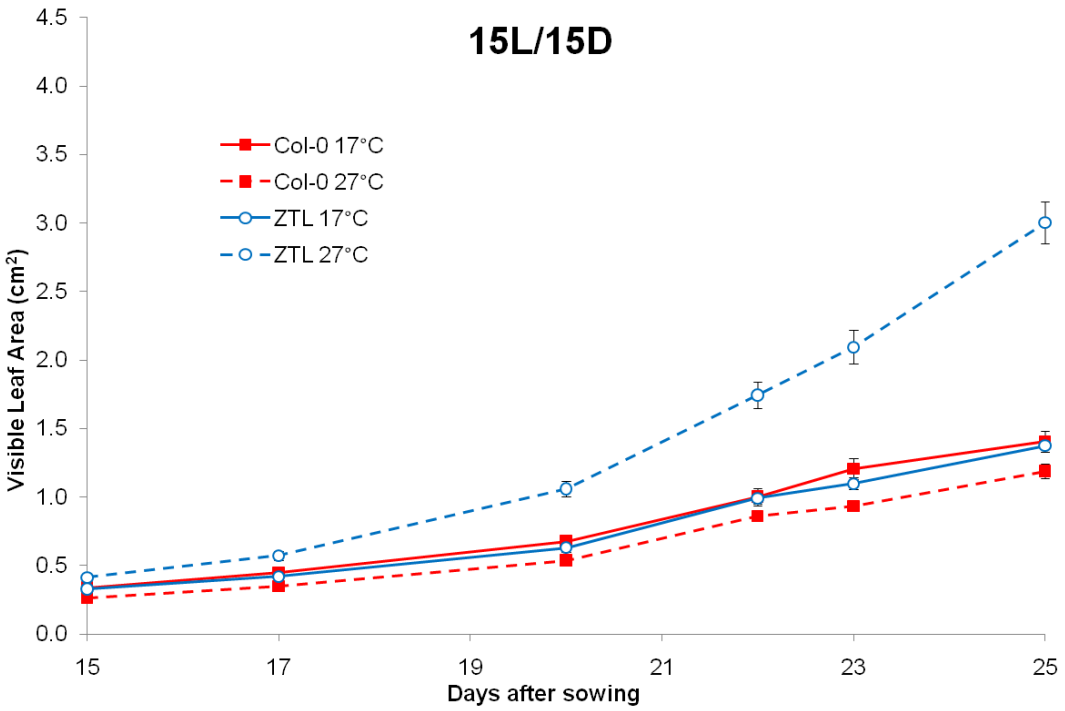


Figure 4-8. Visible leaf area of Col-0 and *ztl* plant lines 15 to 25 day after sowing, grown at 15h L/15h D light cycle and 17ºC or 27ºC. Data is mean ±1SEM. At day 25 in ANOVA test comparing *ztl* to Col-0, **P<0.01 at 17°C and 27°C. Figure representative of three independent replicates.

Fresh weight results (Figure 4-9) are consistent with visible leaf area results. Between 17 and 27°C, both Col-0 and *ztl* plants had a greater fresh weight at high temperature, with ANOVA P-values of **P<0.001 for both lines tested. Matching within the same temperature the wild-type against the mutant highlights that *ztl* plants are always heavier than Col-0, about 30% more at 17°C (**P=0.003) and about 60% more at 27°C (**P<0.001). Therefore the difference between the two genotypes is almost doubling with temperature.

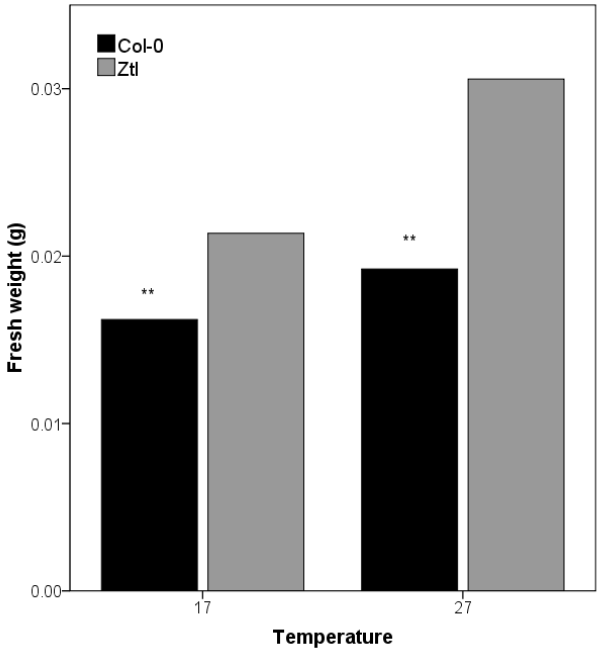


Figure 4-9. Fresh weight of Col-0 and *ztl* plant lines 25 day after sowing, grown at 15h L/15h D light cycle and 17ºC or 27ºC. Data is mean, in ANOVA test comparing *ztl* to Col-0 **P<0.01 at 17°C and 27°C. Figure representative of three independent replicates.

Similar trends are followed by dry weight results (Figure 4-10). The only difference found, analysing 17°C against 27°C results, is that Col-0 plants have the same dry weight (P=0.144) but for the *ztl* line weight is always greater at higher temperature (**P<0.001). Juxtaposing the two lines within each temperature, shows *ztl* plants having more chlorophyll than Col-0, 25% more at 17°C (*P=0.045) and up to 125% at 27°C (**P<0.001). Therefore differences increased dramatically with the rise of experimental temperature.

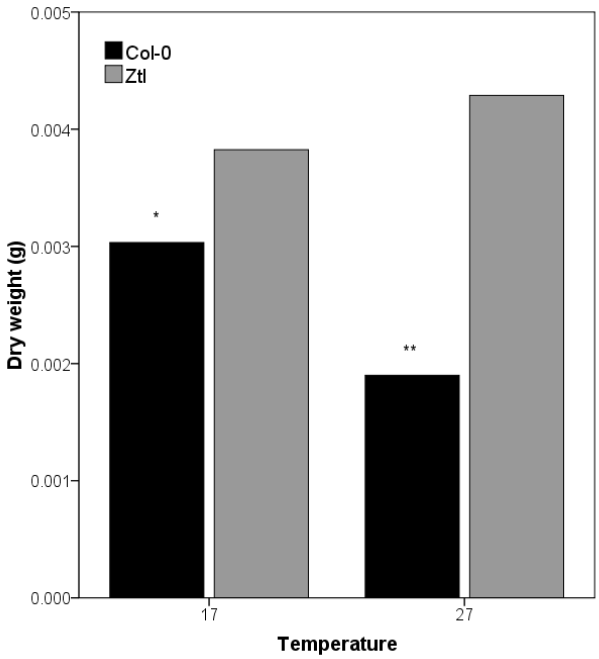


Figure 4-10. Dry weight of Col-0 and *ztl* plant lines 25 day after sowing, grown at 15h L/15h D light cycle and 17ºC or 27ºC. Data is mean, in ANOVA test comparing *ztl* to Col-0 *P<0.05 at 17°C and **P<0.01 at 27°C. Figure representative of three independent replicates.

At first glance, one could think total chlorophyll results on fresh weight (Figure 4-11) are following the tendency described up to now: this is true only in part. Actually over the two temperatures *ztl* plants had the same chlorophyll content, with ANOVA test reporting a P-value of 0.078. On the other side, Col-0 chlorophyll was found to be higher at 17°C rather that at 27°C (**P<0.001). Comparing within each temperature the two lines tested, results in *ztl* being about 25% greater than Col-0 at 17°C (**P=0.003) and about 80% times greater at 27°C (**P<0.001). It is clear that the differences just described increase significantly with the rise of temperature.

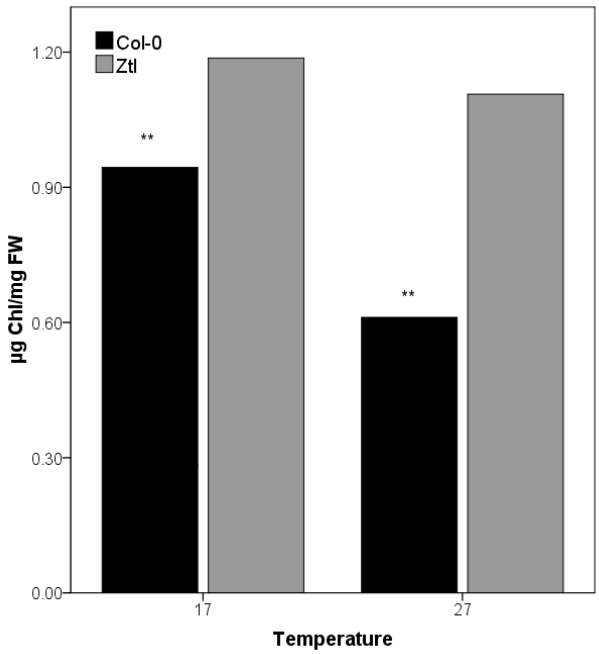


Figure 4-11. Total chlorophyll content (on fresh weight) of Col-0 and *ztl* plant lines 25 day after sowing, grown at 15h L/15h D light cycle and 17ºC or 27ºC. Data is mean, in ANOVA test comparing *ztl* to Col-0 **P<0.01 at 17°C and 27°C. Figure representative of three independent replicates.

Total chlorophyll quantification results on a dry weight basis (Figure 4-12) could be assimilated to the ones from chlorophyll on a fresh weight basis. When comparing 17°C to 27°C both wild-type (P= 0.270) and mutant (P= 0.512) have the same weight at the two temperatures tested. Balancing Col-0 against *ztl* within each temperature shows that *ztl* has more chlorophyll than Col-0 at both temperatures. In particular about 55% more at 17°C (**P=0.003) and about 70% more at 27°C (*P=0.016): the magnitude of the differences just presented increased with temperature.

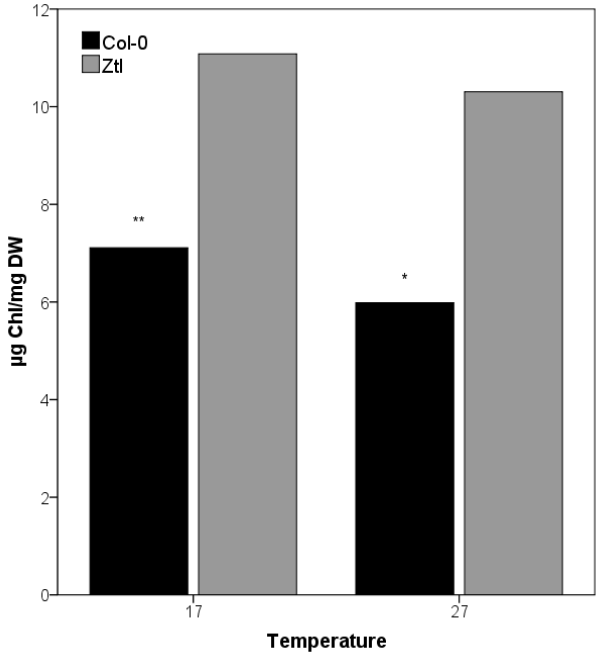


Figure 4-12. Total chlorophyll content (on dry weight) of Col-0 and *ztl* plant lines 25 day after sowing, grown at 15h L/15h D light cycle and 17ºC or 27ºC. Data is mean, in T-Test comparing *ztl* to Col-0 **P<0.01 at 17°C and *P<0.05 at 27°C. Figure representative of a single replica.

Summary of Results

Col-0 and *ztl* in 15hL/15hD light cycles

Leaf Area - 25 days				15LD
Different?	**How?**	**P value**	**Diff.**	
Yes **	*ztl* > Col-0	0.005	0.096	**17°C**
Yes **	*ztl* > Col-0	<0.001	0.220	**27°C**
Yes				> with T°
Yes **	27 > 17	0.001	0.095	**Col-0**
Yes **	27 > 17	<0.001	0.218	***ztl***

Fresh Weight				15LD	Dry Weight			
Different?	**How?**	**P value**	**Diff.**		**Different?**	**How?**	**P value**	**Diff.**
Yes **	*ztl* > Col-0	0.003	0.061	**17°C**	Yes *	*ztl* > Col-0	0.045	0.072
Yes **	*ztl* > Col-0	<0.001	0.172	**27°C**	Yes **	*ztl* > Col-0	<0.001	0.243
Yes				> with T°	Yes			
Yes **	27 > 17	<0.001	0.101	**Col-0**	No	-	0.144	-
Yes **	27 > 17	<0.001	0.211	***ztl***	Yes **	27 > 17	<0.001	0.120

Chl FW				15LD	Chl DW (T-Test)			
Different?	**How?**	**P value**	**Diff.**		**Different?**	**How?**	**P value**	**Diff.**
Yes **	*ztl* > Col-0	0.003	0.053	**17°C**	Yes **	*ztl* > Col-0	0.003	0.195
Yes **	*ztl* > Col-0	<0.001	0.241	**27°C**	Yes *	*ztl* > Col-0	0.016	0.240
Yes				> with T°	Yes			
Yes **	17 > 27	<0.001	0.223	**Col-0**	No	-	0.270	-
No	-	0.078	0.035	***ztl***	No	-	0.512	-

Table 10. Summary of results from the assays done on Col-0 and *ztl* plant lines, grown in 15h L/15h D light cycles and 17ºC or 27ºC. "P value" is probability from Analysis of Variance and "Diff." is the center value from Tukey test, both done as described in 2.5.4 Data analysis. ">with T°" indicates whether the difference between genotypes is increasing with the rise of temperature. *=P<0.05, **=P<0.01

Descriptive Statistics of all replicates

Col-0 and *ztl* in 15hL/15hD light cycles

Leaf Area 25d		N		Mean		SE Mean		St Dev		Variance	
	Replicate	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C
Col-0	1	20	20	1.4055	1.1901	0.0883	0.0577	0.3951	0.2582	0.1561	0.0667
	2	35	38	0.5680	0.8161	0.0420	0.0447	0.2485	0.2758	0.0618	0.0761
	3	40	40	1.0285	1.2546	0.0724	0.0731	0.4581	0.4624	0.2099	0.2138
ztl	1	20	20	1.3754	3.0041	0.0571	0.1982	0.2556	0.8863	0.0653	0.7856
	2	36	37	0.9916	1.2630	0.0554	0.0654	0.3323	0.3980	0.1104	0.1584
	3	40	40	1.0535	1.9360	0.0609	0.1217	0.3849	0.7698	0.1482	0.5926

Fresh Weight		N		Mean		SE Mean		St Dev		Variance	
	Replicate	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C
Col-0	1	40	40	0.0299	0.0323	0.0012	0.0012	0.0073	0.0077	0.0001	0.0001
	2	35	38	0.0162	0.0192	0.0014	0.0010	0.0080	0.0064	0.0001	0.0000
	3	40	40	0.0282	0.0412	0.0019	0.0023	0.0121	0.0146	0.0002	0.0002
ztl	1	40	40	0.0309	0.0569	0.0011	0.0027	0.0069	0.0170	0.0001	0.0003
	2	36	37	0.0214	0.0306	0.0011	0.0012	0.0063	0.0073	0.0000	0.0001
	3	39	40	0.0289	0.0474	0.0014	0.0027	0.0090	0.0172	0.0001	0.0003

Dry Weight		N		Mean		SE Mean		St Dev		Variance	
	Replicate	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C
Col-0	1	23	23	0.0034	0.0030	0.0002	0.0001	0.0009	0.0007	0.0000	0.0000
	2	18	9	0.0030	0.0019	0.0005	0.0002	0.0020	0.0006	0.0000	0.0000
	3	10	10	0.0040	0.0044	0.0006	0.0005	0.0019	0.0016	0.0000	0.0000
ztl	1	24	31	0.0038	0.0055	0.0002	0.0002	0.0009	0.0012	0.0000	0.0000
	2	8	18	0.0038	0.0043	0.0004	0.0004	0.0011	0.0015	0.0000	0.0000
	3	10	10	0.0041	0.0053	0.0005	0.0006	0.0014	0.0019	0.0000	0.0000

Chl FW		N		Mean		SE Mean		St Dev		Variance	
	Replicate	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C
Col-0	1	5	5	0.9441	0.6115	0.0169	0.0317	0.0377	0.0709	0.0014	0.0050
	2	5	5	0.7921	0.5121	0.0205	0.0315	0.0459	0.0705	0.0021	0.0050
	3	5	5	1.6311	0.8446	0.0759	0.0369	0.1697	0.0826	0.0288	0.0068
ztl	1	5	5	1.1868	1.1066	0.0655	0.0575	0.1465	0.1286	0.0215	0.0165
	2	5	5	0.8333	0.8753	0.0551	0.0403	0.1232	0.0901	0.0152	0.0081
	3	5	5	1.8020	1.4367	0.0763	0.0302	0.1705	0.0675	0.0291	0.0046

Chl DW		N		Mean		SE Mean		St Dev		Variance	
	Replicate	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C
Col-0	1	5	5	7.1105	5.9862	0.6118	0.6938	1.3681	1.5514	1.8716	2.4069
ztl	1	5	5	11.0824	10.3057	0.6697	1.1313	1.4975	2.5296	2.2424	6.3988

Table 11. Descriptive Statistics of all the independent experimental replicates of the assays performed on Col-0 and *ztl* lines. Plants were grown in 15h L/15h D light cycles and 17ºC or 27ºC.

4.2.3 - 10h L/10h D – Col-0 performs better than ztl

The third set of experiments comparing the performance of the *ztl* mutant against the wild-type Col-0 was conducted in 10h L/10h D cycles. Although this particular light cycle matches neither the *ztl* mutant nor the wild-type Col-0, assays results and the plants' phenotype (Figure 4-13) match quite closely the one seen in 12h L/12h D cycles (Figure 4-1). At 17°C wild-type plants are slightly bigger that *ztl* mutant seedlings, and at 27°C this difference is even greater.

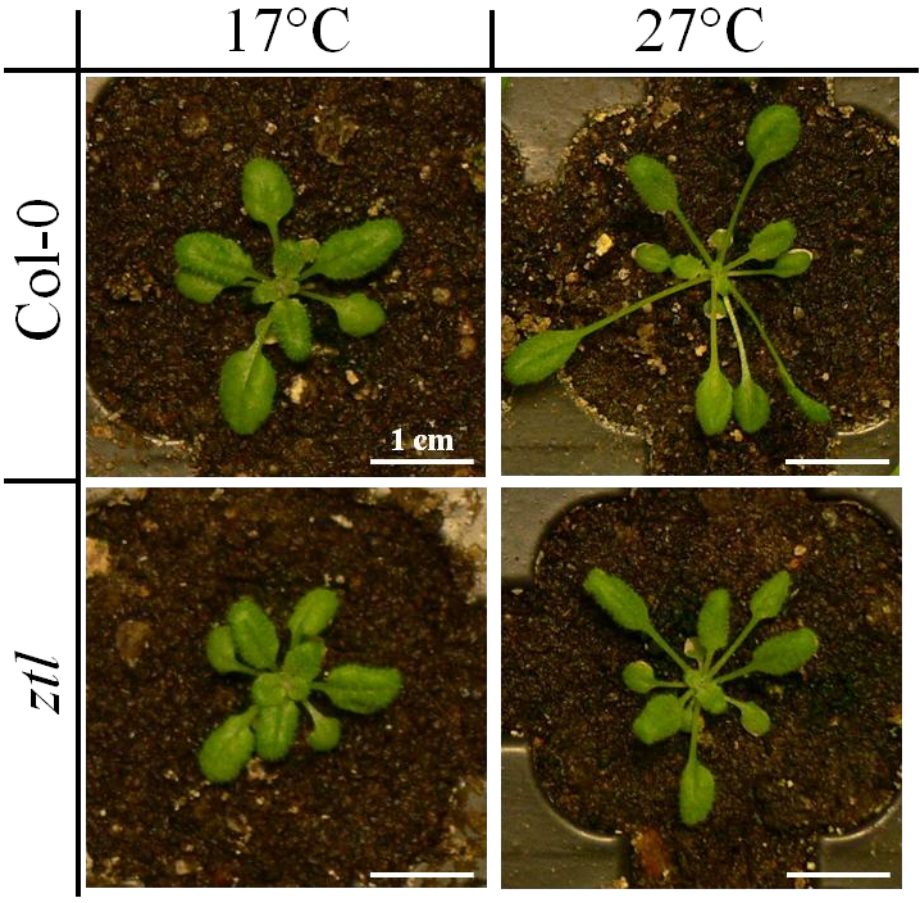


Figure 4-13. Col-0 and *ztl* plants, grown at 17ºC and 27ºC, in 10h L/10h D light cycles. The photo was taken 25 day after sowing, white bars equal to 1cm.

Visible leaf area (Figure 4-14) quantitative results support the visual phenotype. Over the two temperatures both Col-0 and *ztl* had a greater leaf area at higher temperature (**P<0.001). Comparing Col-0 against *ztl* shows the wild-type has a greater leaf area than the mutant, and an increasing difference with the rise of temperature. In particular Col-0 was found to be about 1.2 times greater at 17°C (**P= 0.008) and 1.3 times greater at 27°C (**P<0.001).

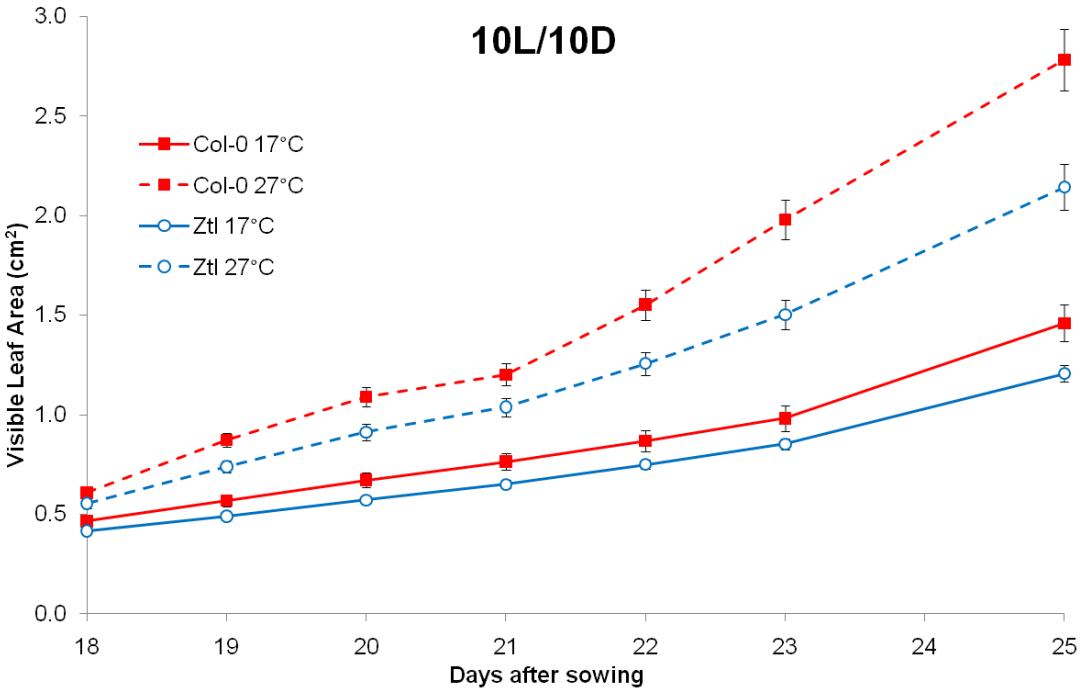


Figure 4-14. Visible leaf area of Col-0 and *ztl* plant lines 15 to 25 day after sowing, grown at 10h L/10h D light cycle and 17ºC or 27ºC. Data is mean ±1SEM. At day 25 in ANOVA test comparing *ztl* to Col-0, **P<0.01 at 17°C and 27°C. Figure representative of two independent replicates.

Fresh weight (Figure 4-15) follows the same trend as visible leaf area. Between 17 and 27°C both genotypes had a greater weight at higher temperature (**P<0.001). A comparison of the two lines within the same temperature highlights Col-0 being always heavier than *ztl*, with a dramatic increase of this difference at higher temperature. In fact at 17°C the difference is smaller, although significant (**P=0.001), but at 27°C Col-0 is almost double than *ztl* (**P<0.001).

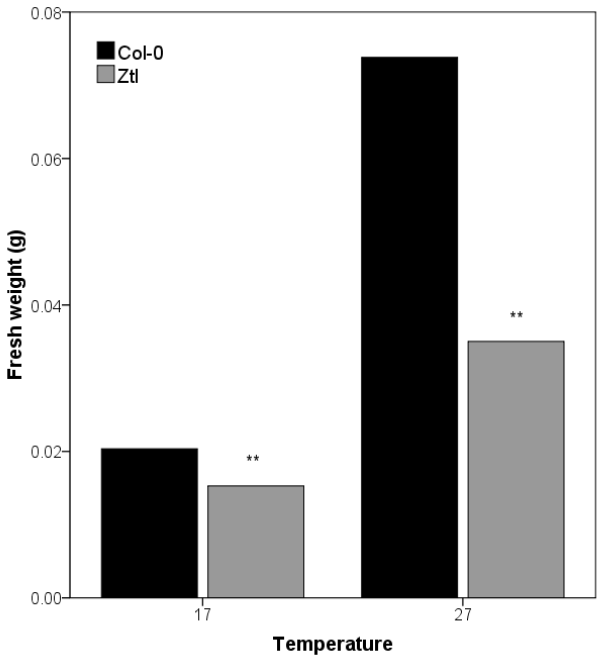


Figure 4-15. Fresh weight of Col-0 and *ztl* plant lines 25 day after sowing, grown at 10h L/10h D light cycle and 17ºC or 27ºC. Data is mean, in ANOVA test comparing *ztl* to Col-0 **P<0.01 at 17°C and 27°C. Figure representative of two independent replicates.

Dry weight (Figure 4-16) roughly correlates with the previous assays. When Comparing 17°C to 27°C, both wild-type and mutant had a greater weight at high temperature (**P<0.001). Matching Col-0 against *ztl* within the same temperature leads at 17°C to no difference (P= 0.742) and at 27°C to Col-0 being definitely a better performer than *ztl* (**P<0.001), with almost double the weight. Therefore the only difference from previous assays was found at 17°C.

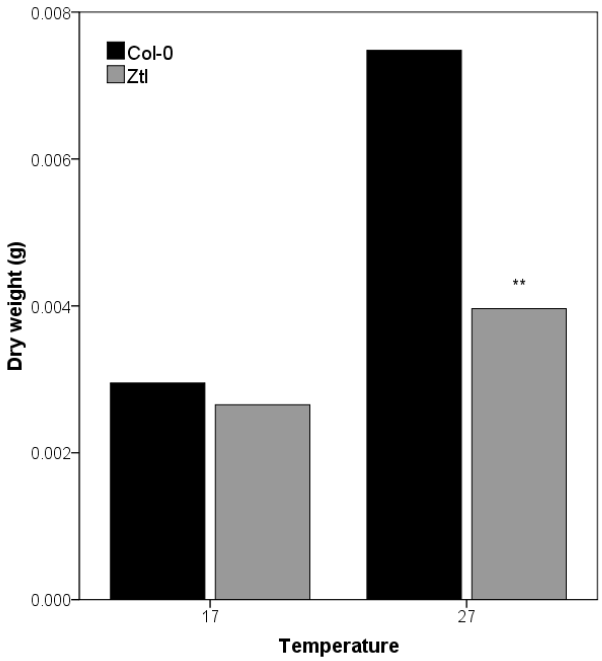


Figure 4-16. Dry weight of Col-0 and *ztl* plant lines 25 day after sowing, grown at 10h L/10h D light cycle and 17ºC or 27ºC. Data is mean, in ANOVA test comparing *ztl* to Col-0 P>0.7 at 17°C and **P<0.01 at 27°C. Figure representative of two independent replicates.

Chlorophyll assays reported strikingly different results from the other assays. This could be explained by the fact that both plants are growing in an "adverse" light/dark cycle, and this could be more effective in altering chlorophyll production than overall plant growth. For total chlorophyll on a fresh weight basis (Figure 4-17), comparing 17°C to 27°C shows no difference at all, in wild-type (P= 0.284) or the mutant (P= 0.220). Moreover, juxtaposing the two genotypes within the same temperature leads again at 17°C to no difference (P= 0.439) and at 27°C to *ztl* having a greater chlorophyll concentration than Col-0 (**P<0.001). The difference obviously increased with temperature, but in the opposite direction of previous assays.

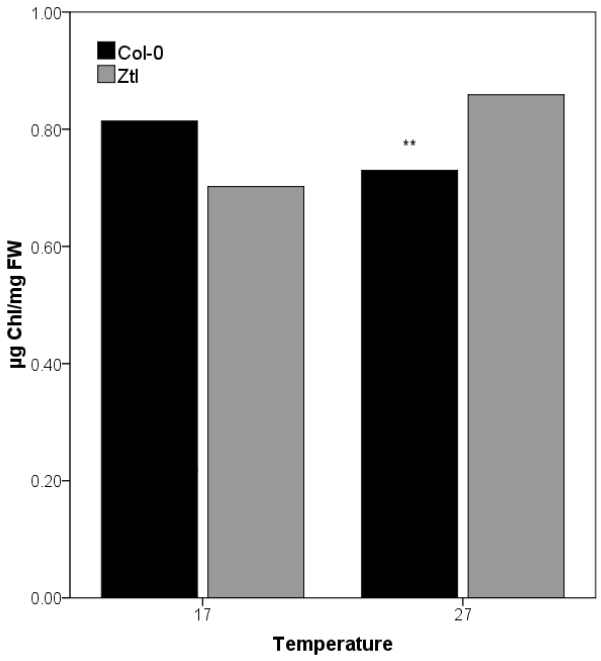


Figure 4-17. Total chlorophyll content (on fresh weight) of Col-0 and *ztl* plant lines 25 day after sowing, grown at 10h L/10h D light cycle and 17ºC or 27ºC. Data is mean, in ANOVA test comparing *ztl* to Col-0 P>0.43 at 17°C and **P<0.01 at 27°C. Figure representative of two independent replicates.

Total chlorophyll on a dry weight basis (Figure 4-18) again did not match results from the previous assays. Analysing 17°C against 27°C results in no differences, neither for Col-0 (P=0.070) nor for *ztl* (P=0.480). Furthermore, comparing within each temperature the two lines tested, results in Col-0 and *ztl* lines having the same chlorophyll content, both at 17°C (P=0.165) and at 27°C (P=0.385). Only one replicate was done for this assay in these experimental conditions and therefore might have led to less accurate results.

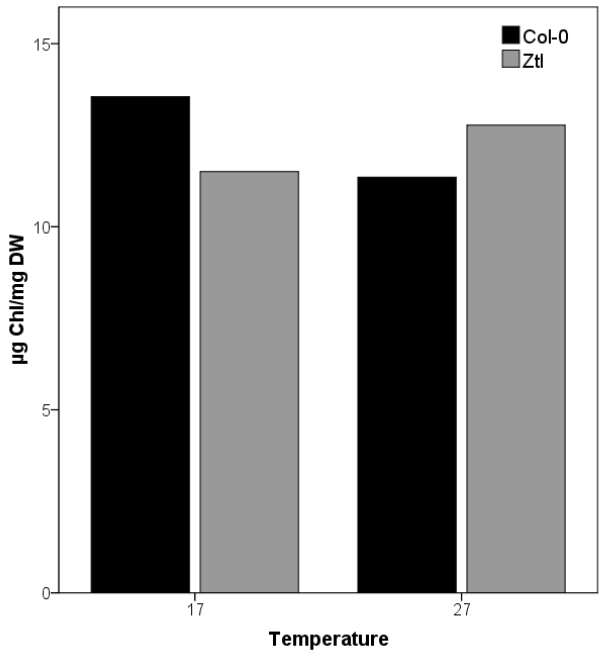


Figure 4-18. Total chlorophyll content (on dry weight) of Col-0 and *ztl* plant lines 25 day after sowing, grown at 10h L/10h D light cycle and 17ºC or 27ºC. Data is mean, in T-Test comparing *ztl* to Col-0 P>0.16 at 17°C and P>0.38 at 27°C. Figure representative of a single replica.

Summary of Results

Col-0 and *ztl* in 10hL/10hD light cycles

Leaf Area - 25 days				10LD
Different?	**How?**	**P value**	**Diff.**	
Yes **	Col-0 > *ztl*	0.008	0.079	**17°C**
Yes **	Col-0 > *ztl*	<0.001	0.158	**27°C**
Yes				> with T°
Yes **	27 > 17	<0.001	0.341	**Col-0**
Yes **	27 > 17	<0.001	0.262	***ztl***

Fresh Weight				10LD	Dry Weight			
Different?	**How?**	**P value**	**Diff.**		**Different?**	**How?**	**P value**	**Diff.**
Yes **	Col-0 > *ztl*	0.001	0.071	**17°C**	No	-	0.742	-
Yes **	Col-0 > *ztl*	<0.001	0.233	**27°C**	Yes **	Col-0 > *ztl*	<0.001	0.230
Yes				> with T°	Yes			
Yes **	27 > 17	<0.001	0.444	**Col-0**	Yes **	27 > 17	<0.001	0.364
Yes **	27 > 17	<0.001	0.284	***ztl***	Yes **	27 > 17	<0.001	0.134

Chl FW				10LD	Chl DW (T-Test)			
Different?	**How?**	**P value**	**Diff.**		**Different?**	**How?**	**P value**	**Diff.**
No	-	0.439	-	**17°C**	No	-	0.165	-
Yes **	*ztl* > Col-0	<0.001	0.111	**27°C**	No	-	0.385	-
Yes				> with T°				
No	-	0.284	-	**Col-0**	No	-	0.070	-
No	-	0.220	-	***ztl***	No	-	0.480	-

Table 12. Summary of results from the assays done on Col-0 and *ztl* plant lines, grown in 10h L/10h D light cycles and 17ºC or 27ºC. "P value" is probability from Analysis of Variance and "Diff." is the center value from Tukey test, both done as described in 2.5.4 Data analysis. ">with T°" indicates whether the difference between genotypes is increasing with the rise of temperature. *=P<0.05, **=P<0.01

Descriptive Statistics of all replicates

Col-0 and *ztl* in 10hL/10hD light cycles

Leaf Area 25d		N		Mean		SE Mean		St Dev		Variance	
	Replicate	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C
Col-0	1	20	20	0.9729	2.6169	0.0959	0.1282	0.4289	0.5732	0.1839	0.3286
	2	40	40	1.4594	2.7823	0.0927	0.1562	0.5860	0.9879	0.3434	0.9760
ztl	1	20	20	0.7592	1.8147	0.0702	0.2032	0.3139	0.9087	0.0986	0.8257
	2	40	40	1.2075	2.1433	0.0415	0.1136	0.2623	0.7182	0.0688	0.5159

Fresh Weight		N		Mean		SE Mean		St Dev		Variance	
	Replicate	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C
Col-0	1	39	38	0.0204	0.0738	0.0011	0.0032	0.0067	0.0199	0.0000	0.0004
	2	40	40	0.0351	0.0753	0.0021	0.0046	0.0134	0.0289	0.0002	0.0008
ztl	1	38	41	0.0153	0.0350	0.0008	0.0021	0.0052	0.0137	0.0000	0.0002
	2	40	40	0.0327	0.0557	0.0012	0.0032	0.0074	0.0200	0.0001	0.0004

Dry Weight		N		Mean		SE Mean		St Dev		Variance	
	Replicate	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C
Col-0	1	32	28	0.0030	0.0075	0.0002	0.0005	0.0011	0.0027	0.0000	0.0000
	2	11	10	0.0044	0.0068	0.0004	0.0009	0.0014	0.0030	0.0000	0.0000
ztl	1	24	19	0.0027	0.0040	0.0001	0.0003	0.0007	0.0013	0.0000	0.0000
	2	10	10	0.0043	0.0051	0.0003	0.0006	0.0010	0.0018	0.0000	0.0000

Chl FW		N		Mean		SE Mean		St Dev		Variance	
	Replicate	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C
Col-0	1	5	5	0.8139	0.7296	0.0832	0.0542	0.1860	0.1213	0.0346	0.0147
	2	5	5	2.1560	2.0490	0.1006	0.0971	0.2249	0.2172	0.0506	0.0472
ztl	1	5	5	0.7021	0.8588	0.0826	0.0517	0.1846	0.1157	0.0341	0.0134
	2	5	5	2.9625	2.8813	0.0993	0.0713	0.2220	0.1595	0.0493	0.0255

Chl DW		N		Mean		SE Mean		St Dev		Variance	
	Replicate	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C
Col-0	1	6	6	13.5503	11.3530	0.5096	0.9051	1.2483	2.2171	1.5583	4.9157
ztl	1	5	6	11.5064	12.7749	1.1320	1.2380	2.5313	3.0326	6.4074	9.1964

Table 13. Descriptive Statistics of all the independent experimental replicates of the assays performed on Col-0 and *ztl* lines. Plants were grown in 10h L/10h D light cycles and 17ºC or 27ºC.

4.2.4 - Light cycles comparison within genotype – Col-0 and ztl

Previously in this chapter data was presented quantifying the increasing importance of circadian resonance at high temperatures, comparing the mutant line *ztl* and its wild-type Col-0. The aim of this subchapter instead is to compare each plant line to itself, across three light/dark cycles: 12h L/12h D, 15h L/15h D and 10h L/10h D. Due to time and equipment constraints it was not possible to design tailored experiments for this comparison, therefore data from experiments presented earlier in this chapter were used.

All the performance experiments presented however, were designed to compare the mutant line with its wild-type over two temperatures and in a given light/dark cycle. To do so plants were germinated and grown in the very same conditions until moved into the experimental environment, set at a single light/dark cycle and two temperatures (one per cabinet, total of two cabinets). This tactic was found to be necessary, as plants germinated in the same growth room successively showed a fairly wide variability in the physiological traits assayed, especially in the magnitude of the result values retrieved. Analysis of Variance (ANOVA), as described in 2.5.4 Data analysis, normally allows this variability to be taken into account and removed from the comparison. However, to remove variability, the comparison sought needs to be done within a single experiment. In the case of the comparison described in this subchapter, a minimum of six cabinets would have been required (three light cycles x two temperatures). Because of the described variability across successive experiments, and statistical analysis limits, the comparison here attempted may not be as valuable as if was done on data produced for the purpose. Massonnet *et al.*, 2010 recently probed the reproducibility of data in independent experiments, underlining the difficulty of a tight control over environmental plant growth conditions.

To have an idea of the differences, results obtained from successive performance experiments were grouped by light cycle and temperature, then averaged all together (Figure 4-19). Across all assays it is clear that in 15h L/15h D (15LD from now on) Col-0 plants performed poorly compared to the other two light cycles, both at 17°C and at 27°C. At first glance, in 10LD plants seem to have a similar performance to12LD. A closer examination however reveals that in dry weight and chlorophyll on a dry weight basis

(Figure 4-19, panels C and E respectively), Col-0 plants grown at 12LD have greater values. This might be interpreted as due to differential water content at different light cycles: in particular, plants would have retained more water at 10LD, and therefore distorted assay results on fresh tissue. In conclusion at 12LD Col-0 plants seem to have a greater performance than at 10LD and 15LD. This is confirmed by looking at a single experiment per light cycle, selected to represent the trends found and plotted in Figure 4-20. In these experiments Col-0 plants performed definitely better at 12LD than the other two light cycles, supporting the hypothesis that having an internal clock matched to the external environment benefits the plant, especially in higher temperatures. Plants however can sustain high temperatures until these become far too stressful. When growth temperature was pushed up to 33°C, Col-0 plants showed a reduced leaf area, weight and chlorophyll content, even in favourable 12LD light cycles (Panels A, B and D in Figure 4-19 and Figure 4-20).

Results from all performance experiments for *ztl* line were grouped by light cycle and temperature in Figure 4-21. Looking at the grouped results, it is not easy to identify a definite trend across the three light cycles tested. In the majority of assays (Figure 4-21, panels B, C and E) *ztl* plants seem to perform better at 12LD, while minor differences were found in the other two light cycles. Plotting a single experiment per light cycle however, makes it is easier to spot some important differences. In all assays except chlorophyll on a fresh weight basis (Figure 4-22, panel E), *ztl* plants perform best at 15LD and at higher temperature the difference is even larger. There is then a downward trend in plant performance when moving from 15LD, to 12LD and finally to 10LD: this trend is particularly visible at 17°C and in leaf area, fresh weight and chlorophyll on a fresh weight basis (Figure 4-22, panels A, B and D respectively). These trends, although not as clear as for Col-0, seem to suggest once again that circadian resonance is important for plant performance with the increase in growth temperatures.

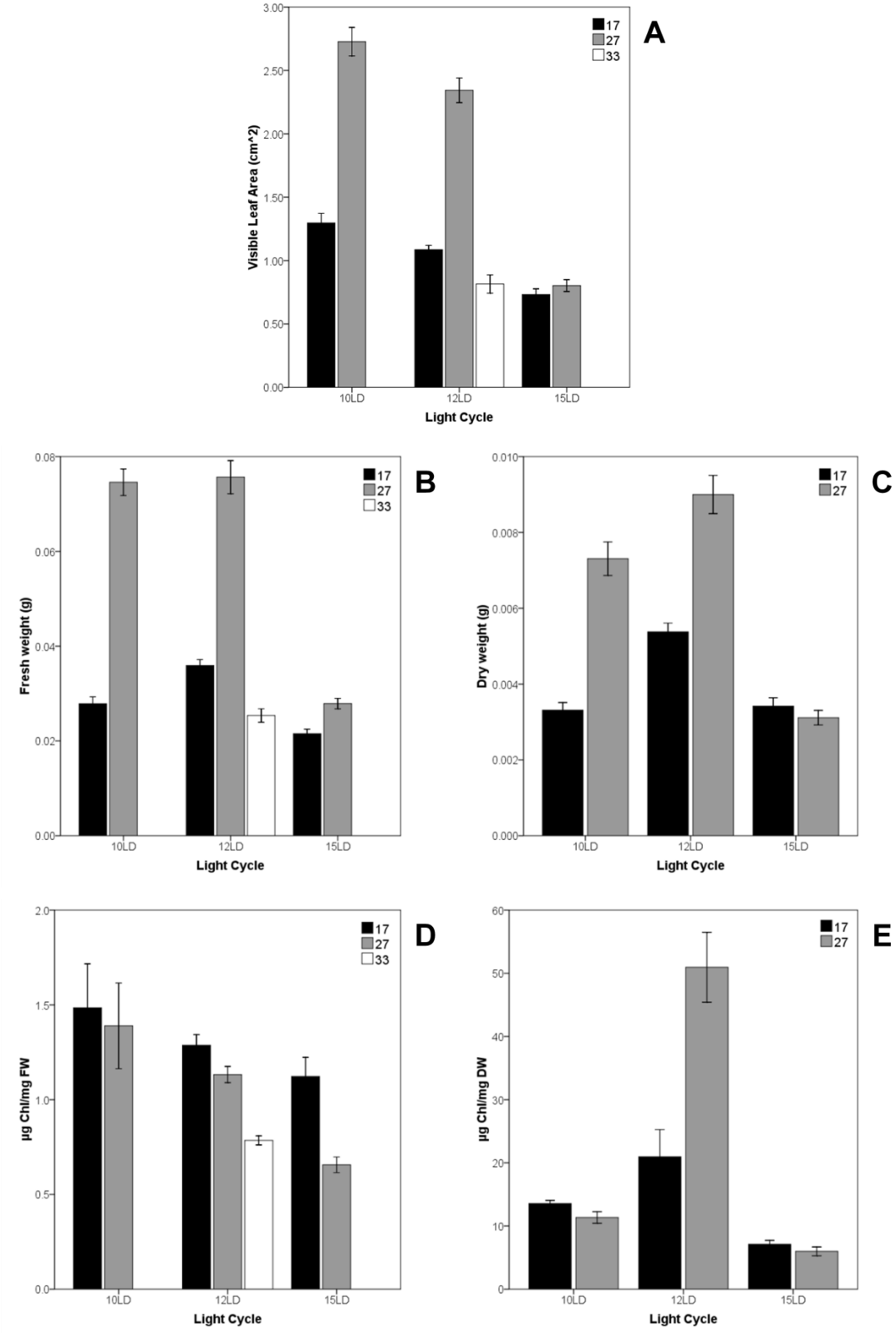


Figure 4-19. Col-0 plants 25 day after sowing, grown at 10h:10h, 12h:12h or 15h:15h Light/Dark cycles and 17, 27 or 33°C. Each bar is the mean of all experiments performed for the specific growth condition. Error bars are ± 1 SEM. Visible leaf area at day 25 (**A**), fresh (**B**) and dry (**C**) weight, total chlorophyll content on fresh (**D**) and dry (**E**) weight.

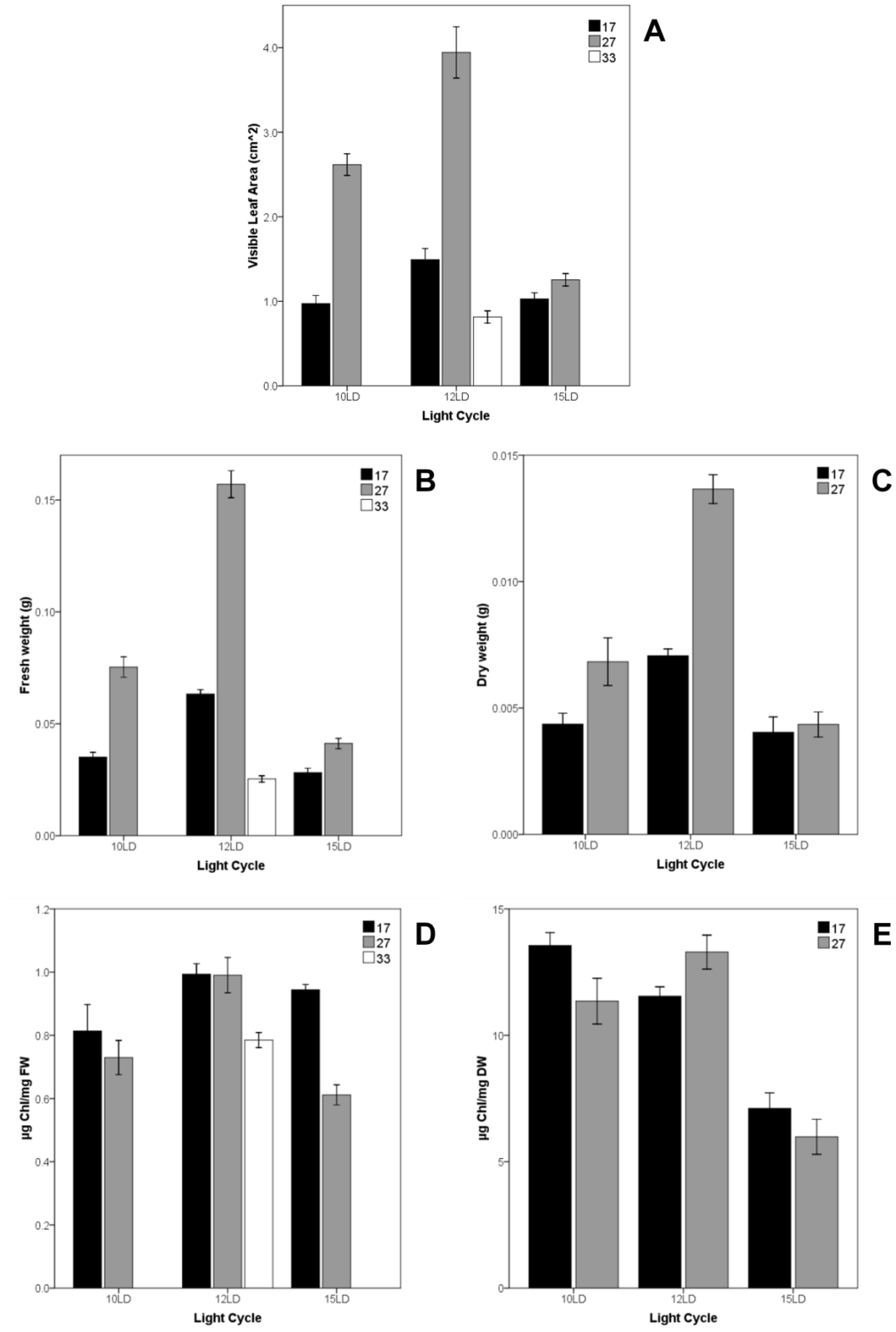


Figure 4-20. Col-0 plants 25 day after sowing, grown at 10h:10h, 12h:12h or 15h:15h Light/Dark cycles and 17, 27 or 33°C. Each bar is the mean of only one selected performance experiment for the specific growth condition. Error bars are ± 1 SEM. Visible leaf area at day 25 (**A**), fresh (**B**) and dry (**C**) weight, total chlorophyll content on fresh (**D**) and dry (**E**) weight.

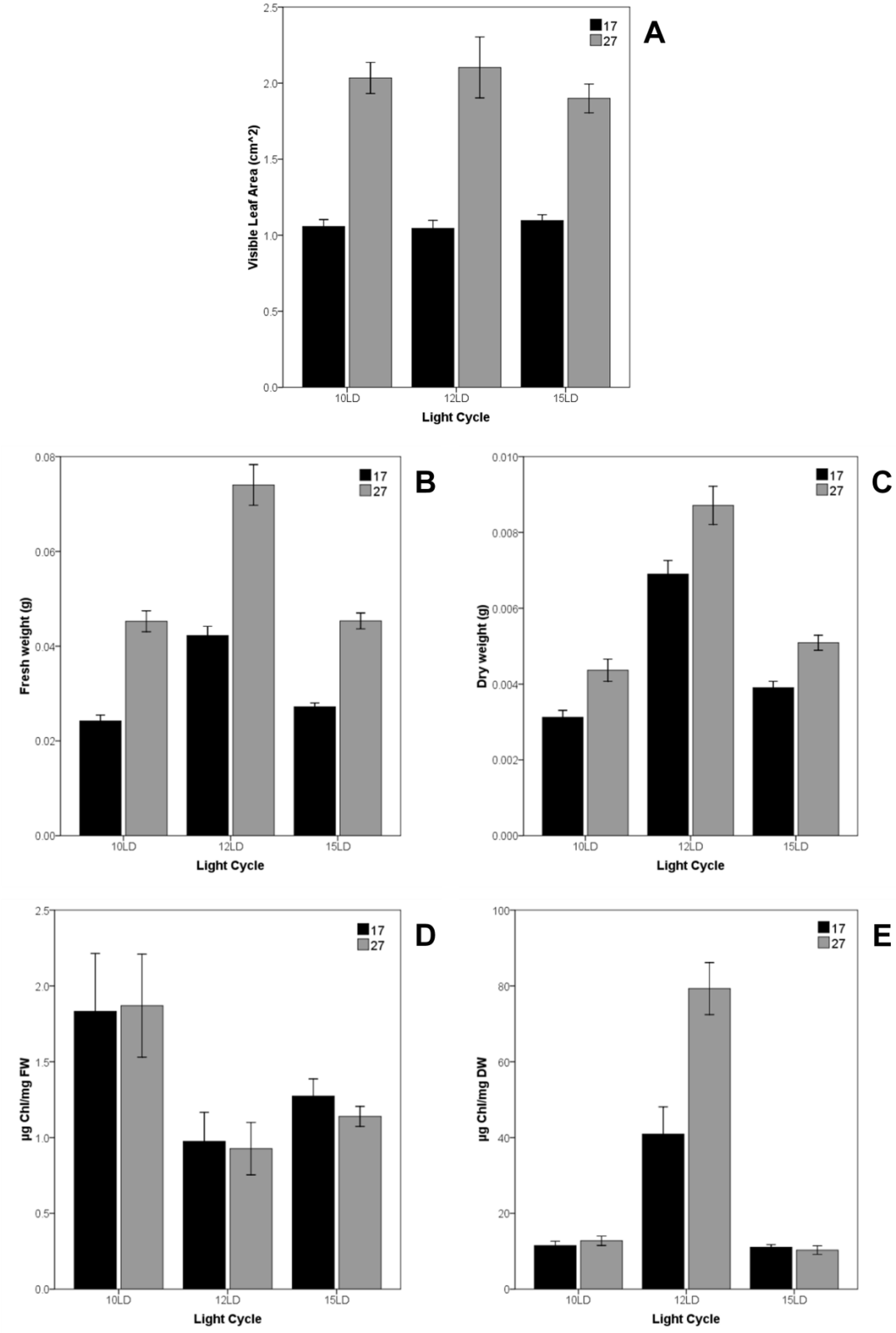


Figure 4-21. *ztl* plants 25 day after sowing, grown at 10h:10h, 12h:12h or 15h:15h Light/Dark cycles and 17 or 27°C. Each bar is the mean of all experiments performed for the specific growth condition. Error bars are ± 1 SEM. Visible leaf area at day 25 (**A**), fresh (**B**) and dry (**C**) weight, total chlorophyll content on fresh (**D**) and dry (**E**) weight.

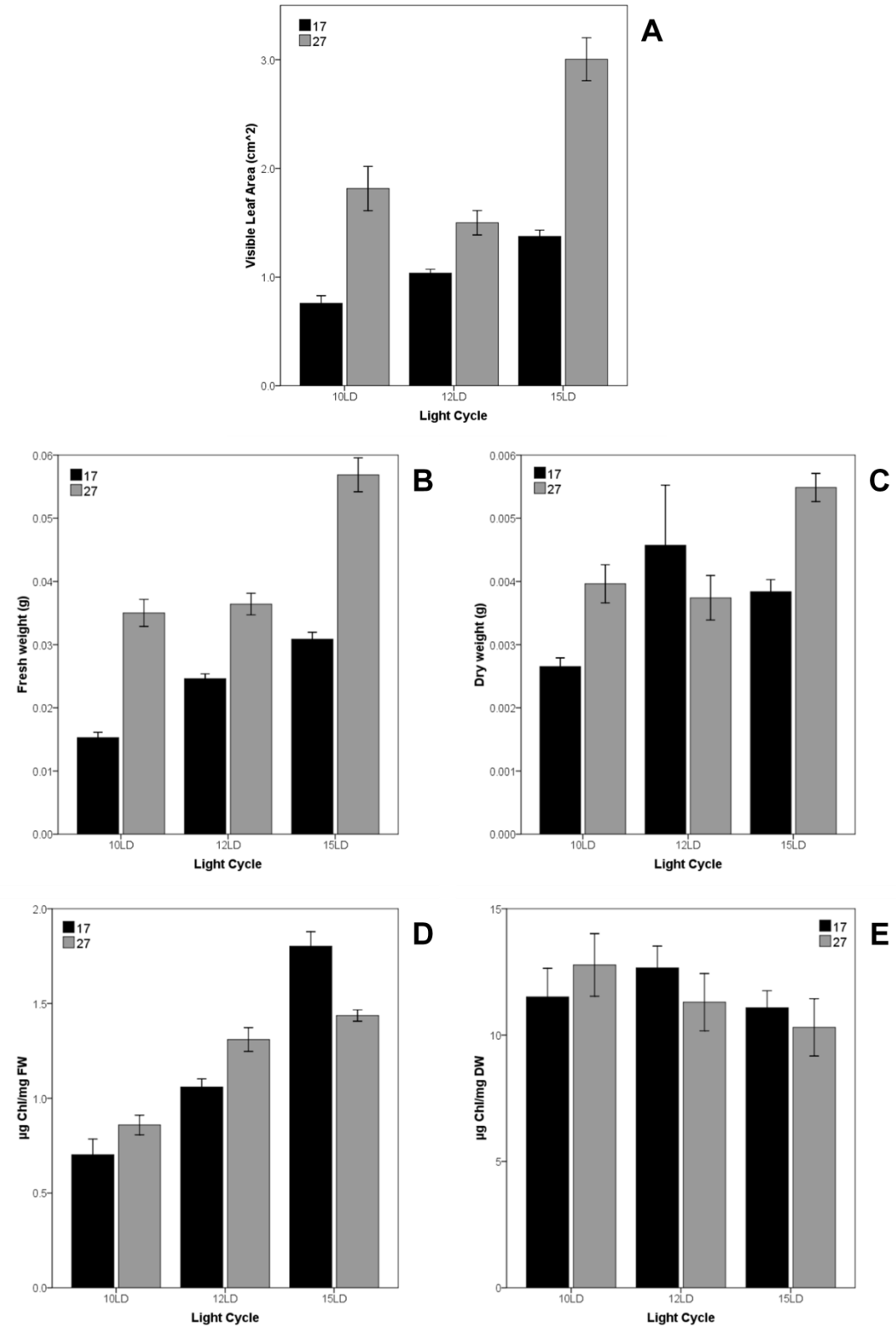


Figure 4-22. *ztl* plants 25 day after sowing, grown at 10h:10h, 12h:12h or 15h:15h Light/Dark cycles and 17 or 27°C. Each bar is the mean of only one selected performance experiment for the specific growth condition. Error bars are ± 1 SEM. Visible leaf area at day 25 (**A**), fresh (**B**) and dry (**C**) weight, total chlorophyll content on fresh (**D**) and dry (**E**) weight.

4.3 Discussion

Previous reports (Dodd *et al.*, 2005) demonstrated that plants with a robust and accurate clock, in circadian resonance with the environment, increased their photosynthesis and growth. In this chapter it was shown that the clock is even more important at higher temperatures. Col-0 and *ztl* plants were grown in 12h L/12h D, 15h L/15h D or 10h L/10h D light cycles and compared at two temperatures, 17°C or 27°C. In 12LD light cycles and 17°C, wild-type (Col-0) plants with a normal period of 24 hours were identical to mutant plants *ztl*, with a long period of 30 hours. Increasing the temperature highlighted the differences between the two lines: Col-0 performed significantly better than the mutant *ztl*. Moving on to 15LD cycles almost reversed the scenario: the line in circadian resonance was *ztl* and in fact performed better than Col-0 even at 17°C, while at 27°C the difference increased even more. Finally in 10LD the two lines, both non-resonant with the environment, behaved in similar way to 12LD.

It could be argued that the difference described might be just due to an increased growth rate at higher temperature: in my opinion this is not the case. In 12LD and 27°C there is a significant difference between the two lines, but this is less apparent at 17°C, where instead it should be found if there was a proportionally reduced growth rate. Moreover, at 15LD the percentage difference between *ztl* and Col-0 increases dramatically with temperature from around 25% at 17°C to 60, 80 or even 125% (Figure 4-9, Figure 4-11 and Figure 4-10 respectively). These percentage differences should instead remain constant if there was an increased growth rate, as the two lines share the whole genome but a single mutation.

The last comparison between *ztl* and its wild-type Col-0 was within the single genotype, across the three light cycles tested. As in the published results by Dodd *et al.*, 2005, Col-0 plants performed poorly at 15LD compared to 12LD in all assays executed. Similarly, at 10LD plant performance was poor when compared to 12LD: in this case however the assays which gave closer results were dry weight and chlorophyll on a dry weight basis. All cases demonstrate an advantage for plants of growing in an environment resonant with their internal clock period. Results presented here are a proof that the

resonance advantage is even greater at high temperature: the magnitude of the response in fact, is much higher at 27°C than at 17°C. Finally, a very similar trend was found across the light cycles for the *ztl* line. Performance was greatest in 15LD, where the mutant period matches the external environment, decreased with shortening of the length of the light cycle to 12LD and then to 10LD. Once again, differences were larger at 27°C, stressing the increased importance of having an accurate clock when the temperature increases.

CHAPTER 5 - An accurate clock (*toc1* line)

5.1 Introduction

In the previous chapter (page 70) it was shown the increased importance for an *Arabidopsis* plant of being in synch with the environmental light/dark cycles when the temperature rises. The lines used to demonstrate this were the long period *ztl* mutant line and its wild type Col-0. I here intend to extend the research on the correlation between circadian resonance and high temperature, using the short period (20h) mutant *toc1* and its own wild-type line C24. Plants were germinated in standard conditions (21°C and 12L:12D) and then transferred to either 17°C or 27°C in 12h L/12h D or 10h L/10h D cycles. Based on our previous experiment we would predict that *toc1* plants perform better than C24 wild type in 20h days and vice versa C24 plants perform better than *toc1*in 24h days.

The mutant *toc1-1* line (Timing of CAB2-1) was identified among a population of EMS mutagenized lines in a screening for altered expression of the *CAB2:LUC* transgene (Millar *et al.*, 1995). *toc1-1* has a consistent shorter period than the wild type, about 20h instead of 24h, not only in the CAB2 reporter but also in several clock controlled outputs including stomatal conductance and early flowering (Somers *et al.*, 1998). Once cloned (Strayer *et al.*, 2000), the *TOC1* gene was found to belong to the *Arabidopsis* pseudo-response regulator family and a homolog of *APRR1* (Matsushika *et al.*, 2000). *TOC1* acts independently from light quality or temperature inputs, proving to be in the central clock mechanism (Somers *et al.*, 1998): this was confirmed by the first model describing a negative transcription and translation feedback loop in *Arabidopsis* (Alabadi *et al.*, 2001). *TOC1* transcripts levels oscillate with a peak in the evening, 12h later and opposite to the partially redundant *LHY/CCA1* genes, activating them through an unknown component X. In turn *LHY/CCA1* act repressing *TOC1* by binding to a promoter region called evening element (EE), and this action is enhanced by activation of repressive chromatin structures (Perales and Mas, 2007) and the targeting for degradation to the 26S proteasome by the ZTL protein (Mas *et al.*, 2003a).

The *toc1-1* line was therefore chosen for these experiments because of its characteristics: it carries a mutation in a central clock component with a strong short period phenotype not altered by temperature changes, it has an intact temperature compensating mechanism and a direct impact on several physiological traits.

5.2 Results

5.2.1 - 12h L/12h D – C24 outperforms toc1

The first set of experiments was conducted in 12h L/12h D cycles, comparing the performance of the *toc1* mutant against the wild-type C24. Plants were germinated in standard conditions (21°C and 12L:12D) and then transferred to either 17°C or 27°C. In the pre-harvesting photo (Figure 5-1) wild-type C24 plants look bigger than *toc1* mutant plants, both at 17°C and 27°C.

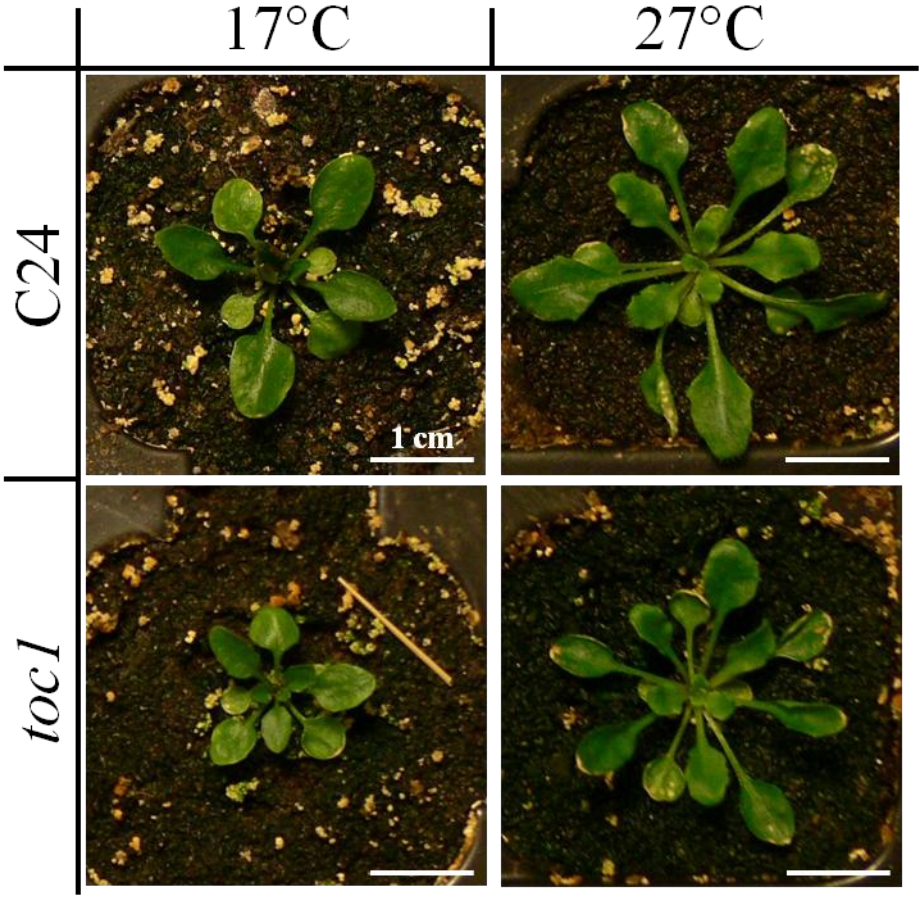


Figure 5-1. C24 and *toc1* plants, grown at 17°C and 27°C, in 12h L/12h D light cycles. The photo was taken 25 day after sowing, white bars equal to 1cm.

This observation is fully confirmed by visible leaf area (Figure 5-2). Over the two growing temperatures C24 plants have the same leaf area, with ANOVA test (at day 25) reporting a P-value of 0.382 when comparing 17°C to 27°C. On the contrary, in *toc1* plants the leaf area increase with temperature is more than twofold (**P<0.001). A comparison between the two lines C24 and *toc1* within each temperature shows at 17°C a twofold difference (**P<0.001) and at 27°C a wild-type still slightly bigger than the mutant (*P=0.012). The magnitude of the differences just described between the two lines however, decreased with rising temperature.

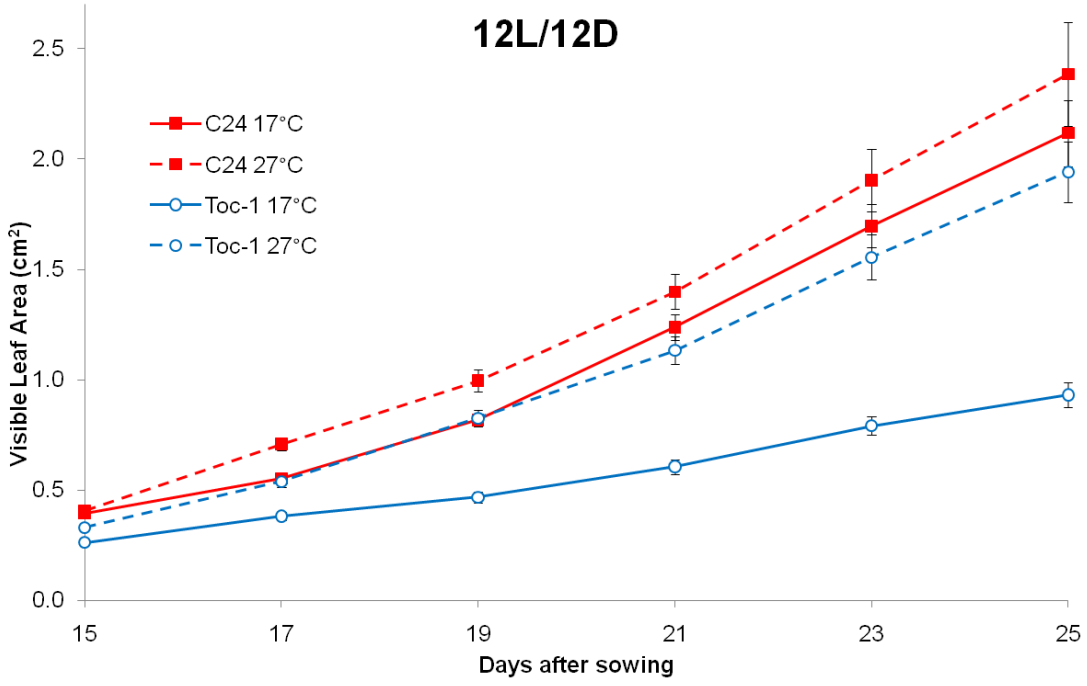


Figure 5-2. Visible leaf area of C24 and *toc1* plant lines 15 to 25 day after sowing, grown at 12h L/12h D light cycle and 17ºC or 27ºC. Data is mean ±1SEM. At day 25 in ANOVA test comparing *toc1* to C24, **P<0.01 at 17°C and *P<0.05 at 27°C. Figure representative of three independent replicates.

Likewise, fresh weight (Figure 5-3) shows a similar trend to visible leaf area. Between 17 and 27°C C24 plants have a comparable fresh weight, with a P-value of 0.526 in the ANOVA test. The fresh weight of *toc1* plants, on the contrary, rises with an increase of temperature (**P<0.001). Comparing within each temperature, results in C24 being always 1.5 times greater than *toc1*, with an ANOVA P-value of less than 0.001 at both temperatures. As for visible leaf area, the differences just described between the two lines do not increase but decrease with the increase in temperature.

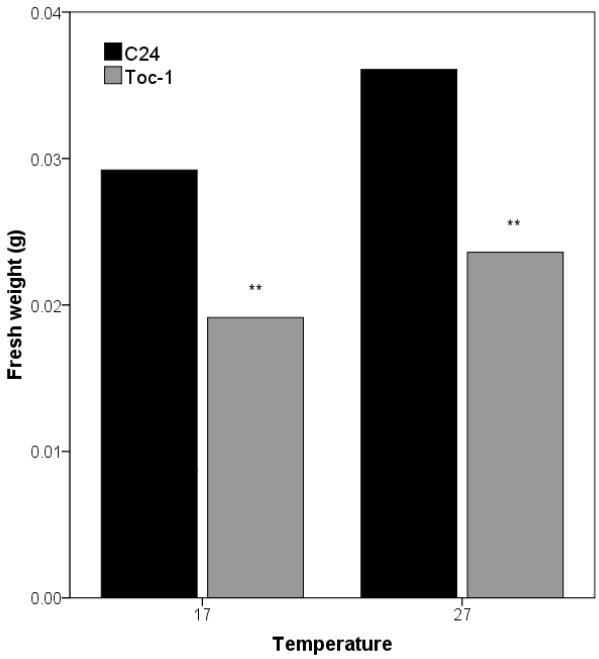


Figure 5-3. Fresh weight of C24 and *toc1* plant lines 25 day after sowing, grown at 12h L/12h D light cycle and 17ºC or 27ºC. Data is mean, in ANOVA test comparing *toc1* to C24 **P<0.01 at 17°C and 27°C. Figure representative of three independent replicates.

Results from the dry weight assay (Figure 5-4) follow less tightly the plants' visible phenotype. When comparing 17°C with 27°C, C24 plants have the same dry weight (P=0.930) and in like manner *toc1* dry weight is equal, with ANOVA test reporting a P-value of 0.504. Matching C24 weight against *toc1* within each temperature shows no difference at 17°C (P=0.123) but at 27°C there is an increased weight of the wild-type versus the mutant line (*P=0.017). It is clear that the difference between the two lines occurs after the increase of temperature.

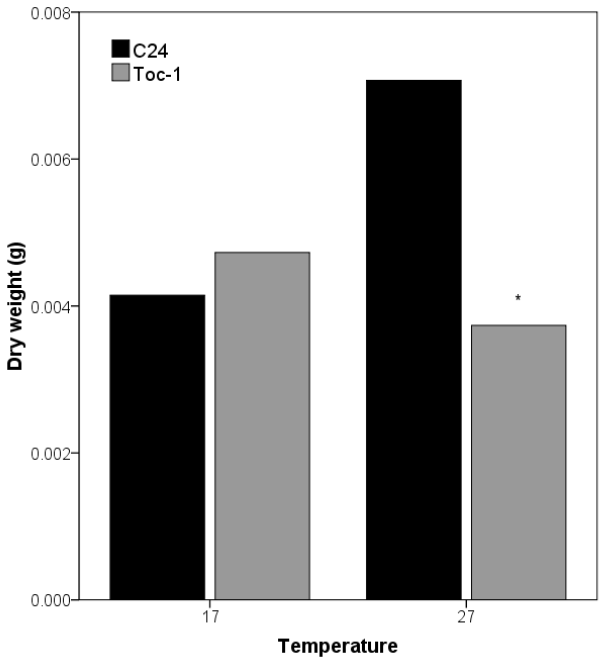


Figure 5-4. Dry weight of C24 and *toc1* plant lines 25 day after sowing, grown at 12h L/12h D light cycle and 17ºC or 27ºC. Data is mean, in ANOVA test comparing *toc1* to C24 P>0.12 at 17°C and *P<0.05 at 27°C. Figure representative of three independent replicates.

Measuring total chlorophyll content on fresh weight (Figure 5-5) resulted in uniform data. Over the two growing temperatures C24 plants had the same chlorophyll content, with ANOVA test reporting a P-value of 0.243; *toc1* chlorophyll however was found to be more concentrated at 27°C than at 17°C (*P=0.018). Balancing wild-type chlorophyll content against the mutant within each temperature led to equivalent results: There was no difference between the lines, either at 17°C (P=0.378) or at 27°C (P=0.677).

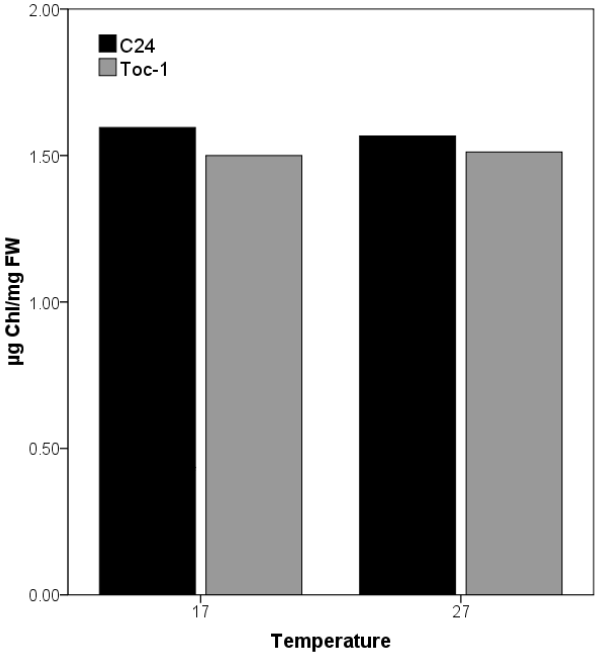


Figure 5-5. Total chlorophyll content (on fresh weight) of C24 and *toc1* plant lines 25 day after sowing, grown at 12h L/12h D light cycle and 17ºC or 27ºC. Data is mean, in ANOVA test comparing *toc1* to C24 P>0.37 at 17°C and P>0.67 at 27°C. Figure representative of three independent replicates.

Total chlorophyll content on dry weight (Figure 5-6) diverges in some ways from the rest of the assays performed. When comparing 17°C to 27°C, both C24 and *toc1* plants had a greater chlorophyll content at high temperature, with P-values in the ANOVA of *P=0.02 and **P<0.001 respectively. A comparison between the two lines C24 and *toc1* within each temperature shows at 17°C no difference (P=0.287), while at 27°C a *toc1* line greater than C24 (**P=0.007): the differences between the two lines therefore increased with temperature.

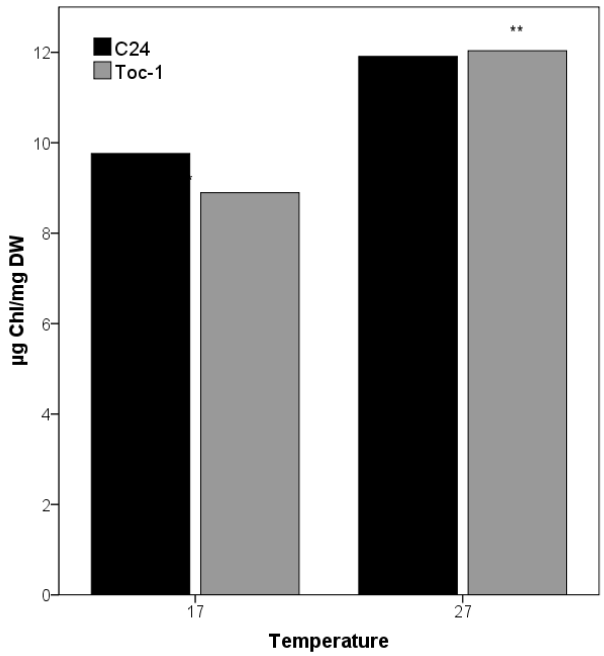


Figure 5-6. Total chlorophyll content (on dry weight) of C24 and *toc1* plant lines 25 day after sowing, grown at 12h L/12h D light cycle and 17ºC or 27ºC. Data is mean, in ANOVA test comparing *toc1* to C24 P>0.28 at 17°C and **P<0.01 at 27°C. Figure representative of two independent replicates.

Summary of Results

C24 and *toc1* in 12hL/12hD light cycles

Leaf Area 25 days				12LD
Different?	**How?**	**P value**	**Diff.**	
Yes **	C24 > *toc1*	<0.001	0.166	**17°C**
Yes *	C24 > *toc1*	0.012	0.060	**27°C**
No				> with T°
No	-	0.382	-	**C24**
Yes**	27 > 17	<0.001	0.126	***toc1***

Fresh Weight				12LD	Dry Weight			
Different?	**How?**	**P value**	**Diff.**		**Different?**	**How?**	**P value**	**Diff.**
Yes **	C24 > *toc1*	<0.001	0.224	**17°C**	No	-	0.123	-
Yes **	C24 > *toc1*	<0.001	0.100	**27°C**	Yes *	C24 > *toc1*	0.017	0.093
No				> with T°	Yes			
No	-	0.526	-	**C24**	No	-	0.930	-
Yes **	27 > 17	<0.001	0.136	***toc1***	No	-	0.504	-

Chl FW				12LD	Chl DW			
Different?	**How?**	**P value**	**Diff.**		**Different?**	**How?**	**P value**	**Diff.**
No	-	0.378		**17°C**	No	-	0.287	-
No	-	0.677		**27°C**	Yes **	*toc1* > C24	0.007	0.213
No				> with T°	Yes			
No	-	0.243	-	**C24**	Yes *	27 > 17	0.020	0.186
Yes *	27 > 17	0.018	0.065	***toc1***	Yes **	27 > 17	<0.001	0.479

Table 14. Summary of results from the assays done on C24 and *toc1* plant lines, grown in 12h L/12h D light cycles and 17ºC or 27ºC. "P value" is probability from Analysis of Variance and "Diff." is the center value from Tukey test, both done as described in 2.5.4 Data analysis. ">with T°" indicates whether the difference between genotypes is increasing with the rise of temperature. *=P<0.05, **=P<0.01

Descriptive Statistics of all replicates

C24 and *toc1* in 12hL/12hD light cycles

Leaf Area 25d		N		Mean		SE Mean		St Dev		Variance	
	Replicate	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C
C24	1	40	40	2.1173	2.3850	0.1501	0.2345	0.9492	1.4829	0.9009	2.1991
	2	20	20	1.5847	2.0594	0.0602	0.0840	0.2692	0.3756	0.0725	0.1411
	3	40	40	1.4249	1.3285	0.0618	0.0620	0.3907	0.3920	0.1527	0.1536
toc1	1	40	40	0.9309	1.9423	0.0558	0.1370	0.3526	0.8662	0.1243	0.7503
	2	20	20	1.4594	1.3999	0.0538	0.0640	0.2404	0.2860	0.0578	0.0818
	3	40	40	1.2663	1.3192	0.0506	0.0450	0.3200	0.2847	0.1024	0.0811

Fresh Weight		N		Mean		SE Mean		St Dev		Variance	
	Replicate	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C
C24	1	40	40	0.0536	0.0529	0.0036	0.0055	0.0228	0.0345	0.0005	0.0012
	2	60	59	0.0292	0.0361	0.0009	0.0014	0.0071	0.0105	0.0001	0.0001
	3	40	40	0.0346	0.0313	0.0014	0.0016	0.0090	0.0099	0.0001	0.0001
toc1	1	40	40	0.0207	0.0433	0.0014	0.0033	0.0087	0.0211	0.0001	0.0005
	2	60	60	0.0191	0.0236	0.0011	0.0013	0.0087	0.0102	0.0001	0.0001
	3	40	40	0.0310	0.0331	0.0013	0.0011	0.0081	0.0072	0.0001	0.0001

Dry Weight		N		Mean		SE Mean		St Dev		Variance	
	Replicate	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C
C24	1	10	10	0.0093	0.0090	0.0004	0.0006	0.0012	0.0018	0.0000	0.0000
	2	19	21	0.0041	0.0071	0.0003	0.0023	0.0013	0.0106	0.0000	0.0001
	3	10	10	0.0072	0.0040	0.0016	0.0006	0.0049	0.0020	0.0000	0.0000
toc1	1	10	10	0.0052	0.0083	0.0002	0.0003	0.0007	0.0009	0.0000	0.0000
	2	18	22	0.0047	0.0037	0.0009	0.0003	0.0037	0.0013	0.0000	0.0000
	3	10	10	0.0068	0.0036	0.0010	0.0002	0.0030	0.0005	0.0000	0.0000

Chl FW		N		Mean		SE Mean		St Dev		Variance	
	Replicate	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C
C24	1	5	5	1.5127	1.4533	0.0376	0.1396	0.0842	0.3122	0.0071	0.0975
	2	4	5	1.0618	1.4460	0.0722	0.0750	0.1444	0.1677	0.0209	0.0281
	3	5	5	1.5954	1.5669	0.0634	0.0586	0.1418	0.1310	0.0201	0.0172
toc1	1	5	5	1.7525	1.6772	0.0489	0.0290	0.1094	0.0648	0.0120	0.0042
	2	5	5	0.8395	1.3570	0.0621	0.0956	0.1389	0.2138	0.0193	0.0457
	3	5	5	1.4997	1.5120	0.0881	0.0297	0.1971	0.0664	0.0388	0.0044

Chl DW		N		Mean		SE Mean		St Dev		Variance	
	Replicate	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C
C24	1	5	5	9.7616	11.9078	0.3205	0.9174	0.7167	2.0515	0.5137	4.2085
	2	5	5	3.3377	6.3665	0.6914	1.0940	1.5460	2.4462	2.3900	5.9839
toc1	1	5	5	8.8950	12.0346	0.1239	0.4011	0.2771	0.8970	0.0768	0.8046
	2	5	5	2.5667	15.4127	0.5551	0.4319	1.2412	0.9658	1.5407	0.9327

Table 15. Descriptive Statistics of all the independent experimental replicates of the assays performed on C24 and *toc1* lines. Plants were grown in 12h L/12h D light cycles and 17ºC or 27ºC.

5.2.2 - 10h L/10h D – C24 performs better than toc1

The second set of experiments comparing *toc1* mutant and its wild-type C24 was conducted in 10h L/10h D cycles. Plants were germinated in standard conditions (21°C and 12L:12D) and then transferred to either 17°C or 27°C. In the test growing conditions (Figure 5-7) wild-type plants look slightly better performers than *toc1* plants, both at 17°C and 27°C.

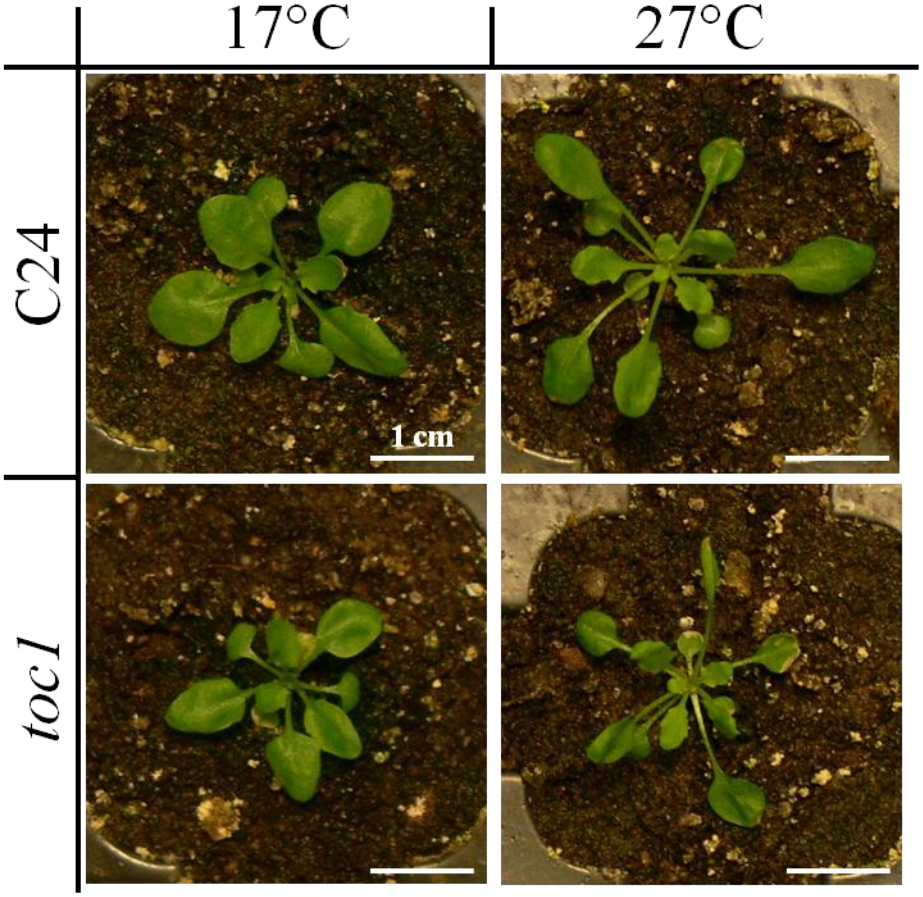


Figure 5-7. C24 and *toc1* plants, grown at 17°C and 27°C, in 10h L/10h D light cycles. The photo was taken 25 day after sowing, white bars equal to 1cm.

Visible leaf area (Figure 5-8) matches what was observed during the initial visual inspection. When comparing 17°C to 27°C, both C24 and *toc1* plants had a greater visible leaf area at high temperature, with P-values in the ANOVA test (at day 25) of **P=0.002 and **P<0.001 respectively. In particular leaf area at 27°C was found to be bigger than at 17°C, more than 2.5 times for *toc1* and more than 2 times for C24. Comparing within each temperature, indicated that C24 was about 1.6 times greater than *toc1* at 17°C (**P<0.001) and about 1.4 times greater at 27°C (**P=0.006). It is clear that these differences decrease with increase of temperature.

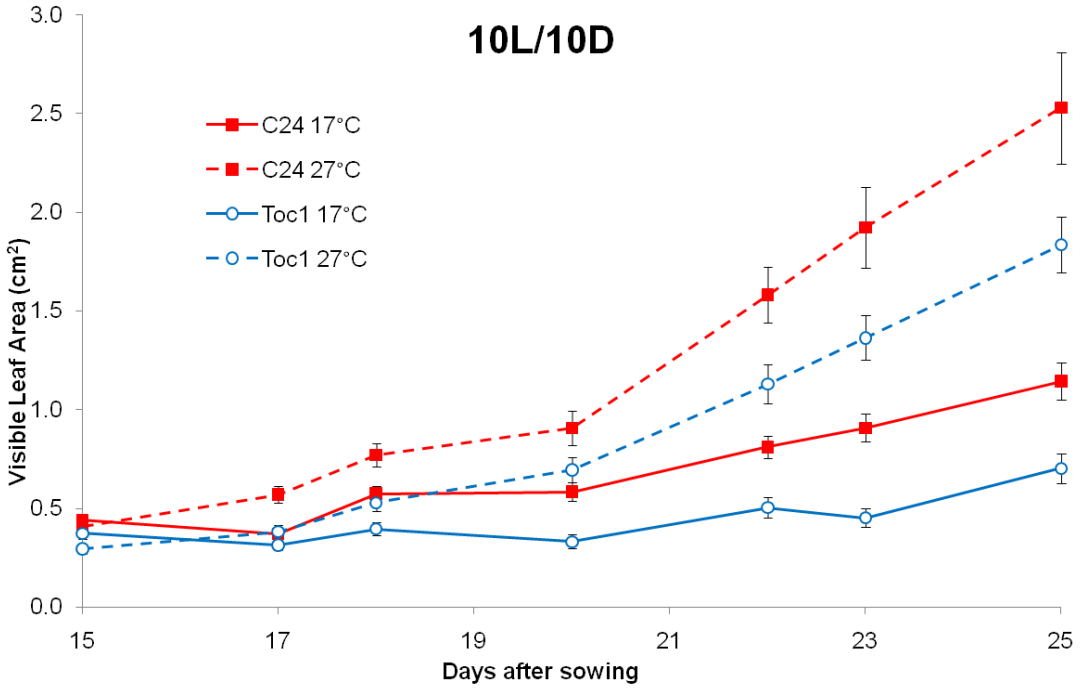


Figure 5-8. Visible leaf area of C24 and *toc1* plant lines 15 to 25 day after sowing, grown at 10h L/10h D light cycle and 17ºC or 27ºC. Data is mean ±1SEM. At day 25 in ANOVA test comparing *toc1* to C24, **P<0.01 at 17°C and 27°C. Figure representative of two independent replicates.

Fresh weight results (Figure 5-9) show an extremely similar trend to visible leaf area. Over the two growing temperatures both C24 and *toc1* mutant plants had a greater fresh weight at 27°C, with ANOVA test reporting significant P-values of less than 0.001. Balancing wild-type fresh weight against the mutant within each temperature, leads to equivalent results, with C24 values being always greater than *toc1* and ANOVA P-values of less than 0.001. Furthermore, the differences between the two lines increased with temperature.

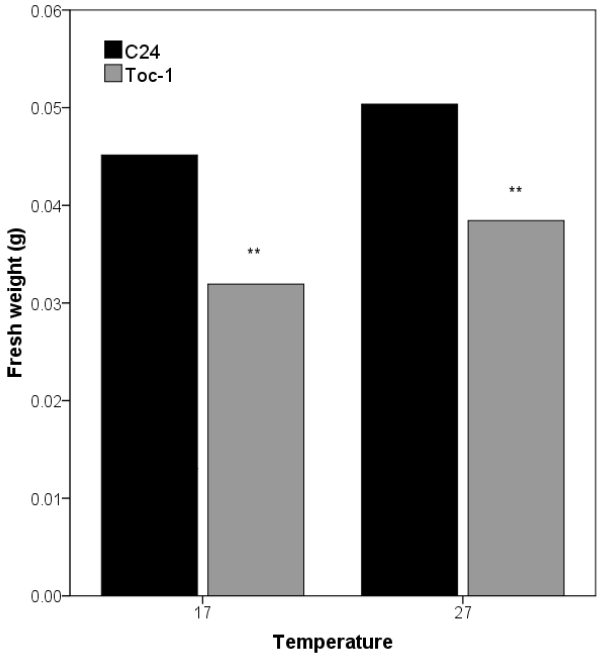


Figure 5-9. Fresh weight of C24 and *toc1* plant lines 25 day after sowing, grown at 10h L/10h D light cycle and 17ºC or 27ºC. Data is mean, in ANOVA test comparing *toc1* to C24 **P<0.01 at 17°C and 27°C. Figure representative of two independent replicates.

Similar are the results obtained from dry weight, presented in Figure 5-10. Comparing 17°C to 27°C, both C24 and *toc1* plants had a greater dry weight at high temperature, with ANOVA P-values of **P<0.001 in either cases. A comparison between the two lines C24 and *toc1* within each temperature shows once again at both temperatures identical results: the wild-type is consistently bigger than the mutant, always with P-values of **P<0.001. The magnitude of the differences increases with the rise of temperature.

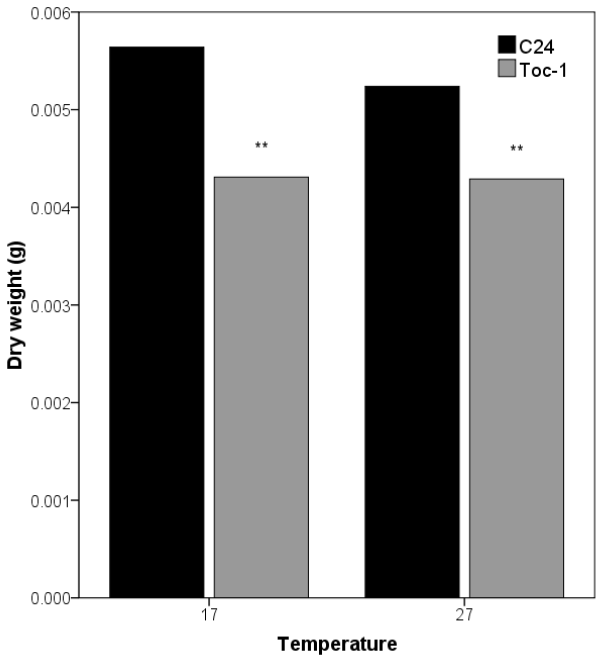


Figure 5-10. Dry weight of C24 and *toc1* plant lines 25 day after sowing, grown at 10h L/10h D light cycle and 17ºC or 27ºC. Data is mean, in ANOVA test comparing *toc1* to C24 **P<0.01 at 17°C and 27°C. Figure representative of two independent replicates.

Total chlorophyll content on fresh weight (Figure 5-11) does not follow as closely the trend described by the previous assays, especially in the comparison between the two lines. Actually no difference was found between C24 and *toc1* when they were compared within the same growing temperature: this is confirmed by the ANOVA P-values, 0.415 at 17°C and 0.371 at 27°C. On the other hand juxtaposing the two temperatures inside each line, shows that at 27°C plants always had more chlorophyll than at 17°C. In fact, the ANOVA P-value for C24 is *P=0.033 and for *toc1* is **P=0.009.

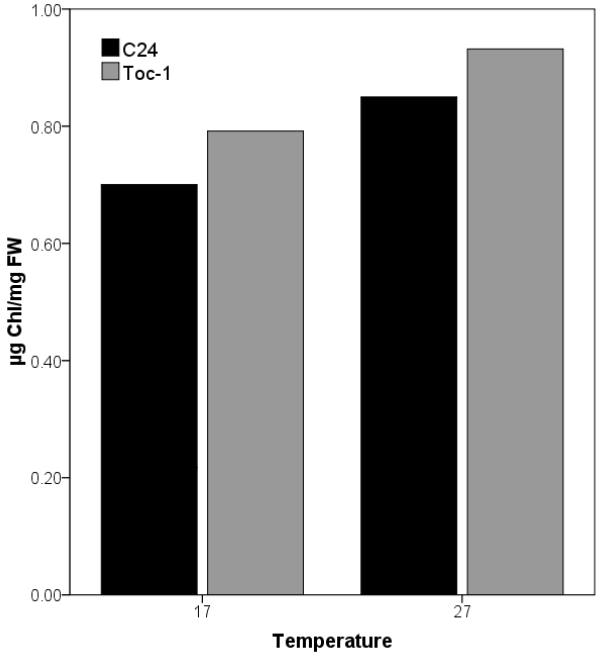


Figure 5-11. Total chlorophyll content (on fresh weight) of C24 and *toc1* plant lines 25 day after sowing, grown at 10h L/10h D light cycle and 17ºC or 27ºC. Data is mean, in ANOVA test comparing *toc1* to C24 P>041 at 17°C and P>0.37 at 27°C. Figure representative of two independent replicates.

Measuring total chlorophyll content on a dry weight basis (Figure 5-12) resulted in data partially diverging from the other assays. Between 17°C and 27°C C24 plants have comparable chlorophyll content, with a P-value of 0.075 in the ANOVA test; *toc1* on the contrary has more chlorophyll at high temperature (**P=0.001). Matching C24 weight against *toc1* within each temperature shows no difference at 27°C (P= 0.841) but at 17°C there is an increased weight in the wild-type versus the mutant line (**P= 0.004). As the only difference statistically valid is at 17°C, there is a decrease in the differences between the two lines with the increase in temperature.

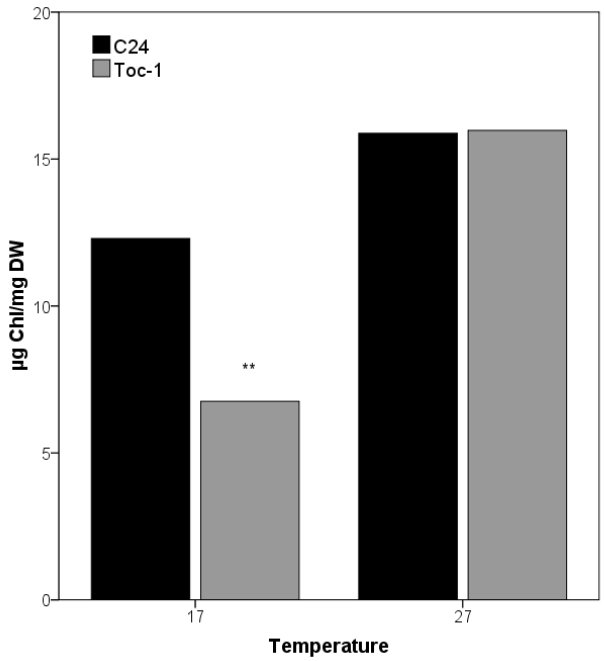


Figure 5-12. Total chlorophyll content (on dry weight) of C24 and *toc1* plant lines 25 day after sowing, grown at 10h L/10h D light cycle and 17ºC or 27ºC. Data is mean, in T-test comparing *toc1* to C24 **P<0.01 at 17°C and P>0.84 at 27°C. Figure representative of one replica.

Summary of Results

C24 and *toc1* in 10hL/10hD light cycles

Leaf Area 25				10LD
Different?	**How?**	**P value**	**Diff.**	
Yes **	C24 > *toc1*	<0.001	0.167	**17°C**
Yes **	C24 > *toc1*	0.006	0.106	**27°C**
No				> with T°
Yes **	27 > 17	0.002	0.126	**C24**
Yes **	27 > 17	<0.001	0.187	***toc1***

Fresh Weight				10LD	Dry Weight			
Different?	**How?**	**P value**	**Diff.**		**Different?**	**How?**	**P value**	**Diff.**
Yes **	C24 > *toc1*	<0.001	0.179	**17°C**	Yes **	C24 > *toc1*	<0.001	0.171
Yes **	C24 > *toc1*	<0.001	0.184	**27°C**	Yes **	C24 > *toc1*	<0.001	0.203
Yes				> with T°	Yes			
Yes **	27 > 17	0	0.229	**C24**	Yes **	27 > 17	<0.001	0.242
Yes **	27 > 17	<0.001	0.234	***toc1***	Yes **	27 > 17	<0.001	0.207

Chl FW				10LD	Chl DW (T-Test)			
Different?	**How?**	**P value**	**Diff.**		**Different?**	**How?**	**P value**	**Diff.**
No	-	0.415	-	**17°C**	Yes **	C24 > *toc1*	0.004	0.258
No	-	0.371	-	**27°C**	No	-	0.841	-
No				> with T°	No			
Yes *	27 > 17	0.033	0.067	**C24**	No	-	0.075	-
Yes **	27 > 17	0.009	0.079	***toc1***	Yes **	27 > 17	0.001	0.381

Table 16. Summary of results from the assays done on C24 and *toc1* plant lines, grown in 10h L/10h D light cycles and 17ºC or 27ºC. "P value" is probability from Analysis of Variance and "Diff." is the center value from Tukey test, both done as described in 2.5.4 Data analysis. ">with T°" indicates whether the difference between genotypes is increasing with the rise of temperature. *=P<0.05, **=P<0.01

Descriptive Statistics of all replicates

C24 and *toc1* in 10hL/10hD light cycles

Leaf Area 25d		N		Mean		SE Mean		St Dev		Variance	
	Replicate	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C
C24	1	20	20	1.1448	2.5291	0.0951	0.2833	0.4254	1.2670	0.1810	1.6053
	2	40	40	1.9167	2.1624	0.1033	0.1085	0.6535	0.6864	0.4270	0.4712
toc1	1	20	20	0.7028	1.8357	0.0748	0.1401	0.3347	0.6266	0.1120	0.3926
	2	40	40	1.4299	1.6018	0.0626	0.0752	0.3956	0.4754	0.1565	0.2260

Fresh Weight		N		Mean		SE Mean		St Dev		Variance	
	Replicate	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C
C24	1	32	35	0.0217	0.0574	0.0024	0.0032	0.0135	0.0188	0.0002	0.0004
	2	40	40	0.0452	0.0504	0.0023	0.0027	0.0145	0.0169	0.0002	0.0003
toc1	1	35	38	0.0123	0.0317	0.0007	0.0019	0.0040	0.0116	0.0000	0.0001
	2	40	40	0.0319	0.0384	0.0014	0.0018	0.0088	0.0117	0.0001	0.0001

Dry Weight		N		Mean		SE Mean		St Dev		Variance	
	Replicate	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C
C24	1	22	23	0.0032	0.0075	0.0003	0.0006	0.0012	0.0028	0.0000	0.0000
	2	10	10	0.0056	0.0052	0.0004	0.0005	0.0013	0.0016	0.0000	0.0000
toc1	1	25	20	0.0022	0.0041	0.0002	0.0003	0.0010	0.0014	0.0000	0.0000
	2	10	10	0.0043	0.0043	0.0004	0.0003	0.0012	0.0011	0.0000	0.0000

Chl FW		N		Mean		SE Mean		St Dev		Variance	
	Replicate	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C
C24	1	5	5	0.7004	0.8499	0.0764	0.0496	0.1708	0.1110	0.0292	0.0123
	2	5	5	2.1578	2.3877	0.0617	0.0949	0.1381	0.2122	0.0191	0.0450
toc1	1	5	5	0.7917	0.9320	0.0559	0.0477	0.1250	0.1066	0.0156	0.0114
	2	5	5	1.6322	1.9539	0.1391	0.0393	0.3109	0.0878	0.0967	0.0077

Chl DW		N		Mean		SE Mean		St Dev		Variance	
	Replicate	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C
C24	1	5	5	12.2962	15.8758	1.1879	0.5609	2.6563	1.2542	7.0560	1.5729
toc1	1	5	5	6.7562	15.9759	0.6212	0.2652	1.3889	0.5930	1.9292	0.3517

Table 17. Descriptive Statistics of all the independent experimental replicates of the assays performed on C24 and *toc1* lines. Plants were grown in 10h L/10h D light cycles and 17ºC or 27ºC.

5.2.3 - Light cycles comparison within genotype – C24 and toc1

Previous subchapters compared performance between the two lines C24 and *toc1*, at two temperatures and in a single light cycle at a time. Here, on completion of preceding work, relative plant performance is measured within each genotype across three light/dark cycles: 12h L/12h D, 15h L/15h D and 10h L/10h D. As explained previously for Col-0 and *ztl* lines (4.2.4 - Light cycles comparison within genotype – Col-0 and ztl) due to time and equipment constraints it was not possible to design tailored experiments for this comparison, therefore data from experiments presented earlier in this chapter were used.

A general idea of the differences for C24 genotype is given by grouping by light cycle and temperature results obtained from successive performance experiment, then averaging them all together (Figure 5-13). Averaging all the experiments, C24 plants seem to perform equally at 10LD and 12LD, with an advantage for 10LD in some assays. This happens in particular in total chlorophyll on a dry weight basis (Figure 5-13 E) both at 17 and 27°C, and in visible leaf area and fresh weight only at 27°C (Figure 5-13, panels A and B respectively). On the other hand, plants at 15LD definitely underperform if compared to 10LD and 12LD, with the only exception of fresh weight at 27°C. Selecting just one experiment for each light cycle leads to a slightly different view of the data (Figure 5-14). While plants at 15LD always perform badly, plants at 12LD now outperform those grown at 10LD, in all assays but total chlorophyll on a dry weight basis (Figure 5-14 E). Overall C24 plants show greater performance at 12LD, although not increasing significantly at 27°C, and not dramatically different from the one shown at 10LD. In this case, to obtain more clear results a tailored experiment would be necessary in the future.

Figure 5-15 groups by light cycle and temperature results from all performance experiments for *toc1* line. In the averaged results, it is quite hard to find a definite trend across the three light cycles. At 27°C performance of *toc1* plants is greater than at 17°C. If only one experiment per light cycle is selected however, a clearer trend is visible in the data. *toc1*plants have a better performance at 10LD cycles than at 12LD or 15LD, and this difference is often increased at higher temperature. Clear examples of this trend are results for leaf area and chlorophyll on a fresh weight basis (Figure 5-16, panels A and D). In conclusion, the evidence seems to suggest that *toc1* plants perform better at 10LD cycles, when their internal circadian period is matched with the external environment.

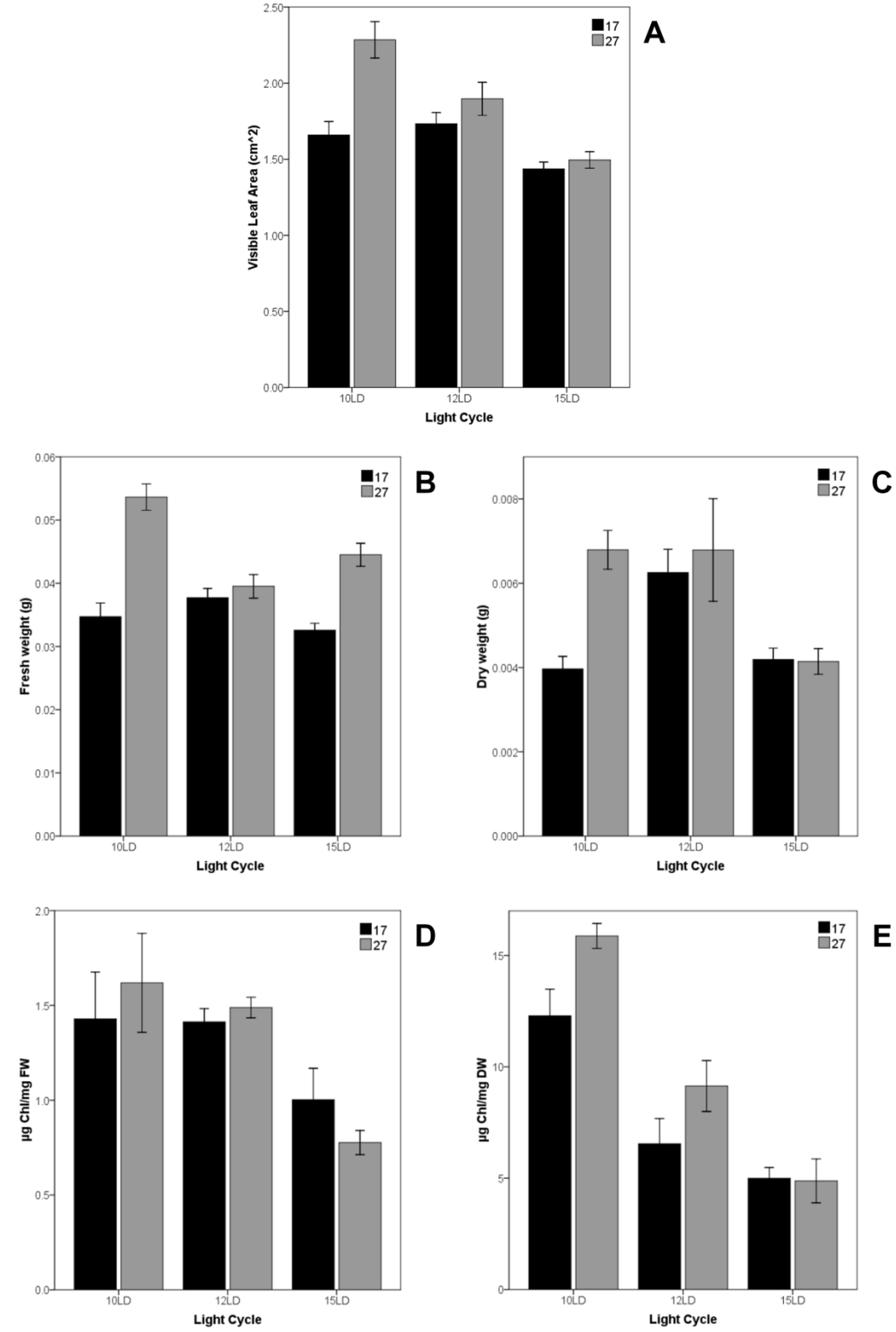


Figure 5-13. C24 plants 25 day after sowing, grown at 10h:10h, 12h:12h or 15h:15h Light/Dark cycles and 17 or 27°C. Each bar is the mean of all experiments performed for the specific growth condition. Error bars are ± 1 SEM. Visible leaf area at day 25 (**A**), fresh (**B**) and dry (**C**) weight, total chlorophyll content on fresh (**D**) and dry (**E**) weight.

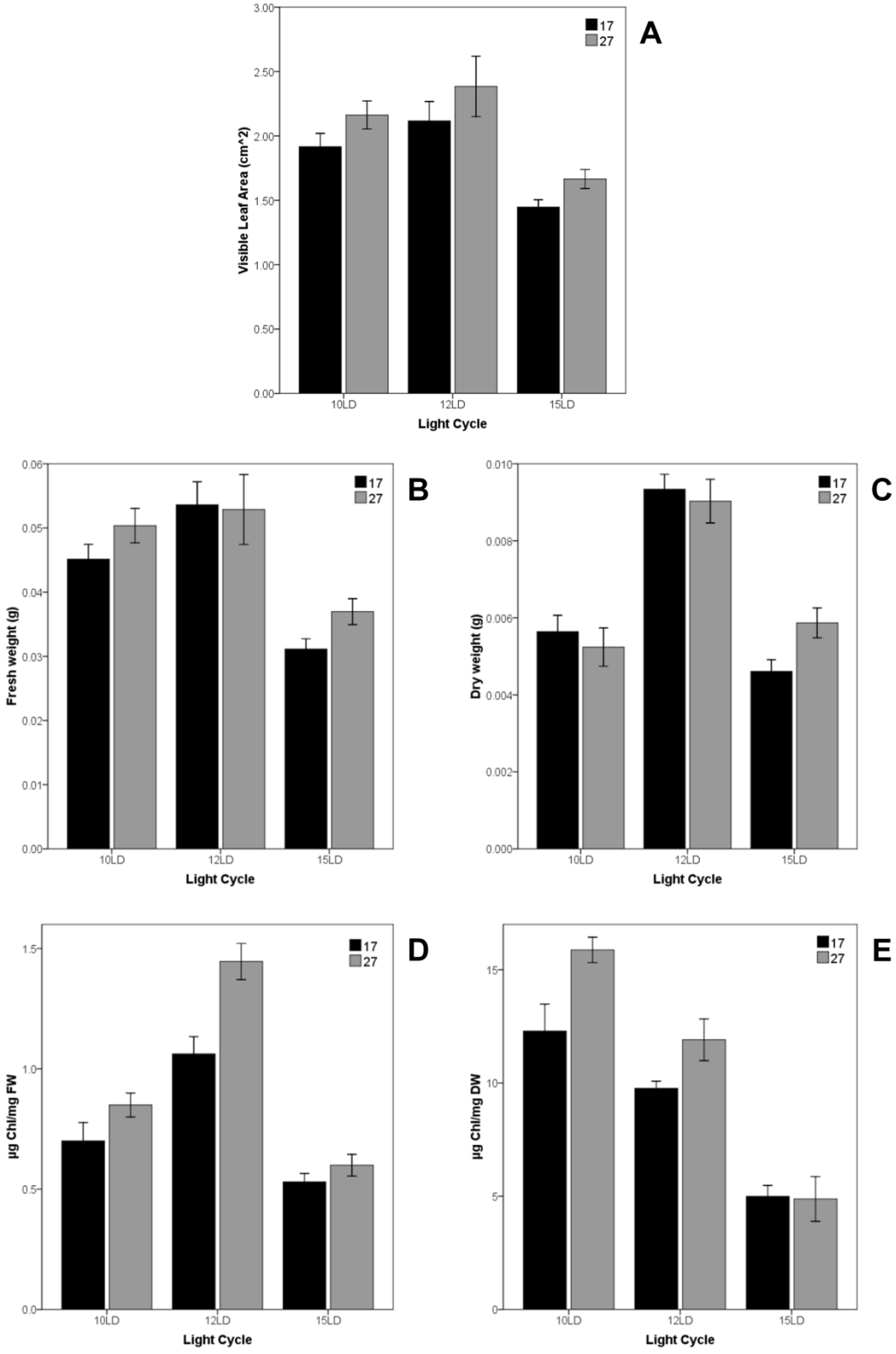


Figure 5-14. C24 plants 25 day after sowing, grown at 10h:10h, 12h:12h or 15h:15h Light/Dark cycles and 17 or 27°C. Each bar is the mean of only one selected performance experiment for the specific growth condition. Error bars are ± 1 SEM. Visible leaf area at day 25 (**A**), fresh (**B**) and dry (**C**) weight, total chlorophyll content on fresh (**D**) and dry (**E**) weight.

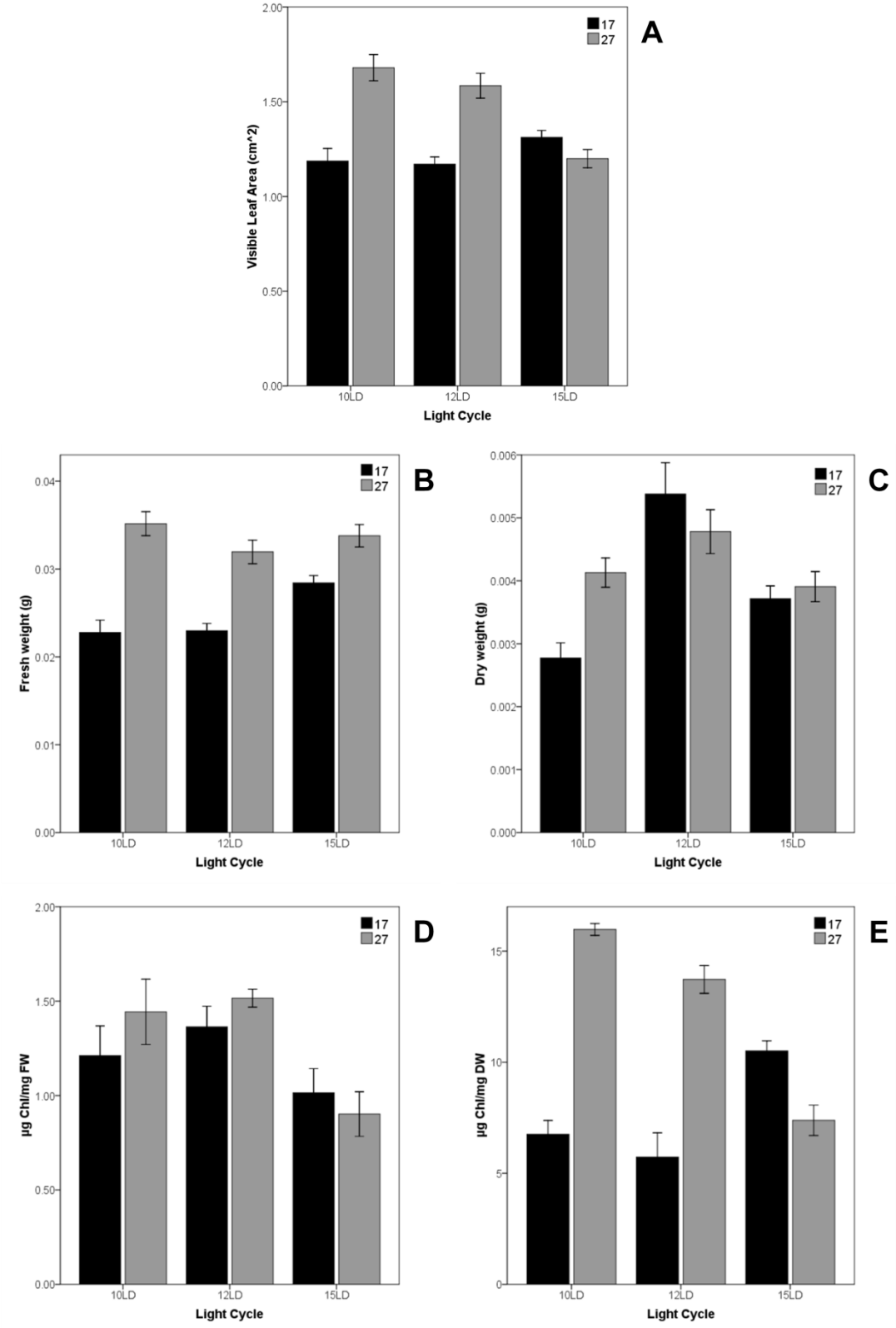


Figure 5-15. *toc1* plants 25 day after sowing, grown at 10h:10h, 12h:12h or 15h:15h Light/Dark cycles and 17 or 27°C. Each bar is the mean of all experiments performed for the specific growth condition. Error bars are ± 1 SEM. Visible leaf area at day 25 (**A**), fresh (**B**) and dry (**C**) weight, total chlorophyll content on fresh (**D**) and dry (**E**) weight.

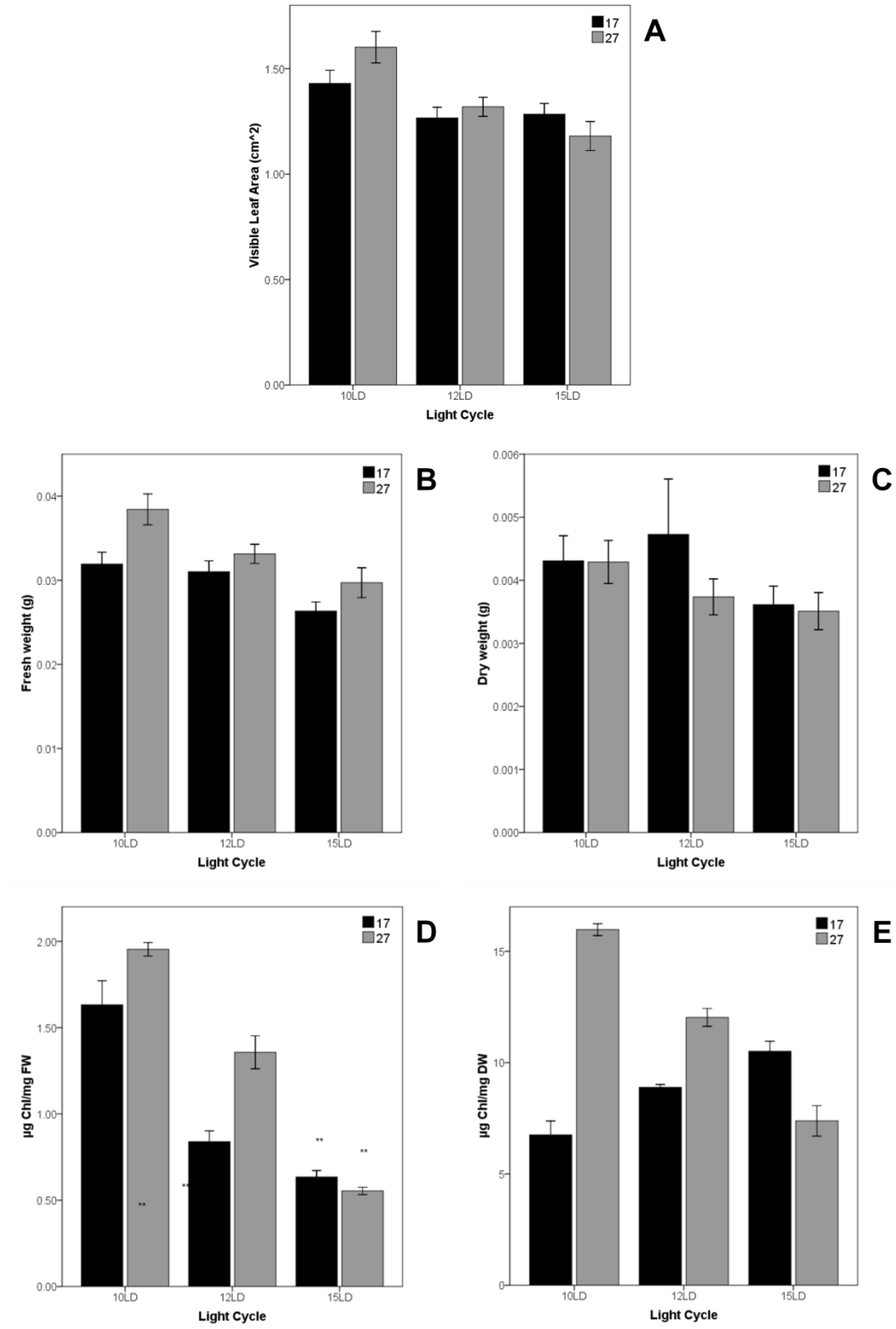


Figure 5-16. *toc1* plants 25 day after sowing, grown at 10h:10h, 12h:12h or 15h:15h Light/Dark cycles and 17 or 27°C. Each bar is the mean of only one selected performance experiment for the specific growth condition. Error bars are ± 1 SEM. Visible leaf area at day 25 (**A**), fresh (**B**) and dry (**C**) weight, total chlorophyll content on fresh (**D**) and dry (**E**) weight.

5.3 Discussion

According to the outcome of other performance experiments regarding circadian resonance (Chapter 4) and to previous reports (Dodd *et al.*, 2005), the experiments described in this chapter were expected to produce specific results. What I expected to see was the wild-type C24 performing better in 12h L/12h D and the mutant *toc1* performing better when grown in 10h L/10h D light cycles. At first glance the results obtained were different from expected data: in almost all cases the wild-type C24 performed better than *toc1*. A closer examination however reveals that while at 12h L/12h D light cycles C24 performance (compared to itself) remains constant between the two temperatures, *toc1* performance rises with temperature. This can be seen in Table 14 (page 113), in the panels of Leaf Area 25, Fresh Weight, Chl FW and Chl DW (where C24 has a less significative P-value at 17°C). The described effect is even magnified at 10h L/10h D light cycles (Table 16, page 121), where *toc1* "Diff." values are higher than at 12h L/12h D. In other words, when *toc1* performance is compared to the wild-type seems not to change, but when is compared to its own performance is actually growing better in 10h L/10h D light cycles and at 27°C.

These considerations are further confirmed by the light cycle comparison (5.2.3 - Light cycles comparison within genotype – C24 and toc1). In fact, little difference was noted in C24 between temperatures; likewise it was highlighted the better performance of *toc1* plants at 10LD cycles than at 12LD or 15LD, increasing with temperature. Overall, *toc1* results match previously published data from Dodd *et al.*, 2005 (weight, leaf area and chlorophyll), completing them with data obtained at higher temperatures. To sum up, the presented data suggests once again that to be able to perform well, plants need to be in circadian resonance with the external light cycle, and this is increasingly important in stressful high temperatures.

CHAPTER 6 - A T° compensated clock (*gi-11* line)

6.1 Introduction

Circadian resonance, described in Chapter 4 and Chapter 5 is not the only way a plant clock can better adapt to predictable environmental stimuli. The need to buffer processes against temperature changes evolved a mechanism known as temperature compensation. This mechanism is a key feature of the circadian clock, giving the ability to maintain an accurate and robust rhythm with a period close to 24h over a broad range of temperatures. The measure for this characteristic is called Q_{10}: it represents the change in reaction rate every 10ºC increase in temperature. The circadian clock rhythm for instance, was found to have a Q_{10} near to 1 between 12°C and 32°C (Somers *et al.*, 1998), therefore it has an internal mechanism able to compensate against temperature changes.

To quantify the importance of temperature compensation in the enhancement of plant growth at high temperatures, the mutant line *gi-11* was used, defective for the temperature compensation mechanism. In particular, the gi-11 null mutant here used is a T-DNA insertion mutation described in Richardson *et al.*, 1998 and Fowler *et al.*, 1999. *GIGANTEA* (*GI*) gene is one of the clock genes identified as the molecular basis of temperature compensation (Gould *et al.*, 2006), therefore an ideal candidate for the aim of the project. The *gi* allele was firstly identified in supervital mutants, late flowering and highly fecund plants by Redei, 1962. *GI* was then characterized (Fowler *et al.*, 1999) as a nuclear protein (Huq *et al.*, 2000) and its multiple roles identified as acting on the circadian clock (Park *et al.*, 1999) upstream of *CONSTANS* (*CO*) in the regulation of flowering by photoperiod (Suarez-Lopez *et al.*, 2001). These two roles are however distinct (Mizoguchi *et al.*, 2005) and not the only ones. In fact, later *GI* was identified as candidate for a temperature specific period QTL involved in temperature compensation (Edwards *et al.*, 2005). In the circadian clock *GI* is maximally expressed in the evening (12h after dawn, Fowler *et al.*, 1999) and acts as part of the evening loop, explaining at least a part of the unknown Y component. *Y*(*GI*) is induced by light, activates the core clock component *TOC1* which in turn negatively regulates Y itself (Locke *et al.*, 2005b). GI also modulates

TOC1 targeting to the 26S proteasome, stabilising in blue light the ZTL protein (Kim *et al.*, 2007). On the other side *GI* plays a key role in the temperature compensation mechanism, extending the temperature range where a robust circadian rhythm can be maintained. This is done in cooperation with other core clock components: *TOC1*, *CCA1* and *LHY*. In high temperatures *LHY* gene expression decreases, balanced by the increase of *TOC1* and *GI* levels. In contrast *CCA1* levels increased in low temperatures, balanced by a reduction of *GI*. Furthermore, the mutants *cca1-11*, *lhy-21* and *gi-11* were unable to compensate for temperature changes (Gould *et al.*, 2006).

For the experiments described here plants were germinated in standard conditions (21°C and 12L:12D), then transferred to either 17°C or 27°C in 12h L/12h D light cycles. Increasing differences between WS and *gi-11* lines were expected to be found with the increase in temperature, with little difference at 17°C and the wild-type performing better than the mutant at 27°C. The anticipated differences however were not found in the assays performed: the overall scenario was found to be quite cryptic and in many cases the *gi-11* mutant performed even better than WS.

6.2 Results

A snapshot of the seedlings just before harvesting (Figure 6-1) highlights some important differences between the lines. At 17°C *gi-11* mutant seedlings are visibly bigger than their wild-type WS, while at 27°C differences are less clear. At high temperature both lines have elongated petioles and smaller leaves, but *gi-11* plants seem generally more stressed and with weaker leaves than WS.

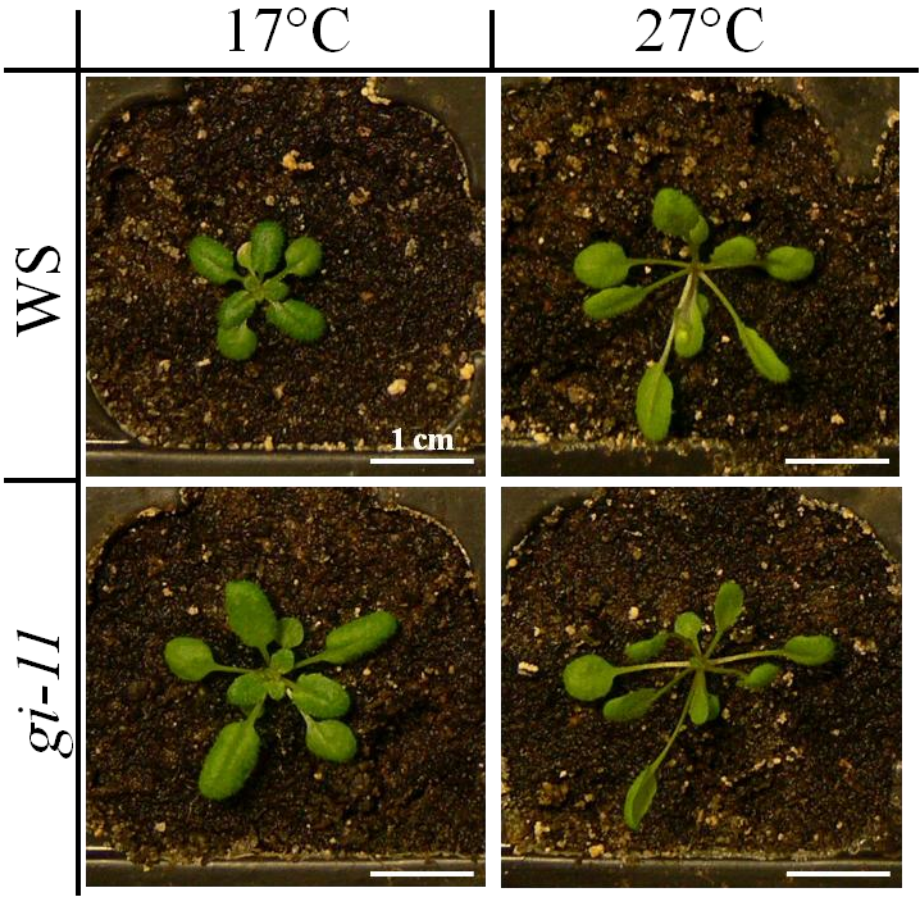


Figure 6-1. WS and *gi-11* plants, grown at 17ºC and 27ºC, in 12h L/12h D light cycles. The photo was taken 25 day after sowing, white bars equal to 1cm.

Quantitative measures of the visible leaf area (Figure 6-2) confirm the initial impression. When comparing 17°C to 27°C, both WS and *gi-11* plants had a greater visible leaf area at high temperature, with a P-value in the ANOVA test (at day 25) of **P<0.001 for both lines. Comparing the two lines within each temperature, results in *gi-11* being about 1.5 times greater than WS at 17°C (**P<0.001) and in no difference at 27°C (P= 0.773). It is clear that the differences decreased with the increase in temperature.

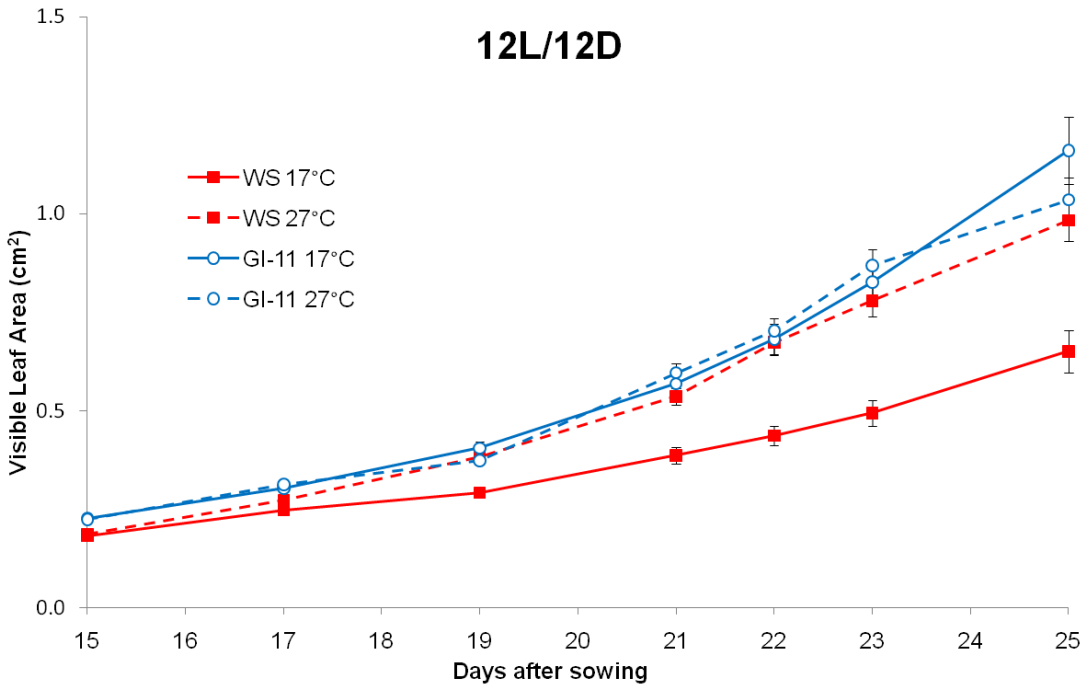


Figure 6-2. Visible leaf area of WS and *gi-11* plant lines 15 to 25 day after sowing, grown at 12h L/12h D light cycle and 17ºC or 27ºC. Data is mean ±1SEM. At day 25 in ANOVA test comparing *gi-11* to WS, **P<0.01 at 17°C and P>0.773 at 27°C. Figure representative of three independent replicates.

Fresh weight results (Figure 6-3) however show a different trend from visible leaf area. Over the two growing temperatures both WS and *gi-11* mutant plants had a greater fresh weight at 27°C, with ANOVA test reporting significant P-values of less than 0.001. Comparing wild-type fresh weight to that of the mutant within each temperature, reveals *gi-11* values being always greater than WS with an ANOVA probability of less than 0.001. Furthermore, the differences between the two lines increased with temperature.

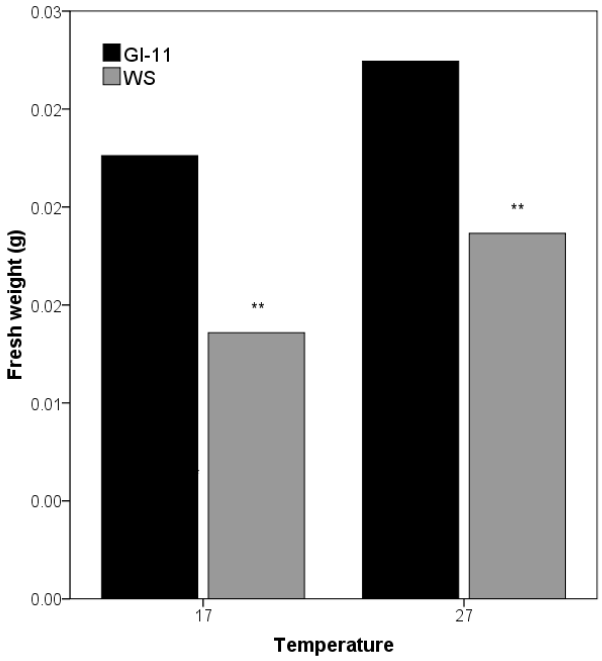


Figure 6-3. Fresh weight of WS and *gi-11* plant lines 25 day after sowing, grown at 12h L/12h D light cycle and 17ºC or 27ºC. Data is mean, in ANOVA test comparing *gi-11* to WS **P<0.01 at 17°C and 27°C. Figure representative of three independent replicates.

Dry weight results (Figure 6-4) differ from the data presented up to now. Between 17 and 27°C WS plants have a comparable dry weight, with a P-value of 0.163 in the ANOVA test. Weight of *gi-11* plants, on the contrary, rises with the increase of temperature (**P=0.001). Comparing within each temperature the two lines tested, results in *gi-11* being greater than WS at 27°C, with an ANOVA P-value of 0.003 and in no difference at 17°C (P= 0.203). Differences just described between the two lines clearly increase with the rise of temperature.

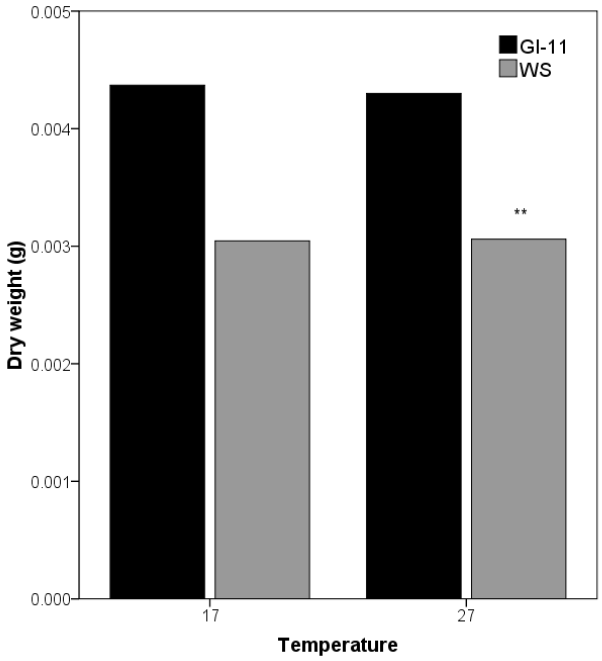


Figure 6-4. Dry weight of WS and *gi-11* plant lines 25 day after sowing, grown at 12h L/12h D light cycle and 17ºC or 27ºC. Data is mean, in ANOVA test comparing *gi-11* to WS P>0.20 at 17°C and **P<0.01 at 27°C. Figure representative of three independent replicates.

Total chlorophyll quantification on fresh weight (Figure 6-5) delivers once more different results, showing completely opposite data to the previous ones. Comparing 17°C to 27°C, for WS plants there was no difference found in chlorophyll concentration (P= 0.579) but for *gi-11* the scenario diverged. At 27°C *gi-11* plants had less chlorophyll than at 17°C, fact confirmed by the ANOVA P-value (**P= 0.002). Matching WS against *gi-11* within each temperature shows no difference at 17°C (P= 0.603) but remarks at 27°C an increased weight in the wild-type versus the mutant line (*P= 0.011). The magnitude of the differences described raised with the temperature increase.

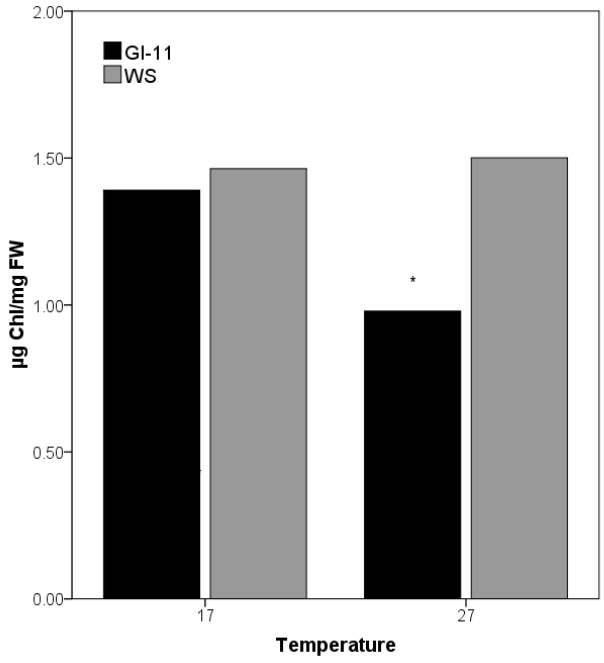


Figure 6-5. Total chlorophyll content (on fresh weight) of WS and *gi-11* plant lines 25 day after sowing, grown at 12h L/12h D light cycle and 17ºC or 27ºC. Data is mean, in ANOVA test comparing *gi-11* to WS P>0.60 at 17°C and *P<0.05 at 27°C. Figure representative of three independent replicates.

In contrast with the results just presented, total chlorophyll quantification on dry weight (Figure 6-6) mirrored what was seen for visible leaf area. Over the two growing temperatures both WS and *gi-11* mutant plants had a greater fresh weight at 27°C, with ANOVA test reporting significant P-values of less than 0.001 for WS and 0.001 for *gi-11*. On the other hand juxtaposing the two lines inside each temperature, shows at 17°C WS plants having more chlorophyll than *gi-11* (**P<0.001) and at 27°C no differences (P=0.897). Therefore differences decreased with the increase in experimental temperature.

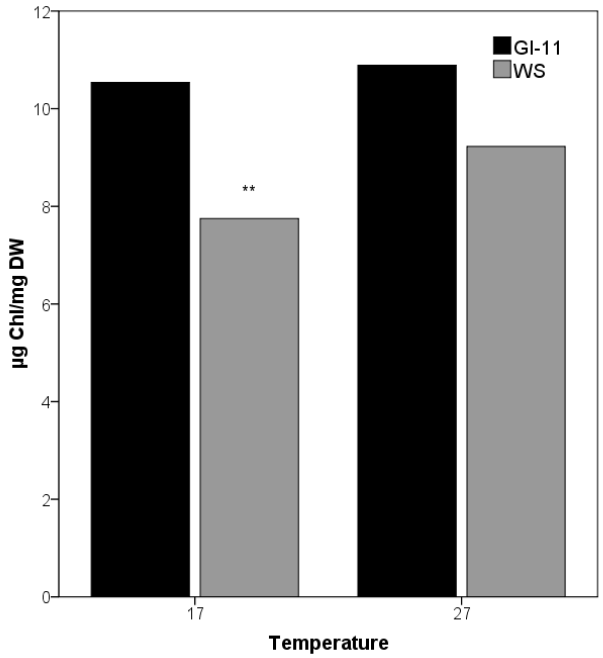


Figure 6-6. Total chlorophyll content (on dry weight) of WS and *gi-11* plant lines 25 day after sowing, grown at 12h L/12h D light cycle and 17ºC or 27ºC. Data is mean, in ANOVA test comparing *gi-11* to WS **P<0.01 at 17°C and P>0.89 at 27°C. Figure representative of three independent replicates.

Summary of Results

WS and *gi-11* in 12hL/12hD light cycles

Leaf Area - 25 days				12LD
Different?	**How?**	**P value**	**Diff.**	
Yes **	*gi-11* > WS	<0.001	0.166	**17°C**
No	-	0.773	-	**27°C**
No				> with T°
Yes **	27 > 17	<0.001	0.259	**WS**
Yes **	27 > 17	<0.001	0.099	***gi-11***

Fresh Weight				12LD	Dry Weight			
Different?	**How?**	**P value**	**Diff.**		**Different?**	**How?**	**P value**	**Diff.**
Yes **	*gi-11* > WS	<0.001	0.137	**17°C**	No	-	0.203	-
Yes **	*gi-11* > WS	<0.001	0.162	**27°C**	Yes **	*gi-11* > WS	0.003	0.135
Yes				> with T°	Yes			
Yes **	27 > 17	<0.001	0.216	**WS**	No	-	0.163	-
Yes **	27 > 17	<0.001	0.241	***gi-11***	Yes **	27 > 17	0.001	0.153

Chl FW				12LD	Chl DW			
Different?	**How?**	**P value**	**Diff.**		**Different?**	**How?**	**P value**	**Diff.**
No	-	0.603	-	**17°C**	Yes **	*gi-11* > WS	<0.001	0.092
Yes *	WS > *gi-11*	0.011	0.063	**27°C**	No	-	0.897	-
Yes				> with T°				
No	-	0.579	-	**WS**	Yes **	27 > 17	<0.001	0.176
Yes **	17 > 27	0.002	0.062	***gi-11***	Yes **	27 > 17	0.001	0.086

Table 18. Summary of results from the assays done on WS and *gi-11* plant lines, grown in 12h L/12h D light cycles and 17ºC or 27ºC. "P value" is probability from Analysis of Variance and "Diff." is the center value from Tukey test, both done as described in 2.5.4 Data analysis. ">with T°" indicates whether the difference between genotypes is increasing with the rise of temperature. *=P<0.05, **=P<0.01

Descriptive Statistics of all replicates

WS and *gi-11* in 12hL/12hD light cycles

Leaf Area 25d		N		Mean		SE Mean		St Dev		Variance	
	Replicate	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C
WS	1	59	59	1.0623	1.8798	0.0623	0.1264	0.4785	0.9710	0.2290	0.9429
	2	60	54	0.8547	1.8590	0.0695	0.1428	0.5380	1.0496	0.2894	1.1017
	3	59	60	0.6500	0.9828	0.0538	0.0518	0.4133	0.4013	0.1708	0.1610
gi-11	1	60	60	1.2625	2.1354	0.0570	0.1070	0.4412	0.8285	0.1946	0.6864
	2	60	58	1.2233	1.4780	0.0746	0.1016	0.5776	0.7741	0.3337	0.5993
	3	60	60	1.1606	1.0348	0.0849	0.0563	0.6577	0.4360	0.4326	0.1901

Fresh Weight		N		Mean		SE Mean		St Dev		Variance	
	Replicate	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C
WS	1	60	59	0.0220	0.0371	0.0014	0.0028	0.0107	0.0212	0.0001	0.0005
	2	59	54	0.0196	0.0386	0.0016	0.0031	0.0119	0.0228	0.0001	0.0005
	3	59	60	0.0136	0.0187	0.0010	0.0010	0.0080	0.0074	0.0001	0.0001
gi-11	1	60	59	0.0261	0.0617	0.0011	0.0030	0.0088	0.0233	0.0001	0.0005
	2	60	58	0.0247	0.0426	0.0015	0.0025	0.0114	0.0193	0.0001	0.0004
	3	60	58	0.0226	0.0274	0.0017	0.0018	0.0130	0.0133	0.0002	0.0002

Dry Weight		N		Mean		SE Mean		St Dev		Variance	
	Replicate	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C
WS	1	10	10	0.0043	0.0043	0.0006	0.0006	0.0019	0.0018	0.0000	0.0000
	2	9	9	0.0024	0.0039	0.0002	0.0004	0.0006	0.0013	0.0000	0.0000
	3	11	10	0.0030	0.0031	0.0004	0.0003	0.0014	0.0008	0.0000	0.0000
gi-11	1	10	10	0.0032	0.0062	0.0002	0.0008	0.0006	0.0027	0.0000	0.0000
	2	10	10	0.0033	0.0048	0.0005	0.0005	0.0015	0.0017	0.0000	0.0000
	3	10	8	0.0044	0.0043	0.0002	0.0003	0.0006	0.0007	0.0000	0.0000

Chl FW		N		Mean		SE Mean		St Dev		Variance	
	Replicate	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C
WS	1	5	5	1.4640	1.5008	0.0565	0.0932	0.1262	0.2084	0.0159	0.0434
	2	5	4	1.3022	1.0994	0.0171	0.0225	0.0382	0.0449	0.0015	0.0020
	3	5	5	1.7408	1.6134	0.2750	0.0466	0.6148	0.1042	0.3780	0.0109
gi-11	1	5	5	1.3908	0.9790	0.0578	0.0099	0.1293	0.0220	0.0167	0.0005
	2	5	5	1.4955	1.2755	0.0732	0.0329	0.1637	0.0735	0.0268	0.0054
	3	5	5	1.3080	1.4128	0.0292	0.0427	0.0653	0.0955	0.0043	0.0091

Chl DW		N		Mean		SE Mean		St Dev		Variance	
	Replicate	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C	17°C	27°C
WS	1	5	5	9.1248	15.8047	0.3852	0.2644	0.8614	0.5911	0.7421	0.3494
	2	5	5	7.3506	12.5243	0.7366	1.5254	1.6471	3.4109	2.7128	11.6343
	3	5	5	7.7487	9.2276	0.5934	1.2586	1.3270	2.8143	1.7608	7.9202
gi-11	1	5	5	10.6765	11.6411	0.2288	0.6691	0.5116	1.4961	0.2618	2.2383
	2	5	5	8.4960	13.4111	0.4509	0.7060	1.0083	1.5788	1.0168	2.4925
	3	5	4	10.5385	10.8908	0.5697	0.2920	1.2738	0.5840	1.6226	0.3411

Table 19. Descriptive Statistics of all the independent experimental replicates of the assays performed on WS and *gi-11* lines. Plants were grown in 12h L/12h D light cycles and 17°C or 27°C.

6.3 Discussion

The results of the experiments described in this chapter, aiming to quantify the importance of temperature compensation in the enhancement of plant growth at high temperatures, were not what was initially expected. Recent studies (Gould *et al.*, 2006) clearly indicated in *GI* a key component of the temperature compensation response, being able to maintain clock rhythmicity in a wider range of temperatures. The two lines involved in the test were therefore expected to grow similarly at 17°C, and differently at 27°C, with the mutant line *gi-11* (with temperature compensation impaired) performing poorly in comparison to the wild-type WS. The experimental data however indicated a more blurred scenario, with the *gi-11* mutant performing better than WS in many cases. Moreover, phenotypic characteristics of the WS background line made it quite difficult to measur visible leaf area accurately: as a matter of fact at 27°C plants had elongated petioles, with leaves often overlapping or turned on one side.

In conclusion mutation of the single *GI* gene seems to be not "enough" to impair plant performance at high temperature. But why it is not enough? At this point can be helpful recalling the roles discovered to date for GI gene in the plant system. Firstly it acts upstream of *CONSTANS* (*CO*) in the regulation of flowering by photoperiod (Suarez-Lopez *et al.*, 2001). If the alteration of this function has an effect, it would certainly be on fitness, expressed as number and viability of seeds produced, and on competition advantage. These effects were the first to be discovered in the sixties by György P. Rédei (Redei, 1962), who identified *gi* as late flowering and highly fecund supervital mutant. In Rédei's article several arguments are presented for and against the hypothesis that *gi* mutant has a real fitness advantage: one for all is that the advantage may be such only in the experimental conditions (long days) and would be lost in real environmental conditions.

The second role discovered for the *GI* gene is within the circadian oscillator, acting to stabilize the rhythm over a broad range of temperatures. A direct consequence of a *gi* null mutation would be a plant unable to compensate its rhythm over temperature changes, resulting in a shorter or longer period. This was tested by Gould *et al.*, 2006,

measuring at 27°C, demonstrated a 1 hour shortening of leaf movement period. The reason for not seeing any difference in the performance experiments just presented could be exactly this: one hour difference in period was not enough to impair significantly the measured phenotypes. A significant and measurable difference from wild-type would be for instance four hours, as seen in previous chapters for the *ztl* and *toc1* mutants. This of course is not a proof of the superfluity of temperature compensation. Other traits may be affected by a lack of clock robustness, leading to reduced plant fitness in the competition for scarce resources in the environment.

CHAPTER 7 - Screen for new mutants

7.1 Introduction

Screens for plants with a different phenotype from the population's average have been done from the beginning of their domestication, or even earlier, when fruit pickers were choosing the best food to eat. With the advent of selective breeding and the green revolution, methods developed to become a well established practice in scientific research. The two main factors impacting on the outcome of a screening program are the composition of the population screened and, directly linked to the first, the method the plants are screened with. A review of genetic screens performed with *Arabidopsis* is in Page and Grossniklaus, 2002. Screening pioneers used populations composed of naturally variable plant lines: differences among lines were due to spontaneous mutations occurring in the natural environment. Natural mutations are still exploited today: analysing the circadian period of leaf movement of Recombinant Inbred Lines (RILs) made from natural accessions, it was possible to identify the *FLOWERING LOCUS C* (*FLC*) gene (Swarup *et al.*, 1999).

An important breakthrough was the discovery that genetic mutations could be induced (Muller, 1930) leading to high frequency mutations and new screen possibilities. In *Arabidopsis* research the first and widely used seed mutagenesis method was EMS, the chemical ethylmethane sulphonate (Koornneef *et al.*, 1982). Successful examples of EMS forward genetic screens in plant circadian research are the discovery of the out of phase 1 (oop1) mutant (Salome *et al.*, 2002) using sulphur dioxide (SO_2) as screen method to select mutants with altered stomatal aperture rhythms and the regulation role of *ELF3* gene in the photoperiodic induction of flowering (Zagotta *et al.*, 1992; Zagotta *et al.*, 1996)

Success in selecting interesting mutants however, is better achieved when the selection procedure is easy and stringent. This has often been made possible by creating a highly specific background line, transforming efficient reporter genes in the plant genome. A commonly used reporter in *Arabidopsis* circadian research is firefly luciferase (LUC), fused to the promoter of a gene of interest. The line is then mutagenised and the progeny

analysed. Examples of the efficiency of the method are the discovery of two important circadian clock mutants, *toc1* (Millar *et al.*, 1995) and *ztl* (Somers *et al.*, 2000; Kevei *et al.*, 2006).

The lines I screened for new mutants, described in this chapter, were generated with yet another system, called activation tagging. Activation tagging involves transforming plants with a T-DNA vector containing multimerized (four copies) transcriptional enhancers from the cauliflower mosaic virus (*CaMV*) *35S* gene and the subsequent generation of a collection of lines to screen (Weigel *et al.*, 2000). This approach has several advantages compared to classic screens based on loss-of-function mutations. It allows the identification of duplicated genes with a redundant role, gene families (Nakazawa *et al.*, 2003) and genes which led to early lethality if absent. Introducing enhancers and not a full promoter leads primarily to an enhancement of the endogenous expression of a gene close to the T-DNA insertion, maintaining any differential expression in leaves and roots, rather than constitutive ectopic expression in all tissues. Finally the candidate gene can be easily isolated by plasmid rescue. In plant research activation tagging has been used effectively in several cases, from rice (Jeong *et al.*, 2002) to *Arabidopsis*, to explore the functions of core clock genes as *GI* (Huq *et al.*, 2000), response to chemical stress (Devi *et al.*, 2006) and salt tolerance (Rus *et al.*, 2001).

The aim of the screen experiments described here is to identify new components of the clock specifically required for function at high temperatures in *Arabidopsis*. Two collections of activation tagged *Arabidopsis* lines were used for the purpose and screened in different experiments: Scheible and Somerville lines and Yokoi, Koiwa, Bressan lines. Before screening these lines it was necessary to optimize the screening conditions. Optimization included finding the best growing conditions in which the wild type plants could grow even and uniform and at the same time the conditions in which it was easier to spot mutant plants amongst the wild types. To do so the phenotype of CCA1ox-o38 plants phenotype was used as reference. When *CCA1* gene, a core clock component, is over-expressed it shows a strong temperature dependent phenotype, exactly what the screen aims to find for new clock components. High light intensity (200 μmol/m2/s at soil level), compost without perlite and a density of 150seeds/pot were found as the optimal conditions to spot CCA1ox-o38 plants among wild-type Col-0 plants at 27°C.

The screen required a series of successive steps, described visually in Figure 7-1. Sown seeds were placed for 72 hours in a 4°C dark cold room, to allow a subsequent synchronised germination, occurred in a 21°C growth room with 12L:12D light cycles. Then the workflow included a first screen (7.2.1 Screen) of all the collection of lines, a second screen (7.2.2 Re-Screen) to confirm that the selected phenotypes are temperature-specific and finally a leaf movement experiment (7.2.3 Leaf Movement), to measure their circadian phenotype. At the end of the screen process, it was possible to isolate four putative circadian mutants, with a leaf movement period sensibly different from the wild type Columbia over the increase of growing temperature to 27°C.

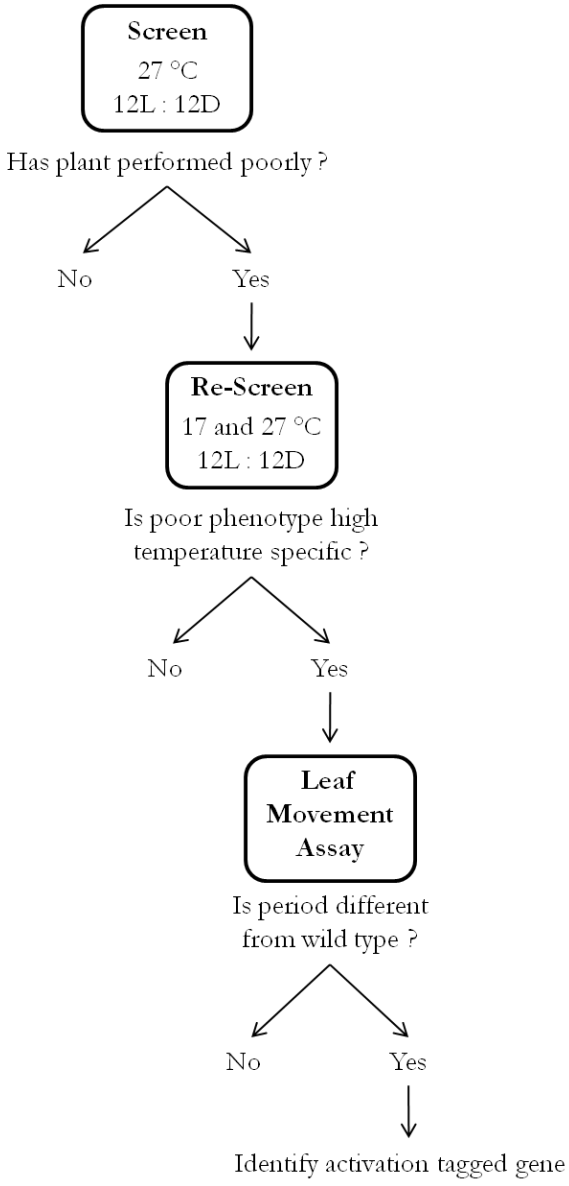


Figure 7-1. Flowchart of the screen experiments.

7.2 Results

7.2.1 Screen

7.2.1.1 Scheible and Somerville lines

The Scheible and Somerville set (N31100) is composed by 208 tubes, each containing a pool of 300-350 activation tagged lines for a total of ~65000 different lines. Three separate experiments were performed, enough to screen all the tubes twice. About 300 seeds were sown for each tube, this implies that not all the lines present in one tube were sown and a further screening experiment would be required to reach saturation. During each experiment the plants were constantly monitored for unusual morphological features and the descriptions inserted in an Excel database. All the different phenotypes selected are listed in Table 20. The most frequent phenotypes selected were the early flowering and long hypocotyl, followed by plants with deformed/missing leaves, small plants and plants with a leaf colour lighter or darker than the average. At the end of the three experiments performed a total of 180 putative mutant plants were transplanted: of these only 145 plants managed to set seeds that were harvested.

Phenotype	**N°**	%
Early flowering	21	11,7
Long hypocotyl	18	10,0
Long hypocotyl, leaves heading up	14	7,8
Deformed/missing leaves	13	7,2
Small plant with long hypocotyl	13	7,2
Small plant with leaves heading up	12	6,7
Small weak plant	10	5,6
Small plant	8	4,4
Small dark green plant	8	4,4
Light green/yellow leaves	6	3,3
Long hypocotyl and long petioles	6	3,3
Long hypocotyl, early flowering	5	2,8
Long weak hypocotyl, leaves heading up	5	2,8
Long weak hypocotyl, leaves heading up, long petioles	5	2,8
Small plant, light green leaves heading up, early flowering	5	2,8
Dark green leaves	5	2,8
Leaves heading up	4	2,2
Small plant with long petioles heading up	4	2,2
Chlorotic plant/leaves	4	2,2
Elongated leaves	4	2,2
Elongated petioles	4	2,2
Small plant with elongated leaves	3	1,7
Dark green plant, early flowering	3	1,7
Total	**180**	**100**

Table 20. Phenotypical description of the putative mutant plants isolated from the screenings performed on Scheible and Somerville lines. The phenotypes are ordered from the most to the less frequent; % is relative to the total number of mutants isolated.

7.2.1.2 Yokoi, Koiwa, Bressan lines

The Yokoi, Koiwa, Bressan set (N31400, N31402, N31404) is a composed by a total of 236 tubes, each containing a pool of 100 activation tagged lines for a total of 23600 different lines. Two separate experiments were performed, enough to screen 196 tubes once and 40 tubes twice. During each experiment plants were constantly monitored for unusual morphological features as for the Scheible and Somerville lines. All the different phenotypes selected are listed in Table 21. The most frequent phenotypes selected were long hypocotyl, weak hypocotyls, early flowering and small plants. At the end of the two experiments performed a total of 222 putative mutant plants were transplanted.

Phenotype	**N°**	%
Long hypocotyl	50	22,5
Early Flowering	42	18,9
Small plant, curved petiols	27	12,2
Weak hypocotyl	19	8,6
Small Plant	17	7,7
Big plant, longs petiols, big leafs	13	5,9
Long weak hypocotyl	11	5,0
Long petioles	9	4,1
Hairy leaves	7	3,2
Long hypocotyl, early flowering	6	2,7
Deformed leaves	6	2,7
Small light green plant	5	2,3
3 cotyledons and 3 leaves	4	1,8
Dark green deformed leaves	3	1,4
Small dark green plant,curled leaves	3	1,4
Total	**222**	**100**

Table 21. Phenotypical description of the putative mutant plants isolated from the screenings performed on Yokoi, Koiwa, Bressan lines. The phenotypes are ordered from the most to the less frequent.

7.2.2 Re-Screen

Seeds harvested from 48 putative mutant lines belonging to the Scheible and Somerville set were used to perform a re-screening experiment. The aim of the re-screen is to determine whether the phenotypes spotted in the screen are expressed at the temperature of 27°C and not at 17°C; if that is the case, the phenotype can be regarded as temperature-specific and therefore interesting. 24 lines showed a phenotype at 27°C not present in the plants grown at 17°C: the two most frequent phenotypes found were early flowering and long hypocotyl. As the re-screen step was able to thin down the number of putative mutant only by 50% (less than expected) each phenotype was given a score on a three colour traffic light basis (Table 22): this is to give a priority in the queue for the following leaf movement experiment, which is a low throughput and laborious analysis. The score was given according to the strength of the phenotype, its similarity to the phenotype found in the screening experiment for the same line and the similarity to other lines selected from the same pool. Six lines which showed a strong and uniform phenotype were forwarded to the following step (leaf movement analysis) to assess their circadian phenotype.

LM?	Putative Mut Line	Phenotype at 27 °C
	01_A_3	
	02_B_4	
	02_B_5	
	03_B_6	1 Plant with long hypocotyl, early flowering
YES	04_B_9	All plants with long hypocotyl
	04_B_10	Early flowering
	05_B_1	Early flowering?
	06_A_8	Long hypocotyl, leaves up
	08_B_2	Early flowering
YES	09_A_4	Long hypocotyl, early flowering
	09_B_1	Early flowering?
	10_A_2	Early flowering
	10_A_3	Early flowering?
	10_B_4	Long weak hypocotyl
	11_A_2	Early flowering?
	11_B_1	few plants with very long hypocotyl
	12_B_2	Long petioles, big leaves
	12_B_3	Early flowering
	13_A_5	Early flowering
YES	13_A_6	Big leaves & petioles, Early flowering
	13_B_2	
	13_B_6	Long petioles, early flowering, big leaves
	14_A_4	
	14_B_5	
	15_A_3	
	15_A_4	
	15_B_3	
	15_B_4	Early flowering
	17_B_2	
	18_B_6	Early flowering
	19_A_5	
	20_B_6	
	20_B_7	
	20_B_8	Early flowering
	22_A_2	
	22_A_4	Twisted leaves at both temperatures
	22_A_5	Early flowering
YES	23_A_4	Strong early flowering on all plants
YES	23_B_6	Light green leaves heading up, long hypocotyl
	23_B_7	Early flowering
	24_A_2	
	24_A_10	Strong early flowering
	25_B_3	
	25_B_5	
	26_A_4	Chlorotic cotyledons
	26_B_4	
	27_A_5	Early flowering
YES	27_B_1	long hypocotyl & leaves up

Table 22. Classification of putative mutant lines according to the Re-Screen results. Three colours were used, referring to lines with a strong (green), weak (yellow) and uncertain (red) phenotype. "LM?" column indicates lines used in leaf movement experiments.

7.2.3 Leaf Movement

A leaf movement experiment was performed with 6 putative mutant plant lines selected from the re-screening experiment (7.2.2 Re-Screen), to measure their circadian phenotype at 17 and 27°C. The wild type Col-2, background of the activation tagged lines screened, was also included. In Table 23 are summarized the results of the experiment.

A	**17°C**			**27°C**		
Line	Leaves	Rhythmic leaves	%	Leaves	Rhythmic leaves	%
Col-2	30	28	93	26	23	88
04_B_9	25	25	100	29	28	97
09_A_4	30	29	97	27	23	85
13_A_6	29	28	97	29	26	90
23_A_4	29	27	93	29	20	69
23_B_6	30	24	80	30	28	93
27_B_1	29	26	90	27	24	89

B	**17°C**				**27°C**			
Line	Period		RAE		Period		RAE	
Col-2	25.38	(0,08)	0.07	(0,01)	25.17	(0,21)	0.10	(0,01)
04_B_9	27.16	(0,30)	0.11	(0,02)	28.62	(0,61)	0.10	(0,01)
09_A_4	25.13	(0,23)	0.13	(0,01)	25.84	(0,21)	0.12	(0,01)
13_A_6	25.28	(0,19)	0.08	(0,01)	25.07	(0,55)	0.17	(0,01)
23_A_4	24.89	(0,15)	0.08	(0,01)	21.94	(0,29)	0.14	(0,01)
23_B_6	26.79	(0,27)	0.15	(0,01)	23.05	(0,43)	0.15	(0,02)
27_B_1	26.60	(0,20)	0.09	(0,01)	22.64	(0,46)	0.27	(0,01)

Table 23. Results of the leaf movement experiment on six putative mutant lines and their background Col-2. **A**) Number of leaves analyzed and rhythmic leaves found. **B**) Period estimates (hours) and Relative Amplitude Errors are weighed means. Weighed Standard Errors are shown in parentheses.

Table 23A shows the number of rhythmic leaves compared to the total number of leaves analyzed. The percentage of rhythmic plants is in most cases lower at 27°C than at 17°C: this is a clear signal that high temperatures affect somehow plants rhythmicity. This could also be due to the presence of more leaves (plants grow faster at 27°C) and therefore to the increased technical difficulty of tracing the rhythm of overlapping leaves. In Table 23B the Relative Amplitude Error, a measure of rhythm robustness varying from 0 (a perfect fit to the cosine wave) to 1 (not statistically significant) shows similar temperature-dependant behaviour. At 27°C RAE is bigger than at 17°C for nearly all lines.

This behaviour is reflected in the distribution of the data points in the scatter plot graphs (Figure 7-2 and Figure 7-3) where the period estimates are plotted against the relative amplitude error at both temperatures. The points at 17°C cluster closer to Col-2 than at 27°C suggesting a reduced robustness of the clock at high temperatures. A closer look at both Table 23B and the graphs allows to note several differences among the lines screened. According to Table 23B the line 04_B_9 has an estimate period close to 27h at 17°C and close to 29h at 27°C, in the graph this is reproduced at 17°C with the data points clustering with a period close to 27h. At 27°C however the points are more spread, with a well marked separation: one third of the points have a period close to 29h and the rest of the data close to Col-2. This might be due to segregation among the plants used for the experiment, a fact already noted in the re-screening experiment. A similar pattern was found in other two lines 23_B_6 and 27_B_1. Both lines at 17°C have a period close to 27h while at 27°C their period is shorter, close to 23h. The data at 27°C are extremely spread, with one fourth of the points remarkably separated from the rest and with a much longer period (32h). The putative mutant lines 09_A_4, 13_A_6 and 23_A_4 at 17°C have a period close to Col-2 (25h) but with a less robust rhythm, as the data points cluster less tightly if compared to Col-0. At 27°C 13_A_6 and 09_A_4 keep a rhythm of 25h while the period of the line 23_A_4 drops to 22h.

To recapitulate, four are the interesting lines identified: 04_B_9 with an estimate period of 27h at 17°C and of 29h at 27°C, 23_B_6 and 27_B_1 with a period of 27h at 17°C and of 23h at 27°C and 23_A_4 with a normal period of 25h at 17°C and a much shorter period of 22h at 27°C.

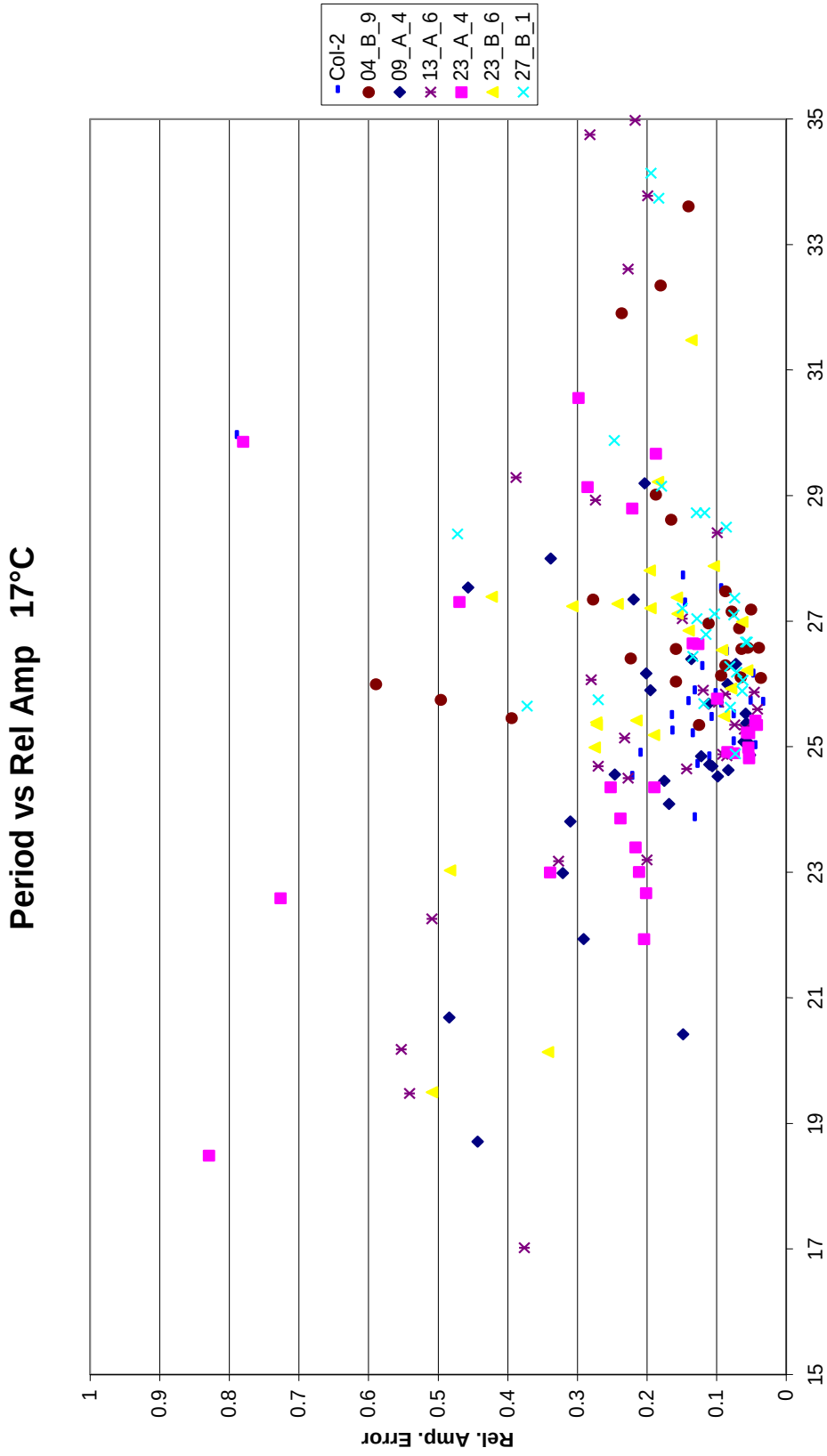


Figure 7-2. Scatter plot graph of the period against the relative amplitude error at 17°C, for all the putative mutants and the background Col-2.

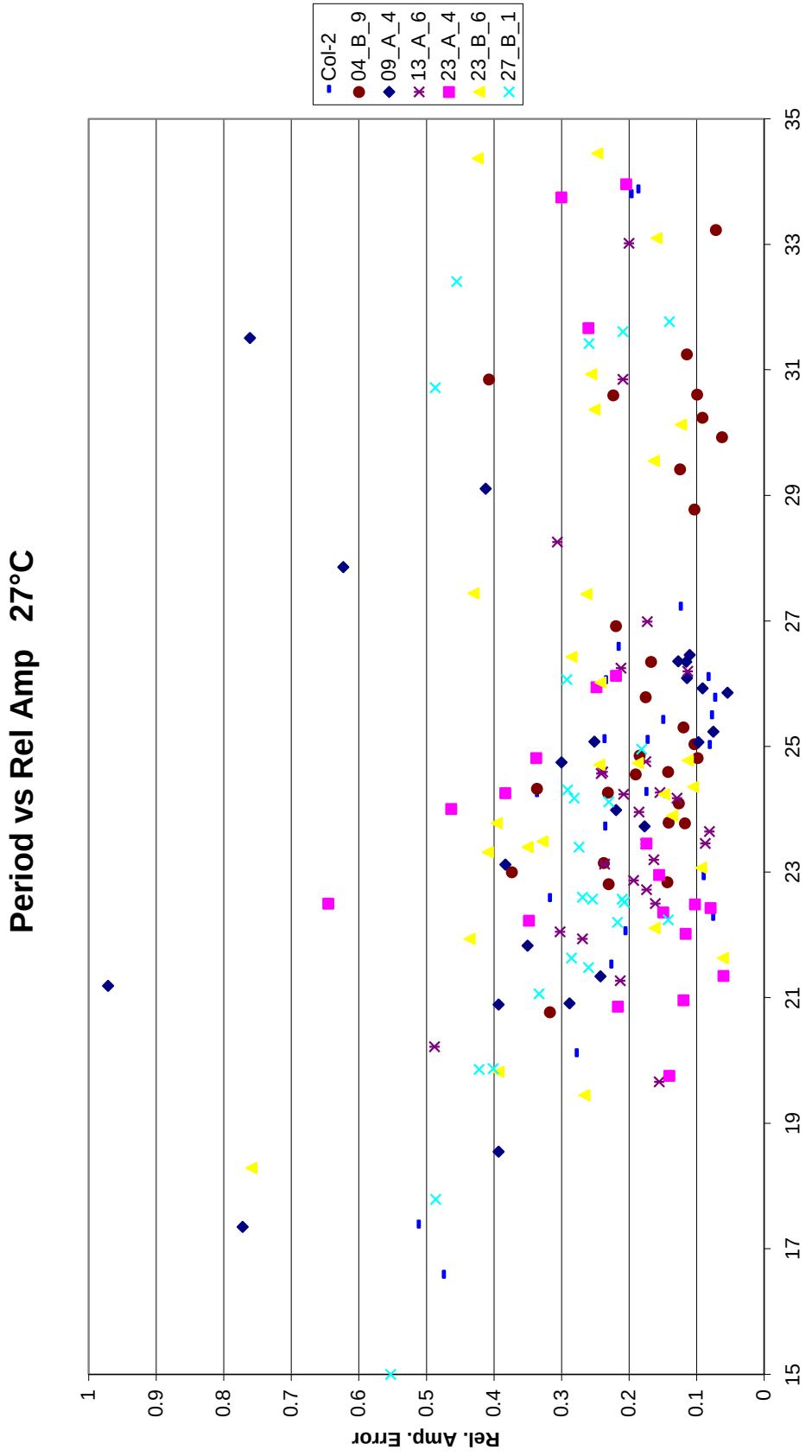


Figure 7-3. Scatter plot graph of the period against the relative amplitude error at 27°C, for all the putative mutants and the background Col-2.

7.3 Discussion

The screening experiments were performed to identify new components of the clock specifically required for function at high temperatures. Nearly 90,000 activation tagged *Arabidopsis* lines were screened, leading to the identification of 408 (0.45%) putative mutant lines, of which only 343 set seed once transplanted. The number of plants selected as different from the wild type clearly depends on a subjective choice: this means that not all the putative mutants with an altered circadian phenotype will be selected and that many of the selected plants will probably not be mutants. Having said this it is important to note that the selection of plants still relied on a method, even though empirical: priority was given to plants which show a phenotype similar to the one of known circadian mutants such as CCA1ox, and plants which flower earlier than the wild type control (another typical trait of circadian mutants).

To narrow down the big number of plants identified, in the second step of the screening process (7.2.2 Re-Screen) plants were grown again at 17°C and 27°C, to confirm the temperature dependent phenotype. During this step two problems occurred. Firstly the number of plants that showed at 27°C a phenotype different from 17°C was bigger than expected: this issue was addressed with a score method developed on a three color traffic light basis as described in Table 22. Secondly the diversity of phenotypes detected within the same line sown, probably due to genetic segregation: a fact confirmed also by leaf movement results (Figure 7-3). This raises the question whether the activation tagged lines used were really homozygous. To overcome the problem, an additional screening step could be introduced before the re-screen, to check the antibiotic resistance of the interesting putative mutant lines. In fact the pSKI15 activation tagging vector construct (Weigel *et al.*, 2000) includes a *BAR* gene for selection on soil with Basta herbicide, and in Yokoi, Koiwa, Bressan lines there is also a Kanamicin resistance. (see Figure 7-4 for a revised flowchart)

The final step of the screen, leaf movement analysis to measure circadian phenotypes, once again raised some issues. During the analysis of the first 6 putative mutant plant lines the wild type Col-2 at 27°C did not show a robust rhythm. In the

scatter plot graph at 27°C (Table 23B) Col-2 data points are not as clustered as they are at 17°C (Table 23A). This led to increased difficulty in the interpretation of the results and was surprising, as Col-0 had previously been used in leaf movement studies and gave robust rhythms of leaf movement at both 17 and 27°C. Despite all the technical difficulties encountered four putative circadian mutants were identified: 04_B_9, 23_A_4, 23_B_6, 27_B_1 for which a closer look is needed to confirm their altered clock period and the location of the activation tagging insertion.

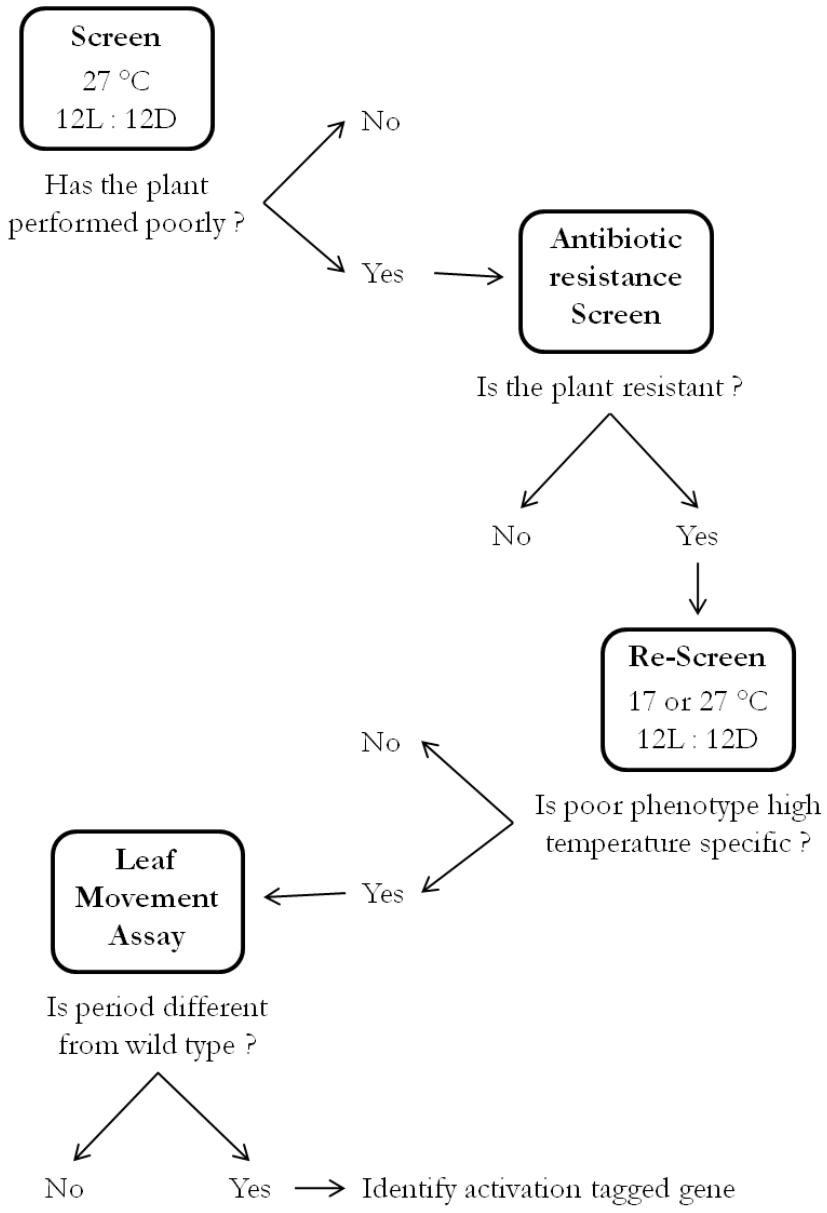


Figure 7-4. Revised flowchart for screen experiments.

The several obstacles described, in particular the need for additional screening steps and the time consuming leaf movement experiments, were magnified by the big numbers involved, leading to the choice of stopping this part of the research. This allowed to dedicate the right amount of resources to the very promising performance experiments described (Chapters 3-5). However to allow a faster future development of this screen I here propose possible future directions. Leaf movement analysis, a fundamental tool used for many years in *Arabidopsis* circadian research, has a low throughput and is extremely time consuming to both setup and to analyze the data. An alternative which could be used to speed up dramatically the circadian screen is delayed fluorescence (Gould *et al.*, 2009). This innovative technique allows the measurement of circadian rhythmicity of any plant, without the need to have its genome transformed with a circadian-regulated luciferase reporter gene. This would give a boost to the screen process, giving the ability to screen hundreds of lines in a few days.

CONCLUSIONS

Hundreds of years passed from the first leaf movements noted in the 18th century to the discovery of the first circadian properties. Unveiling year after year the central role that the circadian clock has in controlling several physiological traits, scientists often imagined this complex mechanism, evolved several times, could be critical for plants' fitness. Only in the last decade however molecular biology allowed to generate plants lacking circadian rhythmicity and to test under laboratory conditions the clock impact on plant fitness (Green *et al.*, 2002). Using the same arrhythmic lines, this study proves that a functional circadian clock is even more important in higher growing temperatures. In fact, performance of *CCA1* over-expressing plants was found to be even lower at 27°C than it was at 17°C. The same increasing difference between the CCA1ox-o38 line and its wild-type Col-0 was measured in transcript and metabolic profiling. A closer look to the expression of the *CCA1* gene however, raised the suspect that part of the measured difference could be due to an altered functionality of 35S promoter used in the transgenic construct. Recent literature confirms this observation, making necessary to reconsider expectations on the expression stability of genes driven by the 35S promoter (Boyko *et al.*, 2010). In particular it would be sensible reviewing results obtained with constructs carrying the 35S promoter and in growth temperatures near and above those tested in this work. Often functionality of useful tools widely used in current laboratory practice as 35S promoter are taken for granted: however more attention is probably needed as unexpected effects can invalidate important experimental results.

Soon after the proof that a functional circadian clock is important for plant fitness, circadian biologists were able to go one step further, showing that even an accurate clock can impact on performance. Plants in circadian resonance with the environment increased their photosynthesis and growth, competing successfully with non resonant plants (Dodd *et al.*, 2005). Using the long (*ztl*) and short (*toc-1*) period mutant lines in this work it was possible to extend that first discovery to demonstrate its increased importance at higher temperature. Both lines used do not carry clock genes expressed by the 35S promoter, excluding the measured phenotypes were somehow generated by a technical artefact.

Particularly clear were *ztl* results, showing no difference between the resonant and non-resonant lines at 17°C, and a significant difference when growing temperature was increased to 27°C. The fact that even an inaccurate clock can impact plant performance at high temperature could be very relevant to field production. Actually, it is unlikely that cultivated crops have a completely arrhythmic clock, as this would totally impair their ability to sense the seasons, making them easily spotted as unfit for production. On the other hand, plants with a non resonant clock could be farmed in normal temperatures without noticing their defect. Increasing global temperatures however would likely highlight their impairment, leading to a reduced field production.

Studying the links between circadian clock and temperature, one cannot neglect the temperature compensation mechanism. While little is known about the molecular temperature sensors in plants, a recent study shed some light on the molecular basis of clock temperature compensation (Gould *et al.*, 2006). The molecular oscillator mechanism was found to change at different temperatures, and the *GI* gene emerged to be a key component of the temperature compensation response, being able to maintain clock rhythmicity in a wider range of temperatures. In this thesis work, using the temperature compensation mutant *gi-11*, it was tested whether plants unable to compensate their circadian clock against temperature performed worse than wild-types when grown at 27°C. It was clear that a little (1hour) variation in the circadian rhythm period generated by the lacking of temperature compensation could not affect significantly the performance of mutant plants. These results however do not completely rule out the possibility that plants lacking circadian clock temperature compensation could underperform at higher temperatures. As the consequences might not be negligible, it would be advisable to further explore the topic, maybe focussing also on longer term effects as flowering time, seed yield and competition for natural resources.

A way to further study the connections between temperature perception, circadian clock and compensation mechanisms would certainly be to find new mutants. A screen to identify new components of the clock specifically required for function at high temperatures was performed as part of this project. At the end of the screen pipeline, four putative circadian mutants were identified, which will need to be further characterised to confirm their altered rhythmicity and eventual position in the current clock model.

Continuation of the current screen would be advisable, certainly using a more highthroughoutput method. Delayed fluorescence is an innovative technique that could to the job. It allows the measurement of the circadian rhythm of any plant, without the need to have its genome transformed with a circadian-regulated reporter gene. Having the ability to screen hundreds of lines in days, the screen performed could be repeated easily. At last, this method could become an invaluable tool to assess the circadian rhythm of crop species, making easier the selection of newly designed crops with selected circadian properties, able to withstand the predicted climatic changes.

To sum up the current study highlights the increased importance, for the model plant *Arabidopsis thaliana,* of having a functional and accurate circadian clock with the increase of environmental temperature. In fact it was demonstrated how a defect in the plant clock functionality can be tolerated at normal temperatures, but impairs the overall plant performance in a more stressful environment. The following diagram (Figure 7-5) summarises the effects of increased growth temperature on the plant lines used, measured in visible leaf area, aerial biomass, chlorophyll, transcript and metabolic profiling. CCA1ox-o38 plants, with a completely non functional clock, showed not only a great reduction in performance, but also greater differences in transcripts and metabolites when compared to the wild type Col-0 line. Plants with altered clock (*ztl* and *toc1*) and the wild type Col-0 performed best at higher temperature when growing in a daily light/dark cycle matching their internal clock period.

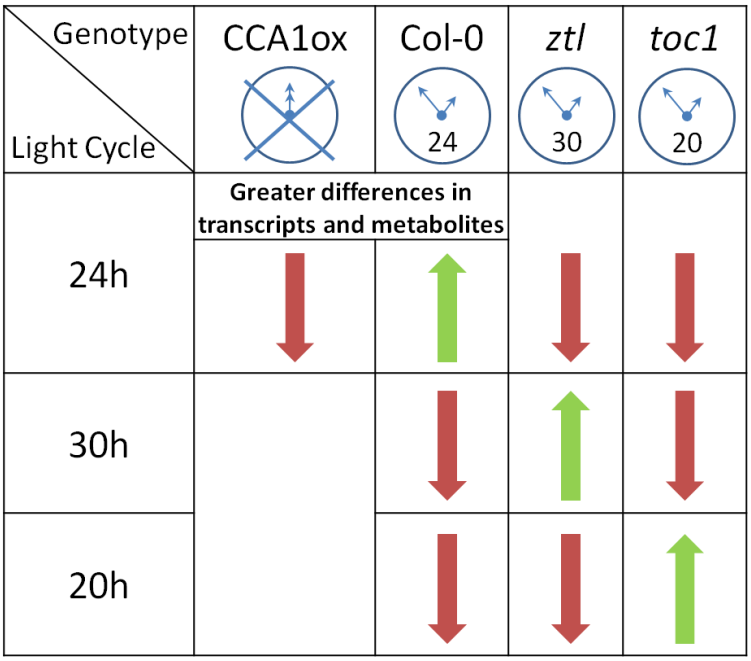


Figure 7-5. Trends measured in visible leaf area, aerial biomass and chlorophyll with the rise of temperature from 17 to 27°C. Clock icons indicate the genotype internal rhythm, crossed means arrhythmic. "Light Cycle" is the length (in hours) of a full light/dark cycle plants were grown in: duration of light and dark was always equal (i.e. 24h = 12hL/12hD). Differences in transcripts and metabolites were measured between Col-0 and CCA1ox o38 plant lines.

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