Investigating the role of an atypical small GTPase in the plant circadian clock

Theses of the Ph.D. dissertation

Péter Gyula

Supervisor: Dr. Ferenc Nagy

Hungarian Academy of Sciences
Biological Research Center
Institute of Plant Biology
Group of Photo- and Chronobiology

University of Szeged
School of Biological Sciences

Szeged
2009

INTRODUCTION

The circadian clock is a biological timing mechanism which provides rhythmicity to gene expression, metabolism and physiology in many organisms. This internal clock helps the organisms to anticipate the most predictable periodic environmental change on Earth: the succession of days and nights, allowing different processes to be scheduled to the most appropriate time of the day. Precise synchronisation of these internal processes to rhythmically changing environmental cues has been shown to enhance fitness of organisms [1].

The genetic circuit underlying the *Arabidopsis* circadian oscillator was initially proposed to function through the reciprocal regulation between the *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*), *LATE ELONGATED HYPOCOTYL* (*LHY*) and *TIMING OF CAB EXPRESSION 1* (*TOC1*) genes [2-4]. The morning-expressed CCA1/LHY Myb transcription factors repress the TOC1 gene; conversely, the evening-expressed TOC1 positively regulates the transcription of CCA1/LHY [5]. TOC1 belongs to the PSEUDO RESPONSE REGULATOR (PRR) protein family, which consists of five members: TOC1/PRR1, 3, 5, 7 and 9 [6]. Recent results of mathematical modelling and experimental approaches have revealed two additional regulatory loops coupled to the CCA1/LHY-TOC1 circuit. The "evening loop" is formed by TOC1 and a hypothetical factor Y, both expressed in the evening. Y positively regulates TOC1, whereas TOC1 represses Y transcription, which is also inhibited by CCA1/LHY. TOC1 promotes CCA1/LHY transcription via another hypothetical component, X [7]. It has been demonstrated that GIGANTEA (GI), a nuclear protein with unknown biochemical function is an essential contributor to Y function [8]. The "morning loop" is formed by CCA/LHY and PRR7/9. CCA1/LHY activates PRR7/9 expression in the morning; conversely, PRR7/9 inhibit CCA1/LHY during the rest of the day [8,9]. The co-ordinated function of the three loops is required to generate the ~24 h basic oscillations in *Arabidopsis*.

This oscillation is synchronised to the environment via periodic light and temperature signals normally associated with the natural day/night cycles. Light signals are perceived by the red/far-red light absorbing phytochrome photoreceptors and the blue light absorbing cryptochromes [10,11] and are transduced to the oscillator through the input pathways. The resetting process, also called as entrainment is essential for setting the phase of the oscillator to the environmental light/dark cycles. The light input also modulates the pace/period of the clock under constant light conditions. Each loop of the plant oscillator contains at least one light-inducible/sensitive component (PRR9, CCA1/LHY, GI) providing a possible molecular mechanism for resetting. The F-box protein ZEITLUPE (ZTL) [12] and protein kinase CK2 [13] represent a different level of regulation, since they are not directly involved in the transcriptional control of clock genes, but they primarily affect the abundance or activity of certain clock proteins. ZTL directs TOC1 for

degradation in a light-dependent manner [14], whereas CK2 modulates the activity of CCA1 via phosphorylation [15].

In my thesis I report about our team effort resulted in the identification and characterisation of a novel clock-associated factor, LIGHT INSENSITIVE PERIOD 1 (LIP1). We demonstrated that LIP1 is a negative factor controlling the light-dependent period shortening of circadian rhythms and light-induced phase resetting during the subjective night in plants and that LIP1 represents the first small GTPase affecting the circadian clock function in plants. Small monomeric GTPases form a large family of eukaryotic proteins with a highly conserved basic biochemical function, which relies on binding and subsequent hydrolysis of guanine nucleotides in a cyclic manner [16]. Small GTPases are molecular switches shuttling between the GDP-bound inactive and the GTP-bound active states. Based on their structural and functional similarities, small GTPases are divided into five subfamilies, RAS, RHO, RAB, RAN, ARF, respectively [17]. LIP1 owns some characteristics of the above mentioned classes, but exhibits remarkable differences which makes this molecule a member of a new, seed plant (Spermatophyta) specific subfamily of small GTPases.

RESEARCH OBJECTIVES

Despite the progression made toward understanding the *Arabidopsis* circadian clock, our knowledge is still incomplete. The present model of the plant circadian clock is quite oversimplified. In order to facilitate building a more complete model we decided to conduct a mutant screen searching for new clock components. We created transgenic plants carrying the *CAB2:LUC* reporter gene. The firefly luciferase gene fused to a circadian controlled promoter allowed us to measure the changes of gene expression pattern in real time in living plants for several days by detecting bioluminescence. These plants were EMS treated so as to introduce random point mutations into the genome. Mutants showing altered circadian gene expression pattern were then selected and genetically mapped to identify the mutated gene responsible for the observed phyenotype. Finally we isolated a formerly unknown clock gene that we named after its circadian behaviour *LIP1* (*LIGHT INSENSITIVE PERIOD 1*). In order to determine its role in the plant circadian clock we estabilished the following workplan:

1. Characterisation of the circadian phenotype of *lip1-1*
2. Mapping the extent of the physiological processes affected by *lip1-1*
3. Identification of the *LIP1* gene
4. Characterisation of the *LIP1* gene expression
5. Investigation of the LIP1 protein
6. Mapping of the LIP1-dependent signaling pathways

METHODS

- Molecular cloning techniques
- Creation and maintainance of transgenic *Arabidopsis thaliana* plants
- EMS mutagenesis
- Genetic mapping, high-throughput genotyping PCR
- *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) under different light and day-length conditions
- Plant total RNA extraction, Northern blotting, Real-time PCR
- Total plant protein isolation, Western blotting
- Various microscope techniques
- Bacterial expression and purification of fusion proteins
- *In vitro* GTPase assay
- Yeast two-hybrid screen and test for protein interactions
- Transient protein expression in plant cells and co-immunoprecipitation of protein complexes

RESULTS

1. In *lip1-1* all the rhythms of the measured circadian outputs are shortened, so LIP1 is not part of the output pathways but a component of the central oscillator.
2. In *lip1-1* the period is the same under all tested light intensities. According to this, LIP1 plays a role in light tuning of the period.
3. In high intensity light the period of *lip1-1* is indistinguishible of the the wild type. We assume that under this condition wild type lacks functional LIP1.
4. The period of *lip1-1* measured in constant darkness is the same as in constant light that supports our belief that in *lip1-1* light has no effect on period.
5. The *lip1-1* is most sensitive to resetting light stimuli around the first half of the subjective night. This indicates that LIP1 is operational only in this part of the day and that the clock gates LIP1 activity.
6. The level of the mRNAs of the central oscillator components are not affected in constant light. Under alternating light/dark conditions the level of *TOC1* is decreased while of the *CCA1*/*LHY* is not affected. We cannot explain this behavior but it was observed by others as well.
7. The hypocotyl of the *lip1-1* is shorter in red and blue light but not in dark or far-red light. This latter is interesting because under these circumstances circadian phenotype can be observed.

Furthermore, hypocotyls are shorter in high intensity light as well, while under these conditions there is no difference in period. According to this, circadian phenotype is independent of the photomorphogenic one in *lip1-1*.

8. Epidermal cells of *lip1-1* are rounded that is the sign of the disruption of the actin cytoskeleton.
9. *lip1-1* is salt sensitive.
10. As a result of the map based cloning it turned out that in *lip1-1* the first half of the coding part of the *LIP1* gene along with the promoter is missing, therefore this allele can be considered as a functional null.
11. The successful complementation of the *lip1-1* phenotype by the *35S* or the *LIP1* promoter driven YFP-LIP1 gene confirmed the result of the mapping.
12. *LIP1* codes for a unique seed plant specific small GTPase. The protein contains several motifs not found in canonical GTPases. The most intriguing difference is the replacement of the catalytic glutamine$_{94}$ for histidine. LIP1 lacks the amino- and carboxi-terminal motifs for lipid modification as well.
13. Computer analysis of the amino acid sequence of the LIP1 protein allowed us to draw a possible way of the regulation of the molecule. According to this, the rate of protein degradation is regulated by stepwise phosphorylation like in the case of other clock proteins.
14. By use of bacterially expressed MBP-LIP1 protein we managed to detect significant GTPase activity of LIP1. It proves that despite of irregularities in amino acid sequence LIP1 is a functional GTPase.
15. We investigated the expression of the *LIP1* gene at multiple levels. Our results indicate that circadian clock does not regulate *LIP1* mRNA. However, we detected a possible circadian regulation in the protein level with a peak at the subjective night. We characterised the intracellular distribution pattern of the YFP-LIP1 fusion protein. The fluorescent signal of the YFP has been detected in the nucleus as well as in the cytoplasm where it is associated with fast moving objects, most probably Golgi vesicules.
16. In a yeast two-hybrid screen we identified a LIP1 interacting protein ROPGEF7. This protein interacts with night specific clock proteins (ZTL, GI, TOC1) as well. All these proteins interact with each other that raises the possibility of the existence of a night specific protein complex.

DISCUSSION

We propose that LIP1 plays a negative role in controlling circadian period and light suppresses this effect in a fluence rate dependent manner. Elimination of LIP1 function (e.g. *lip1-1*) mimics the effect of light and results in a short-period phenotype even in darkness. It follows that in WT plants LIP1 function is fully suppressed at those fluence rates, where *lip1-1* plants display WT periods. We emphasize, however that although the period is less sensitive to light in *lip1-1*, PRCs revealed hypersensitivity to resetting light pulses in the mutant specifically during the first half of the subjective night, producing significantly larger phase delays than in WT. The molecular mechanism by which LIP1 negatively regulates resetting however, remains to be elucidated.

ELF3, ZTL are known clock-associated factors whose function could be paralleled to that of LIP1 in some aspects. ELF3 attenuates resetting light signals similarly to LIP1, but at a slightly later phase. ELF3 negatively regulates period similarly to LIP1, but this is light dependent: WT periods were observed in DD in plants misexpressing ELF3. Moreover, ELF3 probably affects the clock via the transcription of CCA1/LHY. ZTL and XCT has a function in regulating period opposite to that of LIP1. *ztl* mutants show extreme long periods in DD that are dramatically shortened by light. However, ZTL affects the clock at the posttranscriptional level. Based on our data, we propose that LIP1 controls the pace of the clock acting primarily at posttranscriptional level and could be involved in the regulation of the abundance or nucleo-cytoplasmic distribution of its yet unknown target.

Independent of this hypothesis we note that the reduced level of *TOC1* mRNA in LD grown seedlings is consistent with a shorthening of the period in the *lip1* mutant, as strong loss-of-function *toc1* mutants have even shorter periods than *lip1*. Current models of the plant clock mechanism indicate that *TOC1* is both repressed by the morning functions of *LHY* and *CCA1*, and activated by the evening functions including *GI*. It remains to be determined which regulatory mechanism links LIP1 function to the clock circuit.

Light inhibition of hypocotyl elongation is rhythmically gated by the circadian clock, and virtually all clock mutants show aberrations in this photomorphogenic response. Our data indicate a negative role for *LIP1*, throughout the entire fluence rate range tested, in red and blue but not in far-red light-dependent inhibition of hypocotyl growth. Hypocotyl length in dark-grown plants was the same in all genotypes, indicating that the differences observed were indeed light-dependent. The hypersensitivity to red and blue light is also apparent at high fluence rates, where the clock function is not affected by *lip1-1*. Thus our data suggest a separate role for LIP1 in photomorphogenesis. There is only one small GTPase that has been implicated in the regulation of hypocotyl elongation so far. Pra2 from pea is a typical RAB-like small GTPase and was shown to

modulate the synthesis of brassinosteroids in the dark. Misexpression of Pra2 in transgenic tobacco results in a dark-specific hypocotyl phenotype indicating substantially different functions for Pra2 and LIP1 in the regulation of hypocotyl elongation. It is worth mentioning that *lip1* mutant plant has other developmental deficiencies as well. The epidermal cells of *lip1* did not show the well known jigsaw puzzle-like shape but are rounded. This indicates the misregulation of the cytoskeleton, typical of the mutation of ROP GTPases. Mutant plants are also salt sensitive.

LIP1 belongs to a novel subfamily of small GTPases. *In vitro* assays demonstrated significant GTP binding and hydrolysing activity of LIP1, making this protein the first small GTPase with a role in the circadian network of plants. In fact, there are at least two other small GTPase that has been implicated in the function of circadian clocks in any organisms studied so far. Mutations in RAB3a have been suggested to affect the period of behavioural rhythms in mice. However, the core molecular oscillator was not affected, indicating that RAB3a is not functioning in the light input pathway or the oscillator itself, but probably affects the coordination/coupling of rhythm-generating nerve cells. On the other hand, DEXRAS1 has been implicated in shaping the phase-dependent responsiveness of the mammalian circadian clock to photic entrainment cues.

The interaction of LIP1 with a *bona fide* ROPGEF was suprising because it was described earlier that ROPGEFs are specific regulators of the ROP family of GTPases and LIP1 is clearly not a ROP type GTPase, considering its amino acid sequence. It is possible that ROPGEF is not a regulator but actually a downstream effector of LIP1. Alternative explanation is that despite different primary structure, LIP1 conformation mimics ROP. Either way, it may explain the observed typical ROP phenotype, the distorted shape of epidermal cells and maybe salt sensitivity as well.

Analysis of *Arabidopsis* protein sequences identified only one close homolog (named LIP2) of LIP1 sharing the characteristic motifs. Tblastn search of available EST and genomic databases revealed the presence of highly conserved LIP-like sequences in several higher plant taxa, but not in non-plant organisms, suggesting a function for LIP-like molecules that is associated with the physiology of seed plants (Spermatophyte).

SUMMARY

We identified the small GTPase LIP1 as a novel component of the light input pathway of the plant circadian network. We demonstrate that LIP1 function is required for the light-dependent modulation of period and for proper resetting of the clock by light pulses, especially during the early subjective night. Our data show that LIP1 – presumably through regulation of other light sensitive components, i.e. ZTL or GI – limits the degree of phase resetting by light pulses at this time of the circadian cycle, when light is normally not present. It is possible that LIP1 protects the clock from excessive or mis-timed light and, therefore, contributes to the robustness and accuracy of the plant circadian clockwork.

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* Shared first authors

† This publication served as the basis of the dissertation