Analysis of the circadian clock and light signalling function of ZEITLUPE

Ph.D. thesis

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INTRODUCTION

The circadian systems that drive 24h biological rhythms in many organisms evolved as an adaptation to the Earth's rotation and its attendant changes in light and temperature conditions. Plants were the first organisms for which the observation of a circadian rhythm was published. The main advantage of having a daily biological clockwork is that it organizes a temporal program that cues processes to occur at specific phase relationships to the daily environmental cycle. This programming can enable the organisms to anticipate the forthcoming rhythmic changes in the environment and allow them to respond appropriately. Photosynthetic organisms such as plants depend upon sunlight. The availability of this energy is a 24-h cyclic phenomenon, and therefore it is not surprising that plants rhythmically mobilize many processes so as to optimally collect every possible drop of the sun's energy. Consequently, there are many circadian-controlled processes in photosynthetic organisms. In plants, circadian rhythms appear to control gene expression, physiological and biological processes - including circadian rhythms of leaf movements, hypocotyl elongation, stomatal conductance, photosynthetic capacity, chloroplast movements, nitrogen fixation, ion fluxes and many more - and the timing component of photoperiodism, which regulates seasonal reproduction. Clocks with similar properties and regulatory architecture have evolved at least four times, which could indicate that circadian rhythms confer a selective advantage (Young and Kay, 2001); however, the basis for their contribution to fitness remains undetermined (Yanovsky and Kay, 2001; Michael et al., 2003).

Under constant conditions circadian rhythms "free-run" with a period close to, but never exactly 24 h. This free-running behaviour under constant conditions is one of three diagnostic features of circadian rhythms. The other two are temperature compensation and entrainment. The core of the circadian system of every model organism can be described as an oscillator. The components of the oscillator rhythmically regulate their own and each other's expression/activity through one or more negative feedback mechanisms at the transcriptional/translational level, generating a self-sustained oscillation (Young and Kay, 2001). Additional clock-associated factors, which do not carry temporal information and which may not be rhythmic are required to set the period length of this oscillation close to 24h and to adjust other rhythmic properties. The oscillator regulates the expression of overt rhythms through „output" pathways. The phase of the oscillator (subjective time) is set to the objective time of the environment by periodic environmental signals (light and temperature) transduced by input pathways. Light signalling to the plant clock is mediated by several photoreceptors of the phytochrome and cryptochrome families, which themselves are regulated by the clock (Toth et al., 2001). Increasing light fluence rates shorten the

free-running period length in *Arabidopsis* (Millar et al., 1995), likes in many diurnal animals (Aschoff, 1979).

According to the current model (Salome and McClung, 2004), the central oscillator in *Arabidopsis* involves the mutual regulation of three genes: *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*) are morning-expressed genes that repress transcription of *TIMING OF CAB EXPRESSION 1* (*TOC1*). The evening-expressed genes *TOC1, GIGANTEA* (*GI*), *EARLY FLOWERING 3* (*ELF3*) and *EARLY FLOWERING 4* (*ELF4*) are each required for expression of *CCA1/LHY* to reach normal levels on the following morning (Salome and McClung, 2004), though the mechanism of this gene activation is unclear. The majority of known components of the *Arabidopsis* circadian system have been identified by forward genetics, several from limited screens for mutants with altered circadian timing under constant light, and others from flowering time screens. Each gene was represented by one or at most two alleles. Recently, a large-scale screen was reported for mutants with altered rhythmicity of several clock-regulated luciferase (*LUC*) reporter genes under constant light; one of the mutated genes has been identified as an allele of a putative DNA-binding protein gene, *PHYTOCLOCK1* (Onai and Ishiura, 2005). In contrast, a *LUC* screen preceded by a screen for long-hypocotyl seedlings and followed by rapid array-based mapping identified several alleles of *TOC1*, *ELF3*, *ELF4* and the same putative DNA-binding protein gene, here termed *LUX ARRHYTHMO* (Hazen et al., 2005).

ZEITLUPE (ZTL) was the first component of the plant circadian system to be implicated in regulated proteolysis. ZTL, LOV KELCH PROTEIN 2 (LKP2) and FLAVIN BINDING, KELCH REPEAT, F-BOX 1 (FKF1) form a protein family with a unique signature of three protein domains: an N-terminal Period-ARNT-Sim (PAS)-like LIGHT, OXYGEN, VOLTAGE (LOV) domain, a central F-box motif and a C-terminal domain of six kelch repeats that are predicted to form a beta-propeller (Somers et al., 2000; Nelson et al., 2000; Schultz et al., 2001). F-box proteins are important target-specifier components of the Skp1/Cullin/F-box (SCF) type of E3 ubiquitin ligases (Vierstra, 2003). The F-box motif interacts with the Skp1 protein, whereas the C terminal is often responsible for binding the target protein. ZTL is assembled into an SCF^{ZTL} complex *in vivo* together with ASK1 (an *Arabidopsis* SKP1-like protein), AtCUL1 (*Arabidopsis thaliana* CULIN 1) and AtRBX1, which are core components of characterized SCF complexes in *Arabidopsis* (Han et al., 2004). Complex formation requires the presence of a functional F-box domain in ZTL. Transient reduction in AtRBX1 levels phenocopies the long-period phenotype of *ztl* mutants, strongly supporting the role of an SCF^{ZTL} complex in the regulation of circadian rhythms (Han et al., 2004). The proposed function of ZTL is to target the TOC1 protein for degradation (Mas et al., 2003). Surprisingly, TOC1 can interact with ZTL through the LOV/PAS domain and not the kelch

repeats (Mas et al., 2003). However, the mutation in the kelch domain abolished the ZTL-TOC1 interaction, suggesting that the beta-propeller supports protein interactions at the LOV/PAS domain. The unusual LOV/PAS domain of ZTL suggested that *ZTL* function could be light-regulated, because the equivalent domain in other proteins (notably FKF1, Imaizumi et al., 2003) can bind a flavin chromophore that is photoactive in blue light. Circadian rhythms in *ztl* mutants have a long-period phenotype that is light-dependent: circadian period is longer under low than under high fluence rates of constant red or blue light (Somers et al., 2000). This is broadly consistent with the more rapid degradation of TOC1 protein in darkness than in light (Mas et al., 2003). The putative ZTL chromophore was not expected to absorb red light significantly, but a physical interaction was demonstrated between ZTL and the C terminal fragments of phytochrome B (PHYB) and cryptochrome 1 (CRY1) photoreceptor proteins (Jarillo et al., 2001), such that these photoreceptors might be involved in ZTL function. All known *ztl* mutants also had short hypocotyls, because the red-light-controlled inhibition of hypocotyl elongation was hyper-responsive in the mutants; for this phenotype, the *ztl* mutations did not affect blue light responsiveness (Somers et al., 2000 and 2004).

The working model of the *Arabidopsis* oscillator largely conforms to the expected negative feedback loop model that has emerged from studies in other model systems. With the establishment of *Arabidopsis* as a model plant system and the use of luciferase as a non-invasive reporter gene (Millar et al., 1995), plant circadian clock research has gathered speed, although there is still a lot to find out about the function and the relationships of the clock elements identified in plants.

RESEARCH OBJECTIVES

At the beginning of our work only limited information was available about the plant circadian system. Although the core oscillator loop has emerged, it was clear that additional components remained to be identified and that only the outlines of the workings of the *Arabidopsis* clock had been established. The details of resetting by light and temperature were also only incompletely known. The main goal of our work was therefore to perform a large-scale screen to identify new circadian clock mutants. For this, we screened *Arabidopsis* plants for altered circadian behaviour by using a reporter system consisting of the firefly *LUCIFERASE* reporter gene under the control of the *CHLOROPHYLL A/B-BINDING PROTEIN*-encoding gene promoter (*CAB* promoter) that itself is regulated by the circadian clock. The screen was carried out in constant darkness. The 104 mutants recovered identify several new clock-affecting genes. In addition, eleven new *ztl* mutants were identified by our screen. Mutations in all of the three predicted domains of ZTL

protein were recovered, which provided an opportunity to obtain more detailed information on the function of the different domains.

Therefore the main objectives of our work were the following:

1. We wished to examine and compare the circadian, photomorphogenic and flowering phenotypes of the newly identified and the previously described *ztl* mutants. By the characterization of the new alleles of *ztl* we planned to obtain new information on the function of ZTL *in vivo*.
2. Using the yeast two-hybrid system we wanted to test the interaction of the mutated and wild-type ZTL protein with the known interacting partner proteins of ZTL. With this experiment we hoped to define the role of the different protein domains in the formation of functional protein complexes.
3. By comparing the data obtained from the phenotypical tests and the protein interactions in yeast we wished to define the role of the three protein domains of ZTL in the circadian clock and in light regulation.
4. Finally, we wished to quantify the advantage gained by matching the endogenous rhythm with that of the light-dark cycles of the environment, and to identify potential mechanisms by which the circadian clock confers adaptive advantage in light-dark cycles.

METHODS

- Molecular cloning techniques
- Creation and maintenance of transgenic *Arabidopsis thaliana* plants
- EMS mutagenesis
- Plant total RNA extraction, Northern blotting
- Total plant protein isolation, Western blotting
- Yeast two-hybrid test for protein interactions
- In vivo luciferase enzyme activity measurements in intact seedlings
- Determination of period length of circadian rhythms by using BRASS software
- Measuring quantitative traits of plants (hypocotyl length, flowering time, chlorophyll accumulation, leaf size, plant weigh, CO_2 fixation) under different light, temperature and day-length conditions

RESULTS AND DISCUSSION

1. To identify new components of the *Arabidopsis* circadian system, we screened ~46,000 M2 seedlings from two EMS-mutagenised populations, recovering 104 mutants with altered temporal patterns of *CAB:LUC*(+) reporter gene expression. Among the mutants we identified two new *timing of cab 1* (*toc1*), one *early flowering 3* (*elf3*), two *gigantea* (*gi*) alleles and eleven new alleles of *ZTL*. Six mutant lines were identified that did not map close to any already known clock-associated genes.
2. Sequencing of the *ZTL* gene from the eleven *ztl* mutants revealed one mutation within the LOV/PAS domain, one in the F-box domain and nine new mutations within the kelch repeat domain of the protein. The positions of the mutated residues in the ZTL kelch repeats mapped on the external surface of the β-propeller structure of this domain. Mutations were recovered in all of the three predicted domains of ZTL protein, which provided an opportunity to obtain more detailed information on the function of the different domains.
3. We studied circadian clock function in the *ztl* mutants under constant light and temperature conditions (free-running conditions). The rhythmic expression of well-characterized promoter*:LUC*+ fusions (*CAB2*, *COLD CIRCADIAN CLOCK REGULATED 2* (*CCR2*) or *CCA1*) and the leaf movement rhythm were examined in the *ztl* mutants under different free-running conditions. All the *ztl* alleles displayed long-period phenotypes and lowered amplitude of rhythms under all conditions. We concluded from these experiments that ZTL is involved in circadian regulation in both light and darkness, therefore ZTL is not a light input component of the clock.
4. We found that the strength of the circadian phenotype of the new *ztl* mutants is light-dependent. The circadian periods of the mutants are longer under all light intensities examined, but the difference between the wild-type and mutant periods is more pronounced under low than under high fluence rates of constant red or blue light. Consequently, in agreement with a previous publication (Somers et. al, 2000), we observed that the circadian clock of the *ztl* mutant plants shows reduced sensitivity to red and blue light, with the exception of the *ztl-21* (LOV/PAS) mutant, the clock of which shows normal (wild-type-like) sensitivity to red light. The period length of *ztl-21* differs from the wild-type by ~3.5 hours throughout the entire intensity scale of red light, which suggests that the LOV/PAS domain is not involved in the determination of the red light sensitivity of the clock.
5. In addition of the circadian function ZTL is also involved in the control of hypocotyl elongation by red light (Somers et. al, 2000). At high fluence rates of red light the inhibition of hypocotyl elongation was enhanced in all *ztl* mutants - except in *ztl-21* (LOV/PAS), which showed normal

response to red light. In blue light there was no significant alteration in the hypocotyl elongation response in the mutant plants compared to wild-type. It follows, that the role of ZTL in red light regulation of hypocotyl elongation does not require an intact LOV/PAS domain.

6. We observed that mutations in the kelch or the F-box domain delayed flowering in long days, as previously reported for the null allele *ztl-3* (Kim et al., 2005) and the kelch mutation *ztl-1* (Somers et al., 2000). In contrast, flowering time in long days was unaffected in the *ztl-21* (LOV/PAS) mutant, which led to the conclusion that the LOV/PAS domain is not essential for the photoperiodic control of flowering by ZTL.
7. Previously we demonstrated that ZTL affects the circadian period of light-grown plants transferred to constant darkness, however we had no information about the function of ZTL in plants which had never seen light. Light-grown plants initially have a light-adapted complement of photoreceptors, some of which are thought to remain active for hours to days in darkness. To test the dependence of *ZTL* function on photoreceptor signaling, *ztl* mutant and wild-type seedlings were grown in constant darkness, under thermo cycles. The circadian rhythms of *CCR2:LUC+* activity were tested in constant darkness in the dark-grown seedlings and compared to seedlings grown under light/dark (LD) cycles. We observed that temperature cycles, similarly to LD cycles, can properly entrain the mutants' clocks. After entrainment with temperature cycles, in constant darkness, *ztl* mutants showed an identical, long period like after entrainment with photo cycles, indicating that ZTL regulates the circadian system without ongoing photoreceptor signalling. To assess the impact of *ztl* mutations on the temperature compensation of the clock, we determined the period length of mutant and wild-type plants in constant light keeping them at different temperatures. We observed that the *ztl* mutants show increased sensitivity to temperature changes: with increasing temperatures the period of the mutant plants increased faster than in wild-type plants. This means that ZTL is involved in the temperature compensation mechanism of the clock, although it is not an essential component.
8. ZTL protein levels were measured in the mutant plants by Western analysis. The results showed that all mutants tested expressed at least the wild-type level of the protein, except for *ztl-31*. *ZTL* mRNA levels showed little or no effect of the mutations, therefore the alteration in protein levels in the mutants could be the result of a change in protein stability. Two mutations (*ztl-22* and *ztl-27*) caused elevated levels of ZTL in the mutant plants as compared to wild-type. It is possible that both mutations inactivate the protein, which results in slower degradation of the ZTL protein, making it more stable, and this may have led to the observed higher levels of ZTL in these two mutants.
9. The domain structure of the ZTL protein family, together with published data, suggests that ZTL function might be understood at the molecular level in terms of its physical interactions

with degradation targets, the SCF complex and the phytochrome and cryptochrome photoreceptors. To test this notion, representative mutations located in the three different domains of ZTL were introduced into the full length ZTL cDNA. The corresponding mutant proteins were expressed in yeast and tested for interaction with ASK1, TOC1 and the N-terminal domain (PHYBN, amino acids 1-621), C-terminal domain (PHYBC, amino acids 645-1272) or full-length phytochrome B. During the experiment we made the following observations:

- the mutation in the F-box domain reduced the interaction between ASK1 and ZTL;
- the kelch mutant protein ($ZTL^{ztl\text{-}27}$) cannot interact with TOC1 and shows reduced ability to bind ASK1;
- the LOV/PAS mutant protein does not interact with TOC1 and shows strongly reduced binding activity to ASK1;
- neither of the three examined mutations (*ztl-21*, *ztl-22*, *ztl-27*) influenced the strength of the interaction between ZTL and PHYB-C; full length PHYB and the N-terminal functional PHYB fragment cannot bind ZTL; it follows that the *in vivo* significance of the ZTL-PHYB-C interaction detected *in vitro* is unclear.

10. We compared the data obtained from the phenotypical tests and the yeast two-hybrid experiments and established that the ZTL protein has a dual role:
    - first, ZTL is involved in the regulation of the period length and amplitude of the circadian rhythm; this function of ZTL is independent of light;
    - second, ZTL is an important component of red light signalling: it is involved in the adjustment of the red light sensitivity of the clock, the regulation of red light-dependent hypocotyl elongation and the photoperiodic induction of flowering.
11. The functions of the three domains of ZTL protein can be summarized as follows:
    - Mutation in the LOV/PAS domain caused the loss of the circadian, light-independent function of ZTL. In contrast, the LOV/PAS mutant protein can normally fulfil ZTL function in light regulated processes. Accordingly, the LOV/PAS domain and its interaction with TOC1 are not essential components of the red light signalling function of ZTL. It is possible that ZTL functions in light regulated processes by targeting substrates other than TOC1 to the proteolytic degradation mechanism. The LOV/PAS domain is indispensable for the light-independent circadian clock function of ZTL.
    - The F-box mutation affected both functions of ZTL. This domain is involved in binding ASK1, an element of the SCF complex, and probably also interacts with other ASK proteins.

- All kelch mutations caused the complete loss of ZTL function. The kelch repeat domain is probably required to maintain the proper structure of the entire ZTL protein, allowing normal interactions with both TOC1 and ASK proteins. This structural role might depend on intramolecular interaction of the other ZTL domains with the surface of the beta-propeller.

12. Using *ztl* and *toc1* mutant plants we wished to quantify the advantage gained by matching the endogenous rhythm with that of the light-dark cycles of the environment, and to identify potential mechanisms by which the circadian clock confers advantage in light-dark cycles. We hypothesised that matching the endogenous clock period (τ) with the period of the exogenous light-dark cycle (T) (so called 'circadian resonance') provides a quantifiable advantage due to optimization of phase relationship between clock-controlled biology and exogenous day-night cycles. To test this hypothesis, we compared the performance of wild-type plants with that of lines with mutations that alter clock period length in a range of environmental period lengths that were either matched or unmatched to the endogenous clock period. Our data demonstrated that the circadian clock allows plants to increase net photosynthesis. We showed that the circadian clock underlies a 50 % improvement in *Arabidopsis* productivity. This may be achieved by correct anticipation of dawn and dusk, and the synchronization of the synthesis of light harvesting complex proteins and chlorophyll, both of which are unstable in their unbound state. Failure to correctly match the endogenous rhythm to the environmental rhythm leads to reduced leaf chlorophyll content, reduced assimilation, reduced growth and increased mortality. We propose that optimisation of these parameters by circadian resonance may represent one of mechanisms that has selected for circadian clock function in evolution.

CONCLUSIONS

The performed mutant screen resulted in the identification of new clock components and new mutant alleles of already known clock elements, including eleven new alleles of the *ZTL* gene. We isolated mutations in all three predicted functional domains of ZTL, which provided an opportunity to obtain detailed information on the function of the different protein domains *in vivo*. The main result of our work is that we could separate the two functions of ZTL by analysing the mutant phenotypes and the interaction capability of the mutant proteins with known interacting partner proteins. Performing two distinct functions ZTL is most probably involved in targeting different substrates to proteosomal degradation. In the regulation of the circadian clock's period, ZTL acts mainly through the direction of TOC1 degradation, whereas in light signalling ZTL plays a role in the degradation of other, currently unknown proteins. Although it was previously published that ZTL can interact with the C-terminal part of PHYB, we have no evidence that any *ztl*

mutant phenotype is due to impaired interaction with this photoreceptor; it is also possible that the ZTL-PHYBC interaction detected *in vitro* has no effect *in vivo*.

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Data from the 1st and 2nd publications were discussed in the Ph.D. dissertation.