

**Analysis of dynamic changes in gene expression
under the control of the circadian clock in
Arabidopsis**

by

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Declarations

This thesis describes the first (experimental) part of interdisciplinary project where statistical and mathematical methods are applied to analyse experimental data. The work involved setting up a luminescence assay of genetically modified *Arabidopsis* seedlings and the analysis of the resulting data. The materials and the imaging protocol were provided by Mark Spensley.

Abstract

The circadian clock is a mechanism by which organisms anticipate of the daily rhythm of environmental factors like light and temperature. It enables them to adapt their physiology or behavior to rhythmical changes in the environment. According to the current model, the circadian clock consists of a central oscillator, realised as a negative regulatory feedback loop, input pathways, which transmit environmental signals to set the clock to local time, and output pathways, through which the central oscillator regulates other clock dependent genes leading to overt and observable rhythms.

The central oscillator of the *Arabidopsis* clock is believed to consist of a negative feedback loop where the transcription factors LHY and CCA1, whose expression peaks in the morning, repress the transcription of the *TOC1* gene, which peaks in the evening. The TOC1 protein in turn induces the transcription of *LHY* and *CCA1* genes in the morning by a yet unknown mechanism.

To investigate how the transcription of the *LHY* gene is regulated the *LHY* promoter was coupled with the luciferase (*LUC*) gene and introduced into *Arabidopsis* plants. The luminescence generated by the activity of luciferase allows the measurement of the activity of the *LHY* gene.

Several proteins are known, which supposedly are involved in the regulation of *LHY* transcription. Phytochrome interaction factor-3 (PIF3) is a transcription factor which binds to a G-box motif on the *LHY* promoter and also forms complexes with TOC1.

The role of PIF3 in the regulation of the *LHY* gene was tested by comparing transcription patterns of plants carrying a *pif3-1* loss of function mutant to that of wild type plants.

Abbreviations

Protein names:

LHY Long elongated hypocotyl

CCA1 Circadian clock associated 1

TOC1 Timing of CAB expression 1

PIF3 Phytochrome interacting factor 3

PHY Phytochrome

Other abbreviations:

LL constant light conditions (Light-Light)

LD light-dark cycle

EE evening element, a DNA binding site with the sequence AAATATCT

CTR CT rich region.

G-box the DNA-binding site for PIF3 (CACGTG)

bHLH basic helix-loop-helix protein; a class of transcription factors including PIF3

MOAC Molecular Organisation and Assembly in Cells (Doctoral Training Centre)

On notation: Names of proteins will be upper case, names of genes will be upper case and in italics, names of mutant genes will be set in lower case and in italics. For example LHY would denote the protein, *LHY* and *lhy* the gene and mutated gene respectively.

Chapter 1

Introduction

1.1 Introduction to Circadian clocks

The life of many organisms is driven by rhythms like the sleep-wake cycle of mammals, leaf movement or flowering in plants. Organisms keep track of passing time even in the absence of environmental signals. With respect to the daily rhythm this phenomenon is referred to as circadian. Biological clocks maintaining circadian rhythms have been described for organisms ranging from bacteria and fungi to plants and animals. In multicellular organisms certain cells can act as time keeper cells. These time keeping cells can actually be dispersed in different parts of the organism. All clocks seem to be implemented as autoregulatory genetic networks.

The general design scheme, which seems to be applicable to all known clocks, features a central negative feedback loop, called the central oscillator, that drives the oscillation. Environmental signals like light and temperature changes reach the central oscillator by certain input pathways, so that the clock can be entrained to local time. The central oscillator drives the overt rhythms of the organism through output signaling pathways.

1.2 Research on circadian clocks

The circadian clocks of several model organisms have been described in more detail. Earliest genetic screening studies were done in *Drosophila* (see [Scully 2000] for a review of the *Drosophila* clock). Homologues of clock genes of the fly have also been found in a variety of vertebrates including in mice and humans. Another model organism for clock research is the fungus *Neurospora*, where the clock regulates important aspects of its growth and development like sporulation. The core mechanism of this clock evolves around the genes *FREQUENCY*

(*FRQ*), *WHITE COLLAR-1* (*WC-1*) and *WHITE COLLAR-2* (*WC-2*). Interestingly these gene are not related to the clock genes in *Drosophila*. It is hoped that the imminent complete sequencing of the *Neurospora* genome will facilitate further research of its clock mechanism.

Clocks have also been found and studied in cyanobacteria like *Synechococcus elongatus*. Here the central feedback loop was found to involve the genes *kaiA*, *kaiB* and *kaiC*, also so far without any identified homologues in other clock genes. In the recent years *Arabidopsis thaliana* has emerged as a model organism for the plant world. Studies of the plant clock in *Arabidopsis* revealed yet more clock genes not related to the clock genes of other organisms.

In the light of these findings it was suggested that the circadian clock in the different organisms might have arisen independently. Though they still seem to share certain design features, e.g. a central oscillator based on an autoregulatory genetic network. For a detailed review on the current state of the research and further references see [Young 2001].

1.3 *Arabidopsis* as a model organism for circadian clocks in plants.

While circadian clocks has been studied for a long time, only recently *Arabidopsis thaliana* has emerged as a model organism for the plant world. It has several features which make favour its use in the laboratory. It is a small plant with a short life cycle, which makes it easy to grow in a laboratory environment. Further its genome is fairly simple and fully sequenced, which facilitates genetic screening and gene identification.

Several clock components have been identified and basic models have been proposed, but a detailed understanding of the molecular dynamics of the clock is still lacking (see [Carré 2002], [Salomé 2004] for detailed reviews).

The following working model for the core mechanism of the *Arabidopsis* clock has been proposed ([Alabadi 2001], see also fig 1.1):

1.4 Working model for the *Arabidopsis* clock

Light at dawn stimulates the expression of the *LHY* (Long Elongated Hypocotyls) and *CCA1* (Circadian Clock Associated 1) genes. Both gene products repress the expression of the gene

TOC1 (Timing of CAB expression 1) by binding to its promoter. This leads to low *TOC1* protein levels during the day. These low *TOC1* levels in turn decrease the activity of the *LHY* and *CCA1* promoters by an unknown mechanism. Low *LHY* and *CCA1* protein levels then allow increased *TOC1* expression in the evening and accumulation of the gene product over night. High *TOC1* protein levels in the morning finally trigger again the expression of the *LHY* and *CCA1* genes. The *LHY* and *CCA1* proteins in are thought to regulate several output pathways, i.e. inducing the expression of morning genes while repressing those of evening genes.

This simple model does not explain the complex behavior of the clock. For example, the knock out of the *TOC1* gene does not abolish circadian rhythmicity. Also a knock out of either *LHY* and *CCA1* leads to a shortening of the period, but does not abolish clock function. A double mutant of these genes turns arrhythmic, but only some time after release from entrainment, suggesting the existence of related genes that can compensate for the loss of function of *CCA1* and *LHY*. Further there are other genes, which have a strong effect on the clock. For example in mutants of the *ELF3* gene the clock function is disrupted in constant light [Salomé 2004]. These results indicate that this model can only be used as a starting point to investigate the additional layers of regulation and other clock components.

This project focuses on the question of how the *TOC1* protein and other clock components such as *ELF3* regulate the expression of the *LHY* gene.

1.5 The regulation of *LHY* transcription and the role of *PIF3*

Apart from *TOC1*, there are several proteins, which supposedly act on the *LHY* promoter, including PHYTOCHROME INTERACTING FACTOR-3 (*PIF3*), GIGANTEA (*GI*) and EARLY FLOWERING 3 (*ELF3*).

The exact mechanism how *TOC1* acts on *LHY* and *CCA1* promoters is yet unknown. *TOC1* does not bind to DNA by itself, so it was suggested that regulation takes place via associated transcription factors, like *PIF3*.

PIF3 belongs to the class of bHLH transcription factors, which bind to the G-box, a DNA motif common in the promoter regions of light regulated genes like *LHY*. That the *TOC1* protein is known to associate with *PIF3* and that further *PIF3* binds to a G-Box on the *LHY* promoter sequence suggests that *PIF3* mediates the regulation of the *LHY* gene by the *TOC1*

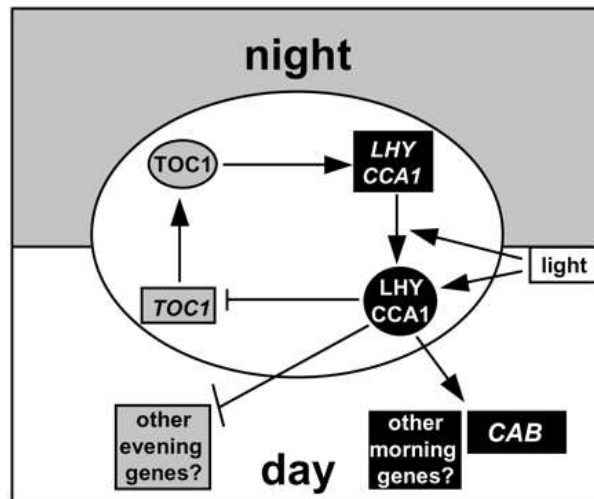


Figure 1.1: Working model of the central oscillator of the *Arabidopsis* clock, taken from [Alabadi 2001]. Circular and rectangular boxes indicate roughly when in the day the protein and the transcription level respectively peak during the day. Light activates the gene expression of *LHY* and *CCA1* at dawn. The proteins *LHY* and *CCA1* regulate the expression of *cab* and other morning genes. *LHY* and *CCA1* repress *TOC1* and maybe other evening genes. The expression levels of *LHY* and *CCA1* gradually fall over the day, so that the level of *TOC1* transcripts peaks in the evening corresponding to a minimum in *LHY* and *CCA1* transcripts. Through a yet unknown mechanism *TOC1* induces the expression of *LHY* and *CCA1*, which peaks again in the early morning.

protein.

PIF3 also interacts with the far red light absorbing (Pfr) form of phytochrome B (PHYB), a photoreceptor for red light responses. The red light triggers the transformation of phytochrome from its inactive red light absorbing (Pr) form to the active Pfr form. Also it has been shown that PIF3 is degraded by these phytochromes after their activation by red light [Park 2004]. In the light of these findings it was suggested that PIF3 mediates the entrainment of the clock by light [Martinez-Garcia 2000]. See figure 1.2 for a general model of the role of PIF3.

1.6 Previous work: analysis of the *LHY* promoter

To investigate how the *LHY* gene is regulated a cis analysis of the *LHY* promoter was carried out. The complete promoter region of *LHY* was linked to the firefly luciferase reporter gene and the heterologous terminator sequence of nopaline synthase (nos). See fig. 1.3 for a scheme of the promoter construct.

When the luciferase enzyme interacts with its substrate luciferin, it produces luminescence which can be measured by photon counting cameras. The strength of the luminescence

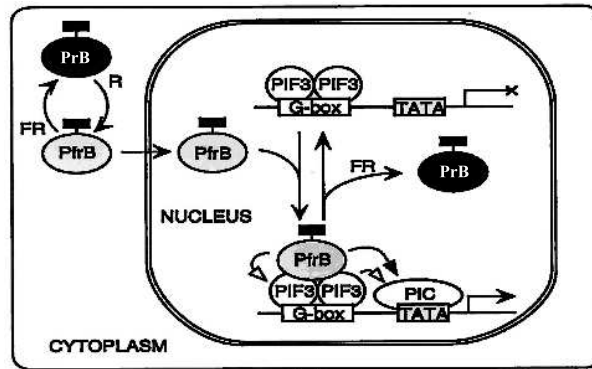


Figure 1.2: Model depicting the PIF3 mediated gene regulation by phytochrome B (PHYB). Red light induces PHYB to change from its inactive form Pr (PrB) into its active form Pfr (PfrB) and is translocated into the nucleus. There it binds to PIF3, which is presumptively bound to the G-box sequence on the promoter as a dimer. In the case of the *LHY* promoter this has the effect of inducing transcription, though details are unknown. Adapted from [Martinez-Garcia 2000].

can be assumed to be proportional to the amount of luciferase in the plant. So introduction of these constructs into *Arabidopsis* plants allows the measurement of activity of the *LHY* gene. Changes in the amount of luciferase mRNA are immediately reflected in the activity of luciferase, so measurement of the gene activity happens in real time. This promoter construct was able to reproduce the typical *LHY* expression pattern.

The overall promoter length was 929 bp, counting base pairs upstream of the starting point of transcription (position +1). Certain promoter regions important for regulation were identified by deletion analysis. It turned out that the upstream sequence starting from position -231 was enough to reproduce the normal *LHY* expression behavior. Cutting the promoter to a length of 128bp was still sufficient to produce rhythmic expression, though with a different wave form. The data is shown in fig. 1.5, a schematic of the promoter constructs is shown in fig. 1.4 a) and c).

In the respective promoter regions, two characteristic sequence patterns were identified. A CT rich region (CTR) stretches from position -38 to +74. Replacing this region with a 35S cauliflower mosaic virus weak promoter (fig. 1.4 d)) destroys circadian regulation of the -128 promoter construct in constant light (LL) (data not shown). The second region of interest is a G-box sequence (CACGTG) at -168. Surprisingly a mutation in the G-box (fig. 1.4 b)) does not produce the altered waveform of the -128 promoter construct, but destroys rhythmic behavior under constant light (fig 1.6).

This suggested that rhythmic transcription of *LHY* might stem from two different rhyth-

mic signals converging on the *LHY* promoter.

It is not yet known what factors act on these sites and how they influence the expression of *LHY*. There may also be more not yet identified binding sites on the *LHY* promoter. One question of interest is through which site TOC1 acts on the *LHY* promoter and whether PIF3 is involved in this process.

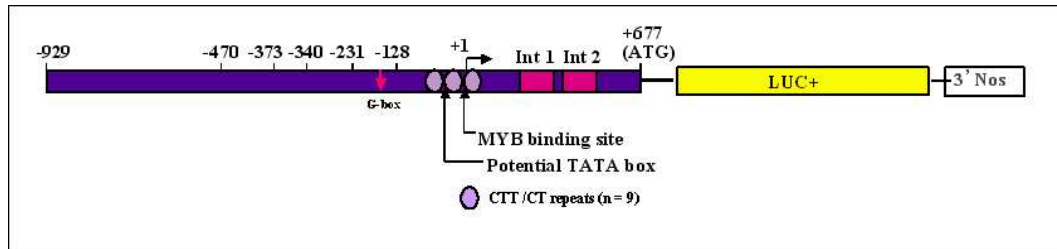


Figure 1.3: Diagram of the -929 *lhy:luc* promoter construct. Note the indicated GBOX and CT, CTT rich regions. The indicated bp position correspond to the cleaving site of a restriction enzyme.

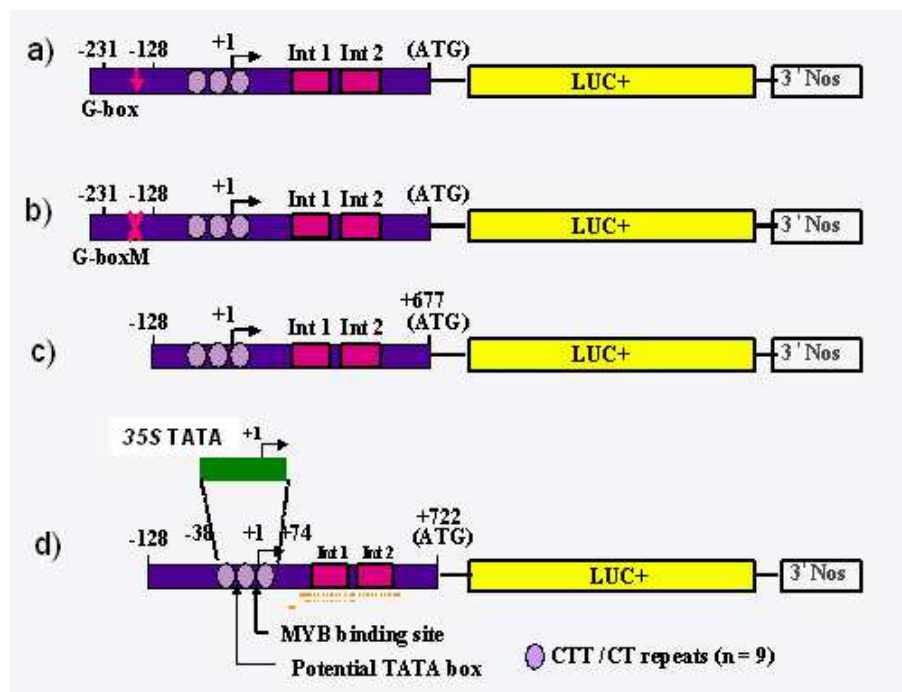


Figure 1.4: Promoter constructs from the *LHY* promoter. a),c) 5' deletion constructs; b),d) mutated constructs. a) -231 *LHY:luc*, b) -231 GBOXm *LHY:luc*, c) -128 *LHY:luc*, d) -128 CTRm *LHY:luc*.

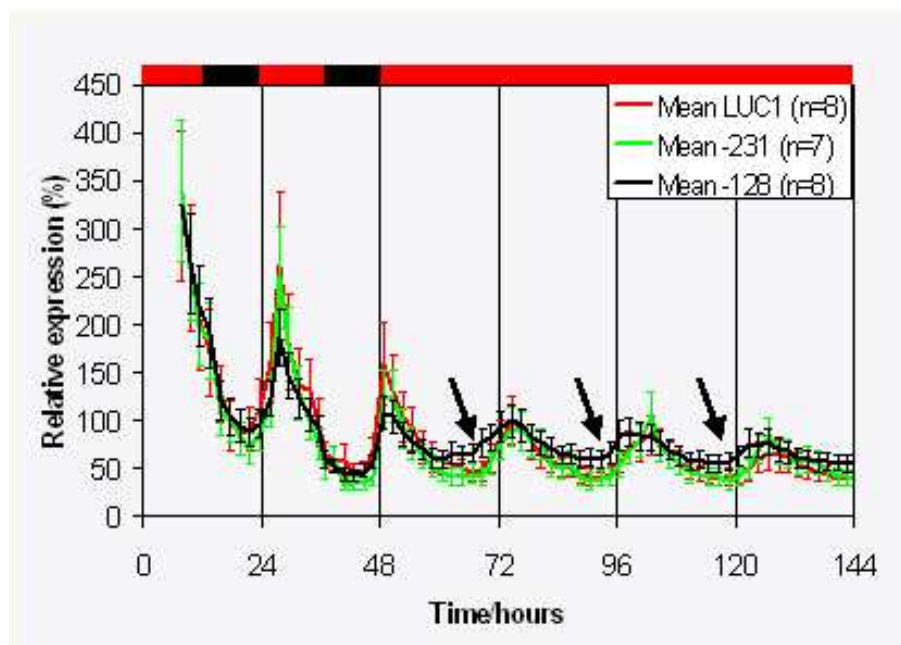


Figure 1.5: Imaging data for *LHY:luc* deletion constructs (-231 and -128) in comparison with the full length (-929, here denoted as LUC1) promoter. The red-black bar indicates light-dark periods. The y-axis is in photocounts per seedling. The arrows indicate where the curve corresponding to the -128 *LHY:luc* promoter rises significantly ahead of the other two. Plants were grown under 12h light - 12h dark (LD) cycles of white light for 7 days, then transferred to red light conditions for imaging. Light-dark cycles were maintained for the first 48h. Then plants were held in constant light.

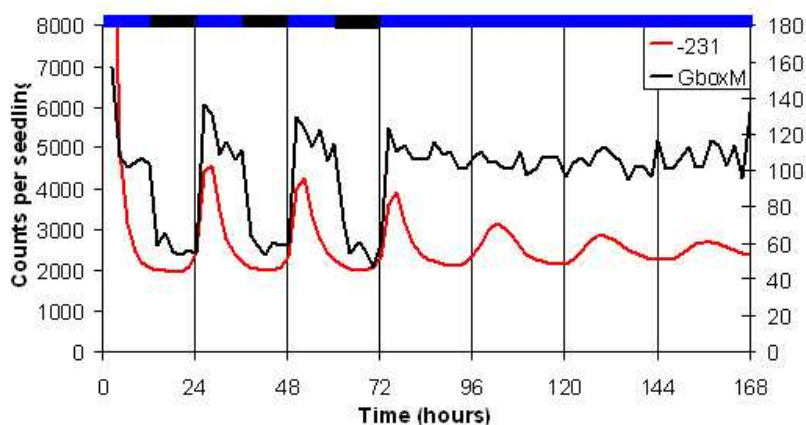


Figure 1.6: Imaging data comparing the -231 to the -231 GBOXm *LHY:luc* promoter construct. The blue-black bar indicates light-dark periods. The right hand y-scale corresponds to the mutated -231 GBOXm construct, the left hand scale to the -231 construct. It can be seen that for the mutated construct the expression pattern becomes arrhythmic in constant light. After 7 days of LD entrainment in white light, plants were transferred to blue light conditions for imaging. Light dark cycles were maintained for 72h. Then plants were held in constant light.

1.7 Aim of the project: the role of the PIF3 transcription factor

1.7.1 Overall aim of a work in progress

This thesis is a first part of a work in progress with the overall goal to elucidate how the *LHY* gene is regulated and what factor act through which site on the *LHY* promoter.

Up to now, two regulatory elements have been identified on the *LHY* promoter. This suggests that the promoter is acted upon by at least two different signals, which might stem from two independent feedback loops. We would like to associate the different candidate factors, which are known to act on the *LHY* promoter as mentioned in section 1.5, with a specific promoter site. The candidate have up to now characterised as follows [Salomé 2004]:

TOC1	Mutations in <i>TOC1</i> to not disrupt rhythms but lead to shortened periods, further the expression levels of <i>LHY</i> and <i>CCA1</i> are very low. The <i>TOC1</i> gene is under clock control and its transcription peaks in the evening.
GI	A nuclear protein with no obvious functional domains. <i>GI</i> mRNA and protein are clock regulated with peaks at dusk. The expression of <i>LHY</i> and <i>CCA1</i> is strongly reduced in <i>gi</i> mutants.
ELF3	Mutations in <i>ELF3</i> lead to loss of circadian rhythms. Plants overexpressing <i>ELF3</i> show a low sensitivity to light signals.
PIF3	A bHLH transcription factor that binds to TOC1 and also to the G-Box on the <i>LHY</i> promoter. <i>PIF3</i> itself is not transcribed rhythmically.

1.7.2 Monitoring *LHY* gene activity

Expression of *LHY*:luciferase constructs is measured in plants where a gene of a candidate regulator is either overexpressed or knocked out.

After synchronising and adapting the rhythm of the plants to a 12h-12h light-dark cycle (LD) (entrainment), they are released into constant light (LL) conditions. This allows to study how the clock reacts when it can no longer rely on light-dark cycles to drive its rhythms.

1.7.3 The role of the PIF3 transcription factor

The focus of this project is on the role of TOC1 and PIF3 in the regulation of the *LHY* promoter. It is known that PIF3 acts on the G-Box of that promoter and further that PIF3 binds to TOC1. Therefore we expect TOC1 to act through the G-Box.

To test this hypothesis, *Arabidopsis* seedlings carrying the -929 *LHY*:luc promoter construct and the *PIF3* loss of function mutant *pif3-1* were imaged for several days. Assuming the PIF3 mediated signal is the only one acting on the G-Box, the resulting data should be comparable to that of wild type plants carrying the -231 GBOXM *LHY*:luc promoter construct.

Chapter 2

Materials and Methods

2.1 Materials

The wild type ecotype Columbia (Col) of *Arabidopsis* was obtained from The Arabidopsis Stock Centre.

The 5' upstream sequences of the *lhy* gene were fused to the ATG start codon of a luciferase (*luc*) reporter construct and to terminator sequences from the nopaline synthase (*nos*) gene. The resulting construct referred to as -929 *lhy:luc* is depicted in figure 1.3 This construct was made by Jac-Yean Kim in the Carré laboratory.

The promoter construct was introduced into the Columbia ecotype of *Arabidopsis*. These wild type plants were crossed with other plants containing the *pif3-1* loss of function mutation to produce a -929 *lhy:luc* / *pif3-1* genotype. This was done by Mark Spensley in the Carré laboratory.

2.2 Methods

2.2.1 Plant growth for seed harvesting

Plants were grown on a 1:1 mixture of compost (B&Q plc, UK) and vermiculite (Silvaperl, UK). The soil was soaked with water containing 2g/litre of insecticide (Intercept, Scotts,UK) before the seedlings were transferred to soil. Plants were grown in the greenhouse under 16 hour photoperiods.

2.2.2 In-Vitro cultures

Seeds were sterilized with 50% (w/v) bleach (Fisher Scientific) and 0.01% (v/v) Tween 20 (Aldrich Chemical Co., UK) for 10 minutes and then rinsed with sterile distilled water four times. Seeds were sown on MS-agar medium [4.2g/litre of Murashige and Skoog powder (Sigma), 1% (w/v) agar, pH adjusted to 5.3-5.7 with 1M KOH, 3% (w/v) sucrose]. Seeds were stratified at 4°C for 4-5 days to synchronize germination.

2.2.3 Luminescence assay with photon-counting cameras

Two weeks before imaging, seeds were sterilized, sowed on MS medium containing 3% (w/v) sucrose and stratified as described above (see section 2.2.2). Plants were then grown for 7 days under $80 \mu\text{molm}^{-2}\text{s}^{-1}$ of white light in temperature-controlled incubators (Sanyo electronic Co., Japan). The photoperiod was 12h of light and 12h of darkness. Plants were kept at a constant temperature of 22°C. For imaging plants were transferred to red light conditions. During imaging light-dark cycles were maintained for 72h, then plants were kept in constant light. One day before, and then on the day when imaging started plants were pre-sprayed with the luciferase substrate luciferin (BIOSYNTH AG, Switzerland) at a 5mM concentration in 0.01% Triton X-100, to remove luciferase activity accumulated prior to the first luciferin treatment. Luminescence was imaged for 10 or 25 minutes every 2 hours for 5-6 days using a photon-counting camera (Hamamatsu). The luminescence levels were quantified from the images using MetamorphTM software (Universal Imaging).

Chapter 3

Results

In a comparative luminescence assay (see methods 2.2.3) 109 seedlings of wild type *Arabidopsis* and 52 seedlings carrying the *pif3-1* loss-of-function mutation were imaged to assess the activity of the *LHY* gene. All seedlings carried the -929 *LHY:LUC* promoter construct (fig. 1.3). Plants were entrained in white light for 7 days and then transferred to red light for imaging. Normal LD conditions were maintained for the first 72 hours. After that plants were kept in constant light.

The luminescence signal of the seedlings was averaged among the two sets of seedlings. Figure 3.1 shows the averaged luminescence intensities for the two sets seedling sets after background correction.

Images were taken every two hours for 158 hours, resulting in 80 images. While the image was taken (20min) the lights were turned off. The time required for each image leads to bound on the time resolution of the time series. If images were taken at smaller intervals, this would distort the desired constant light condition.

It must be pointed out, that noise levels in this assay are an issue. Even though imaging took place in a dark room shut off from any sources of light, the highly sensitive CCD (charge coupled device) camera picked up a varying amount of background signal, due to thermal noise and cosmic rays. Though thermal noise was is largely reduced by cooling the CCD camera to $-50^{\circ}C$, a substantial amount of noise still remains.

The first observation and the most important one is, that the signal pattern of the mutant plant is very close to that of the wild type plants. While the wave patterns are almost identical, we see a slight difference in signal strength. In particular in the first three days under the LD regime the overall signal of the mutant plant seems to be weaker compared to that of the wild

type. Though due to the high noise ratio it may be doubted that this observation is significant. If it were, it would be evidence for a decreased ability of the *pif3-1* mutants to react to the red light signal after the dark period.

Secondly, we note that the clock continues to function in both mutant and wild type plants. Rhythms with a steady period and wave form are persist throughout the imaging period.

Thirdly, we observe a change in amplitude, which concerns both wild type and mutant plants in a uniform manner. The first three peaks of both curves show a clear downward trend. This can be explained by the degradation of the luciferine, the substrate for luciferase, that was sprayed on the seedlings before imaging. As the application of luciferin was not renewed during imaging, it was degraded over time by the activity of luciferase. Also we expect a loss of amplitude after the plants are transferred from LD to LL conditions, as the plant now lacks information from the dark to light transition at dawn. Under LL conditions the amplitude is indeed lower and the peaks become wider at the base.

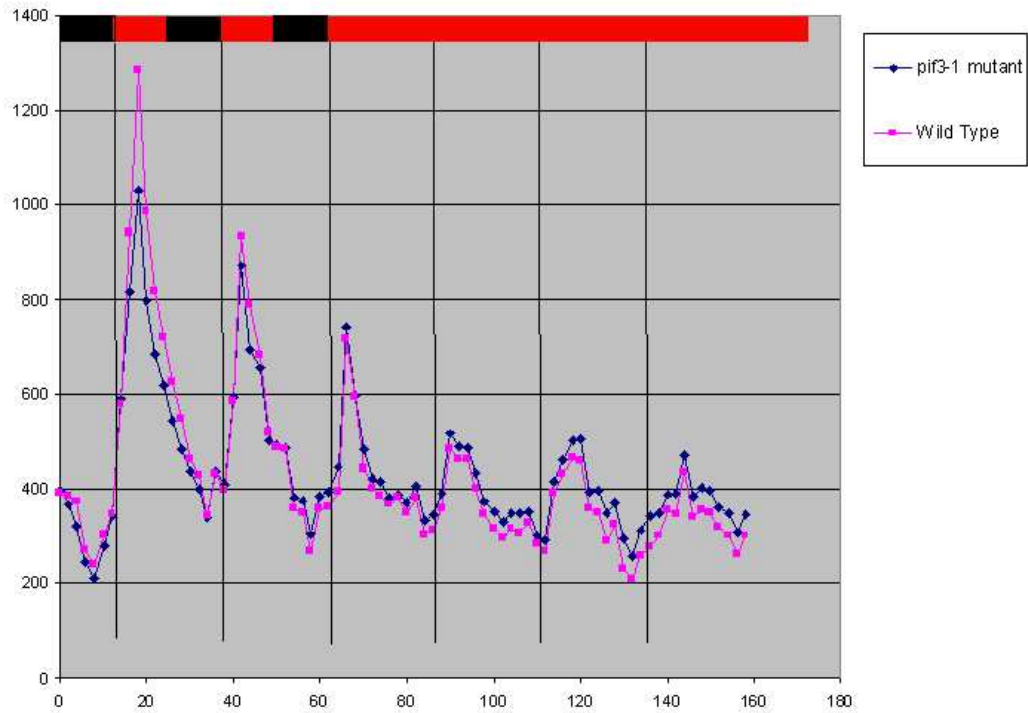


Figure 3.1: Time series of luminescence of a set 109 wild type seedlings and 52 seedlings carrying the *pif3-1* loss-of-function mutation. All seedlings carried the -929 *LHY:LUC* promoter construct. Red and black bars at the top indicate the periods of red light and darkness respectively. The signal strength for the two different sets of seedlings were averaged (meas of the standard error: 155 for wild type, 169 for the mutant) and the respective background signal subtracted. 80 images were taken with a time resolution of 2h. Plants were entrained in white light for 7 days and then transferred to red light for imaging. Normal LD conditions were maintained for the first 72 hours. After that plants were kept in constant light.

Chapter 4

Discussion of Results

Previous experiments suggested two different functions of PIF3 in the *Arabidopsis* clock. Its interaction with the red light activated PHYB makes it a candidate for mediating the resetting of the clock by light at dawn. Secondly, due its interaction with TOC1 and the G-Box on the *LHY* promoter it was suggested that mediates the induction of *LHY* transcription by TOC1 at dawn. Both processes are very likely to involve a number of different proteins and also show a certain degree of redundant components.

The mechanism of light resetting of not yet understood. Apart from the PHYB interacting PIF3 [Martinez-Garcia 2000], several other factors have been identified. PIL1, PIL2, PIL3, PIL4 and PIL6 (PHYTOCHROM-INTERACTING FACTOR 1,2,3,4 and 6) all belong to a certain subfamily, which also contains PIF3, of a large family (150 members) of bHLH transcription factors. All of them are known to interact with TOC1 and have been suggested to be involved in the circadian clock and in light-signal transduction pathways [Fujimori 2004]. Of these PIL4 and PIL6 are under clock control. Also this bHLH transcription family seems to exhibit a large degree of redundancy and combinatorial flexibility in forming active complexes [Toledo-Ortiz 2003]. This might explain why a single PIF3 knock out lacks a distinctive phenotype.

Not only does PIF3 seem to have very closely related factors, also TOC1, which is also known as ARABIDOPSIS PSEUDO-RESONSE REGULATOR-1 (APRR-1) has close relatives, which share a lot of its structural features: APRR1, APRR3, APRR5, APRR7 and APRR9 are all under clock control [Yamashino 2003].

Also in the induction of *LHY* transcription there most likely several factors involved. For in example in gi mutants *LHY* and *CCA1* expression is very low [Park 1999]. This could either

be explained by GI being involved in the expression of *TOC1*, then low TOC1 levels would lead to reduced levels in LHY and CCA1, or GI could interact with TOC1 to induce the expression of *LHY* and *CCA1*. Though in the latter case possibly further factor would be needed, as GI shows no DNA binding activity.

Further there is strong indication that there exist multiple oscillators in *Arabidopsis* which may or may not be involved the regulation of the *LHY* gene. It is known that the output genes *CAB* and *PHYB* show distinctively different rhythms under the same light conditions.

The results of this experiment yield no novel clues about the role of PIF3. They rather support earlier findings, that even though PIF3 is likely to be involved in light induction and *LHY* regulation, it is not essential for clock function. The *pif3-1* mutation was neither sufficient to disrupt circadian rhythms, nor could a significant change in the waveform be observed.

Chapter 5

Conclusion

5.1 *Arabidopsis* clock

The exact role of PIF3 still remains unclear, though a number of clues are available. The lack of a distinctive phenotype of a loss-of-function mutants highlights the complexity and high degree of redundancy of the *Arabidopsis* clock. So it might be useful to keep in mind the difficulties and pitfalls associated with investigating such an intricate and finely tuned mechanism such as a circadian clock as have been discussed in [Lazebnik 2002].

The suggested existence of several oscillators is an exciting notion, but does not makes things easier. It raises the additional question whether these oscillators function predominantly independently or if they are coupled. In the latter case one would like to know how and to what degree the interlocking of the oscillators takes place and what effect it has on observed rhythms. Since then any output gene under clock control could receive signals from either oscillator or signals from several oscillators could converge onto its promoter. These considerations are interesting in the light of the fact that the promoter of the *LHY* gene has at least two distinct active sites mediating rhythmic expression.

So it should be stressed that the simple model introduced earlier (fig. 1.1) can only be a first step or starting point from which the architecture of the clock can be explored. More sophisticated models have already been proposed to incorporate recent findings [Salomé 2004], though a complete picture is still missing.

5.2 Future work

Further experimental work focusing on the role of ELF3 and TOC1 in particular are underway and may yield more clues on their role in the regulation of the *LHY* gene and their connection to the identified promoter sites.

More important clues could be gained from biochemical binding studies to find out whether any of the candidate proteins mediate the formation of complexes, which can bind to the respective *LHY* promoter sites. If relevant complexes could be identified their structure itself might yield clues about *LHY* regulation.

It will further be important to identify the exact nature of how any protein induces the transcription of *LHY*. One possibility would be that the candidate proteins or the complexes they form mediate the release of transcriptional repressors. Alternatively the respective complexes would actively promote transcription by binding to DNA. An assay like Chromatin Immunoprecipitation, that could determine whether the respective complex is bound to the *LHY* promoter during expression would be able to distinguish between those alternatives.

The technique of the luminescence array has proven very useful, but the implementation so far suffers from several drawbacks. One important issue is the quantification of the data. The imaging signal is measured in photocounts capturing the luminescence of the luciferase-luciferin interaction. This makes it very difficult to deduce what the acquired data actually mean in terms of gene expression, as the exact relationship signal strength of the luminescence and actual mRNA or protein levels is unclear and so far not quantifiable.

Secondly, degradation of luciferin over time strongly influences the signal strength. This typically results in a downward trend of the amplitude, which has to be taken into account when information on differences in amplitude is wanted. In addition there is a certain variability in the amount of luciferin applied to the plants and also in the size and growth dynamics of the seedlings. This results in high variances in the data and makes it difficult to compare data between experiments.

Thirdly, as mentioned earlier, there is the thermal noise due to the high sensitivity of the camera. Even though cameras are cooled down to about -50°C , a certain level of background noise persists.

Some of these issues will be tackled in a follow up project. It will be looked at how

filtering techniques can be employed to computationally reduce the noise in the images. Certain artifacts of the imaging process like the decrease of the signal due to luciferin degradation can be removed by statistical smoothing and detrending techniques. This might facilitate the extraction the important features from data and also make data from different experiments easier to compare. For example these techniques will allow to get a objective estimate of the period length of the oscillations. Given data of reasonable quality it might also be possible to identify and distinguish between certain characteristic waveforms.

Information on the actual transcription rates might be gained by fitting the imaging data to a suitable model, which describes the assumed relationship of luminescence to *LHY* transcription rate, defined by a set of stochastic differential equations. Should these techniques be employed successfully they are sure to increase the quality of the information gained from the experiments.

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