The Function of the RNA-Binding Protein CHLAMY1 in the Circadian Clock and its Temperature Integration Process

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Content

Abbreviations

aph VIII	aminoglycoside phosphotransferase gene
APS	ammonium peroxide sulfate
AtGRP	*A. thaliana* GLYCINE-RICH RNA-BINDING PROTEIN
BMAL	BRAIN AND MUSCLE ARNT-LIKE
bHLH	basic Helix-Loop-Helix
bp	base pair
BSA	BOVINE SERUM ALBUMINE
CAB	CHLOROPHYLL A/B BINDING
CaMK	CALCIUM/CALMODULIN-DEPENDENT KINASE
cAMP	cyclic adenosine monophosphate
CBF	C-REPEAT BINDING FACTOR
CCA	CIRCADIAN CLOCK ASSOCIATED
ccgs	clock controlled genes
CCR	COLD AND CIRCADIAN REGULATED
CCTR	CIRCADIAN CONTROLLED TRANSLATIONAL REGULATOR
cDNA	complementary DNA
CELF	CUG-BP-ETR-3-like factors
cGMP	cyclic guanosine monophosphate
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHX	cycloheximide
CK	CASEIN KINASE
CKI-7	N-(2-Aminoethyl)-5-chloroisoquinoline-8-sulfonamide
CLK	CLOCK
COP	CHLAMYOPSIN
CRY	CRYPTOCHROME
CYC	CYCLE
Da	dalton
dATP	deoxyadenosine triphosphate
DBT	DOUBLETIME
dCTP	deoxycytidine triphosphate
DD	dark/dark = continuous darkness
dGTP	deoxyguanosine triphosphate
ddH_2O	double distilled water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	2'-desoxynucleosid-5'-triphosphate
DREB	DEHYDRATION RESPONSIVE ELEMENT BINDING
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
EE	evening element
ELF	EARLY FLOWERING
ERF	ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR
EST	expressed sequence tag
FASPS	familial advanced sleep phase syndrome
FRQ	FREQUENCY
g	gram
GC	guanine/cytosine
GI	GIGANTEA
GFP	GREEN FLUORESCENT PROTEIN
h	hour
HSP	HEAT SHOCK PROTEIN
ICE	INDUCER OF CBF/DREB EXPRESSION
ID	Identifizierungsnummer
JGI	Joint Genome Institute of the Department of Energy
k	kilo, thousand
KAI	"rotation" (Japanese)
KH	lysine homology domain
λ	lambda, wave length
l	liter

LB	Luria Bertani medium
LBP	LUCIFERIN BINDING PROTEIN
LD	light/darkness
LHC	LIGHT HARVESTING COMPLEX
LHY	LATE ELONGATED HYPOCOTYL
LL	light/light = continuous light
LUX	LUX ARRHYTHMO
m	milli, 1/1000
M	molar
MAPK	MITOGEN-ACTIVATED PROTEIN KINASE
µ	micro, 1/1000000
min	minute
mRNA	messenger RNA
mt	mating type
NII	NITRITE REDUCTASE
OA	okadaic acid
OD	optical density unit
ORF	open reading frame
p.a.	*pro analysi*
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PDA	Piperazindiacrylylacrylamid
PDP1ε	PAR DOMAIN PROTEIN 1ε
PER	PERIOD
PhIC	Phosphatase Inhibitor Complex
PIC	Proteinase Inhibitor Cocktail
PK	PROTEIN KINASE
PP	PROTEIN PHOSPHATASE
PRC	phase response curve
PRD-4	PERIOD-4
PRR	PSEUDO-RESPONSE REGULATOR
psa	photosystem I gene family
Q_{10}	temperature coefficient
QTL	quantitative trait loci
RBC	RUBISCO, RIBULOSOBISPHOSPHATE CARBOXYLASE
RLUC	RENILLA LUCIFERASE
RNAi	RNA interference
RNA	ribonucleic acid
roc	rhythm of chloroplast
ROS	reactive oxygen species
RRM	RNA recognition motif
RT	room temperature
s	second
SDS	sodium dodecylsulfate
Ser	serine
SGG	SHAGGY
sil	silenced
τ	tau, period
TA	Tris-acetate
TAP	Tris-acetate-phosphate
TE	Tris-EDTA
TEMED	N;N;N',N'-Tetramethylethylendiamin
Thr	threonine
TIM	TIMELESS
TOC	TIME OF CAB EXPRESSION
Tris	2-Amino-2-hydroxymethyl-propane-1,3-diol
TTP	thymidine triphosphate
UG	uracil/guanine
UTR	untranslated region
UV	ultraviolet
V	volt
v/v	volume/volume

VRI	VRILLE
VVD	VIVID
WC	WHITE COLLAR
WCC	WHITE COLLAR COMPLEX
WT	wild-type
w/v	weight/volume
WW	protein binding domain
Ø	diameter

1. Introduction

Our planet is known to continually move through the space. Within 24 h it performs a complete rotation on its axis, within 365,25 days it makes a circuit around the Sun, within about 225 - 250 million years together with the Sun revolves around the centre of our galaxy. Moreover, the Earth's only natural satellite, the Moon revolves around it within 29,54 days. All these processes cause periodical variations of the environmental conditions on Earth, such as light intensity and spectrum, temperature, air pressure, moisture and the magnetic field. During evolution most if not all organisms from bacteria to mammals adapted to these changes by developing biological rhythms.

Biological rhythms are periodically repeating fluctuations of behavioural and physiological aspects in living organisms. They can significantly vary in their period and thus can be divided into different categories. Infradian rhythms have a period longer than 24 h. Among them, there are low-frequency ones, such as circannual (about a year) or seasonal rhythms (Kortner and Geister, 2000; Danks, 2005), which are related to the rotation of Earth around the Sun, or even longer 11 years rhythms (Chizhevsky, 1936), which are related to periodical changes of the Sun activity. Another group of rhythms pertained to infradian are lunar rhythms (Zimecki, 2006) caused by the Moon rotation around the Earth that have a period of about 29 days. Also, there are some recent evidences that other kinds of infradian rhythms with a period of 7, 5 and 3,5 days do exist in some biological systems (Freeman, 1994; Díaz-Sandoval, 2008). Ultradian rhythms have a period shorter than 24 h. When the period is about 1 h, the rhythms are called circahoralian (Brodsky, 2006). But the period can be also shorter than an hour, as for example the second long heart beat rhythm (Silbernagel and Despopoulus, 1991), or longer than 1 h, as a 3 - 5 h rhythm of growth hormon production (Wagner et al., 1998).

The daily rotation of the Earth on its axis causes the appearance of 24 h long biological rhythms called diurnal. Some of them could simply occur in response to environmental factors, such as light or darkness. If the rhythm persists under constant conditions of light and temperature, it is called circadian. The term “circadian” taken from the Latin words *circa* "around" and *dies* "day", means "approximately a day". It was introduced first by Franz Halberg about 50 years ago (Halberg et al., 1959). The history of observations of such processes is much longer and started more then 250

years ago by the French astronomer Jean Jacques d'Ortous de Mairan, who noted that a heliotrope plant (probably *Mimosa*) sustained rhythms of leaf movement in continuous darkness (De Mairan, 1729). Unfortunately, de Mairan hesitated to conclude that heliotropes have internal clocks, because he could not rule out other possible factors, such as changes in temperature or magnetic forces. Nevertheless, de Mairan's experiment led further generations of researchers to perform circadian experiments with plants. In the next 150 years, Charles Darwin had been one of those, who pursued de Mairan's findings and reverted to the investigation of circadian clocks. Darwin designed an apparatus for measuring leaf movements, and quantified, graphed, and published his results in *The Power of Movement in Plants* (Darwin, 1880). Since that time scientists all over the world more and more often adverted to this topic until the *Cold Spring Harbor Symposia on Qualitative Biology* at 1960 that seems to define the moment when researchers from widely different fields discovered that they all were studying the same process. That meeting laid the groundwork for the field of chronobiology. In 1970s the *Society for Research in Biological Rhythms* was founded with C.S. Pittendrigh as leader. Members of this organizaion did basic research on all types of organisms, plants as well as animals.

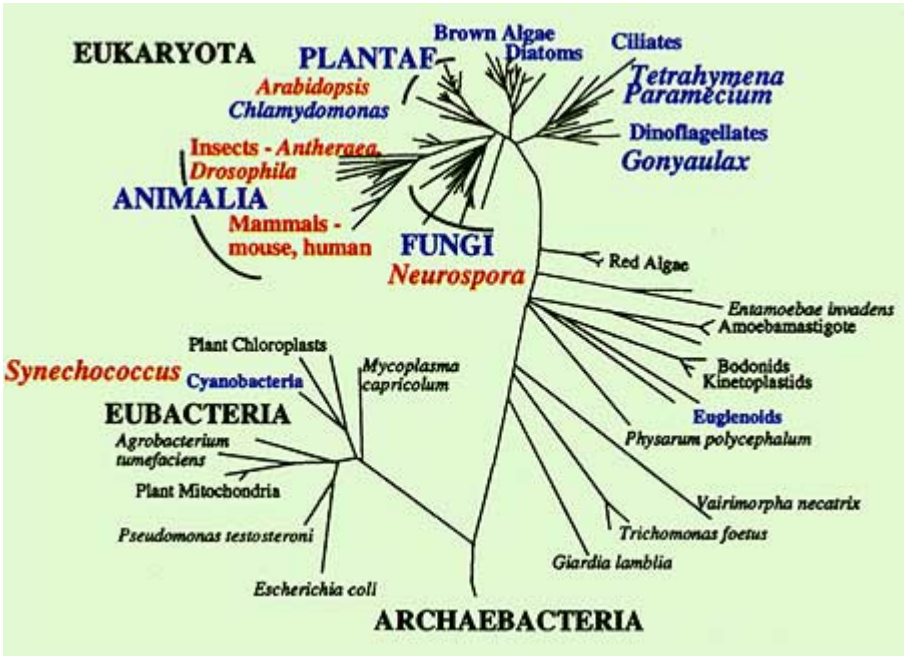


Figure 1. Circadian systems in the universal tree of life (reprinted from Dunlap, 1999)

The diagram shows the range of organisms, in which circadian systems have been investigated. The circadian rhythms of the groups in blue have been studied at a physiological level. The groups in red represent those, in which the clock mechanism has been studied at a genetic and molecular level.

As shown in Figure 1 circadian rhythms are found in many organisms from cyanobacteria to mammals. They involve multiple behavioural, physiological and metabolic processes as, for example, nitrogen fixation in cyanobacteria (Chen et al., 1998), bioluminescence of the marine dinoflagellate *Lingulodinium polyedrum* formerly called *Gonyaulax polyedra* (Hastings and Sweeney, 1957; Morse et al., 1990), photosynthesis and respiration in higher plants (McClung, 2006), spore formation in the mould *Neurospora crassa* (Dunlap and Loros, 2006), locomotor activity of fruit fly *Drosophila melanogaster* (Saunders, 1997), blood pressure or body temperature in mammals (Ishida et al., 1999).

1.1 Physiological properties of circadian rhythms

In spite of the high variety, circadian rhythms have a number of common physiological features. The first is the already mentioned above property to persist under constant conditions of light and temperature, maintaining the period of about 24 h that takes place due to an internal timekeeping mechanism called circadian clock. For example, mice synchronised by light/dark (LD) cycles continue to exhibit rhythmic locomotor activity in dark/dark (DD) conditions with a period close to 24 h for about 100 days (Pittendrigh and Daan, 1974).

In order to remain synchronized with the environment, circadian clocks are able to reset by exposure to an external stimulus, a property called entrainment (Johnson et al., 2003). The consequences of entrainment are that the period of the biological rhythm becomes equal on average to that of the entraining stimuli and that a stable phase relationship is established between the entraining and entrained oscillations. An environmental stimulus that can act to entrain circadian clocks is called a *Zeitgeber* ("time giver"). The most important *Zeitgeber* is the light/dark cycle (Aschoff, 1999). For example, everyone who once flied from Europe to America or the other way around has experienced it in form of a jet lag. But beside light/dark cycles, there are other non-photic *Zeitgebers*. Among them, cycles in temperature are known to efficiently synchronize the clock (Rensing and Ruoff, 2002). But also food availability or social cues can act as *Zeitgebers* (Roenneberg and Merrow, 1998; Harmer et al., 2001).

Another key feature of circadian rhythms, *phase response curve* (PRC), illustrates how the magnitude of phase shifts induced by single stimuli depends on

the time at which the stimuli is given. This means that a pulse of an external "time-giving" stimulus applied to an organism under free-running condition can shift the phase of the rhythm either delaying, advancing or having no effect on it. PRCs are specific for each particular organism (Hastings, 1960; Johnson,1999).

A critical property of circadian rhythms is temperature compensation that is the stability of the clock period over a wide range of physiological temperatures. Biochemical reactions have a Q_{10} of ~ 2, meaning that the rate of reaction doubles with a temperature increasing by 10°C. The period of circadian clocks is much less sensitive to temperature, having a Q_{10} from 0,8 to 1,3 (Hastings and Sweeney, 1957; Pittendrigh and Caldarola, 1973), thus they can be even overcompensated.

1.2 Principles of clock architecture

The simplest model shows a circadian system as a linear progression consisting of three main components: an input pathway linking the clock to the outside world, an endogenous core oscillator keeping the clock going and an output pathway regulating periodic functions inside the cell (Figure 2A). The core oscillator is based on clock genes that establish rhythms even in absence of external stimuli by controlling the expression of themselves and each other *via* molecular feedback loops.

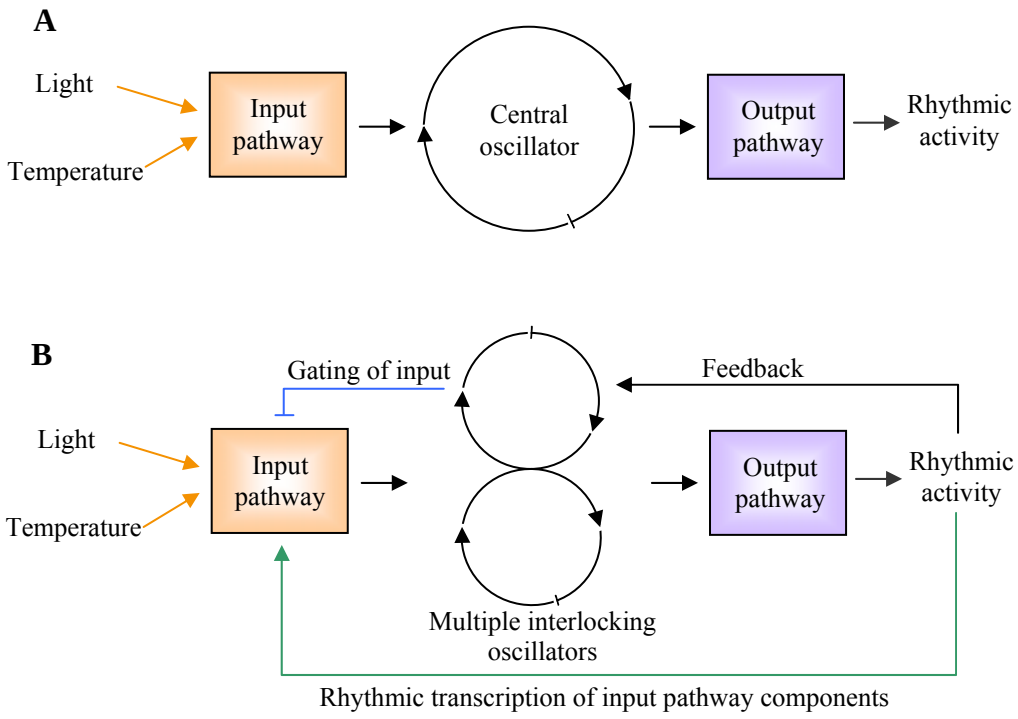


Figure 2. Schematic representation of circadian clock structures (reprinted from Gardner et al., 2006)

A. A model depicting division of the clock into an input pathway, a central oscillator and an output pathway. B. An elaborated description of the clock, consisting of multiple core oscillators, a gated input pathway and outputs, which feed back into the input and the central oscillator.

However, this model is an oversimplification, because many components of the input pathway are themselves outputs of the clock (Devlin and Kay, 2001) and rhythmic outputs may feed back to affect the functioning of the oscillator (Harmer et al., 2001). Moreover, in some organisms the main oscillator was shown to be composed of multiple interlocking loops (Figure 2B) (Paranjpe and Sharma, 2005; Gardner et al., 2006).

Such a complexity of circadian clocks has been suggested to be necessary to impart flexibility to circadian oscillators and also to provide stability and protection against casual perturbations.

1.3 The main components of the circadian clock in different organisms

1.3.1 The central oscillator

Within the last three decades, detailed studies of the circadian rhythms in different model organisms have shown that in spite of a wide variety of particular molecular components all so far known systems have similar regulatory mechanisms. As already mentioned before, an oscillator was found to be based on molecular feedback loops comprising of positive and negative regulatory elements. The positive elements such as the heterodimeric protein complex WHITE COLLAR-1/WHITE COLLAR-2 (WCC) in *N. crassa*, CLOCK/CYCLE (CLK/CYC) in *D. melanogaster* or CLOCK/BMAL in mammals (Wijnen and Young, 2006) are transcriptional activators carrying basic Helix-Loop-Helix (bHLH) DNA-binding motives that are able to enhance the transcription of clock genes by binding to specific sequences (e.g. E-box) in their promoter.

There are different types of genes that can be activated by positive elements. One group of them, so called clock-controlled genes (ccg) participate directly or indirectly in the output pathway. To another group belong genes, whose protein products play the role of negative elements in the feedback loops. These are proteins

such as KaiC in cyanobacteria (Williams, 2007), FREQUENCY (FRQ) in *N. crassa* (Brunner and Schafmeier, 2006), the PERIOD/TIMELESS (PER/TIM) protein complex in *D. melanogaster* (Hardin, 2005) or the PER1/2, CRYPTOCHROME 1 and 2 (CRY1, CRY2) in mammals (Ko and Takahashi, 2006). For this purpose, they undergo some posttranslation modifications in the cytoplasm, as, for example, heterodimerisation and/or phosphorylation by specific kinases allowing them to enter the nucleus, where they interact with the positive elements of the feedback loops changing their conformation and thus making their binding to their own promoterand activation of their own genes impossible.

As already mentioned before, the oscillator is often represented by several interconnecting molecular feedback loops. In some organisms like *N. crassa*, *D. melanogaster* or mouse positive elements from one loop can take part also in another one. For example, in the circadian system of *D. melanogaster* the heterodimeric complex CLK/CYC in addition to *per* and *tim* activates also genes of the basic-leucine zipper transcriptional factor PAR DOMAIN PROTEIN 1ε (PDP1ε) and the basic-leucine zipper transcriptional repressor VRILLE (VRI) that both regulate *clk* transcription, thus locking the second interconnected feedback loop (Hardin, 2005). In case of *N. crassa* the central transcriptional activator complex WCC not only forms a second feedback loop regulating expression of own transcriptional repressor VIVID (VVD) (Heintzen et al., 2001), but also seems to play an important role in the light input pathway.

The circadian system of *A. thaliana* was found to be slightly different. Its oscillator contains at least three interconnected feedback loops and each of them includes the MYB-like transcriptional factors LATE ELONGATED HYPOCOTYL (LHY) and CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) acting as negative elements. The role of positive elements in these loops play TIME OF CAB EXPRESSION 1 (TOC1, also known as PSEUDO-RESPONSE REGULATOR 1), and the other PRRs, and the MYB transcription factor LUX ARRHYTHMO (LUX) (Hazen et al., 2005). Moreover, some other elements like GIGANTEA (GI) (Mizoguchi et al., 2005) or EARLY FLOWERING 4 (ELF4) (Kikis et al., 2005) were found to be parts of that feedback loops, but, so far the exact role of all these components in the clock still stays unclear.

Kinases and phosphatases play a very important role in the functioning of circadian oscillators (Gallego and Virshup, 2007). Working as antagonists in all so

far known feedback loops, they regulate the phosphorylation level of some negative elements, thus controlling their stability and ability to establish protein-protein interactions. CASEIN KINASE 1 and 2 (CK1, CK2) as well as their antagonists PROTEIN PHOSPHATASE 1 and 2A (PP1, PP2A) that are highly conserved components of the circadian system were shown to play a role in *A. thaliana*, *N. crassa*, *D. melanogaster* and mammalian clocks. Thus, it was shown, for examle, that the balance between DOUBLETIME (DBT), the *D. melanogaster* homolog of CK1, and PP2A activities regulates the phosphorylation and stability of the central clock component CLK (Kim and Edery, 2006). Furthermore, some other kinases and phosphatases were found as, for example, the GLUCOSE SYNTHASE KINASE 3 homolog SHAGGY (SGG) phosphorylating TIM in *D. melanogaster* (Martinek et al., 2001) or CALCIUM/CALMODULIN-DEPENDENT PROTEIN KINASE 1 (CAMK1) and PERIOD-4 (PRD-4) phosphorylating FRQ in *N. crassa* (Yang et al., 2001; Pregueiro et al., 2006) as well as PROTEIN PHOSPHATASE 5 working in a complex with CRY and CK1 in mammals (Partch et al., 2006).

1.3.2 The input pathway

1.3.2.1 Temperature

Temperature can affect circadian system by different means. From one side, temperature changes can probably activate some common signal transduction pathways. For example, they were shown to affect membrane properties (Yatvin and Cramp, 1993), ion (particularly calcium) levels, and second messenger concentrations (cAMP and cGMP) (Rensing and Ruoff, 2002). At the same time, temperature signals may have a direct influence on clock mechanism *via* acceleration or slowing of enzyme-regulated processes involved in transcription, processing of gene products (Diernfellner et al., 2005), translation as well as posttranslational modifications of proteins (e.g., phosphorylation/ dephosphorylation).

In the past years, first molecular components were identified from some model organisms that are involved in the mechanism of temperature compensation and/or entrainment by temperature cycles. In *N. crassa*, it was shown that the expression of one of the key components of its circadian system, FRQ, is thermally regulated at the translational level and in addition involves thermosensitive splicing (Liu et al., 1997; Diernfellner et al., 2005; Dunlap, 2006). Further, the PAS/LOV

protein VVD controls temperature compensation of circadian clock phase (Hunt et al., 2007). In *A. thaliana*, quantitative trait loci (QTL) were mapped for period and amplitude of leaf movement. All of the period QTL are temperature specific, suggesting that they may be involved in temperature compensation. Already mentioned before GI, a protein involved in flowering, and the F-box protein ZEITLUPE were identified as strong candidates for two of the QTL (Edwards et al., 2005). A later approach showed that several key clock components of *A. thaliana* are involved in temperature sensing including GI, TOC1, CCA1 and LHY (Gould et al., 2006). In cyanobacteria, it was found that the central clock components KAI A, B and C are not only able to trigger circadian phosphorylation of KAI C *in vitro* in the presence of adenosine triphosphate, but that this rhythm is also temperature compensated (Nakajima et al., 2005). A model for this *in vitro* process has been developed recently (Mori et al., 2007).

1.3.2.2 Light

The mechanisms of the clock entrainment by light/dark cycle are investigated more profound at the molecular level. Thereby, photoreceptors play an important role. Some of them as CRYs in *D. melanogaster* (Ceriani et al., 1999) and *Arabidopsis thaliana* (Lin, 2002), WC-1 in *N. crassa* (Lee at al., 2003), PHOTOTROPINs in plants (Kasahara et al., 2002) or MELANOPSIN in mammals (Hanifin and Brainard, 2007) are blue light photoreceptors, while PHYTOCHROMEs acting in plants are red/far-red reversible photoreceptors (Nagy and Schafer, 2002). The further way of the light signal transduction varies in different organisms. It can lead to transcriptional activation, like in case of *N. crassa* (Dunlap and Loros, 2004), or stimulate degradation of some central oscillator components, like in case of *D. melanogaster* (Ceriani et al., 1999). Moreover, even the presense of a photoreceptor is not obligatory. For example, up to now no "true" photoreceptor involved in the circadian system has been identified in cyanobacteria, where the clock is found to be sensitive to changes in cellular redox state caused by photosynthesis (Ivleva et al., 2005).

1.3.3. The output pathway

Circadian output is often regulated at the transcriptional level involving so called clock-controlled genes (*ccg*s). It is known, for example, that clock-controlled genes

can represent from 1 - 5% like in D. *melanogaster*, but can comprise close to 100% of the genome like in the case of cyanobacteria (Allada et al., 2001).

One example, where the regulation occurs at the transcriptional level in several so far studied plants, are proteins of the LIGHT-HARVESTING COMPLEX (*lhc*), also called *cab* (CHLOROPHYLL A/B BINDING protein; reviewed in Piechulla, 1999). A motif called evening element (EE) was identified in the promotor of the *lhcb1* (*light harvesting chlorophyll a/b binding protein 1*) gene of *A. thaliana* that is recognized by the circadian transcription faktor CCA1 (Wang and Tobin, 1998). The circadian controlled binding of CCA1 to the EE in the *lhcb1* promotor is responsible for the rhythmical expression of this gene. Such EE motifs were identified in 31 genes of *A. thaliana* that are all known to be rhythmically expressed (Harmer et al., 2000).

But regulation of circadian output can also occur at the translational level. For example, in *D. melanogaster* an RNA-binding protein LARK was identified that is shown to be associated with many different RNAs in the central nervous system and that has an important role in the circadian control of population eclosion (Huang et al., 2007). Two closely related clock-regulated RNA-binding proteins, AtGRP7/CCR2 and AtGRP8/CCR1, (*A. thaliana* GLYCINE-RICH RNA-BINDING PROTEIN/COLD AND CIRCADIAN REGULATED) were shown to mediate posttranscriptional control in the output *A. thaliana* clock (reviewed in Schöning and Staiger, 2005). Another RNA-binding protein CIRCADIAN CONTROLLED TRANSLATIONAL REGULATOR (CCTR) was found that is correlated with the rhythmical expression of LUCIFERIN-BINDING PROTEIN in the dinoflagellate *Lingulodinium polyedrum* (Mittag et al., 1994).

1.4 The circadian system of *Chlamydomonas reinhardtii*

1.4.1 *C. reinhardtii*, a eukaryotic model organism

C. reinhardtii is a eukaryotic unicellular biflagellate green alga that lives commonly in fresh water and soil. It has a number of qualities that make it a very useful and popular model organism to study various aspects of cellular and molecular biology (Harris, 2001). *C. reinhardtii* can be quickly grown both in liquid and on solid medium. Being a photosynthetic organism it can live autotrophically, but at the same time it can live without light in heterotrophic conditions using acetate as carbon

source. Thus, mutants with serious damages of photosynthetic apparatus are viable, which gives big advantages for the research of the biogenesis and function of the chloroplast and of its photosynthetic machinery (Rochaix, 2004). The presence of the flagella and the basal body and the investigation of their protein composition and function with respect to their relevance for human diseases are also of high interest (Rosenbaum and Witman, 2002; Snell et al., 2004). Such an organelle as the eyespot allows using *C. reinhardtii* for the research of a primitive visiual system to study the mechanism(s) of the light signalling pathway (Kateriya et al., 2004).

A number of valuable molecular tools have been developed for *C. reinhardtii*. Numerous mutants of this alga are available. *C. reinhardtii* was the first photosynthetic eukaryote, which allowed stable transformation of the nuclear (Debuchy et al. 1989; Kindle et al. 1989), chloroplast (Boynton et al. 1988), and mitochondrial (Randolph-Anderson et al. 1993) genomes. For efficient and stable nuclear transformation, a variety of techniques have emerged, including particle gun bombardment (Debuchy et al., 1989; Kindle et al., 1989), agitation with glass beads (Kindle, 1990), or electroporation (Shimogawara et al., 1998). Moreover, some other tools were developed including reporter genes with optimized codon usage such as *c-gfp* (green fluorescent protein) (Fuhrmann et al., 1999) and *c-rluc* (Renilla luciferase) (Fuhrmann et al., 2004), dominant antibiotic selection markers of sulfometuron methyl, paromomycin and cycloheximide resistance (Kovar et al., 2002; Sizova et al. 2001; Stevens et al. 1996), and strong promoter systems like the *hsp70a/rbcS2* tandem promoter (Schroda et al., 2000; Sizova et al, 2001) or the *psaD* promoter (Fischer and Rochaix, 2001). The application of RNAi (RNA interference) has been established as a method for controlled reduction of protein expression (Fuhrmann et al., 2001; Schroda, 2006).

A big step forward for the use of *C. reinhardtii* as a model system was the sequencing of its entire nuclear (Merchant et al., 2007), chloroplast (Maul et al., 2002) and mitochondrial (Gray and Boer, 1988) genomes and the generation of a comprehensive EST library that currently comprises more than 200,000 ESTs (Asamizu et al. 1999, 2000; Shrager et al., 2003).

1.4.2 The circadian clock of *C. reinhardtii*

C. reinhardtii exhibits a number of already well characterized circadian rhythms. The first one described more then 35 years ago by Victor Bruce (Bruce, 1970) is the

rhythm of phototaxis, which is the active movement of the alga towards a light source. The circadian rhythm of phototaxis in wild type reaches its maximum during subjective day and its minimum during subjective night. The phototactic rhythm can be automatically measured (Mergenhagen, 1984; Johnson et al., 1992). The persistence of the phototactic rhythm under constant conditions was even proven in outer space, where the rhythm continued for at least 6 days in conditions of microgravity (Mergenhagen and Mergenhagen, 1987). Several mutants of *C. reinhardtii* were isolated, which have either a shorter (Bruce, 1972) or a longer period (Bruce, 1974) of the phototactic rhythm. They have been named *per*, but, although they have the same nomenclature as the *per* mutants in *Drosophila* and mammals, it is rather unlikely that the same proteins are defect since there is no evidence for a PER homologue in *C. reinhardtii* (Mittag et al., 2005)

Another circadian rhythm exhibited by *C. reinhardtii* is the rhythm of chemotaxis to nutrients that was studied by using the chemoattractant ammonium (Byrne et al., 1992). The cells were shown to have maximal activity of swimming towards the nitrogen source in the middle of the night phase.

There are also other circadian rhythms in *C. reinhardii* that peak during the night phase. One of them is the rhythm of cell stickiness to glass (Straley and Bruce, 1979) that reflects alteration to the cell surface. Another rhythm is the cell division cycle, which was studied first by Bruce (1970) and later by Goto and Johnson (1995). Recently, Nikaido and Johnson (2000) discovered an additional circadian rhythm in *C. reinhardtii*, the rhythm of its sensitivity to UV radiation. It was found that survival of cultures exposed to UV light reaches a minimum at the day-night switch.

1.4.3 Molecular components of the circadian system of *C. reinhardtii*

Although the molecular mechanisms underlying circadian clocks in various organisms are to a great extend conserved, those regulating the circadian clocks in *C. reinhardtii* seem to be different. Extensive search in the *C. reinhardtii* genome database (JGI) for potential homologues of genes that are known to encode components of the circadian clock in other organisms revealed that there are no obvious homologues, except for the kinases (CK1, CK2, SGG) and phosphatases (PP1, PP2A) (Mittag et al., 2005). At the same time, there were two CRY-like proteins found in *C. reinhardtii* (Mittag et al., 2005). One of them was shown to be

more closely related to the CRY of *A. thaliana* where it is known to act in the input pathway as a photoreceptor, while the other is more related to the mammalian kind of CRY, which acts as a component of the main oscillator. Therefore, it was hypothesized that *C. reinhardtii* may have an "amalgam" clock (Breton and Kay, 2006).

Very recently, an insertional mutagenesis approach highlighted several components of the plastid-driven circadian clock of *C. reinhardtii* (Matsuo et al., 2008).

1.4.4 The circadian RNA-binding protein CHLAMY1 of *C. reinhardtii*

Several years ago, a heteromeric RNA-binding protein entitled CHLAMY1 was discovered in the green alga *C. reinhardtii* that seems to be involved in its circadian system. It was found as a functional homolog of the CIRCADIAN CONTROLLED TRANSLATIONAL REGULATOR (CCTR) from the marine dinoflagellate *Lingulodinium polyedrum*. The CCTR is known to recognize specifically and bind in a circadian manner to a 22-nt sequence carrying seven UG-repeats situated in the 3'-untranslated region (3'-UTR) of luciferin binding protein mRNA (*lbp*) whose protein is a main component of the bioluminescence controlling part of the output pathway in circadian system of *L. polyedrum*. It was found that the level of LBP is circadian controlled being 10-fold higher during the night- comparing to the day-phase, but the level of *lbp* mRNA appeared to be constant over the circadian cycle. Therefore, it was suggested that the circadian expression of LBP is regulated at the translational level (Morse et al., 1989), which was the first example of such a type of regulation. Later it was also found that the oscillations of LBP are in opposite to the binding activity of the CCTR to *lbp* mRNA suggesting that the CCTR functions as a translational repressor (Mittag et al., 1994).

In the *C. reinhardtii* circadian system, a CCTR homolog CHLAMY1 was found. It also recognizes specifically and binds in the circadian manner to a sequence of at least seven UG tandem repeats (Mittag, 1996). But unlike the CCTR, the maximal binding activity of CHLAMY1 occurs at the beginning of the night-phase. Several mRNAs have been identified bearing UG tandem repeats from 7 to 16 in their 3'-UTRs, to which CHLAMY1 can specifically bind (Waltenberger et al., 2001). It was shown recently that the presence of such a UG-repeat in the 3'-UTR of an mRNA can not only mediate circadian rhythmicity of its translation, but it can

also determine its acrophase (Kiaulehn et al, 2007). Biochemical purification and characterization of CHLAMY1 revealed a novel type of RNA-binding protein that is composed of two different subunits (Zhao et al., 2004), whose interaction are necessary for the RNA binding (Figure 3). One of them named C1 (Figure 3A) has three lysine homology domains and a protein-protein interaction domain (WW). The other one called C3 (Figure 3B) belongs to the proteins of the CELF (CUG-BP-ETR-3-like factors) family and bears three RNA recognition motif domains.

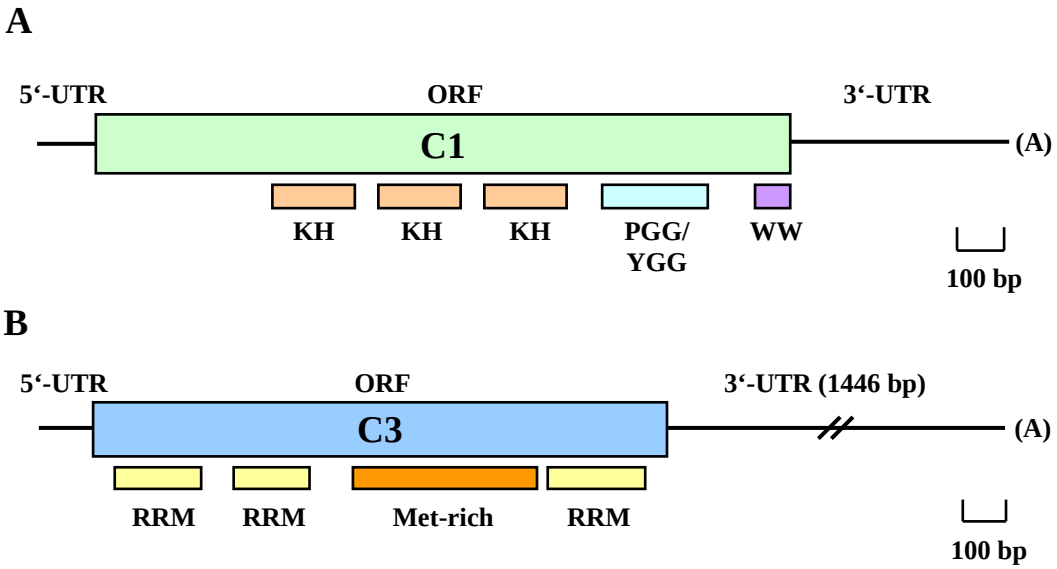


Figure 3. Domain architecture of the subunits of CHLAMY1 encoded by the *c1* and *c3* cDNA, respectively (reprinted from Zhao et al., 2004)

Positions of the open reading frames (ORF), 5'- and 3'-untranslated regions (UTR) and the poly-A tail (A) are shown. A. cDNA encoding the C1 subunit: KH - lysin homology domain, PGG/YGG - region which is rich in glycine, proline, and tyrosine, WW - protein-protein interacting domain,. B. cDNA encoding the C3 subunit: RRM - RNA recognition motive, Met-rich - region, which is rich in methionine and glycine.

The subunits C1 and C3 have theoretical molecular masses of 45 and 52 kDa, respectively, and are present in nearly equal amounts during the circadian cycle. It was shown that at the beginning of the subjective night both subunits can be found in protein complexes of 100 to 160 kDa (Zhao et al., 2004). However, during subjective day when binding activity of CHLAMY1 is low, the C1 subunit is in addition present in a high-molecular-mass protein complex of more than 680 kDa. These data indicate posttranslational control of the circadian binding activity of CHLAMY1.

1.5 Main aims of the PhD work

One major goal of this thesis was to examine the function of the C1 subunit of CHLAMY1 in the circadian system of *C. reinhardtii*. For this purpose, it was planned to silence the expression of this subunit using an RNAi approach. Thereby, it was of interest to study if the silencing of the C1 subunit could have any effect on the phase, period or amplitude of a representative circadian rhythm in *C. reinhardtii*. As described before, CHLAMY1 is known to control some mRNAs like, for example, *nii1* by binding to the $(UG)_{\geq 7}$-repeats in its 3'-UTR. Thus, measurement of NII activity was planned in a silenced C1 strain to study the influence of C1 on its direct output target. At the same time, a suitable physiological rhythm that can be well analyzed is the already mentioned phototactic movement of *C. reinhardtii* that can be automatically measured over a period of seven days. Analysis of the effects on the NII activity and phototaxis rhythms in C1 silenced transgenic strains was expected to enhance the understanding of the role of CHLAMY1 in the circadian system of *C. reinhardtii*.

In all so far studied model organisms the molecular components are involved in a complicate network of interconnected interactions and feedback loops that drive the clock oscillation. Very often changes in the expression of any clock component change the expression of other components, with regard to their phase or amplitude of expression (Lee et al., 2000). Therefore, it was also of interest to analyze if silencing of C1 could affect also the expression of the C3 subunit in parallel.

Another major goal of my work was to investigate the mechanism of temperature integration, a prerequisite for entrainment by temperature cycles and temperature compensation, by analysing the potential involvement of the RNA-binding protein complex CHLAMY1 in those processes. Therefore, it was planned to check the expression level of both C1 and C3 at different temperatures (18 - 28°C), which are within the physiological range for *C. reinhardtii*. If the expression of any of the two subunits would be temperature dependent, it was planned to characterize the underlying molecular mechanism(s). Moreover, a comparable approach in the long period clock mutant *per1* was planned.

2. Materials and Methods

2.1 Materials

2.1.1 Devices

Device description	Producer
Autoclave Varioklav®	H+P Labortechnik, Munich
Thermostat/Incubator (KB-53)	WTB binder, Tuttlingen
Electroblot PerfectBlue™, Semi-Dry-Electroblotter SEDEC M	PEQLAB Biotechnologie GmbH, Erlangen
Horizontal electrophoresis unit Hoefer HE 33, Mini horizontal submarine unit	Amersham Pharmacia Biotech., Freiburg
Vertical electrophoresis unit 1: Maxi-Gel-System, 010- 400, 17x18 cm	Biometra, Göttingen
Vertical electrophoresis unit 2: Ettan Dalt Six	GE Healthcare, Munich
Isoelectric Focusing System, Ettan IPGphor	GE Healthcare, Munich
-86°C Ultra-Low Temperature Upright Freezer	Thermo Forma, Marietta, OH USA
Cooling centrifuge Hermle Z323K	Hermle, Wehingen
Minishaker 1: Vortex Mixer VM-300	NeoLab, Heidelberg
Minishaker 2: Vibrofix VF 1	Electronic Bachhofer, Reutlingen
Minishaker 3: Vortex-Genie®2	Scintific Industries, New York, USA
Rotation shaker Roto-Shake Genie™	Scientific Industries, Inc., USA
Pocking platform shaker GFL 3015	Burgwedel
PCR machine/ Thermocycler PTC-100™	MJ Research Inc./Biozym, Oldendorf
Photometer Helios beta	Thermo Elektron Corporation, San Jose, CA USA
Table centrifuge: Eppendorf 5415D	Eppendorf, Munich
Ultracentrifuge Avanti ™ J-30 I	Beckmann Instruments Inc., Palo Alto, CA USA
Vacuum concentrator Savant Speed vac® Plus SC 110	Savant, Holbrook, NY
Freezing container „Mr. Frosty-Box“ Cryo, 1C; Nalgene	Nunc GmbH & Co. KG, Wiesbaden

2.1.2 Chemicals

All used chemicals had p.A. quality and were purchased in the following companies: Roth (Karlsruhe), Sigma (Steinheim), Merck (Darmstadt) und AppliChem (Darmstadt).

2.1.3 Enzymes

The enzymes were used with specific buffer systems supplied by the corresponding companies.

- **Restriction enzymes** were bought from New England Bioscience (Frankfurt), Roche (Mannheim) and Jena Bioscience (Jena).
- **Klenow (large) fragment of DNA Polymerase I** (5 unit/µl, NEB, Frankfurt).
- **T4 Ligase** (1 unit/µl and 5 units/µl; Roche, Mannheim).
- **RNase A** (0,9 units/µl; Roth, Karlsruhe): 100 mg were dissolved in 10 ml 50 mM sodium acetate buffer (pH 4,8). For inactivation of DNAse activity the solution was cooked for 15 min in water bath and then immediately transferred on ice.
- **Protease Inhibitor (PIC)** (Complete™ Protease Inhibitor Cocktail Tablets; Roche, Mannheim): A tablet was dissolved in 2 ml extraction buffer 1 or 2 for C. reinhardtii. This stock solution is 25x concentrated.
- **Phosphatase Inhibitor Cocktail 1 (PhIC 1)** (DMSO solution) and **Phosphatase Inhibitor Cocktail 2 (PhIC 2)** (aqueous solution), (Sigma, Taufkirchen).
- **Lambda Protein Phosphatase** (400 units/µl, NEB, Frankfurt).

2.1.4 Kits and consumables

Product	Producer
„GenElute™ Gel Extraction Kit“	Sigma (Taufkirchen)
„QIAquick® PCR Purification Kit“	Qiagen (Hilden)
"High Pure Plasmid Isolation System"	Roche (Mannheim)

Glass beads for the transformation of *C. reinhardtii* (Ø 0,45–0,5mm)	Braun (Melsungen)
Glass beads for the protein crude extract preparation (Ø 0,25–0,3 mm)	Sartorius AG (Göttingen)
„PCR Cloning Kit (Blunt end)“	Roche (Mannheim)
„GC-RICH PCR System“	Roche (Mannheim)
4 x Roti-Load	Roth (Karlsruhe)
Gel blotting paper (460 x 570 mm)	Roth (Karlsruhe)
Cellulose-acetate filter Sartorius SM 11200	Sartorius (Göttingen)
Bio-Rad Protein Assay	Bio-Rad (Munich)
Protran® BA 85 Nitrocellulose	Schleicher & Schüll Bioscience GmbH (Dassel)
Roti®-PVDF Transfermembranen	Roth (Karlsruhe)
Fuji Medical X-Ray Film 100 NiF 13x18 cm, Super RX	Fujifilm, Fischer-Sehne Medica IMA (Nordhausen)
Immobiline DryStrip pH 3-10, 18 cm	GE Healthcare (Munich)
MemCode™ Reversible Protein Stain Kit For Nitrocellulose Membrane	Pierce (Rockford, USA)
Okadaic acid	Calbiochem (Darmstadt)
CKI-7 (N-(2-Aminoethyl)-5-chloroisoquinoline-8-sulfonamide)	Toronto Research Chemicals Inc. (USA)
Cycloheximide	Sigma-Aldrich (Taufkirchen)

2.1.5 *C. reinhardtii* strains

Strain	Reference	Genotype	Cultivation medium
SAG73.72	Collection of alga cultures at the University of Göttingen	*wt, mt+*	TAP
CLS31-8	Johnson und Suzuki (Nashville, USA), personal communications	*arg7-, mt-*	TAP^{+}-Medium; 50 mg/ml Arginin
CLS31-10	Johnson und Suzuki (Nashville, USA), personal communications	*arg7-, mt+*	TAP^{+}-Medium; 50 mg/ml Arginin
C1117	*Chlamydomonas* center at Duke University, (Bruce et al., 1972)	*per-1, mt+*	TAP

2.1.6 *Escherichia coli* strains

Strain	Reference	Genotype	Medium
XL1-Blue	Bullock et al., 1987	*recA1,endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac [F'proAB lacIqZ ΔM15 Tn10 (Tet r)]*	LB-Medium

2.1.7 Vectors

Vector	Reference
pBluescript KS+	Stratagene (La Jolla, USA)
pCAP^{S}	Roche (Mannheim)

2.1.8 Recombined plasmids

In this work the following plasmids were used:

pCS30

The plasmid contains *c1* cDNA from position 689 to 2734 (EST: AY505473; Zhao et al., 2004).

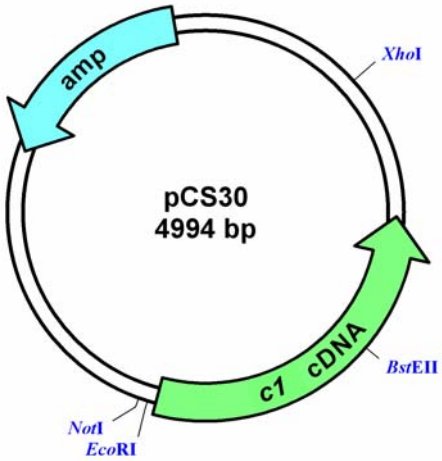


Figure 4. Restriction map of pCS30

c1 cDNA is depicted as the green arrow, the gene of ampicillin resistance (amp) as the cyan arrow.

pGG1

The plasmid contains *ck1* cDNA from position 698 to 1533 (EST: AV390567, AB091079; Gessner and Mittag, personal communication, Schmidt et al., 2006).

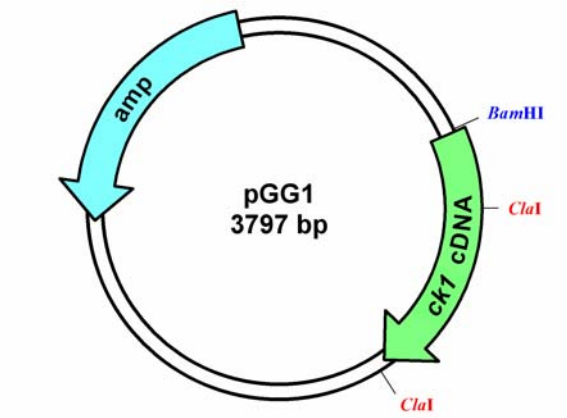


Figure 5. Restriction map of pGG1

ck1 cDNA is depicted as the green arrow, the gene of ampicillin resistance (amp) as the cyan arrow.

pSI103

The plasmid contains the paromomycin resistance gene *aphVIII* under control of the strong *hsp70a/rbcs2* tandem promoter (Sizova et al., 2001). The tandem promoter consists of the *hsp70a* promoter and the *rbcs2* promoter along with the first intron of the *rbcs2* gene, containing an enhancer. Downstream of the *aphVIII* gene the *rbcs2* 3'-UTR is situated.

Figure 6. Restriction map of pSI103

The *aphVIII* gene for paromomycin resistance is depicted as the blue box, the *hsp70a* promoter as the green box, the *rbcs2* promoter with the first intron of the *rbcs2* gene as the beige box. The *rbcs2* 3'-UTR is shown as grey arrow. The gene of ampicillin resistance (amp) is presented as cyan arrow.

2.1.9 Oligonucleotides

Name	Sequence
OMM232	5'-AGGTATGCGTGCACAAAGTC-3'
OMM249	5'-ATGAGCACCGTCTTGAGACTG-3'
OMM267	5'-TGTGAGTTGGTGCGAATGAG-3'
OMM269	5'-AGCGGATGAGGTCCTCAATG-3'
S1	5'-GCTATGACTCACCCGGACGG-3'
S2	5'-CTTAAGAATTCGTCCCACGG-3'

2.1.10 Molecular standards

2.1.10.1 DNA standards

Ladder 1: Lambda DNS/ *BstE*II-Digest, NEB (Frankfurt, Germany)

Fragments: 8454 bp, 7242 bp, 6369 bp, 5686 bp, 4822 bp, 4324 bp, 3675 bp, 2323 bp, 1929 bp, 1371 bp, 1264 bp, 702 bp, 224 bp, 117 bp.

Ladder 2: 1 kb DNA Ladder, NEB (Frankfurt, Germany)

Fragments: 10000 bp, 8000 bp, 6000 bp, 5000 bp, 4000 bp, 3000 bp, 2000 bp, 1500 bp, 1000 bp, 500 bp.

2.1.10.2 Protein standards

SDS-PAGE Molecular Weight Standard, Broad Range (Bio-Rad, Munich)

Proteins: Myosin (200 kDa), ß-Galactosidase (116,25 kDa), Phosphorylase B (97,4 kDa), Serumalbumin (66,2 kDa), Ovalbumin (45 kDa), Carbonicanhydrase (31 kDa), Trypsininhibitor (21,5 kDa), Lysozyme (14,4kDa), Aprotinin (6,5 kDa).

2.1.11 Antibodies

2.1.11.1 Primary antibodies

For this work the following antibodies were used:

- antibodies directed against C1 and C3 subunits of CHLAMY1 (Zhao et al., 2004);
- anti-CK1 peptide antibodies (Schmidt et al., 2006).

2.1.11.2 Secondary antibodies

As a secondary antibody, monoclonal anti-rabbit immunoglobulin G clone RG-96 peroxidase conjugate (Sigma-Aldrich, Taufkirchen) was used.

2.1.12 Cultivation media

All media were prepared from distilled water that has conductivity less than 0,05 µS/cm. Media were sterilised by autoclaving for 40 min at 121°C and 1 bar, and stored at 4°C.

2.1.12.1 *E. coli* cultivation media

Medium	Composition
LB medium	25 g LB powder (Luria Bertani, ready to use product, Sigma); dissolved in 1 l ddH_2O
LB agar plates	1 l LB-Medium; 1,5% (w/v) Agar
Selection medium	10 ml ampicillin (10 mg/ml; Roth, Karlsruhe) per 1 l LB-Medium
SOC medium	2% (w/v) Bactotrypton; 0,5% (w/v) Yeast extract; 8,6 mM NaCl; 2,5 mM KCl; 10 mM $MgCl_2$; after sterilisation add 20 mM sterile filtered glucose; store at -20°C

2.1.12.2 *C. reinhardtii* cultivation media

Medium	Composition
TAP medium (Harris, 1989)	2,42 g Tris ultra pure; 25 ml Filner's Beijernicks Solution; 1 ml 1 M (K)(PO_4)-buffer pH 7; 1 ml Trace Mineral Solution; bring volume to 1 l with ddH_2O; adjust pH to 7,0 with acetic acid
TAP regeneration top agar	TAP medium with 0,5% (w/v) agar
TAP agar plates	TAP medium with 2% (w/v) agar
TAP^+ with NH_4Cl buffer	2,42 g Tris ultra pure; 1 ml 1 M (K)(PO_4)-buffer pH 7,0; 1 ml Trace Mineral Solution; 1 ml 1 M $MgSO_4$ x 2 H_2O solution; 25 ml salt Solution with NH_4Cl; dissolve in 1 l ddH_2O; adjust pH to 7,0 by acetic acid; add 50 mg

	arginine
1x TAP^+ without NH_4Cl buffer	2,42 g Tris ultra pure; 1 ml 1 M (K)(PO_4)-Puffer pH 7,0; 1 ml Trace Mineral Solution; 1 ml 1 M $MgSO_4$ x 2 H_2O
Full medium agar plates	50 ml phosphate buffer for full medium; 50 ml salt solution for full medium; 1 ml Trace Mineral Solution; 1 g yeast extract; 2 g potassium acetate; bring the volume to 1 l with ddH_2O; 2% (w/v) agar
Minimal medium	50 ml phosphate buffer for full medium; 50 ml salt solution for full medium; 1 ml Trace Mineral Solution; bring the volume to 1 l with ddH_2O
Selection medium	50 µg/ml paromomycin in a medium

2.1.13 Buffers and solutions

All buffers and solutions were prepared with ddH_2O und when necessary sterilised by autoclaving for 30 min at 121°C and at 1 bar air pressure.

2.1.13.1 Solutions for *C. reinhardtii* cultivation media

Solution	Composition
Phosphate buffer	14,34 g K_2HPO_4; 7,26 g KH_2PO_4; dissolve in 1 l ddH_2O
Filner's Beijernicks Solution for TAP medium	3,2 g NH_4Cl; 0,4 g $CaCl_2$ x 2 H_2O; 0,2 g $MgSO_4$ x 7 H_2O; dissolve in 200 ml ddH_2O
Salt solution for TAP^+ without NH_4Cl	0,4 g $MgCl_2$ x 7 H_2O (or 0,37 g $MgCl_2$ x 6 H2O); 0,26 g $CaCl_2$ x 2 H_2O; dissolve in 100 ml ddH_2O
Salt solution for full- and minimal media	8 g NH_4Cl; 1 g $CaCl_2$ x 2 H_2O; 2 g $MgSO_4$ x 7 H_2O; dissolve in 1 l ddH_2O
1 M (K)(PO_4) buffer for TAP medium	57,06 g K_2HPO_4 x 3 H_2O; 34,02 g KH_2PO_4; dissolve each in 250 ml ddH_2O; to 250 ml K_2HPO_4 add KH_2PO_4 (ca. 150 – 170 ml) until pH 7,0 will be achieved
Salt solution for TAP^+ with NH_4Cl	1,6 g NH_4Cl; 0,4 g $MgCl_2$ x 7 H_2O; 0,26 g $CaCl_2$ x 2 H_2O; dissolve in 100 ml ddH_2O
Trace Mineral Solution (Hutner et al., 1950)	2,2 g $ZnSO_4$ x 7 H_2O; 1,14 g H_3BO_3; 0,506 g $MnCl_2$ x 4 H_2O (or 0,414 g $MnCl_2$ x 2 H_2O);

	0,499 g $FeSO_4$ x 7 H_2O; 0,161 g $CoCl_2$ x 6 H_2O; 0,157 g $CuSO_4$ x 5 H_2O; 0,110 g $(NH_4)Mo_7O_{24}$ x 2 H_2O; dissolve one by one in ddH_2O; then add 5 g Na-EDTA and hit to 100°C, cool down, adjust pH to 6,5-6,8 by 10% KOH, fill up to 100 ml with ddH2O, sterilise by filtration. The colour of the solution should be clear green and change in some days to violet

2.1.13.2 Solutions for gel electrophoresis and standard molecular biological methods

Solution	Composition
40x TA	48,4 g Tris ultra pure; 11,4 ml concentrated acetic acid; 0,18 g Na-EDTA (or 40 ml 0,5 M EDTA solution, pH 8); dissolve in 250 ml ddH_2O; adjust pH to 8,1 by acetic acid
10/1 TE buffer	10 mM Tris pH 8,0; 1 mM EDTA
50/10 TE buffer	50 mM Tris pH 8,0; 10 mM EDTA
Lysis buffer 1 for the isolation of a plasmid DNA	1 N NaOH; 20% (w/v) SDS
Potassium acetate buffer	3 M potassium acetate, pH 4,8
Lysis buffer 2 for the isolation of a genomic DNA	2% (w/v) CETAP; 100 mM Tris-HCl pH 8,0; 1,4 M NaCl; 20 mM EDTA

2.1.13.3 Buffers and solutions for protein biochemical experiments

Buffer	Composition
Extraction buffer 1	10 mM Tris pH 7,5; 80 mM NaCl; 1 mM EDTA; 1% glycerol
Extraction buffer 1 with PIC and DTT	10 mM Tris pH 7,5; 80 mM NaCl; 1 mM EDTA; 1% glycerol; 0,2 mM DTT; 2% (v/v) PIC
10 mM phosphate buffer, pH 7	50 ml 20 mM Na_2HPO_4 adjust pH to 7 with 20 mM NaH_2PO_4, bring volume to 100 ml with ddH_2O
Extraction buffer 2 (for 2-DE)	10 mM phosphate buffer, pH 7; 14 mM DTT
Extraction buffer 3 (for NII activity test)	50mM Tris pH 8; 5mM EDTA; 14mM dithiothreitol

Neuhoff stain solution	0,5% (w/v) amidoblack 10 B extra; 90% methanol; 10% acetate acid
Neuhoff destain solution	90% methanol; 10% acetate acid
TCA solution	10% (w/v) TCA; 0,1% (w/v) DTT; in acetone
Washing solution	0,1% (w/v) DTT; in acetone
Rehydration buffer	8 M urea; 0,5% (w/v) CHAPS; 20 mM DTT; 0,2% IPG-buffer; 10% glycerol; bromphenol blue
Equilibration buffer 1	50 mM Tris-HCl pH 8,8; 6 M urea; 30 % glycerol; 2% SDS; 1% DTT
Equilibration buffer 2	50 mM Tris-HCl pH 8,8; 6 M urea; 30 % glycerol; 2% SDS; 4% iodacetamide; 0,05% bromphenol blue
2,5x resolving gel buffer, pH 8,9	1,875 M Tris-HCl, pH 8,9; 0,25% (w/v) SDS
Resolving gel (9%)	7,5 ml 30% (w/v) acrylamid/0,8% (w/v) bis-acrylamid; 6,25 ml 2,5x resolving gel buffer; 11,25 ml ddH_2O; 20 µl TEMED; 312 µl 10% (w/v) APS
Resolving gel (10%) for 2-DE	375 mM Tris-HCl, pH8,8; 10% (w/v) acrylamid/0,3% (w/v) bis-acrylamid; 0,02% Na-thiosulfate; 0,0004% (v/v) TEMED; 0,005% (v/v) 10% (w/v) APS
Resolving gel (12%)	10 ml 30% (w/v) acrylamid/0,8% (w/v) bis-acrylamid; 6,25 ml 2,5x resolving gel buffer; 8,75 ml ddH_2O; 20 µl TEMED; 312 µl 10% (w/v) APS
5x stacking gel buffer, pH 6,7	0,3 M Tris-phosphate buffer, pH 6,7; 0,5% (w/v) SDS
Stacking gel (5%)	1,65 ml 30% (w/v) acrylamid/0,8% (w/v) bis-acrylamid; 2 ml 5x stacking gel buffer; 6,15 ml ddH_2O; 10 µl TEMED; 156 µl 10% (w/v) APS
5x electrophoresis buffer 1	0,5 M Tris; 1,92 M glycerol; 0,5% (w/v) SDS
10x electrophoresis buffer 2	0,25 M Tris; 1,92 M glycerol; 1% (w/v) SDS
Coomassie methanol free solution	0,2% (w/v) Coomassie Brillant Blue R250; 45% (v/v) 96% ethanol; 10% (v/v) acetic acid
Distaining solution	20% (v/v) 96% ethanol; 10% (v/v) acetic acid

2.1.13.4 Buffers for immunochemical experiments

Buffer	Composition

Semi-dry transfer buffer	25 mM Tris; 192 mM glycine; 20% Methanol (v/v); pH 8,3
Ponceau solution	0,2% (w/v) Ponceau-S; 3% (v/v) trichloracetate
Blocking buffer	1x TBS-Tween 20, pH 7,5; 1% (w/v) Slim-Fast® Milchshake-Powder, type chocolate (Slim-Fast, Messel)
10x TBS	200 mM Tris, pH 7,5; 1,5 M NaCl
1x TBS-Tween	10% 10x TBS; 0,05% (v/v) Tween 20
Luminol solution 1	100 mM Tris, pH 8,5; 1% (v/v) Luminol stock solution (0,44 g in 10 ml DMSO, protect from light); 2,2% (v/v) p-cumarin acid
Luminol solution 2:	100 mM Tris, pH 8,5; 0,02% H_2O_2

2.2 Methods

Most of the methods were done according to Sambrook et al. (1989) with slight changes.

2.2.1 Methods for a vector construction in *E. coli*

2.2.1.1 DNA amplification

2.2.1.1.1 Design of oligonucleotides („primers“)

For the primer design the computer program „Genrunner“ (Hastings Software, USA) was used.

2.2.1.1.2 Polymerase chain reaction (PCR)

The PCRs were done using the „GC-RICH PCR System“ (Roche, Mannheim) (s. 2.1.4). According to the supplied protocol two master mixes were prepared. The master mix 1 contained desoxynucleotides dATP, dCTP, dGTP, TTP (final concentration is 200 µM each), a sense primer und an anti-sense primer (final concentration is 200 nM each), genomic DNA (final concentration is from 10 ng to 500 ng), „GC-RICH" resolution solution (final concentration 0,5 M) and ddH_2O. The master mix 2 contained the „GC-RICH“ PCR reaction buffer with DMSO, „GC-

RICH“ PCR enzyme mix and ddH_2O. Both master mixes were pipetted together in a thin-walled PCR tube. The reaction was performed in following conditions: 1x 3 min at 95°C, 10x (30 s at 95°C, 30 s at 57°C, 1 min at 72°C), 25x (30 s at 95°C, 30 s at 57°C, 1 min and an additional cycle elongation of 5 s for each cycle at 72°C), 1x 7 min at 72°C.

2.2.1.1.3 Purification of a DNA product of the PCR

The purification of PCR products was done by two different methods. In one case, it was done using an agarose gel, in another, using „QIAquick® PCR Purification Kit“ (Qiagen, Hilden) (s. 2.1.4).

2.2.1.1.3.1 Purification of DNA fragments using the „QIAquick® PCR Purification Kit“ (Qiagen, Hilden)

According to the supplied protocol, one volume of PCR reaction mix was added to five volumes of supplied PB buffer, mixed well by shaking and transferred to the purification column. After centrifugation of the column for 1 min at 16000 g the supernatant was discarded and the column was washed with PE buffer. After centrifugation of the column for 1 min at 16000 g the DNA was eluted with 30 µl EB buffer. The concentration of the isolated DNA was detected by "dot"-test (s. 2.2.1.2.2).

2.2.1.1.3.2 Separation of DNA fragments in agarose gel

The separation of DNA fragments was performed in a horizontal gel electrophoresis chamber (s. 2.1.1). The agarose concentration depended on the size of the analyzed fragment and varied from 0,7% to 1,3% (w/v). TA buffer (s. 2.1.13.2) was used. The agarose was resuspended in the buffer, warmed up to 100°C till complete melting. When the melted agarose was cooled down to appr. 50°C, ethidium bromide was added to it (to final concentration 0,1 µg/ml), then agarose was transferred to the gel holder with a slots comb and left for polymerisation for minimum 1 h. Polymerised agarose gel was transferred to the gel electrophoresis chamber filled with TA buffer, the comb was removed and DNA probes were loaded to slots. To detect the size of separated fragments, the DNA standard was always loaded in one of the slots. DNA fragments were separated for ca. 1h at 100 V. After electrophoresis, the gel was

analysed in UV transilluminator under UV light with the wavelength 256 nm, and the image was documented.

2.2.1.1.3.3 Elution of DNA fragments from agarose gel

After observation under UV light, gel blocks containing DNA fragments of correct size were excised from agarose gels and the DNA fragments were eluted from the gel by „GenElute™ Agarose Spin Columns (Sigma, Taufkirchen) (s. 2.1.4). According to the supplied protocol, a gel block was transferred to a column pre-washed with 100µl 10 mM Tris-HCl buffer, pH 8. The column was then placed into a collection tube and centrifuged 10 min at 16000 g at RT. The concentration of eluted DNA fragment in the supernatant was detected by a "dot-test" (s. 2.2.1.2.2). If necessary, DNA was concentrated by ethanol precipitation (s. 2.2.1.5.5).

2.2.1.2 Determination of DNA concentration

2.2.1.2.1 Photometric analysis

For the determination of DNA concentration in high concentrated solutions (DNA concentration between 5 and 50 µg/µl) a photometric analysis was used. For this method of quantification, 1 µl of DNA solution was diluted in 499 µl of ddH_2O, and the optical density of the resulted solution was measured with a spectrophotometer (s. 2.1.1) in 1 cm quartz cuvettes in the UV light with two different wavelength: λ=260 nm und λ=280 nm against ddH_2O. The relation OD_{260nm}/OD_{280nm} should be between 1,7 and 2,0, otherwise it means that the DNA solution is contaminated by proteins. A solution with 50 µg/ml DNA has $OD_{260nm}=1$.

2.2.1.2.2 "Dot"-test

For the detection of DNA concentration in low concentrated solutions (DNA concentration between 10 and 100 ng/µl) the "dot"-test was used. For this purpose, 1µl DNA sample was diluted in 8 µl of ddH_2O and mixed with 1 µl ethidium bromide solution (5µg/µl). The fluorescence intensity of the solution was analysed under UV light and compared with those of standard solutions (0 ng/µl, 10 ng/µl, 25 ng/µl, 50 ng/µl und 100 ng/µl).

2.2.1.3 Transformation of *E. coli* by heat shock method

An aliquot of plasmid DNA (1 - 10 µl) was added to 100 µl competent cells and incubated on ice for 45 min. After the incubation, the cells were transferred in to a water bath at 42°C for exactly 90 s and then immediately cooled on ice for 2 min. After the heat shock 500 µl SOC medium (s. 2.1.12.1) were added to cells and they were left for 1 h at 37°C. After recovering cells were transferred on selective LB agar plates (s. 2.1.12.1) and incubated from 14 to 16 h at 37°C.

2.2.1.4 Preparation of competent *E. coli* cells

For preculture, *E. coli* strain XL-blue (s. 2.1.6) was inoculated in 2,5 ml of LB medium (2.1.12.1) and incubated 14 - 16 h at 37°C under permanent shaking. 500µl of preculture were transferred to 100 ml of LB medium and grown at 37°C until an OD_{600} of 0,4 - 0,6 was reached. The following procedures were carried out on ice. *E.coli* cells were harvested by centrifugation in 50 ml centrifuge tubes at 9000 g and 4°C. The pellet was carefully resuspended in 10 ml of prechilled 0,1 M $CaCl_2$ and incubated on ice for 30 min. Then the cell suspension was centrifuged again 5 min at 9000 g at 4°C and the supernatant was removed. The pellet was resuspended in 2 ml of 0,1 M $CaCl_2$/10% glycerol, pippeted to 100 µl aliquots in 1,5 ml plastic tubes and immediately frozen in liquid nitrogen. The competent cells were stored at -80°C before they were used for a transformation. To determine the titer of competent cells they were transformed with 1 µl of control plasmid (pBluescript KS+ (s. 2.1.7) (0,1 µg/ml)). The amount of colonies that appeared on selection plates in 14 h after transformation was counted and the titer was calculated as number of colonies per 1 µg of plasmid DNA.

2.2.1.5 Methods of DNA isolation

2.2.1.5.1 Isolation of plasmid DNA from *E. coli* in small scale

Single colonies of *E. coli* from the selective plates after transformation were re-inoculated in 2,5 ml LB (s. 2.1.12.1) with 50 µg/ml ampicillin and grown 14 - 16 h at 37°C with permanent shaking. 1,5 ml of the obtained cultures were harvested by centrifugation for 1 min at 9000 g at 4°C. The supernatant was discarded and the pellet was resuspended in 100 µl of 10/1 TE buffer (s. 2.1.13.2), containing 15 µl of RNAse A (s. 2.1.3) solution. The cells were destroyed by addition of 150 µl of lysis buffer (s. 3.1.13.2), and thereafter the rests of cell walls, cell proteins and connected

to them genomic DNA were precipitated by addition of 200 µl of potassium acetate buffer (s. 2.1.13.2). After the centrifugation of the suspension for 30 min at 16000 g at 4°C the plasmid DNA containing supernatant was transferred to fresh 1,5 ml plastic tubes and the DNA was precipitated by incubation with equal volume of 100% isopropanol for 1 h at -20°C. DNA was collected by centrifugation for 30 min at 16000 g at 4°C. The pellet was washed once with 70% ethanol, dried in vacuum concentrator for 10 min and dissolved in 30 µl of 10 mM Tris-HCl buffer pH 8.

2.2.1.5.2 High pure plasmid isolation

High pure plasmid isolation was done using High Pure Plasmid Isolation System (Roche, Mannheim) (s. 2.1.4) according to the protocol supplied by the company. 4 ml of *E. coli* culture were centrifuged 30 s at 9000 g at RT. The supernatant was discarded and the pellet was resuspended in 250 µl Suspension Buffer including RNase and mixed well. 250 µl of Lysis Buffer were added, mixed gently by inverting the tube six times and incubated 5 min at RT. 350 µl of chilled Binding Buffer were added, mixed gently by inverting the tube 6 times and incubated 5 min on ice. The suspension was centrifuged for 10 min at 16000 g. The entire supernatant was transferred to the upper reservoir of the filter tube, centrifuged 30-60 sec at 16000 g. The filter was transferred to a new collection tube, washed once with 700 µl Wash Buffer II. The filter was then inserted to a new 1,5 ml microcentrifuge tube, 100 µl Elution Buffer were added and centrifuged 30 sec at 16000 g. The eluted purified plasmid DNA was used for DNA sequencing, performed by Eurofins Medigenomix GmbH (Martinsried bei München).

2.2.1.5.3 Large scale isolation of plasmid DNA from *E. coli*

Single colonies of transformed *E. coli* were inoculated in 100 ml LB (s. 2.1.12.1) with 50 µg/ml ampicillin and grown 14 - 16 h at 37°C with permanent shaking. The cells were harvested by centrifugation in 50 ml centrifuge tubes for 5 min at 9000 g at 4°C. The supernatant was discarded and the pellet was resuspended in 5 ml of 50/10 TE buffer (s.2.1.13.2). The cells were destroyed by addition of 10 ml of lysis buffer (s. 2.1.13.2), and thereafter the rests of cell walls, cell proteins and connected to them genomic DNA were precipitated by addition of 10 ml of potassium acetate buffer (s. 2.1.13.2). After the centrifugation of the suspension for 30 min at 32000 g at 4°C the plasmid DNA containing supernatant was transferred to fresh 50 ml

centrifuge tubes and the DNA was precipitated by incubation with equal volume of 100% isopropanol for 1 h at -20°C. DNA was collected by centrifugation for 30 min at 32000 g at 4°C. The pellet was dried in vacuum concentrator (s. 2.1.1) for 10 min and dissolved in 1 ml of 50/10 TE buffer (s. 2.1.13.2). The coprecipitated RNA was removed by incubation with 15 µl of RNAse A (s. 2.1.3) for 1 h at 37°C.
DNA was extracted by phenol-chloroform extraction (s. 2.2.1.5.4) and precipitated by ethanol (s. 2.2.1.5.5). The pellet was dissolved in 100 µl 10 mM Tris-HCl pH 8.

2.2.1.5.4 Phenol-chloroform extraction

Purification of the isolated DNA from coprecipitated proteins was done by phenol-chloroform extraction. One volume of phenol/chloroform/isoamylalcohol (in the ratio 25:24:1) was added to the DNA solution, intensively shaken for 1 min and centrifuged 5 min at 16000 g at RT. The upper phase was transferred to a new plastic tube, and the extraction step was repeated a second time. After that, the upper phase was mixed with chloroform/isoamylalcohol (in the ratio 24:1), shaken for 1 min and centrifuged for 5 min at 16000 g at RT. The plasmid DNA was precipitated from the upper fraction by ethanol precipitation (s. 2.2.1.5.5).

2.2.1.5.5 Ethanol DNA precipitation

1/10 volume of 3 M sodium acetate, pH 4,8, and 2,5 volumes of absolute ethanol were added to one volume of a DNA solution, mixed and incubated for 2 h at -20°C. After incubation, the solution was centrifuged 30 min at 16000 g at 4°C, the DNA pellet was washed with 70% ethanol, dried in vacuum concentrator for 10 min and resuspended in 10 mM Tris-HCl buffer, pH 8.

2.2.1.5.6 Isolation of genomic DNA from *C. reinhardtii*

C. reinhardtii WT strain SAG73.72 was inoculated in 100 ml TAP medium. At a cell density of $3 \cdot 10^6$ cells per ml, cells were harvested by centrifugation in 50 ml centrifuge tubes for 5 min at 4°C and 3950 g. The cell pellet was washed in 1 ml TAP (s. 2.1.12.2) and frozen in liquid nitrogen. Then, it was carefully homogenised in a mortar with a pistil within 2-3 min. 1 ml of lysis buffer 2 (s. 2.1.13.2) was added to cells and mortared again for 2-3 min. The suspension was distributed to 4 mikrocentrifuge tubes (250 µl per tube) and 750 µl of preheated to 65°C lysis buffer

2 (s. 2.1.13.2) were added to each aliquot. After an incubation for 60 min at 65°C to each tube 700 µl of phenol/chloroform/isoamylalcohol (25:24:1) were added and carefully inverted for 10 min. Then the suspension was centrifuged for 10 min at 4°C and 9300 g and the upper phase, containing DNA, was transferred to new mikrocentrifuge tubes. One volume of isopropanol was added to it and incubated for 15 min on ice. Precipitated DNA was collected by centrifugation for 15 min at 4°C and 15000 g, washed once with 1 ml 70% ethanol and dried at RT in vacuum concentrator for 10 min and resuspended in 300 µl 1/10 TE buffer (s. 2.1.13.2). RNA was removed by incubation of the solution with 15 µl of RNAse A (s. 2.1.3) for 30 min at 37°C. The isolation procedure was followed by a phenol-chloroform extraction and finally genomic DNA was precipitated by ethanol. The pellet was dissolved in 100 µl 10/1 TE buffer (s. 2.1.13.2) for 16 h at 4°C. The DNA concentration was detected spectrophotometrically. The quality was checked by gel electrophoresis of 5 µg isolated genomic DNA on a 0,6% agarose gel (s. 2.2.1.1.3.2).

2.2.1.6 DNA restriction

Restriction enzymes (s. 2.1.3) were used for two cases: a) characterisation of isolated plasmid DNAs and b) preparation of DNA fragments for further cloning steps. In both cases, the plasmid DNA was mixed with a restriction enzyme (in ratio 1 unit of the enzyme per 1 µg DNA), with a reaction buffer corresponding to the enzyme and, if necessary, with BSA and the reaction was carried out for at least 2 h at the appropriate temperature. The resulted DNA fragments were analysed by gel electrophoresis (s. 2.2.1.1.3.2) and, when necessary, eluted from the gel (s. 2.2.1.1.3.3).

2.2.1.7 Polishing of sticky ends using Klenow enzyme

Sticky ends of DNA fragments with recessed 3' termini were, if necessary, filled by the polymerase activity of the Klenow fragment of DNA polymerase I (s. 2.1.3). In case of recessed 5' termini, sticky ends were removed by its exonuclease activity. The reactions were carried out according to the supplied protocol.

2.2.1.8 Ligation

The ligation mix contained the ligation buffer (Roche, Mannheim), T4 DNA ligase and DNA (s. 2.1.3). The DNA fragment and the vector were added to the ligation mix in molar ratio 3:1 for sticky end ligations and 5:1 for blunt end ligations. The reaction proceeded for 4 h at 17°C in the case of sticky end ligation and for 14 h at 4°C in the case of blunt end ligation.

After the incubation time was over, the ligation mix was directly used for the transformation of *E. coli* (s. 2.2.1.3).

2.2.2 Work with *C. reinhardtii* cultures

2.2.2.1 Cultivation of *C. reinhardtii*

C. reinhardtii cells were grown in TAP medium (Harris, 1989) (s. 2.1.12.2) under a 12h light–12h dark cycle (LD 12:12) with a light intensity of 71 μE m^{-2} sec^{-1} (1 E = 1 mol of photons) at 18, 23 or 28°C, as indicated for each experiment. Cells were grown under a LD cycle unless otherwise indicated. The beginning of the light period is defined as time zero (LD0) and the beginning of the dark period is LD12. In some cases, cells were released after LD into constant dim light (LL: 20 μE m^{-2} sec^{-1}) and temperature (23°).

2.2.2.2 Harvest of *C. reinhardtii* cells

C. reinhardtii liquid cultures were harvested by centrifugation for 5 min in 50 ml plastic centrifuge tubes at 4°C and 6000 g. 1 ml of the supernatant was used to resuspend the cell pellet, which was then transferred to 2 ml plastic mikrocentrifuge tubes and centrifuged for 2 min at 4°C and 16000 g. The cell pellet was frozen in liquid nitrogen and stored at -80°C.

2.2.2.3 Cell concentration determination

The concentration of *C. reinhardtii* cells in liquid cultures was detected by counting the cell number in a Thoma cell chamber (height: 0,1 mm; 4x4 small squares with the side length 0,05 mm and the area 0,0025mm²).

2.2.2.4 Autolysin preparation

All procedures were done in sterile laminar box. Two arginine deficient strains of *C. reinhardtii* CLS 31-8 (-) und CLS 31-10 (+) (s. 2.1.5) were grown in 100 ml TAP^+ with NH_4Cl buffer (s. 2.1.12.2) each, until a cell density of 4 x 10^6 to 5 x 10^6 cells per ml was reached. The cultures were centrifuged in 50 ml centrifuge tubes for 5 min at 4°C and 6000 g. The pellets were resuspended each in 80 ml TAP^+ without NH_4Cl buffer (s. 2.1.12.2) and cultivated for 19 h under constant light for gametes production. The ability for mating was checked after 16 - 19 h by mixing 10 µl of the both strains together and analysing their behaviour under the microscope. If the mating behaviour was observed, the cell concentration of the both cultures was determined. Then, the cultures were centrifuged 5 min at 4°C and 6000 g and resuspended in TAP^+ without NH_4Cl buffer (s. 2.1.12.2) to a final concentration of 1 x 10^8 cells per ml. For mating, 10 ml of both cultures were mixed together and incubated under constant light for 2 h. Each 15 min a 10 µl aliquot was taken and mixed with 10µl of 1% Triton X 100. If complete cell lysis was observed in 5 min, the cell suspension was centrifuged for 10 min at 4°C and 16000 g. The autolysin containing supernatant could be directly used for the transformation of *C. reinhardtii* or stored at -20°C.

2.2.2.5 *C. reinhardtii* transformation

Cells of *C. reinhardtii* were grown in 100 ml TAP medium (s. 2.1.12.2) until a cell concentration of 1 - 5 x 10^6 cells/ml was reached. Cells were harvested by centrifugation for 4 min at 4°C and 5000 g. The pellet was dissolved in 10 ml autolysin solution (s. 2.2.2.4) and transferred to a sterile conical flask and incubated under constant light conditions for 1 - 4 h depending on the autolysin activity. Every 15 min, the efficiency of lysis was checked by addition of 10 µl 1% triton to 10 µl of cell suspension. Cells that were ready to be transformed showed about 70 - 80% lysis. After treatment with autolyin, cells were collected by centrifugation in 50 ml tubes for 5 min at 5000 g and 10°C. The pellet was carefully resuspended in 1,8 ml of fresh TAP medium (s. 2.1.12.2). For the transformation, 300 µl of cell suspension were mixed with 20 µg of linearised vector DNA and applied over 0,3 g sterile glass beads in a 0,5 ml microcentrifuge tube. The tube was shaken for 15 sec with a minishaker Vortex-Genie®2 (s. 2.1.1) and cells were immediately transferred to 50 ml plastic tubes, containing 10 ml of fresh TAP medium (s. 2.1.12.2). Cells were incubated for 18 h at constant dim light with a light intensity of 42 µE m^{-2} sec^{-1}.

After incubation cells were harvested by centrifugation for 5 min at 5000 g and 4°C and the pellet was dissolved in 1 ml TAP medium (s. 2.1.12.2). Cell suspension was mixed with 3,5 ml melted regeneration cover agar that had been cooled down to 40°C 0,5% (w/v) and applied on TAP agar plates with paromomycin (s. 2.1.12.2). Colonies of successfully transformed *C. reinhardtii* cells appeared in about 10 days after transformation.

2.2.2.6 Preparation of glass beads for the transformation of *C. reinhardtii*

Glass beads (s. 2.1.4) with a diameter from 0,45 to 0,5 mm were washed with concentrated H_2SO_4. After the discard of the acid the glass beads were rinsed with ddH_2O as long as the pH reached neutral value. Finally, the beads were autoclaved and left to dry for several days. For the transformation, 0,3 g of the glass beads were placed in a 1 ml microcentrifuge plastic tube, once again autoclaved and dried.

2.2.2.7 Storage of *C. reinhardtii* cell cultures in liquid nitrogen

C. reinhardtii culture was grown in TAP medium (s. 2.1.12.2) until the cell concentration reached 1 x 10^6 cells/ml. 25 ml of the culture was harvested by centrifugation for 5 min at RT and 3950 g. The pellet was resuspended in 2,5 ml TAP medium (s. 2.1.12.2). The suspensium was aliquated in CryoTubes (250 µl per tube) and to each 250 µl TAP medium with 6% (v/v) methanol was added. The cryotubes were transferred to freezing container (s. 2.1.1) and incubated for ca. 70 min at -80°C. Frozen aliquots were stored in liquid nitrogen storage. For reanimation, the frozen cells in the cryotubes were placed into a water bath for 2 min at 35°C. Cells were resuspended in 10 ml TAP medium (s. 2.1.12.2) and incubated inverting for 16 h under constant light. After the incubation cells were harvested by centrifugation for 5 min at 5000 g und 4°C. The pellet was resuspended in 0,5 ml fresh TAP medium and inoculated on selective TAP agar plates (s. 2.1.12.2).

2.2.3 Methods for the characterization of wild-type or transgenic strains of *C. reinhardtii*

2.2.3.1 Crude extracts preparation for standard SDS-PAGE

150 µl of precooled extraction buffer 1 (s. 2.1.13.3) with PIC and DTT was added to a frozen *C. reinhardtii* cell pellet and left on ice until it melted completely (about 30 min) by stirring it with a sterile tooth pick. In the mean time, glass beads for protein crude extract preparation (Ø 0,25–0,3 mm) (s. 2.1.4) were transferred into 1,5 ml plastic centrifuge tubes (2/3 of the tube volume) and washed twice with the cold extraction buffer to remove completely air bubbles. The suspension of cells in extraction buffer was applied over prewashed glass beads and intensively shaken three times for 1 min using a Vortex minishaker (s. 2.1.1). In between these steps, the tube was incubated for 2 min on ice each time. Then, the cell suspension was carefully transferred in to a new 1,5 ml plastic centrifuge tube and centrifuged for 15 min at 4°C and 16000 g. The protein containing supernatant was transferred to a new tube and used for further experiments.

2.2.3.2 Crude extracts preparation for 2-DE SDS-PAGE

300 µl of precooled extraction buffer 2 (s. 2.1.13.3) with PIC, DTT, PhIC 1 (1%) and PhIC 2 (1%) (s. 2.1.3) were added to a frozen *C. reinhardtii* cell pellet and left on ice until melted completely (about 30 min) under stirring by a sterile tooth pick. In the mean time glass beads for protein crude extract preparation (Ø 0,25–0,3 mm) (s. 2.1.4) were transferred in to 1,5 ml plastic centrifuge tubes (2/3 of the tube volume) and washed twice with the cold extraction buffer 2 to remove completely air bubbles. The suspension of cells in extraction buffer 2 was applied over prewashed glass beads and intensively shaken five times for 1 min using a Vortex minishaker (s. 2.1.1). In between of these steps each time the tube was incubated for 2 min on ice. Then the cell suspension was carefully transferred in to a new 1,5 ml plastic centrifuge tube and centrifuged for 30 min at 4°C and 16000 g. The protein containing supernatant was transferred to a new 2 ml plastic centrifuge tube. A 5 µl aliquot was taken for the protein concentration determination, the rest was mixed with 4x volume of cool TCA solution (s. 2.1.13.3) and left for a minimum of 16 h at -20°C for precipitation. After the incubation the solution was centrifuged for 30 min at 4°C and 16000 g. The pellet was washed for 1 h with the washing solution (s. 2.1.13.3) at -20°C and afterwards centrifuged for 30 min at 4°C and 16000 g. The protein pellet was dried for 10 min at RT and solubilized for 1 h at RT in the rehydration buffer (s. 2.1.13.3) to a final concentration of 2 µg/µl. The resuspended

sample was then centrifuged for 5 min at 16000 g. The crude extract was used for further experiments.

2.2.3.3 Protein concentration determination

For the protein concentration determination, either „Bio-Rad Protein Assay“ (s. 2.1.4) or the Neuhoff method was used.

2.2.3.3.1 "Bio-rad protein assay"

The determination of the protein concentration determination was done with the „Bio-Rad Protein Assay“ (Bio-Rad, München) (s. 2.1.4) according to the protocol supplied by the company. A protein crude extract (s. 2.2.3.1 or s. 2.2.3.2) was diluted 20 times with ddH_2O and 10 µl of it were mixed with 5 ml 1:5 diluted "Bio-Rad" solution. The reaction was incubated at RT for 5 min and the optical density was detected at λ 595 nm by a spectrophotometer (s. 2.1.1). The protein concentration in the probe was determined using a calibration curve created by measuring of standard BSA solutions (5 - 80 µg).

2.2.3.3.2 Neuhoff method

Protein concentration determination with the Neuhoff method was done as described in Neuhoff et al., 1979. 2 µl aliquots of protein crude extracts (s. 2.2.3.1 or s. 2.2.3.2) were applied on 0,5 x 0,5 cm piece of the cellulose-acetate filter (s. 2.1.4) and incubated in the Neuhoff stain solution (s. 2.1.13.3) for 2 min. After the incubation, the filters were washed 3 times in the Neuhoff destain solution (s. 2.1.13.3) until non-covered parts of filters become white. The filters were dried and desolved in 1 ml of water free DMSO. The optical density was detected at λ 630 nm by spectrophotometer (s. 2.1.1). The protein concentration in the probe was determined using calibration curve created by measuring of standard (1 - 20 µg) BSA solutions.

2.2.3.4 One dimensional protein separation by SDS-PAGE

In this method, proteins were separated by denaturing SDS gel electrophoresis according to their molecular weight (Laemmli, 1970). For the preparation of the gel, a gel chamber was constructed from two glasses and 3/4 of it was filled up with the resolving gel (s. 2.1.13.3). After polymerisation of the resolving gel, the rest of the

chamber was filled up with the stacking gel (s. 2.1.13.3). Before loading of the gel, protein probes were mixed with "4x Roti-Load" (s. 2.1.4), denatured in a boiling water bath for 5 min and centrifuged for 30 sec at 4°C and 16000 g. Electrophoresis buffer 1 (s. 2.1.13.3) was used in this experiment. Electrophoresis was carried out a in water cooling electrophoresis chamber (s. 2.1.1) at 150 V for the first one hour, before the proteins entered the resolving gel, and than at 250 V for about 2 h. When the electrophoresis was finished, the r stacking gel was removed and the resolving gel was used for the further experiments.

2.2.3.5 Coomassie staining

SDS-PAGE gels (s. 2.2.3.4) were incubated in a Coomassie methanol free solution (s. 2.1.13.3) for ca. 12 h at RT. To remove the background coloration, the gels were incubated 3 times for ca. 1 h in distaining solution (s. 2.1.13.3).

2.2.3.6 Immunoblotting (Western blot transfer)

After SDS-PAGE (s. 2.2.3.4 or 2.2.3.10), the resolving gel was transferred to the semi-dry transfer buffer (s. 2.1.13.4) and incubated for 10 min on a rocking platform shaker (s. 2.1.1). In parallel the nitrocellulose membrane (s. 2.1.4) was incubated for 5 min in semi-dry transfer buffer (s. 2.1.13.4). The gel and the membrane were transferred in to the blotting chamber (s. 2.1.1) in following order starting from the cathode: 3 sheets of the moistened gel blotting paper (s. 2.1.4), the nitrocellulose membrane, the gel and again 3 sheets of moistened gel blotting paper. The transfer was carried out for 1,5 h with the electrical current power calculated as 1,5 mA per one cm^2 of the transferring gel.

2.2.3.7 Ponceau S membrane stain

The quality of the Western blot transfer was checked by Ponceau staining. The membrane was incubated in Ponceau solution (s. 2.1.13.4) for 5 min at RT and washed 3 times with ddH_2O. The standard and the electrophoresis front line were marked on the membrane.

2.2.3.8 MemCode™ Reversible Protein Stain Kit

In the case of the 2-DE procedure MemCode Reversible Protein Stain Kit (Pierce) (s. 2.1.4) was used instead of Ponceau staining (s. 2.2.3.7). The membrane staining was done according to the protocol, supplied by the company. The membrane was incubated for 30 sec in the MemCode™ Stain, rinsed 3 times with MemCode™ Destain solution, incubated with MemCode™ Destain solution for 5 min and washed several times with ddH_2O.

2.2.3.9 Immunodetection of proteins

After Western blot transfer (s. 2.2.3.6) the nitrocellulose membrane was used for the protein immunodetection. First, for prevention of unspecific binding of antibodies, the membrane was incubated for 2 h at RT in the blocking buffer (s. 2.1.13.4) on a rotation shaker (s. 2.1.1). The blocked membrane was then incubated with primary antibodies (s. 2.1.11.1) dissolved in the blocking buffer for 16 h at 4°C on the rotation shaker. After the incubation the membrane was washed 3 times for 10 min with TBS-tween buffer (s. 2.1.13.4) and incubated for 1 h at RT with secondary antibodies (s. 2.1.11.2) dissolved in the blocking buffer. Then the membrane was washed again 3 times for 15 min with TBS-tween buffer and once with TBS buffer for 10 min. The secondary antibodies were horse-radish peroxidase conjugated. A chemiluminescent reaction was used for the detection of the antibody binding. The following chemiluminescent reaction was carried out in darkness. The nitrocellulose membrane was incubated for 5 min in the reaction solution containing luminol solution 1 and 2 (s. 2.1.13.4), mixed in a 1:1 ratio. After the incubation, the membrane was packed into a plastic foil and exposed to photofilms (s. 2.1.4). The time of exposure varied from 5 sec to 1 h.

2.2.3.10 Standardized 2-Dimensional Electrophoresis (2-DE)

2-DE was basically done according to Wagner et al. (2004) with some modifications. 150 µl of the protein crude extract for 2-DE (s. 2.2.3.2) were mixed with 300 µl of the rehydration buffer (s. 2.1.13.3) and applied into the strip holder. After the IPG strip (s. 2.1.4) had been placed in the strip holder over the solution and covered with paraffin, rehydration was carried out for 12 h. Then, isoelectric focusing in Isoelectric Focusing System (IPGphor) (s. 2.1.1) was started with the following program: 200 V (1 h), 500 V (1 h), 1000 V (1 h), linear gradient from 1000 to 8000 V (1 h) and 8000 V (6 h). Thereby, current was limited to 0.05 mA per IPG strip.

After the end of this program, the strip was removed from the strip holder and equilibrated for 15 min in the equilibration solution 1 and 15 min in the equilibration solution 2. Each strip was placed on the top of the polyacrylamide resolving gel for 2-DE. Strips were fixed on the gels with 0.5% agarose in electrophoresis buffer 2 (s. 2.1.13.3), and the electrophoresis was performed at 4°C and 2.5 Watt per gel over night (~12 h) using an Ettan Dalt 6 electrophoresis unit (Amersham Biosciences) (s. 2.1.1).

After electrophoresis, immunoblotting (s. 2.2.3.6) was done along with anti-C1 antibodies. Directly after the Western blot, the membrane was stained with MemCode™ Reversible Protein Stain Kit (Pierce, Rockford, USA) (s. 2.1.4) and biggest spots were marked and used as position markers for the comparison of different samples. Then, membranes were further used for antibody incubation.

2.2.3.11 Nitrite reductase (NII) activity test

For the NII assay, harvested cells were washed with 50 mM Tris/5 mM EDTA, pH 8, two times before their storage at -80°C. For extracts, cells were resuspended in the extraction buffer 3 for NII test (s. 2.1.13.3), and lysed by intensive shaking 5 x 1 min with glass beads (Ø 0.25–0.30 mm) (s. 2.1.4) using a Vortex minishaker (s. 2.1.1) at highest speed, incubating for 2 min on ice in between. Cell debris was removed as described in Zhao et al. (2004) and the resulting crude extract was immediately used further. NII activity was determined by a dithionite assay as described by Vega et al. (1980) along with 15 mg of total protein from the crude extract. The reaction was carried out for 20 min at 40°C. The assay involved sodium dithionite as reductant and methyl viologen as electron carrier. Enzymatic activity was followed by measuring colorimetrically (540 nm) (s. 2.1.1) the rate of disappearance of nitrite. One unit of activity was defined as the amount of enzyme that catalyzed the reduction of 1 mmol of nitrite per minute.

2.2.3.12 Densitometry Analysis

Quantifications of the volumes of bands/spots obtained on photofilms after immunodetection of proteins (s. 2.2.3.9) were done with the Image Master™ 2D Elite (vs 4.01) software from Amersham Pharmacia Biotech. For characterization of the silencing level of protein of interest, calibration curves were created that correlate band volumes of the protein of interest from different concentrations of wild-type

protein crude extract. They were used for comparison of the protein expression level in the examined strains. For 2-DE immunoblots of C1, the volume of all spots was set to 100% and compared to the volume of a phosphorylated spot.

2.2.3.13 Phototaxis test

The measurement was done with a self-made phototaxis machine, developed by Mergenhagen (1984). Preparation of cell culture for the assay, phototaxis measurement and data evaluation were done as previously described (Schmidt et al., 2006; Iliev et al., 2006). The measurements were done by M. Fiedler.

3. Results

3.1 The role of the C1 subunit of CHLAMY1 in the circadian system of *C. reinhardtii*

3.1.1 Silencing of the C1 subunit of CHLAMY1

The first part of my work was aimed at investigating the role of the C1 subunit of CHLAMY1 in the circadian system of *C. reinhardtii* by silencing its gene and analyzing the circadian rhythms of phototaxis and nitrite reductase (NII) activity in the modified strains. For this purpose, a RNAi (RNA interference) strategy was applied that is based on the method developed by Fuhrmann et al., 2001. The native *c1* promoter was used in the construct. Thus, the potential promoter regions of the *c1* together with the first three exons and two introns were fused to an inverted corresponding *c1* cDNA part so that a double-stranded RNA will be formed in the cell that triggers silencing.

3.1.1.1 Construction of the *c1* silencing vector with the native promoter

A genomic DNA fragment of 2607 bp containing the potential promoter region of *c1* (1238 bp in front of the AUG including the predicted 122 bp 5'-UTR) and the first three exons and two introns of *c1* (gene model estExt_fgenesh2_kg.C_30171, vs3 of the genome) were PCR-amplified (s. 2.2.1.1.2) using the GC-RICH PCR System kit from Roche (s. 2.1.4) according to the instructions of the kit along with genomic DNA from *C. reinhardtii* (s. 2.2.1.5.6) and the primers (s. 2.1.9) OMM267 (as a sense PCR primer) and OMM269 (as an antisense PCR primer) (Figure 7).

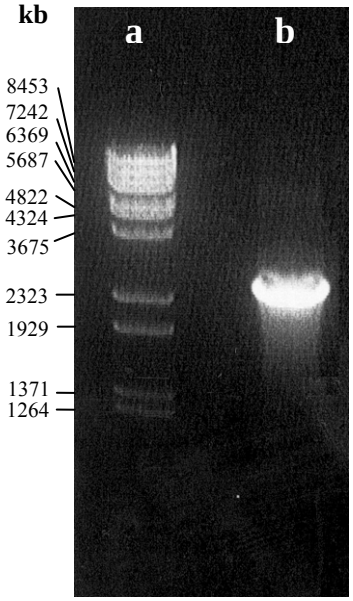


Figure 7. Detection of the *c1* PCR amplified genomic DNA fragment by agarose gel electrophoresis

The PCR reaction was carried out using GC-RICH PCR System from Roche (2.1.4) with OMM267 and OMM269 primers (s. 2.1.9) along with genomic DNA. The product was separated for 1 h at 100 V on a 1% agarose gel (s.2.2.1.1.3.2) and visualized by ethidium bromide staining. a) Ladder: Lambda DNA-*Bst*EII digest (s. 2.1.10.1). b) PCR product, containing the fragment of *c1* genomic DNA together with its potential promoter region.

The amplified genomic DNA was then cloned into the *Mlu*NI of the pCAP^S vector (s. 2.1.7) using a PCR Cloning Kit (Roche) (s. 2.1.4) and following the instructions of the manual. The resulting plasmid was named pOV4 (Figure 8).

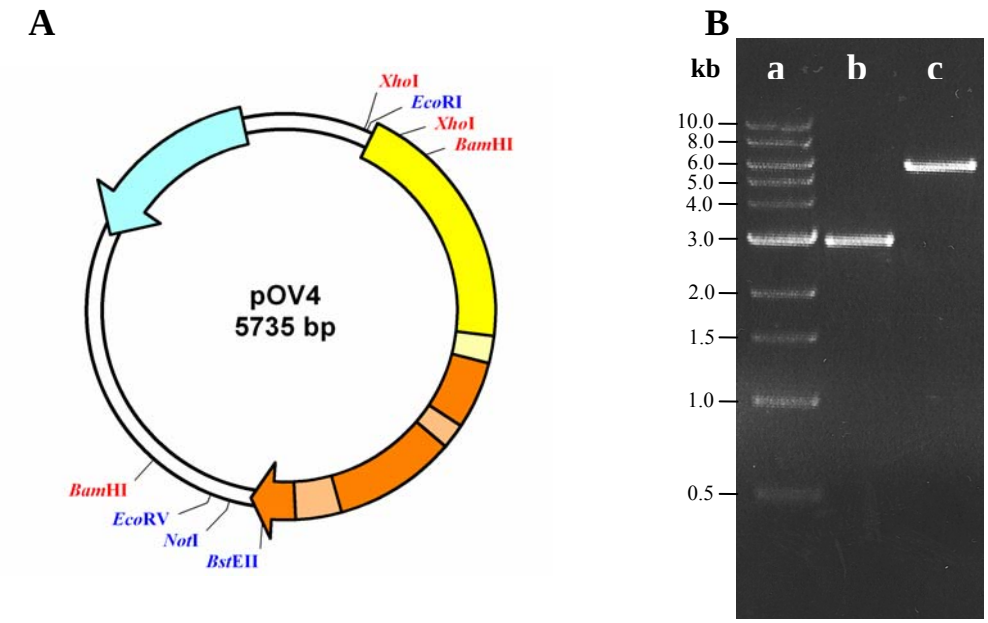


Figure 8. Construction of pOV4

A. Restriction map of pOV4. The *c1* genomic DNA fragment introduced to pCAP^s vector (s. 2.1.7) consists of the potential *c1* promoter region and 5'-UTR (yellow boxes), its first 3 exons (dark orange

boxes) and 2 introns (light orange boxes). The cyan arrow depicts the ampicillin resistance gene of pCAP^{s}. B. Restriction analysis of pOV4. The plasmid was digested (s. 2.2.1.6) with the below mentioned restriction enzymes and fragments were separated on a 1% agarose gel (s.2.2.1.1.3.2) for 1 h at 100 V: a) 1 kb DNA Ladder (NEB) (s. 2.1.10.1); b) pOV4 digested with *Bam*HI; c) pOV4 digested with *Xho*I.

pOV4 was characterized by restriction analysis with the restriction enzymes *Bam*HI and *Xho*I. After separation of the DNA fragments on an agarose gel, the expected bands of 2868 and 2867 bp in the case of the *Bam*HI digest and of 5735 bp in the case of the *Xho*I digest were detected (Figure 8). The absence of point mutations was proven by sequencing of the PCR containing fragment in pOV4 with the primers S1 and S2 (s. 2.1.9).

Thereafter, pOV4 was cut (s. 2.2.1.6) with *Bst*EII and *Not*I. A 957 bp long *c1* cDNA fragment of pCS30 (s. 2.1.8; Zhao et al., 2004) that was digested with the same enzymes was cloned into it, resulting in an insertion of the cDNA in opposite direction to the genomic DNA fragment. The resulting plasmid was named pOV5 (Figure 9).

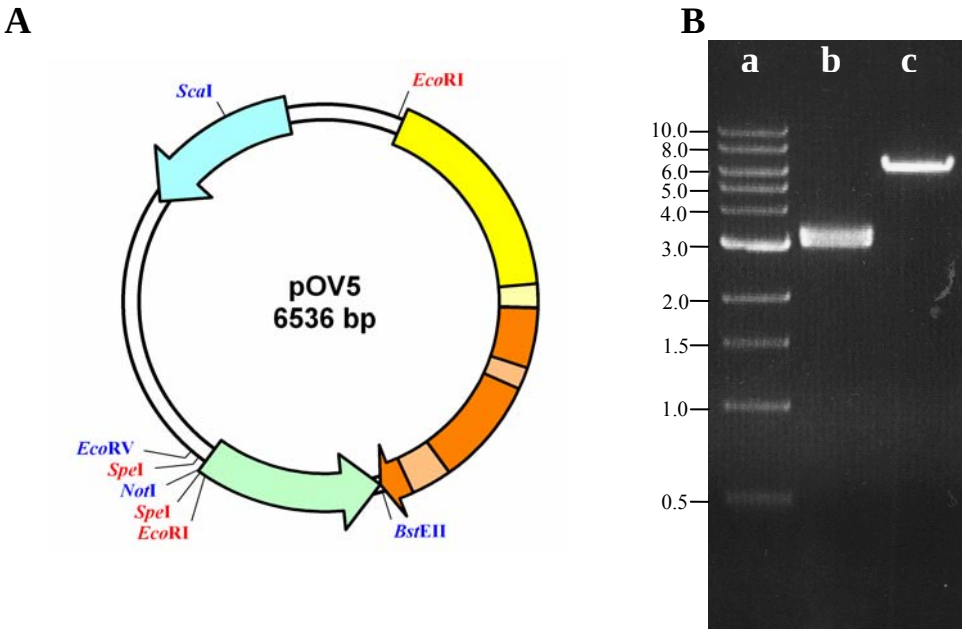


Figure 9. Construction of pOV5

A. Restriction map of pOV5. The *c1* cDNA fragment corresponding to the first 3 exons of *c1* gene (green box) was introduced in reversed orientation directly after the *c1* genomic DNA fragment consisting of the potential *c1* promoter region, its 5'-UTR (yellow boxes), its first 3 exons (dark orange boxes) and 2 introns (light orange boxes). The cyan arrow depicts the ampicillin resistance gene of pCAP^{s} (s. 2.1.7). B. Restriction analysis of pOV5. The plasmid was digested (s. 2.2.1.6) with the below mentioned restriction enzymes and fragments were separated on a 1% agarose gel (s. 2.2.1.1.3.2) for 1 h at 100 V: a) 1 kb DNA Ladder (NEB) (s. 2.1.10.1); b) pOV5 digested with *Eco*RI; c) pOV5 digested with *Eco*RV.

The characterization of pOV5 was done by restriction analysis with the restriction enzymes *Eco*RI and *Eco*RV. After separation of the DNA fragments on an agarose gel, the expected bands of 3505 and 3031 bp in the case of the *Eco*RI digest and of 6536 bp in the case of the *Eco*RV digest were detected (Figure 9).

Further, pOV5 was digested (s. 2.2.1.6) with *Sca*I and *Spe*I in order to introduce a 3864 bp DNA fragment from pSI103 (s. 2.1.8) cut with the same enzymes that contains the paromomycin resistance gene *aphVIII* (Sizova et al., 2001). The resulting plasmid was named pOV6 (Figure 10).

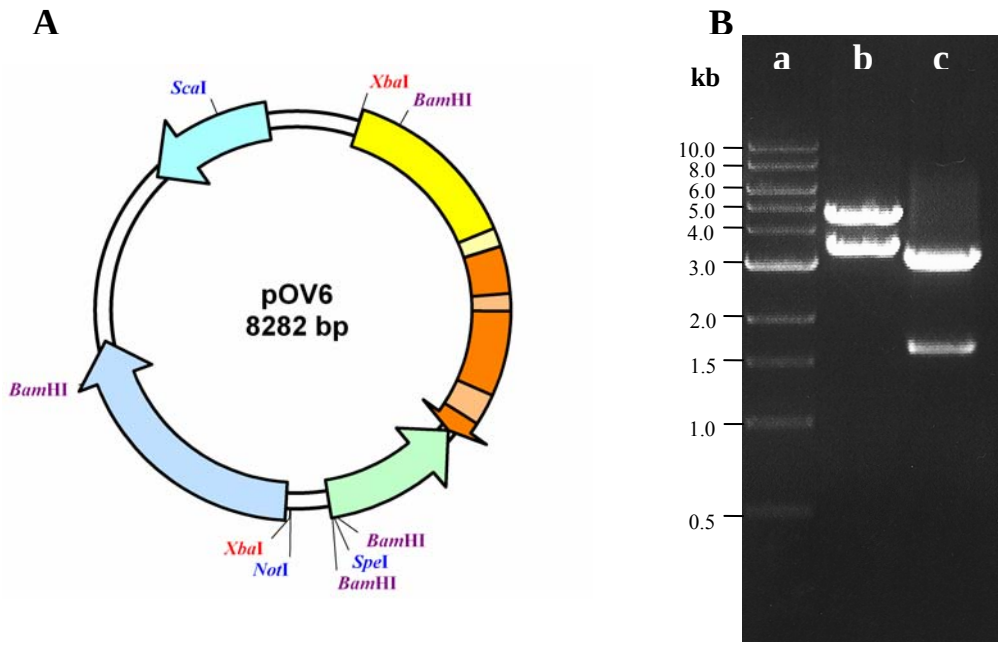


Figure 10. Construction of pOV6

A. Restriction map of pOV6. The paromomycin resistance gene *aphVIII* from pSI103 (blue arrow) was introduced to the plasmid containing the *c1* RNAi construct formed by a *c1* cDNA fragment corresponding to the first 3 exons of the *c1* gene (green box) cloned in reversed orientation directly after the *c1* genomic DNA fragment consisting of the potential *c1* promoter region and its 5'-UTR (yellow boxes), the first 3 exons (dark orange boxes) and 2 introns (light orange boxes). The cyan arrow depicts the ampicillin resistance gene of pCAP^{s} (s. 2.1.7). B. Restriction analysis of pOV6. The plasmid was digested (s. 2.2.1.6) with the mentioned below restriction enzymes and fragments were separated on a 1% agarose gel (s. 2.2.1.1.3.2) for 1 h at 100 V: a) 1 kb DNA Ladder (NEB) (s. 2.1.10.1); b) pOV6 digested with *Xba*I; c) pOV6 digested with *Bam*HI.

pOV6 was characterized by restriction analysis with the restriction enzymes *Xba*I and *Bam*HI. After separation of the DNA fragments on an agarose gel the expected bands of 4498 and 3787 bp in the case of the *Xba*I digest and of 3251, 3172, 1847 and 12 bp in the case of the *Bam*HI digest were detected (Figure 10) with the exeption of the 12 bp fragment.

3.1.1.2 Selection of C1 silenced strains of *C. reinhardtii* transformed with pOV6

C. reinhardtii cells were transformed with pOV6 (s. 2.2.2.5). Transformed strains (C1-sil) grown under selection of paromomycin were used for further analysis. Cells were grown to a cell density of about 1 - 5 x 10^6 cells/mL and protein crude extracts were prepared (s. 2.2.3.1). For comparison, a crude extract from non-transformed wild-type cells was used. Different amounts of proteins from wild-type (100, 50 and 25 µg per lane) were separated on SDS-PAGE (s. 2.2.3.4) and quantitatively compared to proteins from transformed strains (100 µg per lane) after immunoblotting (s. 2.2.3.6) with the anti-C1 antibody. Equal loading was checked by Ponceau staining (s. 2.2.3.7). In total, ca. 150 strains were checked. Silencing down to a level of about 25-30% was observed in some transgenic strains (e.g., C1-sil_{35}), but some strains also showed only silencing down to 40-80% (e.g., C1-sil_{72}; Figure 11, Table 1). The strains depicted in Figure 11 were used for further characterization (s. 3.1.2.2 and 3.1.2.3).

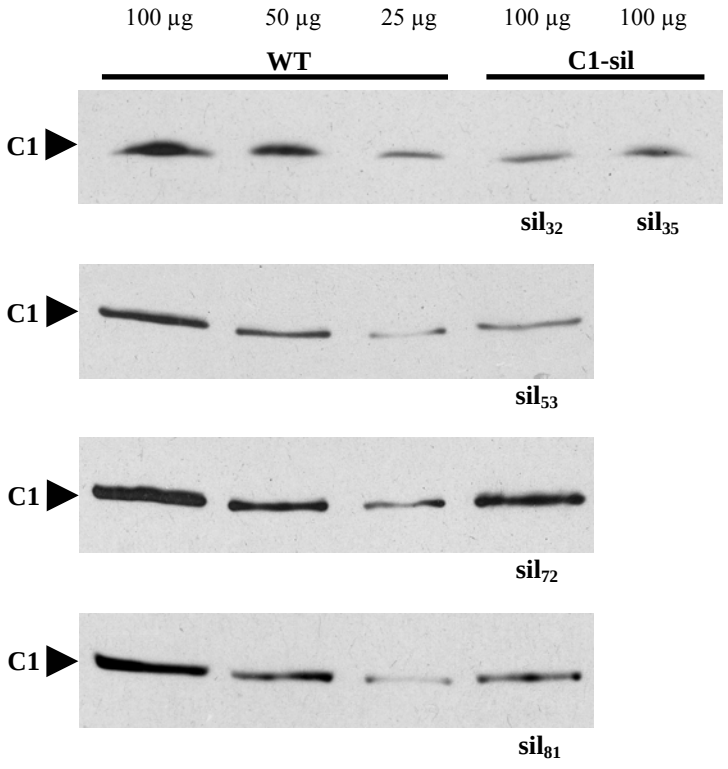


Figure 11. Analysis of C1-sil strains by immunoblotting with anti-C1 antibodies

Different amounts of proteins from a crude extract (s. 2.2.3.1) (100, 50 and 25 µg per lanes) of wild-type (WT) cells were separated on a 9% SDS-PAGE (s. 2.2.3.4) and used for immunoblotting with the anti-C1 antibodies (s. 2.2.3.6) along with protein crude extracts (100 µg per lane) from different C1-silenced strains (C1-sil_{32}, C1-sil_{35}, C1-sil_{53}, C1-sil_{72} and C1-sil_{81}). The position of C1 is indicated by an arrow.

Table 1. Quantification of the C1 expression level in the C1-sil strains

Strain	Level of silencing
C1-sil$_{32}$	26
C1-sil$_{35}$	30
C1-sil$_{53}$	41
C1-sil$_{72}$	74
C1-sil$_{81}$	53

Densitometry quantifications (s. 2.2.3.12) were done. "Level of silencing" indicates the level of C1 expression (%) in different transformed strains determined by a calibration curve, correlating different amounts of C1 from crude extracts of wild-type with those of the silenced strains.

It should be mentioned that the analysis of the strains with a high level of C1 silencing (e. g., C1-sil$_{35}$) was particularly complicated since the level of C1 reverted within a few weeks back to wild-type level. After transformation and selection on paromomycin plates, colonies were always grown up and checked by Western analysis for their degree of silencing. If silencing of C1 was occurring, the same colony was grown up and checked again by Western analysis to verify the result. In most, but not all cases, silencing was still maintained within this time range. But in case of a further repeat one or two weeks later, reversion back to wild-type level was already completed in most analyzed strains.

3.1.2 Characterization of C1-sil strains

3.1.2.1 Determination of the C3 level in the strains with decreased level of C1 expression

In all studied model organisms the oscillatory system is driven by positive and negative feedback loops. Thereby, variations in protein level of any clock component can affect the “normal” expression of other components. For example, in *N. crassa* a knock-out of FRQ leads to decreased levels of WC-1 (White Collar-1; Lee et al., 2000). Also, co-regulation was found in some multiprotein complexes of *C. reinhardtii*, such as components of the photosystems (Wostrikoff et al., 2004; Göhre et al., 2006). Therefore, it was of interest to understand if down-regulation of one

subunit of CHLAMY1 could cause an effect on the “normal” expression of the other subunit. It was examined in the C1-sil strains if the level of C3 was changed in parallel. In strains where C1 was silenced down to 25-30%, a strong co-regulation of the C3 level was observed (e.g., C1-sil$_{32}$ and C1-sil$_{35}$; Figure 12, Table 2), whereby C3 was even more silenced than C1 (below 25%). In contrast, a less pronounced or no co-regulation of the C3 subunit was found in the transgenic strains that had shown a smaller degree of C1 silencing (e.g., C1-sil$_{72}$; Figure 12, Table 2). These data indicate that strong co-regulation of C3 in case of C1 silencing depends on a critical low level of C1 that has to be reached in the cell.

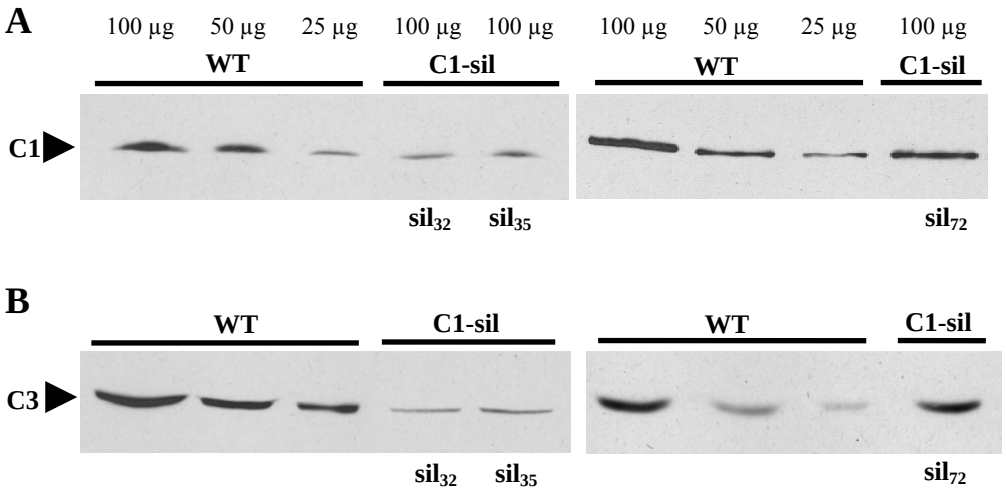


Figure 12. Silencing of C1 below a critical level causes strong co-regulation of C3

A. Different amounts of proteins from a crude extract (s. 2.2.3.1) (100, 50 and 25 µg per lanes) of wild-type (WT) cells and of crude extracts (100 µg per lane) from different C1-silenced strains (C1-sil$_{32}$, C1-sil$_{35}$ and C1-sil$_{72}$) were separated on a 9% SDS-PAGE (s. 2.2.3.4) and used for immunoblotting with the anti-C1 antibodies (s. 2.2.3.6). The position of C1 is indicated by an arrow. B. The same procedure as described before (see A) was carried out, but immunoblotting (s. 2.2.3.6) was done with anti-C3 antibodies. The position of C3 is indicated by an arrow.

Table 2. Quantification of the C3 expression level in the C1-sil strains

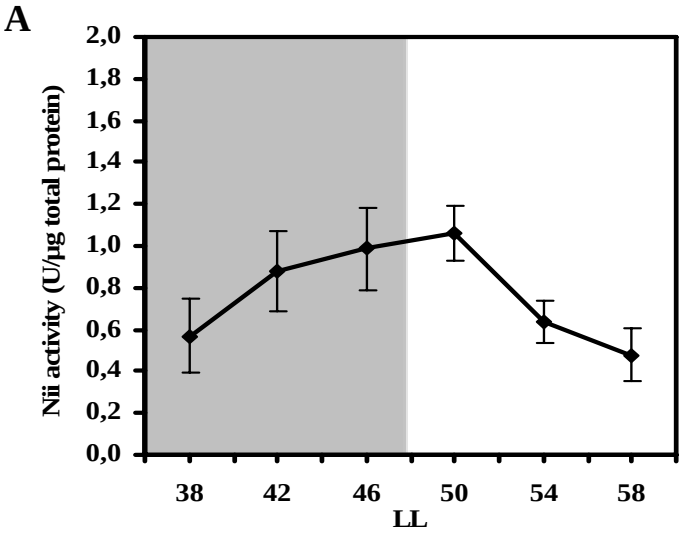
Strain	Level of C1 expression	Level of C3 expression
C1-sil$_{32}$	26	12
C1-sil$_{35}$	30	15
C1-sil$_{72}$	74	83

Densitometry quantifications (s. 2.2.3.12) were done. "Level of C1 expression" (%) was determined in different transformed strains by a calibration curve, correlating different amounts of C1 in crude

extracts from wild-type with those from the silenced strains. The same procedure was done to determine the "level of C3 expression".

3.1.2.2 Measurement of the circadian rhythm of NII activity in strains where the C1 level is reduced

In order to obtain functional information about the role of the C1 subunit in the circadian system of *C. reinhardtii*, it was important to find out if there is a difference in circadian rhythms between wild-type and C1sil strains. Therefore, the rhythm of NII activity was manually measured (s. 2.2.3.11) in a strain where the C1 level was reduced in comparison to wild-type. NII represents one of the key enzymes of nitrogen metabolism. Its activity was shown before to be diurnally regulated with a maximum during the middle of the light period (Pajuelo et al., 1995). *Nii* mRNA bears an UG-repeat in its 3'-UTR that is recognized by CHLAMY1 (Waltenberger et al., 2001). Thus, the influence in the expression level of CHLAMY1 on its direct output targets can be studied. For this purpose, cells that were entrained under a LD cycle were released to constant dim light (LL) and NII activity (s. 2.2.3.11) was measured starting from the second day during subjective night (LL38) till the end of the next subjective day (LL 58) in a four hours interval. In wild-type cells, NII showed a circadian rhythm in its activity, however with rather modest amplitude of about 2. Maximal activity occurred during early day-phase (Figure 13A).



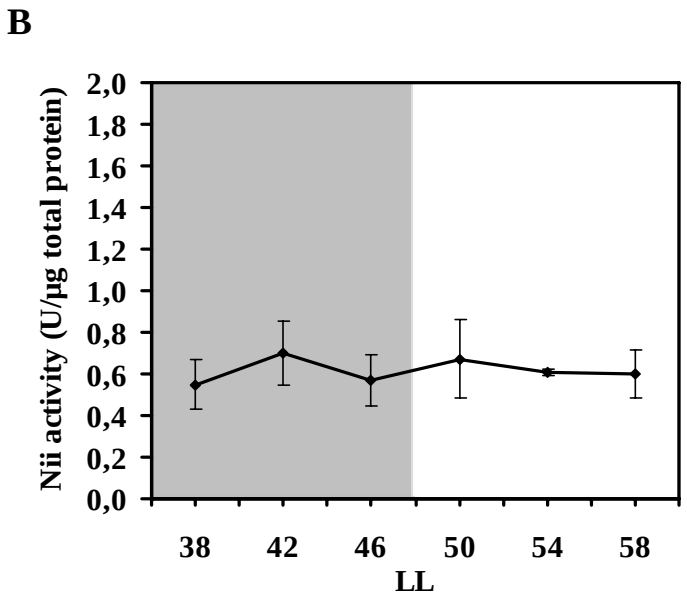


Figure 13. A decrease of the C1 level abolishes the circadian rhythmicity of NII activity in *C. reinhardtii*

Cells were grown under a LD (12:12) cycle and then transferred to constant conditions of dim light (LL 0). After 38 h, cells were harvested every 4 h at the indicated times. Crude extracts were prepared and enzyme activities were determined (s. 2.2.3.11). Error bars represent SEM of 3 independent measurements. Subjective night - gray background; subjective day - white background. A. Wild-type cells SAG 73.72. These measurements were done by E.-M. Schmidt. B. Strain C1-sil$_{81}$.

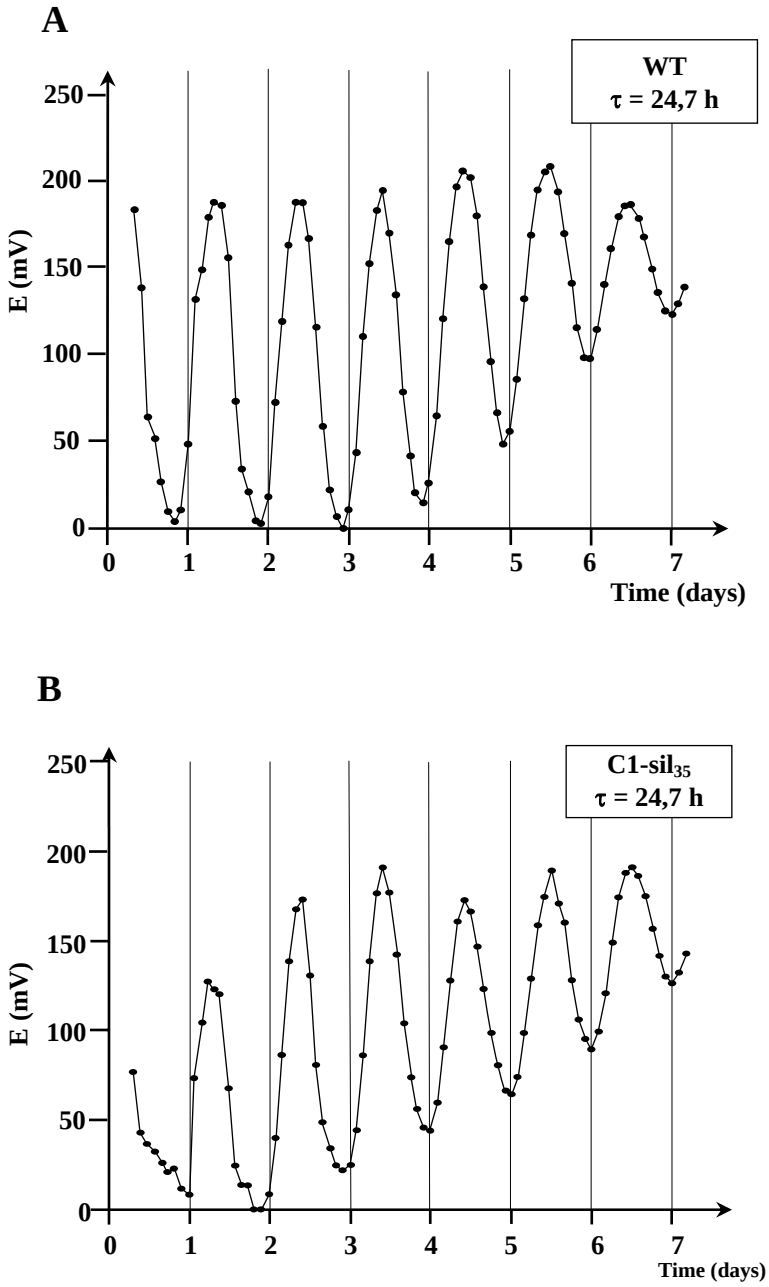
For analyzing NII activity in a C1 silenced strain, a most recent characterized strain was chosen, which is not very strongly silenced to avoid potential reversion during the experiments, as mentioned above. In C1-sil$_{81}$ (Figure 11, Table 1), the C1 level was reduced to about 53%. Immediately after the Western analysis, the strain was used for the measurements of NII activity and it was verified after the experiment that the C1 level was still reduced. In this case, arrhythmicity of NII activity was observed (Figure 13B). These data show that C1 is an essential component of the circadian system in *C. reinhardtii*.

3.1.2.3 Measurement of the circadian rhythm of phototaxis in C1-sil strains

To postulate the role of C1 in circadian system, it was also important to find out if another circadian output rhythm was disturbed in the same way.

First, automatic phototaxis measurements (s. 2.2.3.13) with several C1-sil strains, where C1 was silenced below 40% and C3 was strongly co-silenced, were done directly after the verification of C1 silencing and in all cases they showed wild-

type like behavior of phototaxis. As example, phototaxis of C1-sil$_{35}$ is shown (Figure 14B). However, the wild-type like behavior could be due to the fact that the C1 level had already been reverted to wild-type since every analysis of the silencing level of C1 after the phototaxis assay revealed complete reversion of the C1 level back to wild-type in these strains (Figure 14E, Table 3).



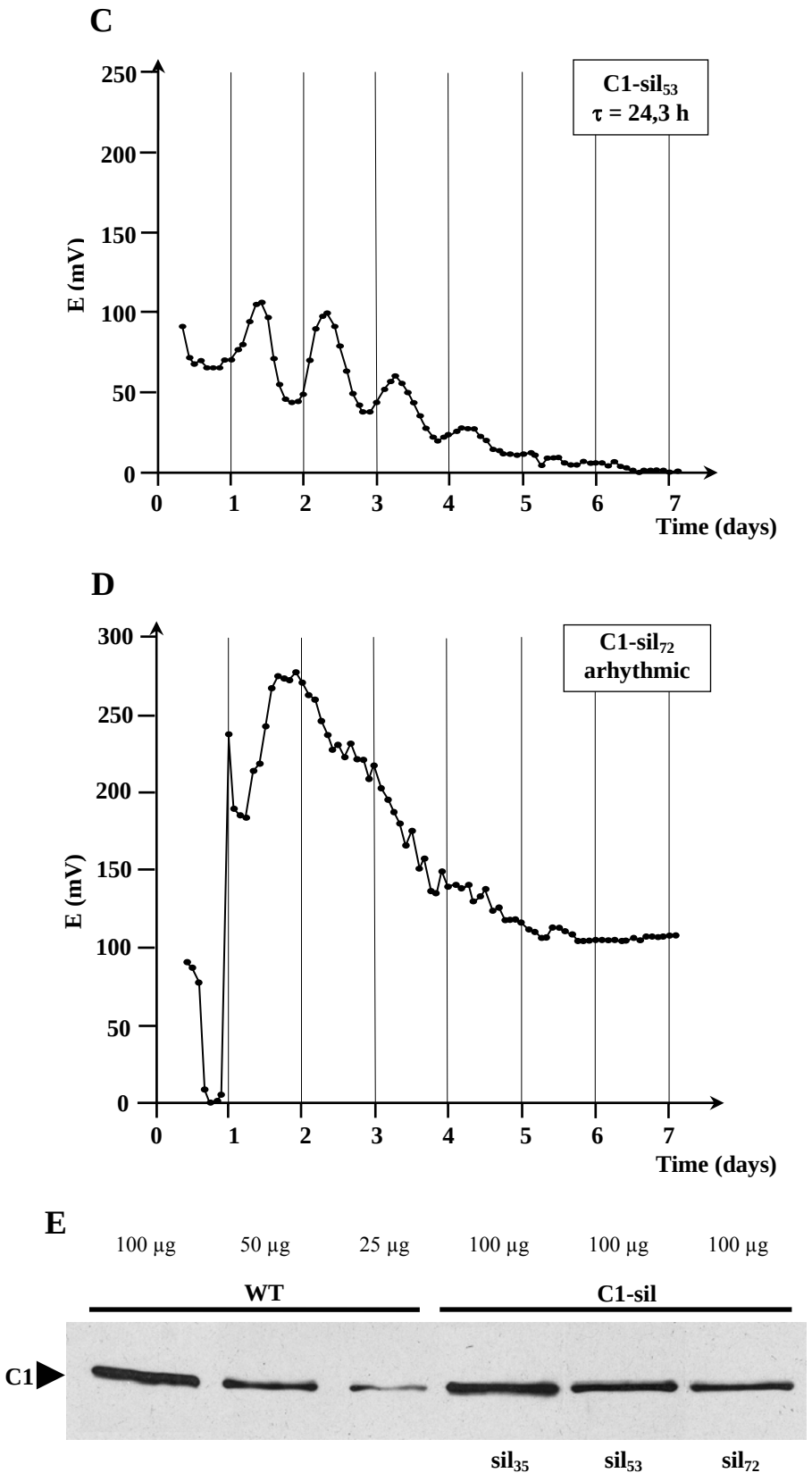


Figure 14. Silencing of C1 can cause arrhythmic behavior

Phototaxis rhythms of wild-type (A) and C1-sil strains (C1-sil_{35} (B), C1-sil_{53} (C) and C1-sil_{72}(D)) were measured (s. 2.2.3.13) using the automated phototaxis-measuring unit developed by Mergenhagen (1984). "E" represents the extinction in mV. Time (days) indicates how long cells were exposed to constant darkness. A. Phototaxis of wild-type cells under free running conditions. The free

running period of 24,7 h is indicated. B. Phototaxis of C1-sil$_{35}$ under free running conditions. The free running period of 24,7 h is indicated. It should be noted that the C1 as well as the C3 levels were fully reverted back to wild-type level when C1-sil$_{35}$ cells were checked after the phototaxis experiment. C. Phototaxis of C1-sil$_{53}$ under free running conditions. The free running period of 24,3 h is indicated. In the case of C1-sil$_{53}$, silencing (~84% of the WT level) was still observed after the phototaxis experiment. D. Phototaxis of C1-sil$_{72}$ under constant conditions showing complete arrhythmicity. In this case, silencing (~70% of the WT level) was still observed after the phototaxis experiment. E. Analysis of the C1 expression level by immunoblotting in C1-sil strains after phototaxis experiment. Different amounts of proteins from a crude extract (s. 2.2.3.1) (100, 50 and 25 µg per lanes) of wild-type (WT) cells were separated on a 9% SDS-PAGE (s. 2.2.3.4) and used for immunoblotting with the anti-C1 antibodies (s. 2.2.3.6) along with protein crude extracts (100 µg per lane) from different C1-silenced strains (C1-sil$_{35}$, C1-sil$_{53}$ and C1-sil$_{72}$). The position of C1 is indicated by an arrow.

Table 3. Quantification of the C1 expression level in the C1-sil strains before and after the phototaxis test

Strain	Before	After
C1-sil$_{35}$	30	107
C1-sil$_{53}$	41	84
C1-sil$_{72}$	74	70

Densitometry quantifications (s. 2.2.3.12) were done. The level of C1 expression (%) "before" (according to Table 1) and "after" the phototaxis test was determined in different transformed strains by a calibration curve, correlating different amounts of C1 in crude extracts from wild-type with those from the silenced strains.

Only in the case of strains where silencing was not as pronounced, reduced levels of C1 could still be found by Western analysis (s. 2.2.3.6) after the phototaxis assay (Figure 14E, Table 3) showing that the cells were still silenced in C1 during the assay. Notably, these transgenic lines showed arrhythmic behavior from the very beginning under constant darkness (C1-sil$_{72}$, Figure 14D) or after three days under constant conditions (C1-sil$_{53}$, Figure 14C). Thus, reduced level of C1 causes the same effect (arrhythmicity) on the phototaxis rhythm as on the rhythm of NII activity (s. 3.1.2.2). These data suggest that C1 is an essential component of the circadian clock in *C. reinhardtii*.

3.2 Investigation of the role of the C1 and C3 subunits of CHLAMY1 in temperature integration

In the first part (s. 3.1) of my thesis it was shown that changes in the C1 expression level can cause arrhythmicity of the phototaxis (s. 3.1.2.3) and NII activity rhythms

(s. 3.1.2.2) demonstrating that this protein is an important component of the oscillatory machinery in *C. reinhardtii*. From the other side, changes in the C3 expression level were shown to lead to a shift in acrophase (Iliev et al., 2006). Thus, it was interesting to analyze if the duo of the C1/C3 subunits of the RNA-binding protein CHLAMY1 could also be involved in temperature integration, a prerequisite for entrainment by temperature cycles and temperature compensation.

3.2.1 Detection of the expression level of the C1 and C3 subunits at different temperatures

To study a possible role of C1/C3 in temperature integration, it was important to check first if their expression is itself temperature sensitive. Therefore, *C. reinhardtii* wild-type cells were grown at different temperatures and C1/C3 expression levels were analyzed. Beside the usual cultivation temperature of 23ºC, a lower (18ºC) as well as a higher temperature (28ºC) were selected that are still in the physiological range of *C. reinhardtii* (Harris, 1989), but differ in total of 10ºC. Cells were harvested during early day (LD2) when the binding activity of CHLAMY1 is low, and at early night (LD14) when it is high (Mittag, 1996). Protein crude extracts were prepared (s. 2.2.3.1) and equal amounts of protein per lane were separated on SDS-PAGE (s. 2.2.3.4) and immunoblotted with anti-C1 antibodies (s. 2.2.3.6) (Figure 15). For comparative immunoblots, equal amounts of proteins per lane were visually checked by Ponceau staining (s. 2.2.3.7) of the membrane. In addition, a duplicate gel was stained with Coomassie (s. 2.2.3.5) to corroborate that similar amounts of proteins were loaded (Figure 15C).

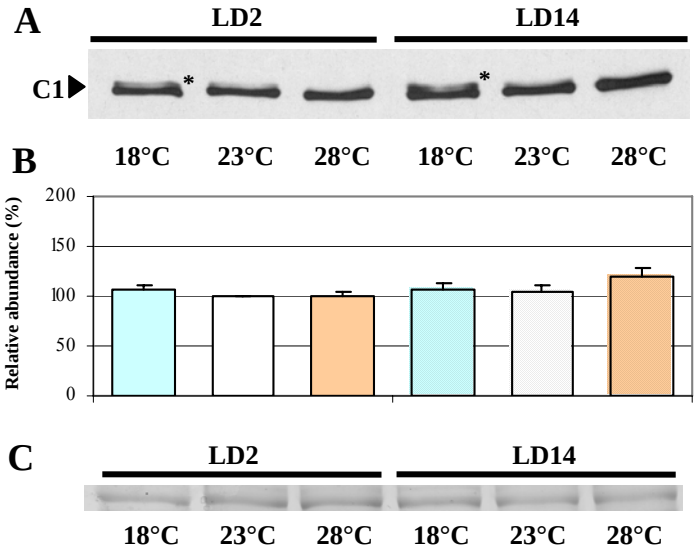


Figure 15. The C1 expression level does not change significantly in wild-type cells grown at different temperatures

A. Cells were grown at 18, 23 and 28ºC and harvested during early day (LD2) and early night (LD14). Crude extracts (s. 2.2.3.1) were prepared and proteins (100 µg per lane) were separated on a 9% SDS-PAGE (s. 2.2.3.4) along with a molecular mass standard and immunoblotted with anti-C1 antibodies (s. 2.2.3.6). * indicates a possible modified form of C1. B. Densitometry quantifications (s. 2.2.3.12) were done with three independent experiments. Thereby, the amount of C1 detected in cells grown at 23ºC and harvested at LD2 was set to 100% and used as reference. C. A random nonspecific Coomassie blue-stained band from a duplicate gel shows that similar amounts of proteins were loaded.

At both time-points, there was no significant change in the level of C1 at the different temperatures visible (Figure 15). However, at a closer look it seemed that there might be a change in a posttranslational modification at the lower temperature, which was analyzed further (see below).

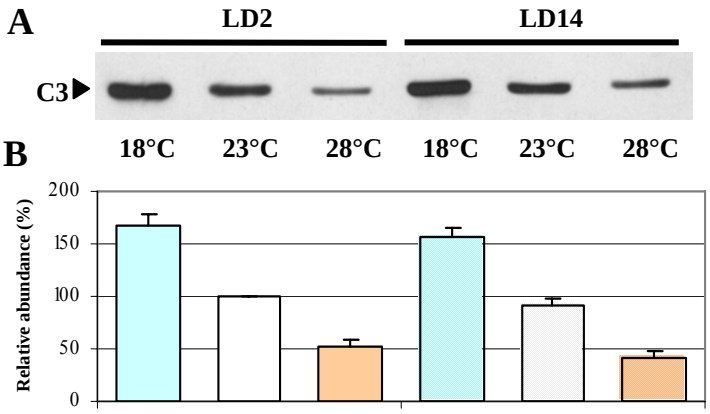


Figure 16. The C3 expression level increases at low temperature in wild-type cells of *C. reinhardtii*

The same procedure as described in Figure 15 was carried out, but immunoblotting (s. 2.2.3.6) was done with anti-C3 antibodies. The loading control is shown in Figure 15C.

In contrast to C1, the level of C3 was significantly changed at the different temperatures as could be seen in immunoblots with anti-C3 antibodies (s. 2.2.3.6) where the same protein crude extracts were used as for the C1 detection (Figure 16). The highest amount of C3 was present at the low temperature and its lowest amount appeared at the high temperature. This was visible at both selected time points. The change in amplitude was ca. three-fold. As was already described before, the equal amount of proteins loaded was visually checked by Ponceau staining (s. 2.2.3.7) of the membrane and, in addition, by Coomassie staining of a duplicate gel (s. 2.2.3.5) (Figure 15C).

3.2.2 Investigation of the possible posttranslational modification of the C1 subunit at different temperatures

To check if C1 might be indeed present in different posttranslational modified forms, proteins from protein crude extract (s. 2.2.3.2) were separated by a standardized 2-DE procedure (s. 2.2.3.10) and immunoblotted with anti-C1 antibodies (s. 2.2.3.6).

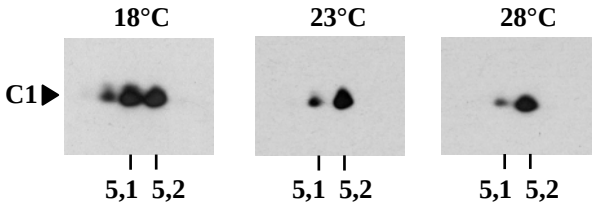


Figure 17. C1 is modified at the low temperature.

C1 immunodetection from proteins of crude extracts (s. 2.2.3.2) separated by 2-DE. Cells were grown at 18, 23 and 28ºC and harvested during early day (LD2). Crude extracts were prepared and proteins (300 µg per assay) were separated on standardized 2-DEs (s. 2.2.3.10). For the first dimension, an IPG strip of pH 3 to 10 was taken, in the second dimension, a 10% SDS-PAGE was used along with a molecular mass standard. The proteins were then immunoblotted with anti-C1 antibodies (s. 2.2.3.6). The positions of pH 5.1 and 5.2 that are close to the theoretical pI of C1 (5.17) are indicated.

At 28ºC, C1 appeared mostly as a single spot on 2-DE at a pH of about 5.2 (Figure 17). That is in accordance with its theoretical isoelectrical point (pI: 5.17). A minor additional spot was present towards the lower pH, indicating that a small part of C1 is present in a posttranslational modified form. At 23ºC and especially at 18ºC, this modified state of C1 was significantly increased. Thus, C1 gets posttranslational modified especially at low temperature.

Since the modified forms of C1 did not show a significant change in molecular mass, but a change towards the acidic pH, it was postulated that phosphorylation could represent the modification. To check for this, the cells were grown again at the low temperature (18ºC) when the state of the posttranslational modification is highest, and the protein crude extract (s. 2.2.3.2) (300 µg) was treated either without (control) or with 1 µl (400 units) lambda protein phosphatase (s. 2.1.3) for 30 min at 30°C. This procedure is known to remove phosphate groups from proteins.

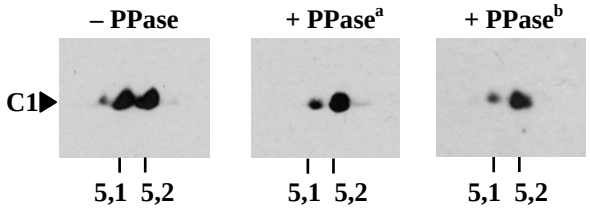


Figure 18. C1 is hyper-phosphorylated at the low temperature.

The same procedure as described before (Figure 17) was undertaken with cells grown at 18ºC (- PPase). In one case (+ $PPase_a$), the extract was treated for 30 min with Lambda PPase (NEB, Frankfurt) (s. 2.1.3) at 30ºC according to the protocol of the supplier. In another case (+ $PPase_b$), the used amount of PPase was increased five times.

Separation of the proteins by 2-DE (s. 2.2.3.10) and immunoblotting (s. 2.2.3.6) with the anti-C1 antibodies showed that the modified forms of C1 were significantly reduced in the phosphatase treated cells (Figure 18; + $PPase^a$). These data suggest that C1 is hyper-phosphorylated at the low temperature. However, there was still a small amount of a modified C1 present even when the amount of lambda protein phosphatase was five times increased for the incubation (Figure 18; + $PPase^b$). This could mean that C1 has an additional posttranslational modification. But it is also possible that phosphorylated C1 that is present in the C1-C3 and in addition in the ≥680 kDa complex during day-phase cannot be fully accessed by the PPase.

Densitometry analysis (s. 2.2.3.12) showed that the phosphorylated degree of C1 increased from ca. 28% at 28ºC to ca. 51% at 18ºC (Table 4).

Table 4. The temperature dependent phosphorylation degree of C1 in wild-type

Strain	Temperature	Percentage	SEM
WT	18°C	50.7*	1.2
WT	23°C	36.5	2.9
WT	28°C	27.8*	2.9

Densitometry quantifications (s. 2.2.3.12) were done with two (or three indicated by *) independent experiments. Percentage indicates the amount (%) of the phosphorylated degree of C1. Thereby, as 100% was taken the volume of all spots.

3.2.3 Investigation of the regulation level of the temperature dependent *c3* expression

To find out if the differential regulation of *c3* occurs at the transcriptional or translational level, an inhibitor protocol was developed (Figure 19).

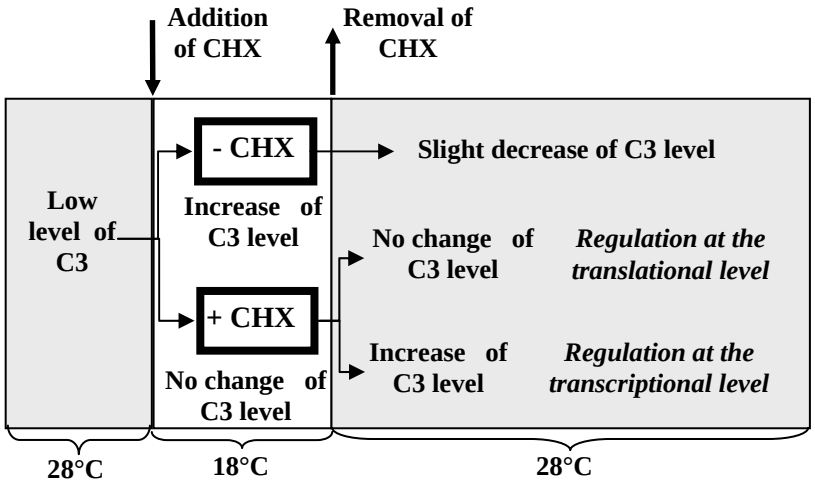


Figure 19. Scheme of the experimental procedure with and without cycloheximide (CHX, s. 2.1.4) treatment

Wild-type cells were grown at 28ºC under a LD cycle and transferred at LD4 to 18ºC by centrifuging them and dissolving them in 18ºC pre-cooled TAP medium (s. 2.1.12.2). In one case, CHX (s. 2.1.4) was added. After 3 h at 18ºC, the two different cell cultures were centrifuged again, washed with TAP medium and then dissolved in 28ºC pre-warmed TAP medium (s. 2.1.12.2) without the inhibitor. Cells were grown for additional 4 h at 28ºC. In the presence of CHX at 18ºC, no translation of *c3* mRNA can occur, but *c3* mRNA may accumulate if the up-regulation of *c3* at 18ºC occurs at the transcriptional level, and thus an increase of C3 protein after removal of CHX can be expected.

For the inhibitor experiment (Figure 19), the cells were grown first at the high temperature where the level of C3 is low. After that, cycloheximide (s. 2.1.4), an inhibitor of translation, was added for a 3 h period and at the time of addition the cells were transferred to 18ºC. In parallel, a control culture without cycloheximide was put to the low temperature. If *c3* up-regulation would be controlled at the transcriptional level, the cells should induce (with or without cycloheximide) and accumulate (in the presence of cycloheximide) *c3* mRNA during this time. In absence of the inhibitor, the produced mRNA should be immediately translated and an increase of C3 protein should occur. This was found after 3h at 18ºC with the control (Figure 20AC).

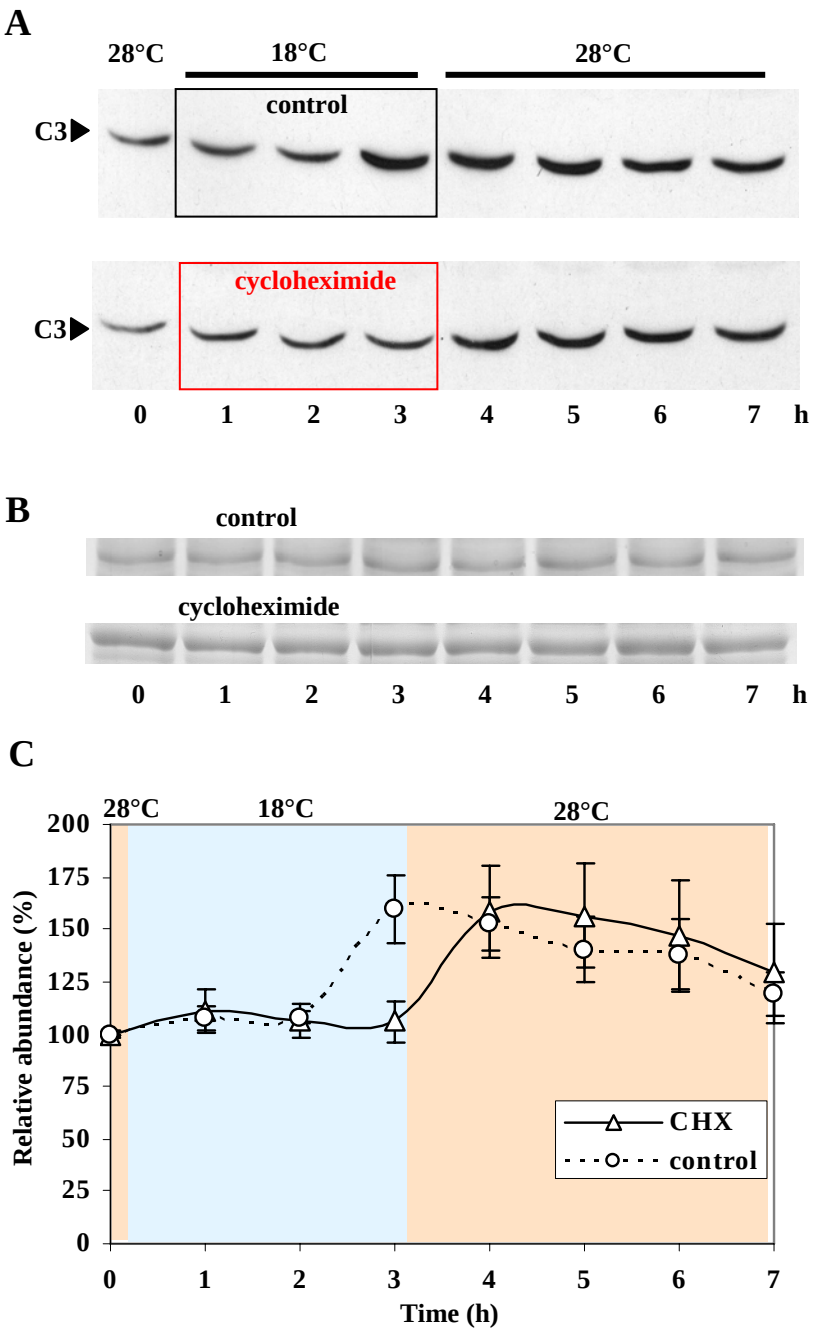


Figure 20. Up-regulation of *c3* occurs at the transcriptional level

A. Immunodetection of C3 levels. Cells from the control and cycloheximide (s. 2.1.4) treated cells were harvested before, in the middle and after the treatment as described before (Figure 19) at the indicated time-points and protein crude extracts were prepared (s. 2.2.3.1). Proteins (50 µg per lane) were separated on a 9% SDS-PAGE (s. 2.2.3.4) along with a molecular mass standard and immunoblotted with anti-C3 antibodies (s. 2.2.3.6). B. A random nonspecific Coomassie blue-stained (s. 2.2.3.5) band from duplicate gels shows that similar amounts of proteins were loaded. C. Densitometry quantifications (s. 2.2.3.12) were done with tree independent experiments. The relative abundances of C3 protein in the control (circles, dash-line) and cycloheximide treated cells (triangles, continuous line) are presented. Thereby, the amount of C3 at time-point 0 (28°C) was set to 100% and used as reference.

In the presence of cycloheximide, this increase was not visible, as expected. After the 3h at 18°C, the inhibitor was removed by washing the cells in TAP medium (s. 2.1.12.2), and the cells were transferred back to 28ºC where the induction of *c3* expression should be significantly reduced and were kept there for additional 4h. In the control, the level of C3 stayed similar after the switch to the high temperature. In case of the culture that had been treated with cycloheximide an increase in the level of protein was visible within the first hour after the switch to the higher temperature (Figure 20AC). Equal amounts of proteins per lane were checked in two duplicate gels that were stained with Coomassie (s. 2.2.3.5) (Figure 20B).

It was concluded that *c3* mRNA was accumulated during the cycloheximide treatment, but could not be translated due to the inhibitor. Once the stimulating low temperature conditions were switched back to 28ºC and the inhibitor was removed from the cells, accumulated *c3* mRNA was translated and caused the increase in C3. This would not occur if *c3* would be regulated at the translational level, because during the inhibitor treatment at the low temperature no *c3* mRNA would be induced and could thus accumulate. Therefore, the up-regulation of *c3* at the low temperature occurs at the transcriptional level.

3.2.4 Detection of potential components playing a role in temperature dependent changes of the C1 and C3 subunits

3.2.4.1 Screening for kinases that are able to phosphorylate C1

It was also interesting to find out which kinase(s) might be responsible for the hyper-phosphorylation of C1 at the low temperature and if the phosphorylation level of C1 could influence C3 expression. Theoretical predictions of phosphorylation sites of C1 were done using internet server (NetPhosK 1.0 server under www.cbs.dtu.dk/services/NetPhosK/; Blom et al., 2004) that highlighted several protein kinases that are able to phosphorylate C1 (Figure 21).

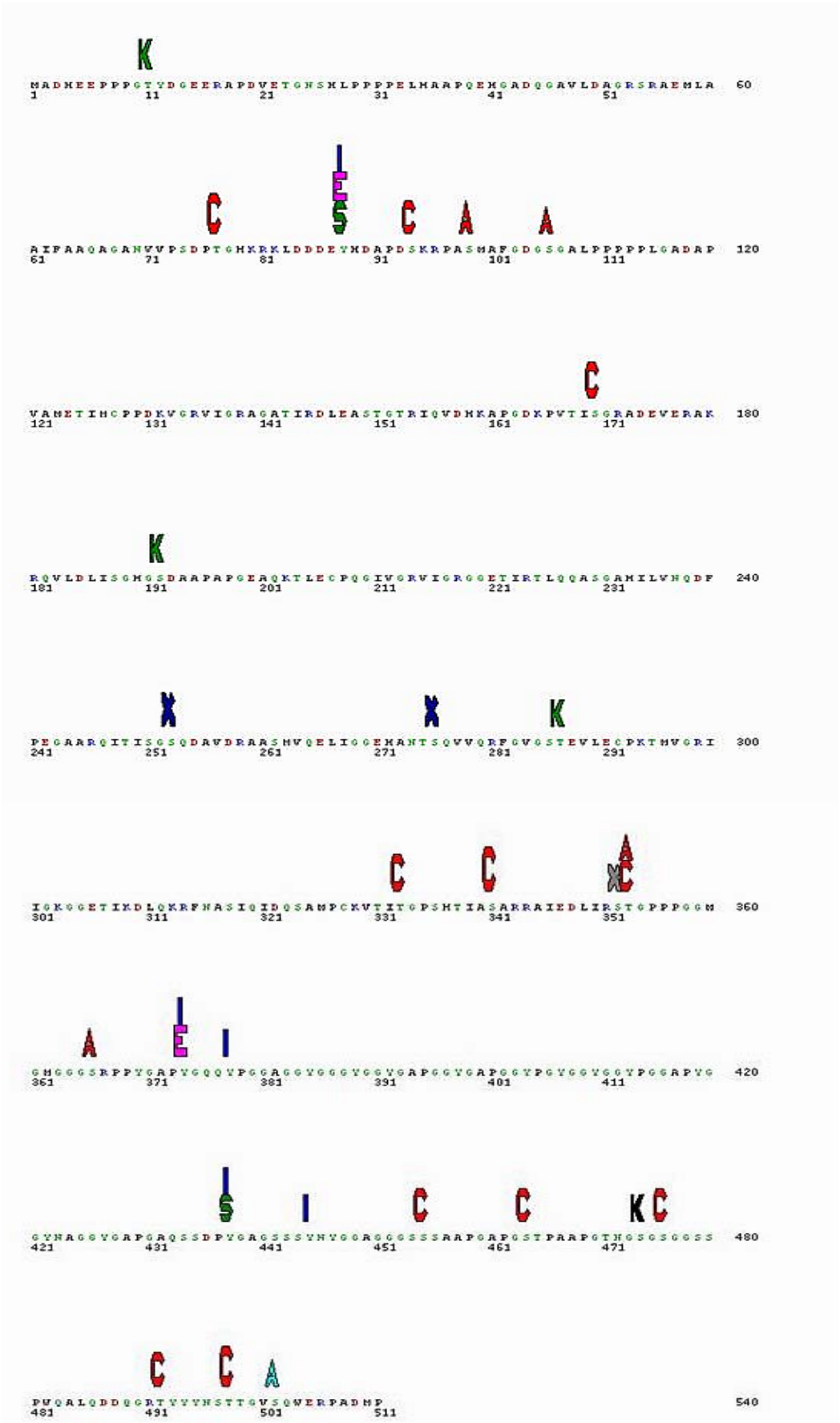


Figure 21. Theoretical predictions of phosphorylation sites of C1

The amino acid sequence of C1 was uploaded to the site www.cbs.dtu.dk/services/NetPhosK/ and analysed for potential phosphorylation sites that can be recognized by the listed below kinases. Potential phosphorylation sites are marked over the sequence with followind letters: green **K** - CKII (CASEIN KINASE II), red **C** - PKC (PROTEIN KINASE C), blue **I** - INSR (INSULIN RECEPTOR KINASE), pink **E** - EGFR (EPIDERMAL GROWTH FAKTOR RECEPTOR PROTEIN KINASE), green **S** - SRC (SARCOME PROTEIN TYROSINE KINASE), red **A** - PKA (PROTEIN KINASE A), blue **X** - DNAPK (DNA DEPENDENT PROTEIN KINASE), grey **X** - GSK (GLYCOGEN SYNTASE KINASE), black **K** -CK1 (PROTEIN KINASE 1), cyan **A** - ATM (ATAKIA-TELANGIECTASIA MUTATED KINASE).

Among several depicted kinases, CK1 was present that was an object of a parallel research carried out in our lab. In that study, it was shown that the CK1 silencing causes period shortening of the circadian clock in *C. reinhardtii* (Schmidt et al., 2006). For the CK1 silencing an RNAi strategy was used.

3.2.4.2 Investigation of the CK1 influence on temperature dependent changes of C1 and C3 subunits

3.2.4.2.1 Construction of the CK1 silencing vector

The genomic DNA 1576 bp fragment containing the potential promoter region of *ck1* (908 bp in front of the AUG including the predicted 258 bp 5'-UTR) and the first four exons and three introns of *ck1* (gene model estExt_gwp_1W.C_200087, vs3 of the genome) was PCR-amplified (s. 2.2.1.1.2) using the GC-RICH PCR System kit from Roche (s. 2.1.4) according to the instructions of the kit along with genomic DNA from *C. reinhardtii* and the primers (s. 2.1.9): OMM232 (as a sense PCR primer) and OMM249 (as an antisense PCR primer) (Figure 22).

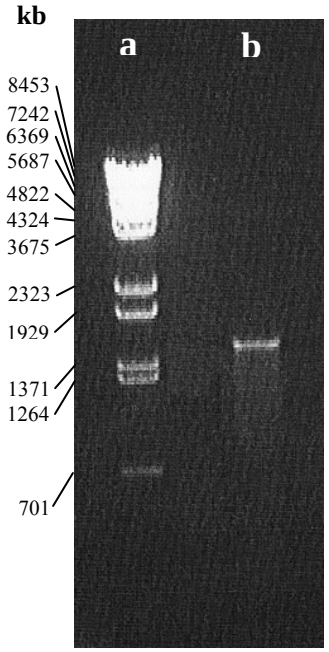


Figure 22. Detection of the *ck1* containing PCR product by agarose gel electrophoresis

PCR reaction was carried out using GC-RICH PCR System from Roche (s. 2.1.4) with OMM232 and OMM249 primers (s. 2.1.9). The product was separated (s. 2.2.1.1.3.2) on a 1% agarose gel for 2 h at 100 V and visualized by ethidium bromide staining. a) Ladder: Lambda DNA-*Bst*EII digest (2.1.10.1). b) PCR product, containing fragment of *ck1* genomic DNA together with its potential promoter region.

The genomic DNA was cloned into the *Mlu*NI of the pCAP^S vector (s. 2.1.7) from the PCR Cloning Kit (Roche) (s. 2.1.4) following the instructions of the manual and the resulting plasmid was named pOV1 (Figure 23).

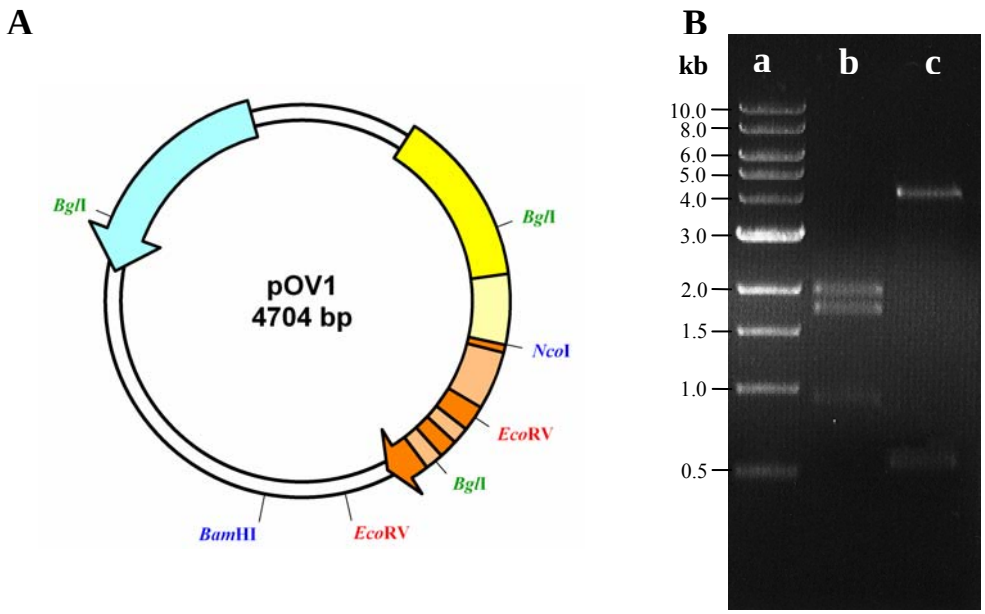


Figure 23. Construction of pOV1

A. Restriction map of pOV1. The DNA fragment, introduced to pCAP^s vector (s. 2.1.7), consists of the potential *ck1* promoter region and its 5'-UTR (yellow boxes), its first 4 exons (dark orange boxes) and 3 introns (light orange boxes). The cyan arrow depicts the ampicillin resistance gene of pCAP^s. B. Restriction analysis of pOV1. The plasmid was digested (s. 2.2.1.6) with the below mentioned restriction enzymes and fragments were separated on a 1% agarose gel (s. 2.2.1.1.3.2) for 1 h at 100 V: a) Ladder: 1 kb DNA Ladder (NEB) (s. 2.1.10.1); b) pOV1 digested with *Bgl*I; c) pOV1 digested with *Eco*RV.

pOV1 was characterized by restriction analysis with the restriction enzymes *Bgl*I and *Eco*RV. After separation of the DNA fragments on an agarose gel, the expected bands of 2009, 1766 and 929 bp (weakly visible) in the case of the *Bgl*I digest, and of 4137 and 567 bp (weakly visible) in the case of the *Eco*RV digest were detected (Figure 23). The absence of point mutations was proved by sequencing of the PCR containing fragment of pOV1 with S1 and S2 primers (s. 2.1.9). Thereafter, pOV1 was cut (s. 2.2.1.6) with *Cla*I and *Bam*HI and a 258 bp long *ck1* cDNA fragment of pGG1 (s. 2.1.8) that was digested with the same enzymes was cloned into it resulting in an insertion of the cDNA in opposite direction to the genomic DNA fragment. The resulting plasmid was named pOV2 (Figure 24).

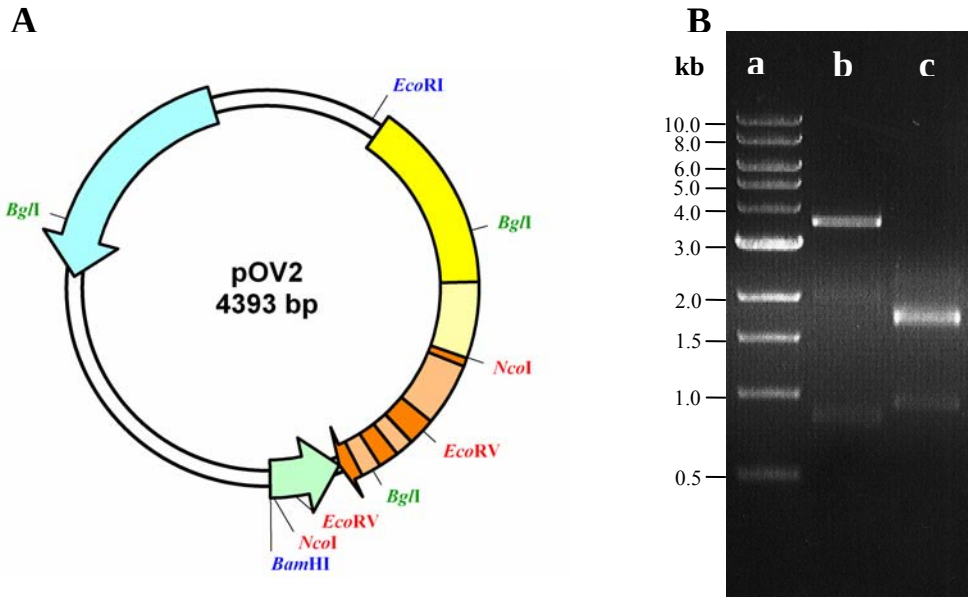


Figure 24. Construction of pOV2

A. Restriction map of pOV2. The *ck1* cDNA fragment corresponding to the first 4 exons of *ck1* gene (green box) was introduced in reversed orientation directly after the *ck1* genomic DNA fragment, which consisted of the potential *ck1* promoter region and *ck1* 5'-UTR (yellow boxes), its first 4 exons (dark orange boxes) and 3 introns (light orange boxes). The cyan arrow depicts the ampicillin resistance gene. B. Restriction analysis of pOV2. The plasmid was digested (s. 2.2.1.6) with mentioned restriction enzymes and fragments were separated on a 1% agarose gel (s. 2.2.1.1.3.2) for 1

h at 100 V: a) Ladder: 1 kb DNA Ladder (NEB) (s. 2.1.10.1); b) pOV2 digested with *Nco*I; c) pOV2 digested with *Bgl*I.

The characterization of pOV2 was done by restriction analysis with the restriction enzymes *Nco*I and *Bgl*I. After separation of the DNA fragments on agarose gel, the expected bands of 3549 and 844 bp (weakly visible) in the case of the *Nco*I digest and of 1766, 1698 and 929 bp (weakly visible) in the case of the *Bgl*I digest were detected (Figure 24).

pOV2 was digested (s. 2.2.1.6) with *Sca*I and *Bam*HI in order to introduce a 3858 bp DNA fragment from pSI103 (s. 2.1.8) partially digested with the same enzymes that contains the paromomycin resistance *aphVIII* gene (Sizova et al., 2001). The resulting plasmid was named pOV3 (Figure 25).

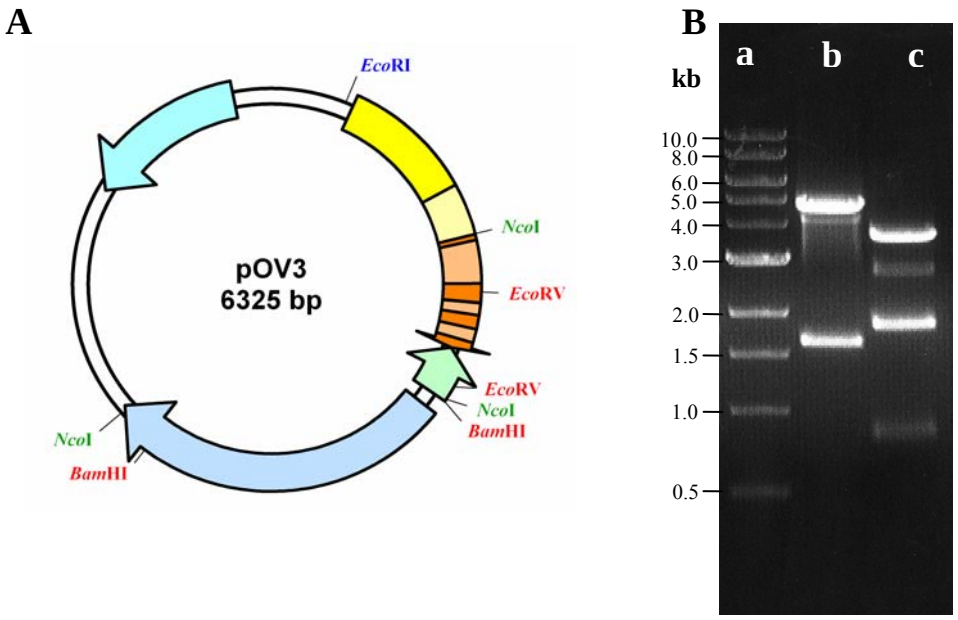


Figure 25. Construction of pOV3

A. Restriction map of pOV3. The paromomycin resistance gene *aphVIII* from pSI103 (blue arrow) was introduced to the plasmid containing the *ck1* RNAi construct formed by the *ck1* cDNA fragment corresponding to the first 4 exons of *ck1* gene (green box) cloned in reversed orientation directly after the *ck1* genomic DNA fragment consisted of potential *ck1* promoter region and *ck1* 5'-UTR (yellow boxes), first 4 exons (dark orange boxes) and 3 introns (light orange boxes) of *ck1* gene. The cyan arrow depicts the ampicillin resistance gene. B. Restriction analysis of pOV3. The plasmid was digested (s. 2.2.1.6) with mentioned restriction enzymes and fragments were separated on a 1% agarose gel (s. 2.2.1.1.3.2) for 1 h at 100 V: a) Ladder: 1 kb DNA Ladder (NEB) (s. 2.1.10.1); b) pOV3 digested with *Bam*HI; c) pOV3 digested with *Nco*I.

pOV3 was characterized by restriction analysis with the restriction enzymes *Bam*HI and *Nco*I. After separation of the DNA fragments on agarose gel the expected bands

of 4698 and 1627 bp in the case of the *Bam*HI digest and of 3636, 1845 and 844 bp in the case of the *Nco*I digest were detected (Figure 25).

Transformation of pOV3 into *C. reinhardtii* cells and characterization of transformed strains was done by G. Gessner.

3.2.4.2.2 Study of the effect of the CK1 silencing or inhibition on temperature dependent changes of C1 and C3 subunits

To check a hypothesis that CK1 could play a role in temperature dependent changes of CHLAMY1 components, it was analyzed if silencing or inhibition of CK1 could affect the phosphorylation of C1 and the expression of *c3*. For this purpose, the specific CK1 inhibitor CKI-7 (s. 2.1.4) was used. In some cases, experiments were complemented with the CK1 silenced strain (CK1-sil$_2$) where CK1 expression level was decreased to ca. 40% in comparison to wild-type.

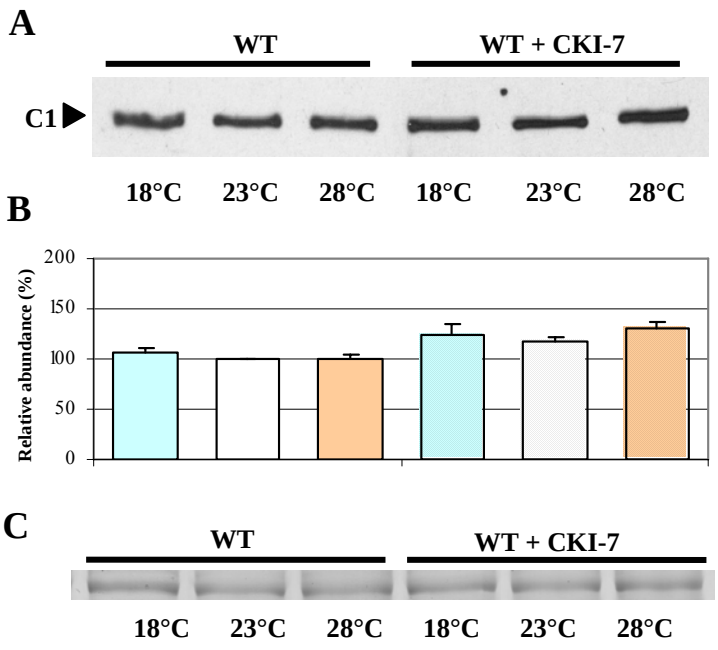


Figure 26. The expression level of C1 does not significantly change in cells where CK1 was inhibited

A. Wild-type cells grown in the absence (WT) or presence of CKI-7 (s. 2.1.4) (WT + CKI-7) were grown at 18, 23 and 28ºC and harvested during early day (LD2). Crude extracts were prepared (s. 2.2.3.1) and proteins (50 µg per lane) were loaded on a 9% SDS-PAGE (s. 2.2.3.4) along with a molecular mass standard and immunoblotted with anti-C1 antibodies (s. 2.2.3.6). B. Densitometry quantifications (s. 2.2.3.12) were done with three independent experiments. Thereby, the amount of C1 detected in WT cells grown at 23ºC without the inhibitor was set to 100%. C. A random nonspecific Coomassie blue-stained band from a duplicate gel shows that similar amounts of proteins were loaded.

The expression level of C1 was rather similar at the different temperatures when CK1 was inhibited by treatment with the CK1 inhibitor, CKI-7 (s. 2.1.4; Preuss et al., 2004) (Figure 26).

It was also analyzed, if the phosphorylation level of C1 was changed in those cells where CK1 was either inhibited or silenced by an RNAi approach (Figure 27).

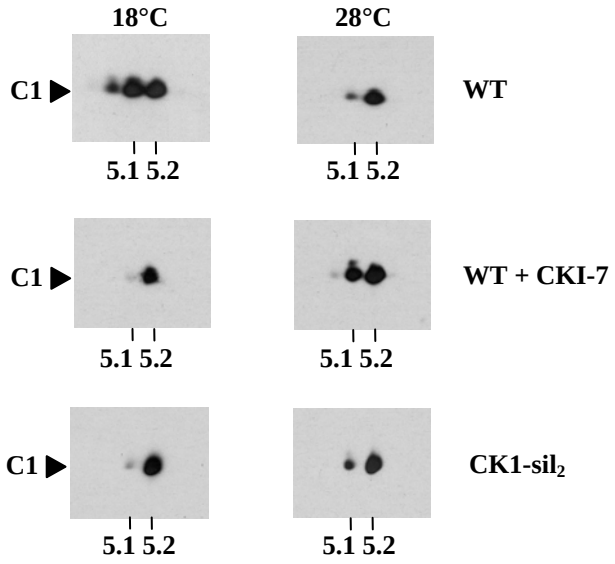


Figure 27. The phosphorylation degree of C1 depends on CK1

WT cells alone (WT) or with the inhibitor CKI-7 as well as the CK1 silenced strain (CK1-sil$_2$) were grown at either 18 or 28ºC and harvested during early day (LD2). Crude extracts were prepared (s. 2.2.3.2) and proteins (300 µg per assay) were separated on standardized 2-DEs (s. 2.2.3.10). For the first dimension, an IPG strip of pH 3-10 was taken, in the second dimension, a 10% SDS-PAGE was used along with a molecular mass standard. The proteins were then immunoblotted with anti-C1 antibodies (s. 2.2.3.6). The positions of pH 5.1 and 5.2 that are close to the theoretical pI of C1 (5.17) are indicated.

Table 5. The temperature dependent phosphorylation degree of C1 in wild-type (WT) and cells where CK1 was either inhibited or silenced

Strain/Inhibitor	Temperature	Percentage	SEM
WT	18°C	50.7*	1.2
WT	28°C	27.8*	2.9
WT/CKI-7	18°C	15.8	8.7
WT/CKI-7	28°C	40.7	1.4
CK1-sil$_2$	18°C	28.6	0.2
CK1-sil$_2$	28°C	44.9	2.2

Densitometry quantifications (s. 2.2.3.12) were done with two (or three indicated by *) independent experiments. Percentage indicates the amount (%) of the phosphorylated degree of C1. Thereby, the volume of all spots was taken as 100%. CKI-7 - specific inhibitor of CK1. CK1-sil$_2$ - CK1 silenced strain (CK1 expression ca. 40% in comparison to WT; Schmidt et al., 2006).

At the low temperature, it was reduced in both cases in comparison to wild-type as analyzed by standardized 2-DE (s. 2.2.3.10) and immunoblotting (s. 2.2.3.6) with anti-C1 antibodies (Figure 27). At the high temperature (Figure 27, Table 5), the phosphorylation level of C1 increased in comparison to wild-type in case of CK1 inhibition with CKI-7, but did not change in case of CK1-sil$_2$. These data suggest that CK1 is involved in C1 phosphorylation at the low temperature. It may directly phosphorylate C1 or activate another kinase that in turn phosphorylates C1. It was surprising that C1 was now 13% more phosphorylated at the high temperature upon full inhibition by CKI-7 (Table 3) in comparison to wild-type. To explain these data, one can assume that CK1 is a member of a temperature dependent signal cascade involving other kinases as will be discussed later.

It was also analysed if the C3 level was changed in CK1 inhibited cells. The up-regulation of *c3* at the low temperature was not as pronounced in the CK1 inhibited cells as in wild-type (Figure 28). Also, it is important to mention that the expression level of *c3* was lower at 18 and 23°C in CK1 inhibited cells compared to wild-type. Therefore, CK1 seems to contribute to the activation of *c3* at the low temperature.

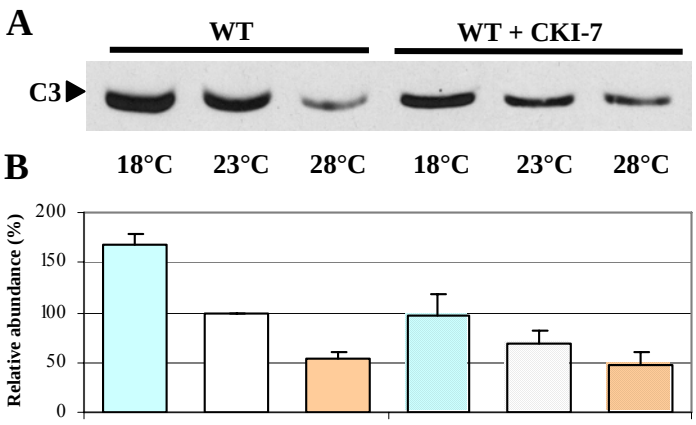


Figure 28. Immunodetection of C3 in WT cells grown in the presence of CKI-7 and in the CK1 silenced strain

The same procedure as described before (Figure 26) was carried out, but immunoblotting (s. 2.2.3.6) was done with anti-C3 antibodies. The loading control is shown in Figure 26C.

3.2.4.2.3 Detection of the expression level of CK1 at different temperatures and its dependency on the activity of Ser-/Thr-phosphatases

It was also interesting to see if CK1 expression might be itself temperature dependent. Therefore, the level of CK1 was analyzed in cells that were grown at the different temperatures. Surprisingly, CK1 was up-regulated at the high temperature (Figure 29). It should be mentioned that in mammalian cells CK1 was shown to auto-phosphorylate itself that results in an inactivation and following degradation of the enzyme (Rivers et al., 1998). For activation of CK1, Ser-/Thr-PPs have to remove the phosphate groups from the enzyme. This was proven by addition of okadaic acid (s. 2.1.4), an inhibitor of Ser-/Thr-PPs that is known to inhibit specifically PP2A, PP4, PP5 and to some part PP1 at low concentrations. In contrast, PP2B is only inhibited at higher concentrations (10 µM) and PP2C and PP7 not at all (Janssens and Goris, 2001). PP1 and PP2A are known to be present in *C. reinhardtii* (Mittag et al., 2005). Blasting (performed by S. Seitz) of the PP4 and PP5 proteins from mouse against the translated *C. reinhardtii* models (genome, version 3) showed in case of PP4 no protein that has any significant hit in *C. reinhardtii* and in case of PP5 two models encoding PP5 like proteins (IDs 31082 and 195748).

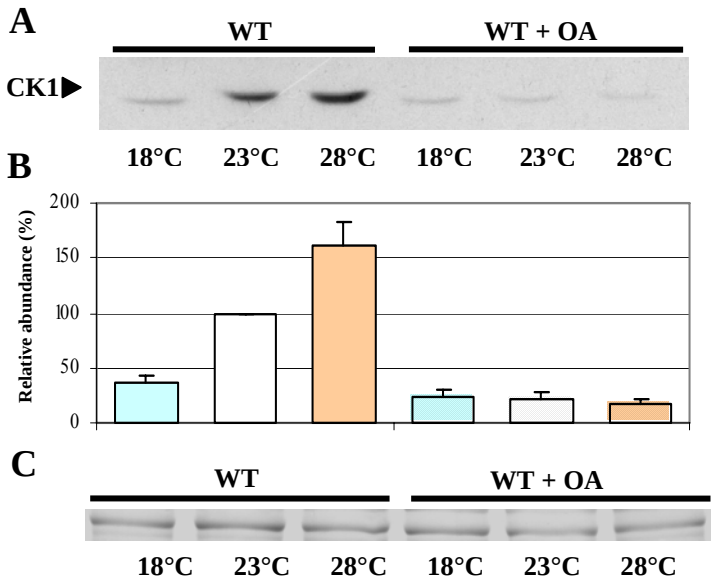


Figure 29. Protein abundance of CK1 is temperature controlled and influenced by PPs

A. Immunodetection of CK1 in WT cells grown in the absence or presence of 1.5 µM Ser-/Thr-PP inhibitor okadaic acid (s. 2.1.4) (WT + OA). Cells were grown at 18, 23 and 28ºC and harvested during early day (LD2). Crude extracts were prepared (s. 2.2.3.1) and proteins (100 µg per lane) were separated on a 9% SDS-PAGE (s. 2.2.3.4) along with a molecular mass standard and immunoblotted (s. 2.2.3.6) with anti-CK1 peptide antibodies (Schmidt et al., 2006). B. Densitometry analysis (s. 2.2.3.12). For quantifications, the amount of CK1 detected in WT cells grown at 23ºC without the inhibitor was set to 100%. C. A random nonspecific Coomassie blue-stained band from a duplicate gel shows that similar amounts of proteins were loaded.

It was checked if addition of okadaic acid at low concentrations (1.5 µM) to the cells would influence the temperature dependent expression of CK1. This was indeed the case (Figure 29). In the presence of okadaic acid, the expression level of CK1 stayed at a similar low level at all observed temperatures. This suggestes that CK1 and at least one of the mentioned above Ser-/Thr-PPs (PP2A, PP1, PP5) act in *C. reinhardtii* in an interconnected way.

3.2.4.3 Investigation of a role of Ser-/Thr-PPs within the circadian system of *C. reinhardtii* and their influence on temperature dependent changes of C1 and C3

3.2.4.3.1 Measurement of the circadian phototaxis rhythm in *C. reinhardtii* culture after addition of okadaic acid

Since inhibition of Ser-/Thr-PPs by okadaic acid influenced temperature dependent CK1 expression (s. 3.5.2.2.2), the treatment with okadaic acid was also used to see if it would influence the temperature dependence of C1 and C3. At first, the addition of okadaic acid to the cell culture was checked to see if it has an influence on phase and/or period of circadian rhythms in *C. reinhardtii*. For this purpose, the circadian rhythm of phototaxis was used as clock output process, since it can be automatically measured over several days (s. 2.2.3.13) (Mergenhagen, 1984). Addition of okadaic acid to an end concentration of 1.5 µM (performed by M. Fiedler) caused a change in period within the first two to three days and then always resulted in arrhythmicity (Figure 30).

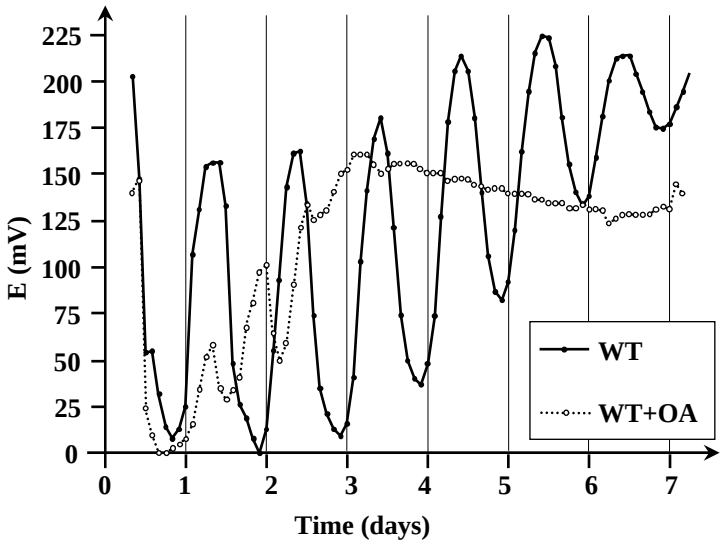


Figure 30. Changes in the phototaxis rhythm of wild-type cells after okadaic acid (OA) treatment

To measure the circadian rhythm of phototaxis, an automated measuring unit was used (s. 2.2.3.13). E represents the extinction in mV. Time (days) indicates how long cells were exposed to constant darkness. The wild-type had a circadian rhythm of phototaxis with an average period of 24.6 h (n = 10; SEM: 0.1). If 1.5 µM OA (s. 2.1.4) was added to the culture medium at the day 0 a period shortened down to 16.5 h [average period: 19.9 h (n = 9; SEM: 0.8)] was observed for first two days and finally arrhythmicity. It should be noted that the cells were motile till day four, but lost motility starting from day five (Wagner et al., 2006).

In several independent experiments with 1.5 µM okadaic acid, the period was in most cases significantly shortened within the first two to three days under constant conditions from 24.6 h (average period wild-type without okadaic acid) down to 16.5 h (average period: 19.9). In one case, the period was increased (up to 42h) during the first two to three days before arrhythmicity occurred. If this is taken into account an average period of 22.3 (n = 10; SEM: 2.6) is given. Such a period lengthening (38h) was also found in one experiment, where a slightly higher amount of okadaic acid (2 µM) was used. The arrhythmic behavior can only be evaluated as clock related till day four as will be discussed later.

3.2.4.3.2 Investigation of PPs involvement into temperature dependent changes of C1 and C3

In the next step, the levels of C1 and C3 as well as C1 phosphorylation were analyzed in okadaic acid treated cells. C1 was rather constantly expressed at the different temperatures in the presence of the PPs inhibitor (Figure 31).

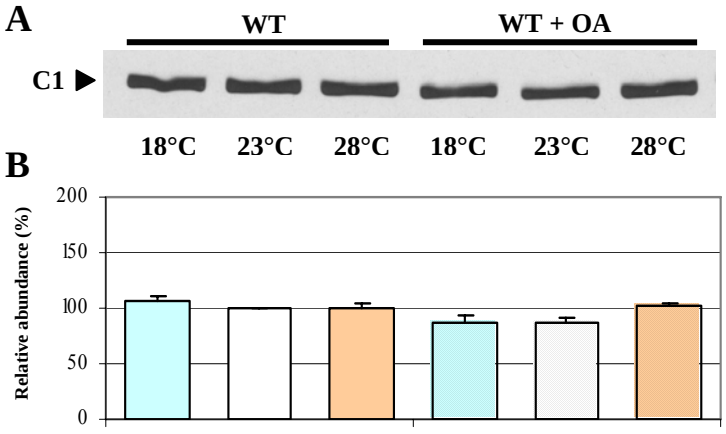


Figure 31. Inhibition of PPs does not significantly affect the expression of C1

A. Cells were grown at 18, 23 and 28ºC in absence (WT) or presence of 1.5 µM okadaic acid (OA; s. 2.1.4) (WT + OA) and harvested during early day (LD2). Crude extracts were prepared (s. 2.2.3.1) and proteins (50 µg per lane) were separated on a 9% SDS-PAGE (s. 2.2.3.4) along with a molecular mass standard and immunoblotted (s. 2.2.3.6) with anti-C1 antibodies. B. Densitometry quantifications (s. 2.2.3.12) were done with three independent experiments. Thereby, the amount of C1 detected in WT cells grown at 23ºC without the inhibitor was set to 100%. Loading control is shown in Figure 29C.

However, the phosphorylation state of C1 changed at the different temperatures in a similar way as seen before with the CK1 inhibition (Figure 32, Table 6). C1 that is only little phosphorylated in wild-type cells at the high temperature appeared to be more phosphorylated in cells grown in the presence of okadaic acid, while at the low temperature its phosphorylation level was reduced.

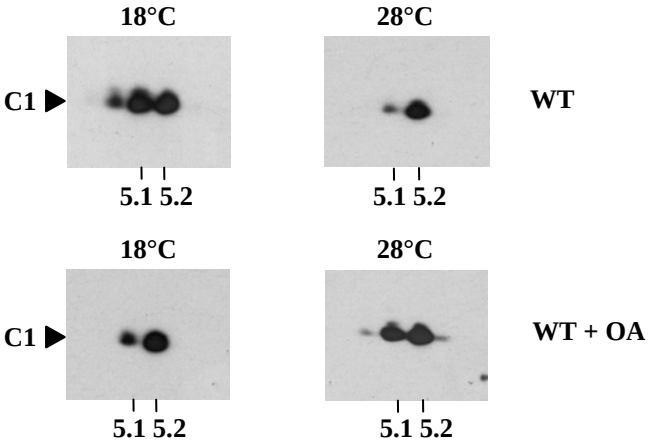


Figure 32. Okadaic acid (OA) changes the phosphorylation pattern of C1 at different temperatures

Wild-type (WT) cells were grown at either 18 or 28ºC in absence or presence of OA (s. 2.1.4) (WT + OA) and harvested during early day (LD2). Crude extracts were prepared (s. 2.2.3.2) and proteins (300 µg per assay) were separated on standardized 2-DEs (s. 2.2.3.10). For the first dimension, an IPG strip of pH 3 to 10 was taken, in the second dimension, a 10% SDS-PAGE was used along with a molecular mass standard. The proteins were then immunoblotted (s. 2.2.3.6) with anti-C1 antibodies. The positions of pH 5.1 and 5.2 that are close to the theoretical pI of C1 (5.17) are indicated.

Table 6. The temperature dependent phosphorylation degree of C1 in wild-type cells and cells treated with okadaic acid (OA)

Strain/Inhibitor	Temperature	Percentage	SEM
WT	18°C	50.7*	1.2
WT	28°C	27.8*	2.9
WT/OA	18°C	28.6	0.2
WT/OA	28°C	44.9	2.2

Densitometry quantifications (s. 2.2.3.12) were done with two (or three indicated by *) independent experiments. Percentage indicates the amount (%) of the phosphorylated degree of C1. Thereby, the volume of all spots was taken as 100%.

Further, the expression level of C3 was different in comparison to wild-type, as judged by immunoblots (Figure 33), where it was significantly increased at 23 and 28°C. These data suggest that at least one of the above mentioned Ser-/Thr-PPs is not only interconnected with CK1, but also with C1 and C3.

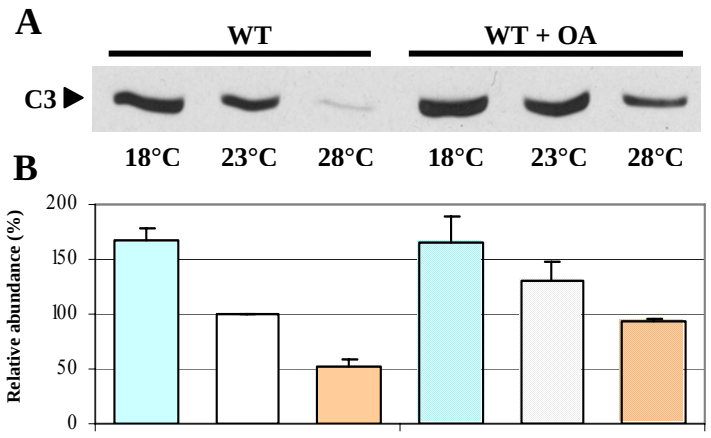


Figure 33. Presence of okadaic acid influences the expression of C3 at different temperatures

The same procedure as described before (Figure 31) was carried out, but immunoblotting (s. 2.2.3.6) was done with anti-C3 antibodies. The loading control is shown in Figure 29C.

3.2.4.4 Studies on temperature integration of C1 and C3 in the *per1* mutant

The *per1* mutant of *C. reinhardtii* has a lengthened period of the circadian phototaxis rhythm (Bruce, 1972). Albeit it has the same name as the *per* mutant from *D. melanogaster*, the protein of *C. reinhardtii* might not be related to PER of *D. melanogaster*. So far, there are no indications which gene/protein is defect in the *per1* mutant. It was of interest to check if the functional temperature related network of C1 and C3 along with Ser-/Thr-PPs and CK1 is maintained or changed in the *per1* mutant.

3.2.4.4.1 Detection of the expression level of C1 and C3 and of the phosphorylation pattern of C1 at different temperatures in the *per1* mutant

In the *per1* mutant, the level of C1 stayed rather constant at the different temperatures (Figure 34), which is similar to wild-type.

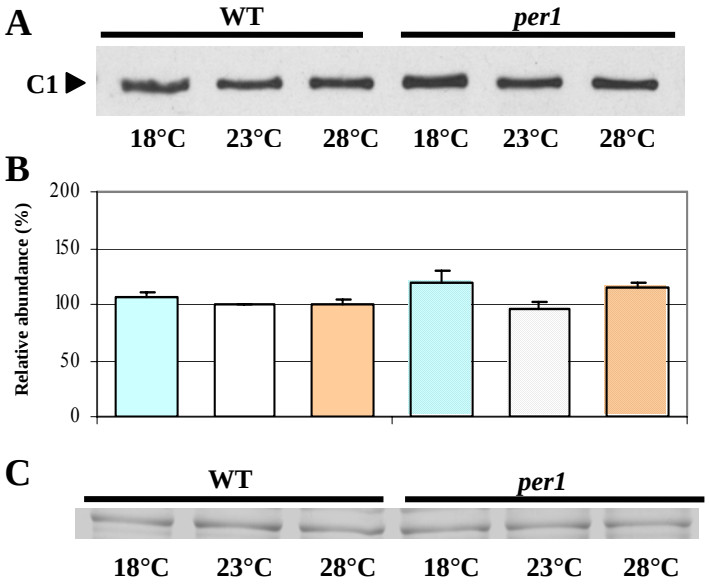
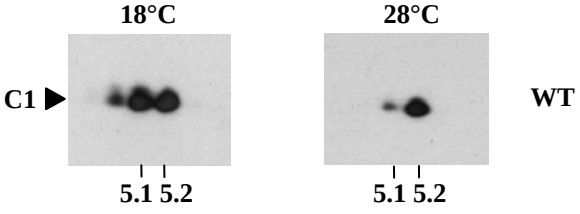


Figure 34. The C1 expression level at different temperatures is not affected in *per1* mutant in comparison to WT

A. Cells of the wild-type (WT) and the *per1* mutant were grown at 18, 23 and 28ºC and harvested during early day (LD2). Crude extracts were prepared (s. 2.2.3.1) and proteins (50 µg per lane) were separated on a 9% SDS-PAGE (s. 2.2.3.4) along with a molecular mass standard and immunoblotted (s. 2.2.3.6) with anti-C1 antibodies. B. Densitometry quantifications (s. 2.2.3.12) were done with three independent experiments. Thereby, the amount of C1 detected in WT cells grown at 23ºC was set to 100%. C. A random nonspecific Coomassie blue-stained band from a duplicate gel shows that similar amounts of proteins were loaded.

However, the phosphorylation status of C1 in *per1* changed in comparison to wild-type. C1 was relatively little phosphorylated at both, low and high temperature (Figure 35, Table 7).

It was also of interest to see if the C3 level is changed in the *per1* mutant. Notably, it was significantly increased in the *per1* mutant compared to wild-type, especially at the high temperature as judged by immunoblots (Figure 36).



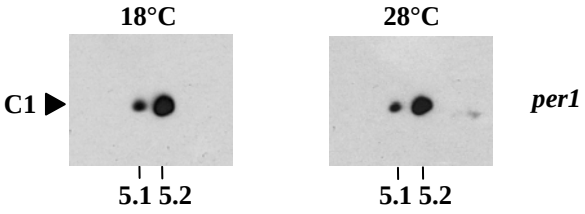


Figure 35. The level of C1 phosphorylation is decreased at all temperatures in *per1* cells comparing to wild-type

Cells of wild-type (WT) and the *per1* mutant were grown at either 18 or 28ºC and harvested during early day (LD2). Crude extracts were prepared (s. 2.2.3.2) and proteins (300 µg per assay) were separated on standardized 2-DEs (s. 2.2.3.10). For the first dimension, an IPG strip of pH 3 to 10 was taken, in the second dimension, a 10% SDS-PAGE was used along with a molecular mass standard. The proteins were then immunoblotted (s. 2.2.3.6) with anti-C1 anti-bodies. The positions of pH 5.1 and 5.2 that are close to the theoretical pI of C1 (5.17) are indicated.

Table 7. The temperature dependent phosphorylation degree of C1 in wild-type (WT) and the long period mutant *per1*

Strain/Inhibitor	Temperature	Percentage	SEM
WT	18°C	50.7*	1.2
WT	28°C	27.8*	2.9
Per1	18°C	25.3	3.3
Per1	28°C	24.0	1.1

Densitometry quantifications (s. 2.2.3.12) were done with two (or three indicated by *) independent experiments. Percentage indicates the amount (%) of the phosphorylated degree of C1. Thereby, the volume of all spots was taken as 100%.

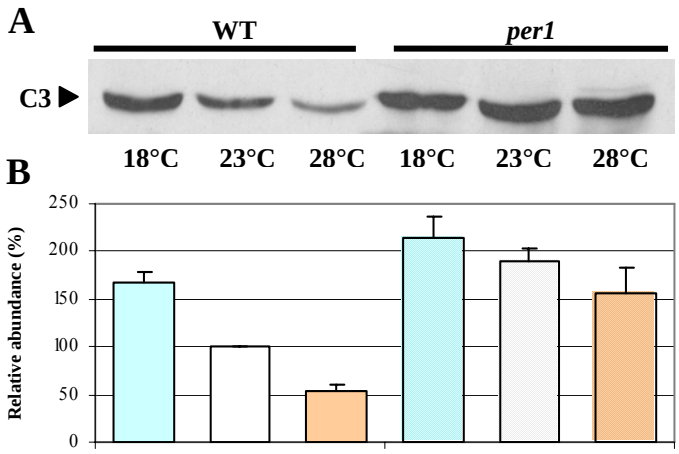


Figure 36. The C3 expression level is increased in the *per1* mutant at different temperatures

The same procedure as described before (Figure 34) was carried out, but immunoblotting (s. 2.2.3.6) was done with anti-C3 antibodies. The loading control is shown in Figure 34C.

Thus, it can be concluded that a) both temperature dependent processes like phosphorylation of C1 and expression of *c3* are altered in the *per1* mutant, and b) the mutant protein in *per1* belongs to a clock related functional network including C1 and C3.

3.2.4.4.2 Investigation of the possible interconnection between PER1 and CK1

It was also analyzed if the temperature dependent regulation of CK1 was changed in the *per1* mutant, but this was not the case (Figure 37). *ck1* expression is still up-regulated at high temperature in *per1*.

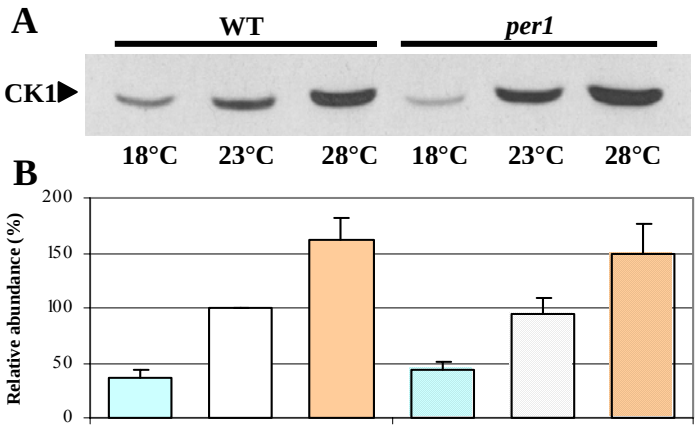


Figure 37. The temperature dependent expression of CK1 is not affected in the *per1* mutant.

A. Cells of the wild-type (WT) and *per1* mutant were grown at 18, 23 and 28ºC and harvested during early day (LD2). Proteins from crude extracts (s. 2.2.3.1) (100 µg per lane) were separated on a 9% SDS-PAGE (s. 2.2.3.4) along with a molecular mass standard and immunoblotted with anti-CK1 peptide antibodies (s. 2.2.3.6). B. Densitometry quantifications (s. 2.2.3.12) were done with three independent experiments. Thereby, the amount of CK1 detected in WT cells grown at 23ºC was set to 100%. The loading control is shown in Figure 34C.

4. Discussion

4.1 The use of RNAi for silencing of C1 and CK1

In the first part of the present study the function of the C1 subunit of the heteromeric RNA-binding protein CHLAMY1 within the circadian system of *C. reinhardtii* was analyzed by silencing of its expression *via* RNA interference approach. The same approach was used also in the second part of the work to silence CK1.

RNAi, also termed posttranscriptional gene silencing (PTGS) in plants or RNA quelling in fungi, has been used first in the nematode *Caenorhabditis elegans* to manipulate gene expression (Fire et al., 1998). It involves double-stranded RNA (dsRNA) intermediates that may specifically affect gene expression at the transcriptional and/or posttranscriptional levels. Knock-down approaches that trigger RNA silencing *via* constructs that express antisense or inverted repeat-containing RNAs have been successfully introduced to *C. reinhardtii* (Schroda et al., 1999) and have been continuously improved since then.

The method that was used in current work has been developed by Fuhrmann et al. (2001) and involves usage of the endogenous promoter. Thus, the potential promoter regions of the *c1* or *ck1* genes together with the first exons and introns were fused to an inverted cDNA parts so that a double-stranded RNA will be formed in the cell that triggers silencing. Such an approach had been also successfully applied in our lab for silencing of C3 subunit of CHLAMY1 (Iliev et al., 2006). Maximal silencing down to about 25% (with C1) or even lower (with CK1 and C3) of wild-type level was observed, which is in the range of other RNAi silencing approaches conducted in *C. reinhardtii* (reviewed in Schroda, 2006). While silencing of CK1 and C3 was relatively stable for many months, when the cells were kept on paromomycin plates, and only little reversion was observed during this time frame, strong silencing of C1 was not stable over time and could only be maintained for a few weeks. Reversion of RNAi strains back to wild-type level over a few months had been observed already before with RNAi and antisense constructs of *C. reinhardtii* (Schroda, 2006). However, the relatively quick reversion of C1 seems to be a particular case of C1, especially when it is silenced below a critical level of about 40% and may be triggered by a specific mechanism within the cell. It can be hypothesized that the presence of a minimal amount of C1 in the cell might be indispensable to the life of *C. reinhardtii*, which would explain the fact that no

strains were found with C1 expression level lower then 25%, even this was indeed the case with other silencing constructs (CK1 or C3) as mentioned before.

4.2 C1, a subunit of CHLAMY1 is essential for an intact circadian clock of *C. reinhardtii*

To examine the influence of the down-regulated C1 subunit, circadian activity of NII as well as phototaxis were measured.

NII was chosen as a direct output target of CHLAMY1, since it is known that *Nii* mRNA bears an UG-repeat in its 3'-UTR (Waltenberger et al., 2001) that is a specific recognition motif of CHLAMY1. NII activity was determined in the C1-sil$_{81}$ strain, where the C1 level was reduced to about 53%, which could be still reproduced after the NII assay. Arrhythmicity was observed in this case, confirming the important role of C1 in the circadian output system of *C. reinhardtii*.

The rhythm of phototaxis was checked as a second circadian output process. In this case, arrhythmic behavior could be found in strains where C1 was only slightly silenced. While C1-sil$_{53}$ still showed circadian behavior for the first three days under constant conditions before it became arrhythmic, C1-sil$_{72}$ had disturbances in its circadian phototaxis rhythm from the very beginning. In these strains, silencing of C1 was still observed after the phototaxis assay had been finished when analyzed by immunoblotting. This is especially impressive when one considers that the level of C1 in these strains was altered very little (70% of the wild-type level in case of C1-sil$_{72}$ and 85% in case of C1-sil$_{53}$).

As was already mentioned, C1 strains that had a more pronounced silencing below 40% (e.g., C1-sil$_{35}$) were not stable over time. Reversion of C1 to its wild-type level occurred within a relatively short period of time and in each case examined, the reversion was already completed when cells were analyzed after the phototaxis experiment. Thus, the wild-type like circadian rhythm of C1-sil$_{35}$, for example, cannot be interpreted in an unambiguous way. It can be assumed that the cells were already reverted before the phototaxis measurement and thus, a wild-type like behavior occurred. It would be rather surprising if slight (down to 70%) silencing of C1 can cause arrhythmicity while stronger silencing of C1 would not. However, this possibility cannot be excluded for sure.

Altogether, these data demonstrate that changes in the C1 level can cause arrhythmicity with regard to both analyzed output rhythms and corroborate its important role as a component that is not only a part of the circadian output pathway, but seems to be at the same time also a part of the central oscillator of the circadian system in *C. reinhardtii*. Such a pleotropic function was already demonstrated before for some central clock components in different organisms. For example, one of the core components in the *A. thaliana* clock, the MYB-related transcriptional factor CCA1 was shown to be also essential for correct output regulation and at the same time represents an important component of the oscillatory system (Ding et al., 2007).

Very recently, Matsuo et al. (2008) reported about the identification of several genes that are essential for normal circadian rhythmicity of a chloroplast bioluminescent reporter. Several putative transcription factor genes encoding MYB (*roc15*, *roc40*, *roc59* and *roc75, roc = rhythm of chloroplast*), zinc-finger (*roc56* and *roc66*), and basic leucine zipper (*roc76*) DNA-binding domain protein genes were included. These data appear to be of special interest also with regard to this work, because one of the detected MYB-containing transcription factor genes, *roc40*, was shown to contain a $(UG)_{\geq 7}$ repeat element in its 3'-UTR. Thus, CHLAMY1 may participate in the circadian clockwork *via* the UG repeat of *roc40*.

4.3 Co-regulation of the C1 and C3 subunits

It was shown before that the interaction of the C1 and C3 subunits of the CHLAMY1 RNA-binding protein complex is necessary for the binding of CHLAMY1 to its RNA targets (Zhao et al., 2004). It is interesting that the two subunits appeared to be interconnected also in their expression level. A decrease in C1 below a critical level (e.g., in C1-sil$_{32}$ and C1-sil$_{35}$) resulted in strong down-regulation of C3. In these cases, the C3 expression was altered with even higher amplitude (15 - 20% comparing to wild-type) than C1 (25-30% of wild-type level). At the same time, the less silenced C1 strains (e.g., Cl-sil$_{72}$ expressing ca. 70% of wild-type level) showed only weak co-regulation of C3 (ca. 80-90%).

In parallel investigations carried out in our institute, it was shown that such a co-regulation also takes place when C1 was overexpressed (Iliev et al., 2006). In contrast, changes in the C3 level had little (C3 overexpression) or no significant (C3

silencing) effect on C1 expression (Iliev et al., 2006). Therefore, C1 seems to be the core unit in the CHLAMY1 complex.

Currently, it is not known by which mechanism this co-regulation occurs, but this subject will be of a great interest in future studies. In the case of the assembly-governed regulation of the complex biogenesis of subunits of PSI and II, it was found that translation is involved in this process and that the 5'-UTRs communicate this regulation (Wostrikoff et al., 2004; Minai et al., 2006). If a comparable mechanism occurs with C1 and C3, there exists the possibility that C1 as an RNA-binding protein might regulate the translation of C3, for example. However, C1 might also activate some yet unknown factor(s) that influence(s) the expression of C3. At the same time, some preliminary experiments carried out in our institute indicate that this co-regulation occurs at the transcriptional level (Seitz and Mittag, unpublished data).

4.4 Temperature changes in the physiological range alter C3 and CK1 protein abundance and the phosphorylation degree of C1

Biochemical reactions typically have temperature coefficients of 2 or more, i.e. their reaction rates double with every 10°C rise in temperature (Snyder, 1908). In contrast, the period length of circadian rhythms is temperature compensated (Q10 close to1; Pittendrigh, 1954; Hastings and Sweeney, 1957). Already in 1956, Colin Pittendrigh and Victor Bruce proposed that temperature compensation of the clock's period should be based on the mutual coupling of two temperature-dependent oscillators with complementary temperature coefficients (Pittendrigh, 1993). Further, temperature cycles beside light cycles are one of the main environmental cues that can synchronize circadian clocks (Rensing and Ruoff, 2002). For control of both processes (temperature compensation and temperature entrainment), certain components of the circadian clock should be able to "sense" or integrate temperature.

In the green alga *C. reinhardtii*, several components were so far shown to be involved in the circadian oscillatory machinery. Reduction of the level of the Ser-/Thr-kinase CK1 results in period shortening and finally arrhythmicity (Schmidt et al., 2006). Mutation of the still unknown PER1 leads to period lengthening (Bruce, 1972). As it was discussed before, the C1 subunit of the RNA-binding protein CHLAMY1 causes arrhythmicity when its expression level is altered. At the same

time, silencing or overexpression of another CHLAMY1 subunit C3 was found to cause shifts in the acrophase of circadian rhythms (Iliev et al., 2006). Very recently, further important components of the *C. reinhardtii* circadian clock represented mainly by transcriptional factors were found, as already mentioned (Matsuo et al., 2008).

In the next part of the current study, temperature changes of 10°C ranging from 18 up to 28°C were shown to alter in different way these clock-relevant components. It should be mentioned that these temperatures are still in the physiological range of *C. reinhardtii* (Harris, 1989) and thus, these clock components can integrate temperature. For true stress related responses, e.g. heat stress, a significantly higher temperature (40°C) should be applied (Schulz-Raffelt et al., 2007).

The temperature dependent regulation of the above mentioned proteins occurs at different levels. In case of C3 and CK1, temperature dependent changes in their abundance occurred at the different temperatures. For C3, it was found that its level increased at low and decreased at high temperature. For CK1, the opposite case was observed: its abundance increased with temperature. In case of C1, the protein amount stayed constant, but posttranslational modifications were altered at the different temperatures.

In further investigations using treatment with lambda PPase, the observed C1 modifications were shown to be phosphorylation. This is of a great interest since phosphorylation is known to play a central role in all so far studied circadian systems. At the same time it should be mentioned that even after treatment with five times higher amount of PPase the modified form of C1 did not disappear completely. This could mean that there is another type of posttranslational modification of C1 occurring at the same time. Another reason could be the 3D conformation of C1 within the CHLAMY1 complex or in the large ≥680 kDa complex that occurs during day time (Zhao et al., 2004) where some phosphorylated sites of C1 may be not fully accessible for the lambda PPase.

It was proposed that the changes of C3 protein level at the different temperatures could be a consequence of its altered expression occuring either at the transcriptional or translational level. A cycloheximide experiment revealed that low temperature treatment resulted in transcriptional activation of C3 expression. An on-line computer analysis of the *c3* promoter (http://alggen. lsi.upc.es/cgi-

bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) predicted a presence of several possible *cis*-acting elements (Figure 38).

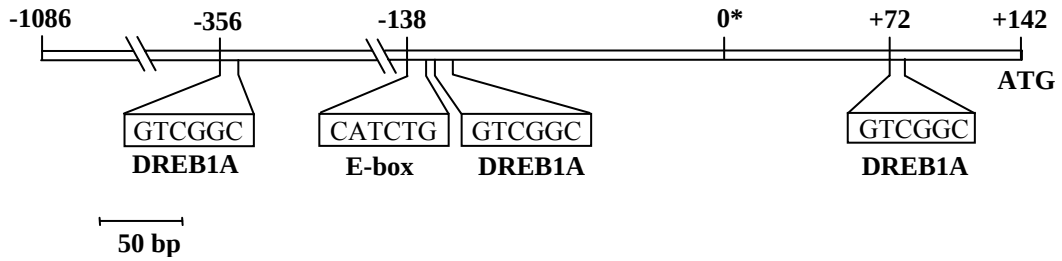


Figure 38. Presence of several possible *cis*-acting elements was predicted in *c3* promoter

A schematic representation of the potential *c3* promoter region including its 5'-UTR is shown from position -1086 to +142. * defines the first nucleotide of the *c3* EST clone (AV641734) and should be close to the +1 of the *c3* mRNA. Bars indicate *cis*-acting elements including an E- as well as three DREB1A-boxes.

First, three DREB1A-boxes were found to be situated in *c3* promoter and 5'-UTR at the positions -356, -138 and +72. These sequences are known to be recognized by DREB (DEHYDRATION RESPONSIVE ELEMENT BINDING) proteins that are important transcription factors in plant abiotic stress response and signal transduction (Agarwal et al., 2006). It should be noted that DREB1A, B and C are also known as CBF3, 1 and 2, respectively (C-REPEAT BINDING FACTOR). They are involved in two separate signal transduction pathways with regard to low temperature or dehydration stress. They belong to the ERF (ETHYLENE RESPONSIVE ELEMENT BINDING FACTORS) family of transcription factors. ERF proteins are a sub-family of the APETLA2 (AP2)/ethylene responsive element binding protein transcription factors that is distinctive to plants. Recently, it was shown that a DREB factor (named PpDBF1) occurs also in the moss *Physcomitrella patens* and is also involved in salt, drought and cold stresses there (Liu et al., 2007). Further members of the cold signaling pathway in higher plants that are in a functional network with the DREB factors include for example ICE1 (INDUCER OF CBF/DREB EXPRESSION1) and its SIZ1- (for SAP and MIZ) mediated SUMOylation (Miura et al., 2007). Interestingly, it has been shown in *A. thaliana* that low temperature induction of CBF1, 2 and 3 is gated by the circadian clock (Fowler et al., 2005). In *C. reinhardtii*, several gene models (genome, version 3) were found that encode

proteins with ERF/AP2-like domains. The two with highest homology to the *A. thaliana* DREB1A and 2A factors have protein IDs 167010 and 205920. However, the reported temperature shift activating DREB1 in higher plants is usually a switch from room temperature (about 22°C) to 4°C or even below. So far, there are no data available if 18°C can already act as stimulating low temperature.

Another *cis*-acting element that could be relevant for the differential expression rate of *c3* is an E-box, which is situated only two nucleotides away from one of the DREB1A-boxes. E-boxes are known relevant *cis*-acting elements for circadian regulation. For example, in *D. melanogaster*, they are situated in the promoter regions of *per* and *timeless* (*tim*). Transcriptional activation of both genes occurs *via* the CLK-CYC heterodimer and is blocked when the PER/TIM complex interacts with CLK-CYC (Yu et al., 2006). But potential homologues to the heterodimer CLK-CYC that recognize the E-box have not been found in *C. reinhardtii* (Mittag et al., 2005). Positive transcriptional regulation by an E-box was also reported in other cases such as with the polyserase gene, where the E-box is situated in the 5'-UTR and is required for maximal promoter activity (Hayama et al., 2007).

Current investigations in our lab showed that replacement of the DREB1A-boxes at positions -130 and +72 or deletion of the one at the position -356 reduced the amplitude of cold-induced *c3* up-regulation, suggesting that these boxes contribute to some extent to the up-regulation (Voytsekh, Seitz et al., under revision). At the same time, replacement of the other *cis*-acting element, the E-box at -138, abolished completely up-regulation of *c3* at low temperature (18°C) showing that it represents the key element for temperature integration of *c3*.

Based on the current data, it seems possible that the different phosphorylation status of C1 at low and high temperature might be involved in the regulation of *c3* at the different temperatures in a direct or indirect way. C1 bears three KH domains that are known as RNA binding domains and binds in combination with C3 to UG-repeat RNAs (Zhao et al., 2004). There are reports that KH-domain-containing proteins can recognize RNA and DNA sequences, as it was shown for example with polyC-binding proteins (Du et al., 2007). Moreover, DNA-binding protein complexes such as NF-kappaB that mediate selective gene regulation can contain KH domain subunits. In case of the NF-kappaB complex, the KH domain ribosomal protein S3 is a subunit of this complex (Wan et al., 2007). These findings open the opportunity

that C1 could be theoretically directly involved in up-regulating of *c3*, for example as part of a DNA-binding protein complex. The finding that *c3* expression is co-regulated depending on the level of C1 discussed before, would be in concert with such a postulation, which has to be carefully analyzed in future.

4.5 CK1 is involved in the temperature dependent phosphorylation changes of C1 and influences the expression level of C3

The hyper-phosphorylation of C1 at low temperature should involve the action of a kinase. Among several predicted kinases, CK1 was present. Since it was already known that silencing of CK1 has an effect on the circadian clock of *C. reinhardtii* (Schmidt et al., 2006), it was of interest to check if silencing or inhibition of CK1 can have an effect on either C1 phosphorylation or *c3* expression. This was indeed the case. CK1 inhibition was shown to affect *c3* temperature dependent expression. It still resulted in the up-regulation of *c3* expression at the low temperature, however with a lower amplitude as compared to wild-type. At the same time, C1 hyper-phosphorylation at 18°C disappeared in cells where CK1 was inhibited by CKI-7 as well as in the $CK1_{sil2}$ strain where CK1 is silenced by RNAi (Schmidt et al., 2006). But one must be careful concluding that CK1 directly phosphorylates C1. The present data do not exclude the possibility that CK1 may activate another kinase that phosphorylates C1 and that could be additionally present in the complex with C1. Notably, at 28°C, C1 became higher phosphorylated in CK1-inhibited cells compared to wild-type. One possibility to explain these data is that another kinase is activated at high temperature by the inhibition of CK1. It is known that kinases can be activated or inactivated upon their phosphorylation (Krupa et al., 2004). If this is indeed the case, a temperature controlled signaling network of clock relevant kinases may exist.

4.6 Ser-/Thr-PPs play an essential role within the circadian system of *C. reinhardtii* and influence temperature integration of C1 and C3

The action of Ser-/Thr-kinases like CK1 is often counter-balanced by Ser-/Thr-PPs such as PP5, PP2A and PP1 (Rivers et al., 1998; Partch et al., 2006; Wijnen and Young, 2006). PP1 and PP2A have been shown to be essential for the clock machinery in *N. crassa* and *D. melanogaster* (Yang et al., 2004; Sathyanarayanan et al., 2004; Fang et al., 2007), and PP5 for the mammalian clock (Partch et al., 2006). Mammalian CK1 is known to auto-phosphorylate itself resulting in its inactivation. The action of a Ser-/Thr-PP is necessary to activate CK1 (Rivers et al., 1998). This PP was recently shown to be PP5 (Partch et al., 2006).

An effect on the phosphorylation pattern of C1 at low and high temperature, similar to one caused by CKI-7, could be seen in cells treated with okadaic acid that is able to affect PP2A, PP4, PP5 and PP1 at the used concentration of 1.5 µM (Janssens and Goris, 2001). This suggests that CK1 and one of the just mentioned PPs are also interconnected in *C. reinhardtii*. Both may influence the activity of a "yet unknown kinase".

It was also shown that addition of okadaic acid to the *C. reinhardtii* cell culture disturbs circadian phototaxis rhythms resulting in complete arrhythmicity on the third day after addition. In the used concentration, okadaic acid was shown before to cause immobility of the cells starting from day five (Wagner et al., 2006). In these experiments, the mobility of the cells was examined under the microscope with three samples taken at day two, four and five after incubation with okadaic acid. On the second and fourth day, nearly all cells were fully motile. On day five, only 10 to 15% of the cells were still motile and about 10% appeared dead. 75 to 80% of the cells had flagella that were still beating, but the cells were not able to move forward. Thus, PP2A and/or PP1 and/or PP5 are essential components of the circadian clock, but are also relevant for flagella movement of *C. reinhardtii*.

Ser-/Thr-PP inhibition by okadaic acid had also effects on the C3 expression, however, in opposite way to those caused by inhibition of CK1. As mentioned before, alterations of the level of C1 (in- or decrease) cause parallel changes in the level of C3 (Iliev et al., 2006). Thus, C1 can directly or indirectly influence C3 expression. It was first hypothesized that this co-regulation may be also present at the different temperatures and may depend on the different phosphorylation levels of C1. The results of CK1 and PP inhibition that convert the phosphorylation level of C1 at low and high temperature in a similar way, but have opposite effects on C3 expression, render this hypothesis unlikely. It seems to be more feasible that CK1

and the PP act on a transcription factor that activates *c3* at low temperature in addition to their influence on C1. It was shown recently that DREB from *Pennisetum glaucum* is a phosphoprotein and that its phosphorylation regulates its DNA-binding activity (Agarwal et al., 2007). There, the phosphorylated PgDREB2A cannot bind to the DREB-box.

As it was already mentioned before, inhibition of CK1 resulted in a decrease of *c3* up-regulation amplitude comparing to wild-type. Inhibition of Ser-/Thr-PPs by okadaic acid had an even profounder effect. The *c3* expression level was now up-regulated, especially at the high temperature. Thus, one can hypothesize that the transcription factor activating *c3* may be in a non-phosphorylated form in wild-type at high temperature through the action of a Ser-/Thr-PP. If this PP is inhibited it may exist in the phosphorylated form and thus may be able to activate *c3*. However, such a postulation has to be carefully examined in future.

The data that have been obtained so far on the temperature dependent regulation of C1 and C3 show that both are part of a complex network involving CK1, at least one of the above mentioned Ser-/Thr-PPs and (a) yet unknown transcription factor(s). Thereby, CK1 and the Ser-/Thr-PPs are interconnected in a temperature dependent way. While CK1 is up-regulated at high temperature in wild-type, this up-regulation is absent in cells treated with okadaic acid where the level of CK1 is always low. Thus, it is challenging to make final conclusions, for example, on the thermally relevant function of the involved kinases or Ser-/Thr-PPs, since their inhibition leads to multiple effects that are hard to dissect in detail. A systems biology approach including modeling may be useful in future to unravel this temperature related signaling network. Also, it will be interesting to find out if such a network exists in other organisms. CK1, for example, was shown to be involved in phosphorylation of components of the oscillatory machinery in different model organisms including *N. crassa*, *D. melanogaster* and mammals (Wjinen and Young, 2006). In humans, a missense mutation (T44A) in the *CKIdelta* gene even results in familial advanced sleep phase syndrome (FASPS) that is in correlation with decreased activity of CK1 (Xu et al., 2005). It can be relatively easy checked if the thermal regulation of CK1 also occurs in these organisms.

4.7 PER1 is part of a functional network including C1 and C3 as well as PP2A and/or PP5

As mentioned earlier, Victor Bruce had isolated *per* mutants that show a significantly lengthened period already in 1972. One of them, *per1*, was included in the present studies to see if there could be a connection between PER1 and the temperature sensing of C1 and C3. In the *per1* mutant, temperature dependent hyper-phosphorylation of C1 as well as *c3* expression are altered, suggesting that PER1 is indeed part of the network regulating temperature sensing of C1 and C3. In case of C1, it was observed to be only little phosphorylated in the *per1* mutant at both 18 and 28°C. This could be due to a missing kinase or increased PP activity. Notably, *c3* expression was significantly increased in the *per1* mutant, both at low and high temperature. But, the up-regulation of CK1 at high temperature was not influenced in the *per1* mutant.

It can be hypothesized that a transcription factor that increases *c3* expression might be activated both at 18 and 28°C in *per1*. This could be achieved, for example, by changing the phosphorylation status of such a transcription factor. The involvement of a PP or a kinase that is altered in the *per1* mutant could also explain the changes in the phosphorylation level of C1 in *per1*. Another possibility is that there is some positive acting mutation in the c3 promoter that causes the increased c3 expression. To check for this, the promoter region of the *c3* gene (positions -874 to +274) was sequenced in the *per1* mutant (performed by D. Iliev). However, there was no difference in comparison to the wild-type sequence depicted from JGI excluding this possibility.

Albeit the nature of PER1 is still unknown, it becomes evident that it is also part of the functional temperature depending network involving C1, C3 as well as PP2A and/or PP5. Future studies will show if the idea of Pittendrigh and Bruce about the mutual coupling of two temperature dependent oscillators might possibly involve CHLAMY1 and PER1 in *C. reinhardtii*.

5. Summary

The research of my thesis focused on the circadian RNA-binding protein CHLAMY1 from the green alga *Chlamydomonas reinhardtii* that consists of two subunits named C1 and C3. CHLAMY1 binds specifically to $(UG)_{\geq 7}$-repeat sequences situated in the 3'-untranslated regions of several mRNAs such as *nitrite reductase 1* (*nii1*).

The role of the C1 subunit within the circadian system was characterized by silencing its gene using an RNAi approach. The expression level of C1 was silenced down to 25 - 85% depending on the transgenic line. As a first indicator of the influence of CHLAMY1 on the circadian output, measurement of NII activity was carried out with a transgenic strain under circadian conditions. In wild-type, NII activity peaks around the beginning of subjective day. In a transgenic strain, where C1 was silenced to ca. 50%, arrhythmicity was observed. Circadian phototaxis, which can be measured automatically, was chosen as a second output rhythm. Also in this case, arrhythmicity was observed immediately (70% silencing) or after the first three days (84% silencing) under constant conditions in transgenic lines. These data indicate a central role of the C1 subunit in the circadian system of *C. reinhardtii*. In addition to these results, a co-regulation between C1 and C3 subunits was found. Thus, silencing of C1 lower than 40% in comparison to wild-type caused strong down-regulation of the C3 subunit in parallel, suggesting a role for C1 as a core subunit within the CHLAMY1 complex.

It was also analyzed if the two subunits play a role in temperature integration, the basis for other key properties of circadian clocks including entrainment by temperature cycles and temperature compensation. It was shown that C1 is hyper-phosphorylated at low (18°C) and hypo-phosphorylated at high temperature (28°C). These temperatures are in the physiological range of *C. reinhardtii*. In case of C3, its expression level was found to be up-regulated at low temperature. An inhibitor experiment with cycloheximide showed that this regulation occurs at the transcriptional level.

Moreover, it was shown that the clock-relevant CASEIN KINASE1 (CK1) and Ser-/Thr-PROTEIN PHOSPHATASEs (PPs) mediate the temperature dependent regulation of C1 and C3. Notably, the expression of CK1 was itself temperature controlled and increased at 28°C. Data obtained from the investigations with the long period clock mutant *per1* showed that temperature integration of both C1 and C3 is

altered there. A low phosphorylation level of C1 as well as a high expression level of C3 was observed at different temperatures in *per1*. Altogether, the obtained data suggest that a temperature controlled functional network of clock-relevant proteins exists in *C. reinhardtii* including C1, C3, CK1, PPs and PER1.

Zusammenfassung

Im Rahmen meiner Arbeit wurde das circadiane RNS-Bindeprotein CHLAMY1 der Grünalge *Chlamydomonas reinhardtii* untersucht, das aus den beiden Untereinheiten C1 und C3 besteht. CHLAMY1 bindet spezifisch an $UG_{\geq 7}$ -Wiederholungen, welche in den 3'-nicht translatierten Regionen von mehreren mRNSs, wie z. B. der *Nitritreduktase1* (*nii1*) mRNS vorkommen.

Zunächst wurde die Funktion der C1 Untereinheit im circadianen System durch einen RNSi-Ansatz überprüft. Das Expressionsniveau von C1 konnte hierbei auf 25 – 85% reduziert werden, abhängig von der transgenen Linie. Um den Einfluss von CHLAMY1 auf den circadianen Ausgang zu bestimmen, wurde als erster Indikator der NII-Aktivitätsrhythmus gemessen. Im Wildtyp erreichte die NII-Aktivität ein Maximum zu Beginn des subjektiven Tages. In einem transgenen Stamm, in dem C1 ca. 50% weniger exprimiert wurde, wurde Arrhytmizität festgestellt. Der Rhythmus der circadianen Phototaxis, den man automatisiert über sieben Tage messen kann, diente als zweiter Indikator. Auch in diesem Fall wurde Arrhythmizität beobachtet, entweder ab dem ersten Tag unter konstanten Bedingungen (transgener Stamm mit 70% C1-Reduktion) oder nach drei Tagen (transgener Stamm mit 84% C1-Reduktion). Diese Daten weisen darauf hin, dass C1 eine zentrale Rolle im circadianen System von *Chlamydomonas reinhardtii* spielt.

Weiterhin wurde eine Koregulation der C1- und C3-Untereinheit gefunden. So zeigten transgene Stämme, in denen C1 <40% reduziert worden war, eine signifikante Reduktion der C3-Untereinheit. Dies weist darauf hin, dass die C1 Untereinheit eine zentrale Einheit im CHLAMY1-Komplex darstellt.

Im Rahmen dieser Arbeit wurde auch untersucht, ob die Expression der beiden Untereinheiten C1 und C3 auf Temperaturunterschiede im physiologischen Rahmen reagiert. Es wurde gefunden, dass sich die Expression von C1 bei niedriger (18°C) und hoher (28°C) Temperatur nicht ändert. Jedoch liegt C1 bei der niedrigen Temperatur hyperphosphoryliert vor, wohingegen es bei 28°C hypophosphoryliert ist. Im Falle von C3 konnte ermittelt werden, dass es bei niedriger Temperatur erhöht exprimiert wurde. Ein Inhibitorexperiment mit Cycloheximid zeigte, dass diese Regulation auf transkriptioneller Ebene erfolgt.

Außerdem wurde gezeigt, dass die Uhren-relevante Caseinkinase 1 (CK1) und Ser-/Thr-Protein Phosphatasen (PPs) die temperaturabhängige Regulation von

C1 und C3 vermitteln. CK1 wird sogar selbst temperaturabhängig exprimiert. Untersuchungen mit der Langperioden-Mutante *per1* weisen signifikante temperaturabhängige Unterschiede der C1-Modulierung bzw. der C3-Expression im Vergleich zum Wildtyp auf. So lag eine Hypophosphorylierung von C1 bei beiden Temperaturen (18 und 28°C) vor, während C3 bei beiden Temperaturen vermehrt exprimiert wurde.

Die Ergebnisse dieser Arbeit lassen auf ein temperaturabhängiges Netzwerk von Uhren-relevanten Proteinen schließen, an dem C1, C3, CK1, Ser-/Thr-PPs sowie PER1 beteiligt sind.

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7. Curriculum Vitae

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Education and Qualification

1989 -1996	Municipal general secondary school №24, Mytishchi
1996 -2001	M.V. Lomonosov Moscow State University, Biological Department Speciality: Physiology Specialization: Plant Physiology Diploma work "Regulatory changes of intracellular pH in the process of activation and germination of pollen grain of *Nicotiana tabaccum L.*". The work was carried out at the chair of Plant Physiology. Grade: excellent. Average grade: 4,88 (maximum mark 5,00; minimum positive mark 3,00)
2001-2003	A scientific position at the Chair of Physicochemical Biology of Biological Department of M.V. Lomonosov Moscow State University
2003-2004	A DAAD fellowship at the Institute of General Botany (FSU Jena, Germany)

Since June, 2004	PhD student at the Institute of General Botany, Friedrich-Schiller-University of Jena Theme of PhD thesis "The function of the RNA-binding protein CHLAMY1 in the circadian clock and its temperature integration process". Supervisor: Prof. Dr. M. Mittag

Languages: Russian (native), English (fluent), German (elementary)

Publications of O. Voytsekh:

All publications indicated with # are related to this PhD work.
All authors indicated with an * have shared first authorship.

1. Matveyeva, N.P., **Voytsekh, O.**, Andreyuk, D.S. and Yermakov, I.P. (2002). Role of H^+-ATPase and alternative oxidase in regulation of intracellular pH at different stages of development of the tobacco male gametophyte. Russian Journal of Developmental Biology 33, 436-443.

2. Matveyeva, N.P., Andreyuk, D.S., **Voytsekh, O.**, and Yermakov, I.P. (2003). Regulatory changes in the intracellular pH and Cl^- efflux at early stages of pollen grain germination *In Vitro*. Russian Journal of Plant Physiology 50, 360-365.

3#. Schmidt, M., Gessner, G., Luff, M., Heiland, I., Wagner, V., Kaminski, M., Geimer, S., Eitzinger, N., Reissenweber, T., **Voytsekh, O.**, Fiedler, M., Mittag, M., Kreimer, G. (2006). Proteomic analysis of the eyespot of *Chlamydomonas reinhardtii* provides novel insights into its components and tactic movements. Plant Cell 18, 1908-1930.

4#. Iliev, D., **Voytsekh, O.**, Mittag, M. (2006). The circadian system of *Chlamydomonas reinhardtii*. Biol. Rhythm Res. 37, 323-333.

5#. Iliev, D.*, **Voytsekh, O.***, Schmidt, E-M., Fiedler, M., Nykytenko, A., Mittag, M. (2006). A heteromeric RNA-binding protein is involved in maintaining phase and period of the circadian clock. Plant Phys. 142, 797-806.

6. Govorunova, E.G., **Voytsekh, O.O.**, Sineshchekov, O.A. (2007). Changes in photoreceptor currents and their sensitivity to the chemoeffector tryptone during gamete mating in *Chlamydomonas reinhardtii*. Planta 225, 441-449.

7#. Kiaulehn, S., **Voytsekh, O.**, Fuhrmann, M., Mittag, M. (2007). The Presence of UG-repeat sequences in the 3'-UTRs of reporter luciferase mRNAs mediates circadian expression and can determine acrophase in *Chlamydomonas reinhardtii*. J. Biol. Rhythms 22, 275-277.

8#. **Voytsekh, O.***, Seitz, S.B.*, Iliev, D., Mittag, M. Both subunits of the circadian RNA-binding protein CHLAMY1 can integrate temperature. Plant Physiol. Under revision.

9#. **Voytsekh, O.***, Seitz, S.B.*, Prager, K., Bösger, J., Iliev, D., Fiedler, M., Mittag, M. Temperature integration of the circadian RNA-binding protein CHLAMY1 includes phosphorylation events and is disturbed in the per1 mutant. In preparation for resubmission.

Oral presentations:

1. PhD meeting within the frame of the DFG Research Group FOR 504, Freiburg, June, 2004.
2. PhD meeting within the frame of the DFG Research group FOR 504, Berlin, March, 2006.
3. Middle German Symposium of Plant Physiology, Leipzig, February, 2007.

8. Eigenständigkeitserklärung

Die geltende Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität ist mir bekannt. Die vorliegende Dissertation habe ich selbständig verfasst und keine anderen als die von mir angegebenen Quellen, persönliche Mitteilungen und Hilfsmittel benutzt.

Bei der Auswahl und Auswertung des Materials haben mich die in der Danksagung meiner Dissertation genannten Personen unterstützt. Sämliche Personen, die mich bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts unterstützt haben, sind in der Danksagung genannt.

Ich habe nicht die Hilfe eines Promotionsberaters in Anspruch genommen und Dritte haben weder mittelbar noch unmittelbar geldwerte Leistungen von mir für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen.

Ich habe die Dissertation noch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht. Ferner habe ich nicht versucht, diese Arbeit oder eine in wesentlichen Teilen ähnliche oder eine andere Abhandlung bei einer anderen Hochschule als Dissertation einzureichen.

Jena, April 2008 .....

Olga Voytsekh

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