Histone modifications and the *Arabidopsis thaliana* circadian clock

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I hereby declare that the above thesis is my own work and neither the substance nor any part of the dissertation has been submitted in the past, nor is being, nor is to be submitted for a degree at this University or any other University.

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LIST OF ABBREVIATIONS

BRASS	Biological rhythms analysis software system
bp	base pair
CCA1	CIRCADIAN CLOCK ASSOCIATED 1
cDNA	complementary DNA
ChIP	chromatin immunoprecipitation
clf	curly leaf
CLOCK	circadian locomoter activity kaput
DD	constant darkness
CTAB	N-cetyl-trimethyl-ammonium bromide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediamiamine tetraacetic acid
FFT-NLLS	fast Fourier transform non-linear least squares
Gcn5	General control non-repressed protein 5
Hd1	histone deacetylase1
IAA	isoamyl alcohol
LHY	LATE ELONGATED HYPOCOTYL
LL	constant
mRNA	messenger RNA
MS	Murashige and Skoog medium
MW	Molecular weight
NASC	The Nottingham Arabidopsis Stock Centre
PCR	polymerase chain reaction
PVP	Polyvinyl pyrrolidone

PVPP	Polyvinyl polypyrrolidone
RAE	relative amplitude error
RT-PCR	reverse transcription-polymerase chain reaction
T-DNA	transfer DNA
TAE	Tris-acetate-ethylenediamiamine tetraacetic acid
Taf1	TATA-binding Protein associated factor 1
TOC1	TIMING OF CAB EXPRESION 1

ABSTRACT

The circadian system has a regulatory role in almost all aspects of a plant's life. In *Arabidopsis thaliana*, almost 36% of the genome has been shown to be circadianly regulated and many genes that are circadianly regulated have been shown to be light responsive or involved in light responses. Rhythmic histone acetylation has been demonstrated in the promoter of *TIMING OF CAB EXPRESSION1 (TOC1)*. Here, I used semi-quantitative Reverse Transcriptase Polymerase Chain Reaction (semi-quantitative RT-PCR) to investigate which enzymes are involved in the rhythmic expression of *TOC1*. I also determined whether loss-of-function histone acetylation and methylation mutants could affect the overall functioning of the circadian oscillator by measuring their circadian leaf movement and delayed fluorescence (DF) rhythms. *GCN5/ HAG1* mutant plants (*gcn5*) exhibited erratic *TOC1* expression in both constant dark (DD) and constant light (LL) conditions. Although *TOC1* expression appeared to be rhythmic in both DD and LL conditions, the waveform of the rhythm was altered in *TATA-binding protein associated factor 1* (*taf1*) mutants. This suggested that TAF1 and GCN5 might play different roles in the rhythmic histone acetylation affecting *TOC1* expression. DF data and leaf movement data indicated that both TAF1 and GCN5 might play a role in the overall functioning of the *A. thaliana* circadian clock. Arrhythmic *TOC1* expression and DF was observed in *histone deacetylase 1* (*hd1*) mutants, suggesting that HD1 is not only involved in the rhythmic histone deacetylation affecting *TOC1* expression but in the overall functioning of the circadian clock. Semi-quantitative RT-PCR, DF and leaf movement studies demonstrated that CURLY LEAF (CLF), a histone methylase is involved in both the histone methylation affecting *TOC1* expression and in the overall functioning of the *A. thaliana* circadian clock.

SECTION 1: Literature Review

1.1 An introduction on to circadian rhythms

The Earth rotates on its axis every 24 hours resulting in any position on the earth's surface alternatively facing towards or away from the sun, producing day and night. These changes affect the behaviour, metabolism and physiology of most organisms and many organisms have developed a time keeping mechanism that allows them to anticipate the regular changes rather than to simply respond to the environment (Millar, 1999). The sleep/wake patterns in animals are an obvious example of these behavioural changes. Another example of a process influenced by these changes in the earth's rotation is changes in the position of organelles in plants (Millar, 1999).

Most organisms including cyanobacteria, plants and other higher eukaryotes have persistent rhythms in gene expression, physiology, and behaviour with periods of approximately 24 hours (Harmer *et al.*, 2001; Young and Kay, 2001; McClung *et al.*, 2002). These rhythms are termed circadian, from the Latin words "circa" (about) and "dies" (day), rhythms. Circadian rhythms are governed and maintained by an endogenous oscillator also termed the biological clock. This clock allows plants to anticipate changes in their environment and therefore offers them a selective advantage. For sessile plants, this ability is necessary to ensure their survival. For example, net photosynthesis has been shown to fall dramatically when internal and external cues diverge (Ouyang *et al.*, 1998; Green *et al.*, 2002; Michael *et al.*, 2003; Dodd *et al.*, 2005). Biological processes can be described as outputs of the circadian clock if they meet three criteria: firstly, the rhythms must persist with a period of approximately 24 hours after an organism is transferred from entraining conditions (an environment that varies

from time of day) to free-running conditions (an unchanging environment). Secondly, environmental cues such as light and temperature can control the phase of the rhythms. Lastly, circadian rhythms are temperature compensated; they occur with approximately the same period across a wide range of temperatures (Harmer, 2009). Circadian rhythms often take the form of sinusoidal waves that can be described in terms of period, phase and amplitude (Figure1)

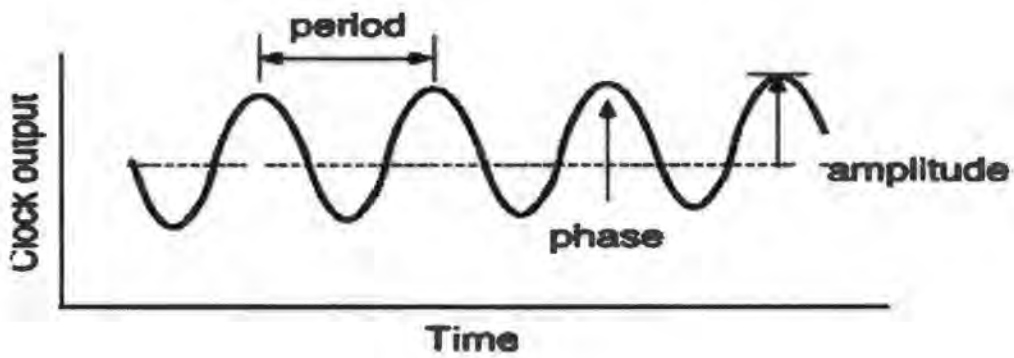

Figure 1. Sinusoidal waves showing critical terms used to define circadian rhythms (From McClung, 2006).

The biological clock of plants can be entrained, or 'reset', by specific environmental signals such as light and temperature, such that the phase of the circadian clock matches the external environment (Harmer *et al.,* 2001). Light and temperature may also affect the rhythmic amplitude of the clock outputs (Rensing and Ruoff, 2002). Availability of nutrients has also been suggested to act as an entraining signal in some organisms (Roenneberg and Merrow, 1998). In *Arabidopsis thaliana* light has been shown to be the predominant environmental signal responsible for the synchronisation of the clock and the external environment (Devlin, 2002).

The circadian system has a regulatory role in almost all aspects of a plant's life: germination, hypocotyl elongation, leaf movement, shade avoidance, flowering time, flower opening, scent production, tuberization, winter dormancy, stomatal opening, photosynthesis, photoprotection and protection from extreme temperature (Yakir *et al.*, 2006). Photoperiodism is the term used to describe a plant's ability to flower in response to changes in the photoperiod: the relative lengths of day and night. Therefore, photoperiodism is a case in which a circadian rhythm is combined with light signaling (Millar, 2004). The circadian clock therefore allows plants to respond to the annual cycle of day length and thus is involved in seasonal changes.

1.2 The plant circadian clock

1.2.1 Organisation of the plant circadian clock

The most simplified conceptual framework of the plant circadian clock consists of the input pathways involved in the perception and transmission of environmental cues to synchronise the central oscillator that generates and maintains rhythmicity through multiple output pathways (Figure 2; McWatters, *et al.*, 2001; Más, 2005).

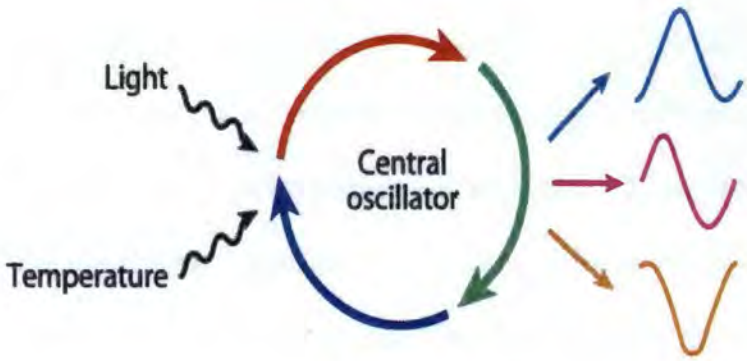

Figure 2. A simplified conceptual framework of the plant circadian system depicting resetting pathways that change the phase of the clock in response to environmental cues such as light and temperature, and variety of rhythmic outputs (From Harmer, 2009).

However several experiments have revealed the existence of a far more complex circadian clock, with multiple feedback loops whereby output elements regulate the central oscillator and the input elements are tightly regulated by the clock (McWatters, *et al.*, 2000; Hall *et al.*, 2002; Thain *et al.*, 2002; Eriksson and Millar, 2003).

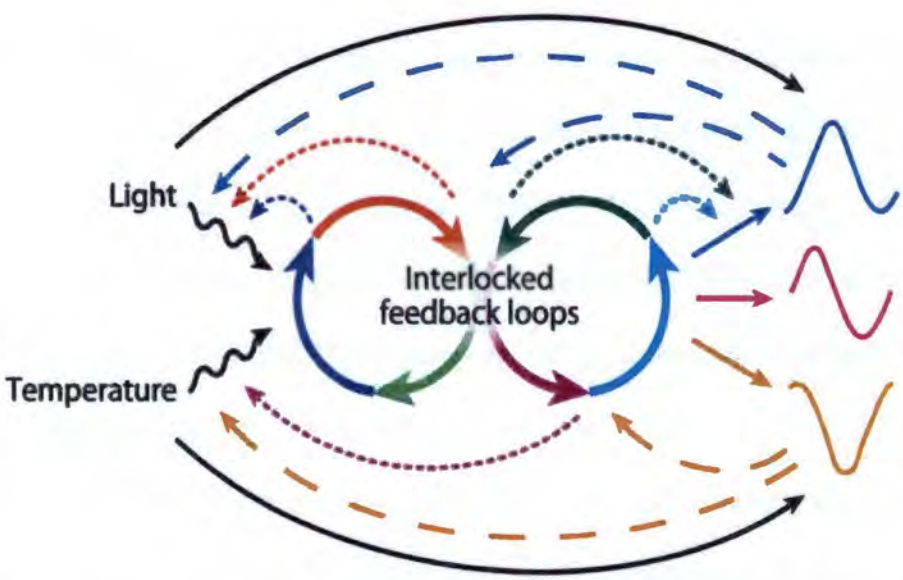

Figure 3. A more complex circadian system in multicellular organisms with multiple clocks showing multiple feedback loops (From Harmer, 2009).

1.2.2 A brief history of circadian clock research in plants

In 1729, a French astronomer de Marian noticed that daily leaf movements in a heliotrope plant (possibly *Mimosa pudica*) persisted in constant dark conditions. He suggested that these rhythms were related to the sleep patterns of bedridden patients (reviewed in: Pittendrigh, 1993). Free running defines the state of a rhythm in the absence of entraining stimuli. In 1832, de Candolle accurately measured the free running period of *M. pudica* leaf movement rhythms to be 22 to 23hours (McClung, 2006). This suggested that the leaf rhythms were not just a response to the environment, but were endogenous. Leaf movement experiments have been exploited by scientists investigating plant endogenous rhythms up to the present day.

Despite these finding, there was still an ongoing debate among scientists on whether daily leaf movements were truly endogenous (McClung, 2006). In 1915, Pfeffer showed that many free running periods of leaf movements were not exactly 24hours (reviewed in: McClung, 2006). This was important in proving that these rhythms were in fact endogenous and not driven by some unknown cue associated with the earth's rotation. It took a while for scientists to agree that circadian rhythms were temperature compensated. The Q_{10} temperature coefficient is a measure of the rate of change of a biological or chemical system as a consequence of increasing the temperature by 10°C (Curiel yuste, *et al.,* 2004). In a typical chemical process, the rate of the reaction doubles with a 10°C increase in temperature (Q_{10} = 2) (Curiel yuste, *et al.,* 2004). However, in 1931 Bünning demonstrated that the period of leaf movement in *Phaseolus coccineus* (Runner bean) exhibited a Q_{10} of only 1.2 (reviewed in: McClung, 2006). By the 1960s, this observation had been extended to other plants as well as animals. It was later observed that an imperfect temperature compensation mechanism at higher temperatures, could lengthen the period insufficiently or too much, while an imperfect

temperature compensation mechanism at low temperatures could shorten the period too little or too much (McClung, 2006).

Output rhythms can give insight into the circadian clock. Apart from leaf movements, other processes such as stomatal movement, photosynthetic activity and germination are some examples of physiological processes under circadian control in plants (Yakir *et al.*, 2006). Studying these and other output rhythms can give us an insight into the control and functioning of the circadian oscillator. When assayed under entraining light/dark cycles, the rhythms usually assume the same period as the changing environment (24 hours). Therefore, the non-24-hour periodicity of the endogenous circadian clock can only be revealed when experiment are done in free-running conditions, i.e. in the absence of any entraining stimuli (Rensing and Ruoff, 2002).

In order to determine leaf movements, plants are first entrained under light/dark cycles and then transferred to constant light conditions. Plants are imaged at time intervals, in our case every 20 minutes, for several days. The period and amplitude of the movement can be determined by tracking the leaf positions in digitized images using the appropriate software. If the leaves still rise and lower rhythmically in the absence of light-dark stimulus with a period of ~24 hours, the rhythms will be considered circadian. Leaf movements therefore assess the integrity of the plant circadian clock. However these experiments are limited to constant light conditions, as images cannot be captured in the dark. Also only growing leaves give can be used as rhythmicity ceases after leaf growth is complete (Somers, 1999).

Many studies have revealed that some nuclear transcripts were under circadian control. In 1985, Kloppstech demonstrated that *CHLOROPHYLL A AND B BINDING PROTEIN* (*CAB2*) transcription was under circadian control (Kloppstech, 1985). This was also demonstrated three years later in *Hordeum vulgaris* (Barley) (Nagy *et al.*, 1988). In 1992, Miller *et al.*, demonstrated that transcription and accumulation of firefly luciferase (LUC) mRNA, could be rhythmically driven by *s*hort fragments of the *A. thaliana CAB2* promoter. They also demonstrated that the LUC protein and therefore the *LUC* mRNA could be detected as rhythmic light emission from individual *A. thaliana* seedlings possessing the *CAB2::LUC* transgene. The use of LUC became a very useful tool as it allowed the first screening of *A. thaliana* clock mutants and testing the effects of various mutations on the functioning of the circadian clock (Millar *et al.*, 1995a). This method had the advantage of being nondestructive, high-throughput and convenient. Although a highly sensitive camera was required to detect the luciferase activity, luciferase had many characteristics that made it a suitable candidate for studying circadian rhythms. Excitation by light was not required for the detection of the luciferase activity (which can perturb the circadian clock) and the light emission by the luciferase enzyme closely tracks the activity of the promoter driving its expression (Millar *et al.*, 1995b; Onai *et al.*, 2004).

The expression of many genes in diverse genetic backgrounds can be detected by the use of quantitative reverse transcriptase polymerase chain reaction (semi-quantitative RT-PCR) assays. This circumvents the need for transgenics (Harmer, 2009). DNA microarrays can also be used to identify more rhythmically expressed genes which may potentially lead to the identification of more clock components (Sato *et al.*, 2003).

Recently, Anthony Hall's group demonstrated that chlorophyll delayed fluorescence (DF) can be used as a tool for the measurement of circadian rhythms in higher plants (Gould *et al.*, 2009). In 1951, Strehler and Arnold discovered DF or delayed light emission in photosynthetic plants. DF is a result of post-illumination emission of light from chlorophyll a, mainly from photosystem II (PSII), as a result of a charge recombination between excited plastoquinone Q_A and the reaction centre chlorophyll P680, leading to the emission of a proton (Rutherford et al., 1984). Using a charged couple device (CCD) camera system that had been developed for the *in vivo* monitoring of promoter::LUC activity, Gould *et al.* (2009) demonstrated that rhythms in DF were regulated by the same biological clock that drove rhythms in other circadian outputs. An advantage of this method is that it is a simple, high-throughput method that uses existing technology and does not require the insertion of a reporter gene thus giving it broad applicability.

1.2.3 Molecular basis for plant circadian clocks

A. thaliana has been used as a model species to study circadian rhythms in plants (Yakir *et al.*, 2007). *A. thaliana* has a small genome of about 125 Mb. Its genome has been sequenced and is smaller than the genomes of most plants. It is highly fecund, and a physically small plant and many individuals can be grown in a small area (*Arabidopsis* Genome Initiative 2000; McWatters, *et al.*, 2001). Generating transgenic plants using *Agrobacterium tumefaciens* as a vector to introduce selected DNA sequences in *A. thaliana* genome is easy (Clough and Bent, 1998). The fully sequenced *A. thaliana* genome has allowed the successful use of forward genetics in identifying components of the circadian clock.

In animals, circadian rhythms were first scientifically determined in 1894 by experiments involving pigment rhythms in arthropods. Later in 1922, daily activity in rats was described (reviewed in: McClung, 2006). Forward genetics is a genetic approach where one starts with a mutation phenotype and works towards identifying the mutated gene. Reverse genetics on the other hand, is the experimental procedure used to investigate the function of a gene or protein. In order to do this, one starts with a cloned segment of DNA or a protein sequence and uses this knowledge to introduce programmed mutations, through direct mutagenesis, back into the genome in order to investigate their functions (Sessions *et al.*, 2002). The use of forward genetics in identifying components of the circadian clock in the 1970s was a breakthrough in the field of circadian rhythms (reviewed in: McClung, 2006). In 1984, the *Drosophila melanogaster* (fruit fly) *period* (*per*) gene was cloned (Bargiello and Young, 1984). Five years later, the *Neurospora crassa* (bread mold) *frequency* gene was cloned (McClung *et al.*, 1989). More clock components including those of *Drosophila, Neurospora* and mammals were elucidated in the 1990s (Dunlap, 1999). In plants, *TIMING OF CAB EXPRESSION1 (TOC1)* was the first clock gene to be cloned (Strayer *et al.*, 2000). Positional gene cloning was shown to be easier in *A. thaliana* than in either *Pisum sativum* (pea) or *Triticum aestivum* (wheat) and therefore, *A. thaliana* became a very powerful system for both molecular cloning and forward genetic analysis techniques (Somerville and Koornneef, 2002).

Most of the components of the plant circadian oscillator have been identified using forward genetics in *A. thaliana*. CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) are both single MYB transcriptional factors and were proposed to function at the core of its oscillator. They were identified because the loss of

function *lhy* mutant plants had a late flowering and an elongated hypocotyl phenotype (Wang and Tobin 1998, Schaffer et al. 1998). Plants with defective mutations in both genes retained their rhythmicity, but showed a short period while constitutive expression of either gene was shown to cause arrhythmia (Green and Tobin, 1999; Schaffer *et al.*, 1998; Wang and Tobin, 1998). Analysis of mutational phenotypes identified the pseudoresponse regulator TOC1, as a central part of the *A. thaliana* oscillator (Millar et al., 1995; Strayer *et al.*, 2000). Constitutive expression of *TOC1* caused arrhythmia in plants (Makino et al., 2002; Más *et al.*, 2003) while *toc1* mutant plants showed a shortened period phenotype in many outputs (Millar *et al.*, 1995; Somers et al., 1998; Strayer *et al.*, 2000). Therefore CCA1, LHY and TOC1 form part of the central oscillator (loop A, Figure 4) in A. *thaliana.* CCA1 and LHY are partly redundant transcription factors and are thought to regulate *TOC1* expression by binding directly to the Evening Element (EE) consensus motif present at the *TOC1* promoter thereby repressing its transcription (Alabadi *et al.*, 2001). Increase in the expression of *TOC1* was predicted to directly or indirectly activate the transcription of *CCA1* and *LHY* (Alabadi *et al.*, 2001). *TOC1* is an evening-phased gene and no DNA-binding domains have been recognised in the TOC1 protein (Strayer *et al.*, 2000). Recent studies done by Pruneda-Paz *et al.*, 2009, have identified CCA1 HIKING EXPEDITION (CHE), a transcription factor that belongs to the class I TCP (TBI, CYC, PCFs) family. CHE interacts with TOC1 and binds to the *CCA1* promoter, establishing a linkage between TOC1 and *CCA1* gene regulation (Pruneda-Paz *et al.*, 2009). Recent experimental and mathematical studies predict an unknown component X (Figure 4) thought to act between TOC1 and the CCA1 and LHY promoters (Locke et al., 2005a). PSEUDO-RESPONSE REGULATOR 7 (PRR7), PRR9, GIGANTEA (GI), (ZEITLUPE) ZTL, PRR3 and an unknown factor Y are proposed to form other loops (Figure 4, Locke *et al.*, 2005b). Recently, PRR9, PRR7 and PRR5 were demonstrated to suppress

CCA1 and *LHY* promoter activities and therefore suppressing their transcription (Nakamichi *et al.*, 2010). Using reporter constructs, PRR5 was also shown to directly regulate to *CCA1* and *LHY* expression (Nakamichi *et al.*, 2010). Additional components of the clock, which include EARLY FLOWERING4 (ELF4) and LUX ARRHYTHMO (LUX), were also identified (Doyle et al., 2002; Hazen et al., 2005). Recent studies have identified LIGHT-REGULATED WD1 (LWD1) and LWD2 as clock proteins that are involved in regulation of photoperiodic flowering in *A. thaliana* (Wu *et al.*, 2008).

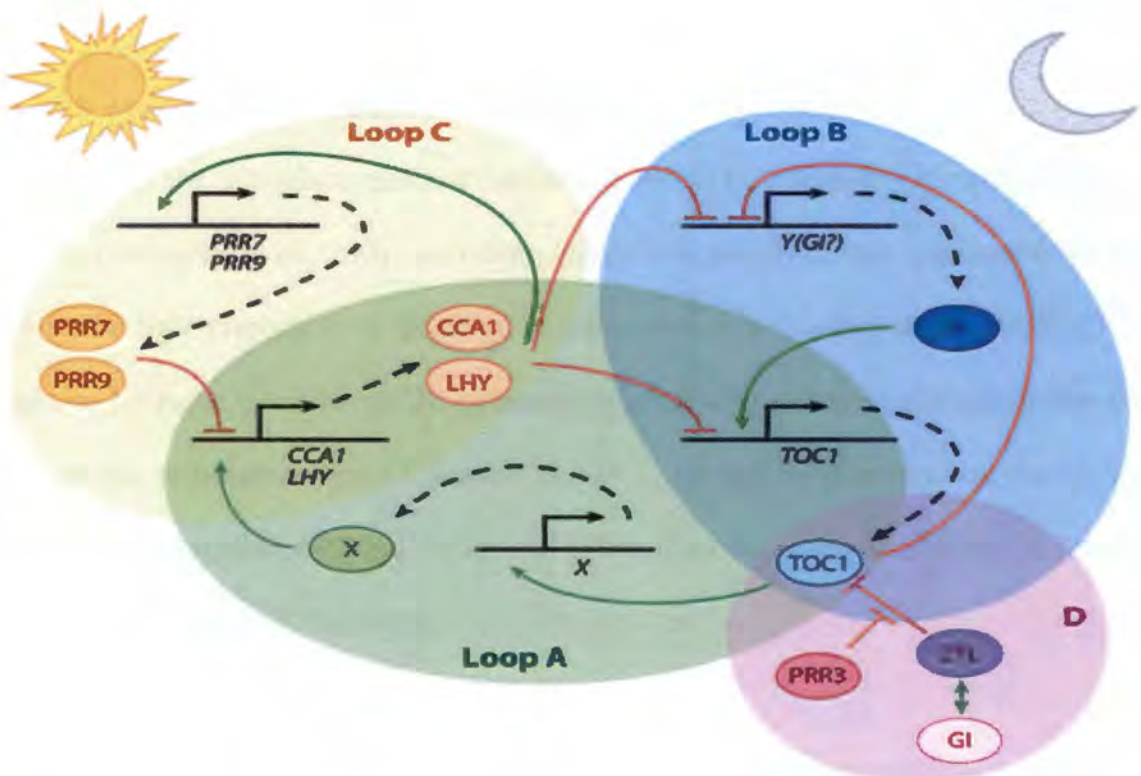

Figure 4. Model of the plant circadian network indicating various loops (A, B, C and D) that make up the circadian clock. Other genes implicated in the function of the clock have been omitted for clarity (From Harmer, 2009)

These transcriptional-translational feedback loops, but not the individual components, have been shown to be conserved between plants, mammals, insects and fungi. It has been

suggested that the reason for this conservation is to enhance the robustness of the network against perturbation (Kwon and Cho, 2007). Sweeney and Haxo, (1961) showed that circadian rhythms in the giant green alga *Acetabularia* persisted for several days after removal of its nucleus, suggesting that transcriptional feed loops may not be crucial in the functioning of the circadian clock in plants. In cyanobacteria, normal clock function has been shown to rely on both rhythmic phosphorylation and transcription (Kitayama *et al.,* 2008). Therefore, the robust circadian rhythms in higher plants may require both the biochemical and transcriptional cycles (Harmer, 2009).

1.2.4 How does the clock regulate gene expression?

Almost 36% of the *A. thaliana* genome has been shown to be circadianly regulated (Yakir, *et al.*, 2006; Covington *et al.*, 2008), and many genes that are circadianly regulated have been shown to be light responsive or involved in light responses (Harmer et al., 2000). This is thought to be made possible by the presence of several promoter motifs associated with phase-specific expression. The EE present in TOC1, GI and LUX as well as the morning element (ME), protein box (PBX), GATA and G-boxes are examples of such motifs (Alabadi *et al.,* 2001; Hudson and Quail, 2003).

An essential property of the clock function is its capacity to maintain rhythms in phase with the environment (Devlin and Kay, 2001). For plants that grow in temperate regions where daily rhythms in light and temperature change through the seasons, day length perception is very important for these plants to regulate developmental transitions such as initiation of flowering (Searle and Coupland, 2004). *A. thaliana* serves as an example of how the information provided by the circadian clock together with the light signals allow plants to

regulate the initiation of flowering when light coincides with the sensitive phase of the oscillatory cycle (Yanovsky and Kay, 2003). In *A. thaliana*, the oscillation of the diurnal transcript and the stability of the protein of *CONSTANS* (*CO*), a flowering component, the photoinducible phase (Suárez-López *et al.*, 2001; Valverde *et al.*, 2004). *CO* induces its target FLOWERING LOCUS T (FT) (Suárez-López *et al.*, 2001). Therefore, the coincidence of CO photoperiodic rhythm relative to the light cycle was proposed to as the basis for the earlier flowering of *A. thaliana* plants under inductive long days (Roden *et al.*, 2002).

1.3 Gene expression, Chromatin organization and histone tails

Eukaryotic genomes are very large and are therefore packaged into a stable structure termed chromatin. The precise organization of chromatin and the regulation of its re-organisation are crucial for cellular processes such as DNA transcription, replication, repair and recombination (Rice and Allis, 2001). As the circadian oscillator is based on a transcription-translation network, regulation of chromatin structure is particularly interesting in terms of regular transcription of key genes.

The Circadian Locomoter activity Kaput (CLOCK) protein has been identified to be part of the mammalian circadian clock. CLOCK and Brain and Muscle ARNT-like protein 1 (BMAL1) form a heterodimer to up-regulate transcription of genes encoding Period 1 (PER1), PER2, Cryptochrome 1 (CRY1) and CRY2 (Hirayama *et al.*, 2005). CLOCK has been identified as a histone acetyltransferase (HAT) (Doi *et al.*, 2006). Even though CLOCK is constitutively expressed, it can only enter nucleus with BMAL1 this gives it circadian activity as BMAL is rhythmically expressed (Ripperger and Schibler, 2006). Recently, Perales and Más (2006) demonstrated rhythmic histone acetylation in the promoter of *TOC1*

in *A. thaliana*. However, the enzymes responsible for this have not yet been identified. In order to investigate this we first need to understand a bit more about chromatin modification and modifiers in plants

The basic repeating structural unit in the chromatin is the nucleosome. Nucleosome core particles consist of a histone octamer core around which 146 bp of DNA are wrapped. The core histones are arranged as a $(H3\text{-}H4)_2$ tetramer and two H2A-H2B dimers are positioned on both sides of the tetramer (Lunger *et al.*, 1997, Wolffe, 1998). The basic structure of the core histones include an N-terminal domain, a globular domain organised by the histone fold and a C-terminal tail. According to Spencer and Davie, 1999, these histone domains mediate histone-histone and histone-DNA interactions. A fifth class of histones binds to the core histones and this causes the stabilization of the higher order compaction of chromatin (Zhou *et al.*, 1998; Spencer and Davie, 1999).

1.3.1 Histone modifications

The chromatin structure is susceptible to various modifications. Swi/Snf (Switch/Sucrose non fermentable) is a yeast nucleosome remodeling complex composed of several proteins-products of the SWI and SNF genes (*SWI1*, *SWI2/SNF*, *SWI3*, *SWI5*, *SWI6*) as well as several other polypeptides (Pazin and Kadonaga, 1997). Mechanisms that have been implicated in this include the participation of ATP-dependent remodeling factors such as Swi/Snf and the nucleosome remodeling factor (NURF) and the involvement of enzymatic activities (Peterson and Workman, 2000; Cheung *et al.*, 2000a). The N-terminal domains of histones are subjected to various histone modifications such as acetylation, methylation, phosphorylation,

ADP-ribosylation, sumoylation and ubiquitination (Figure 5; Spencer and Davie, 1999; Nathan *et al.*, 2003).

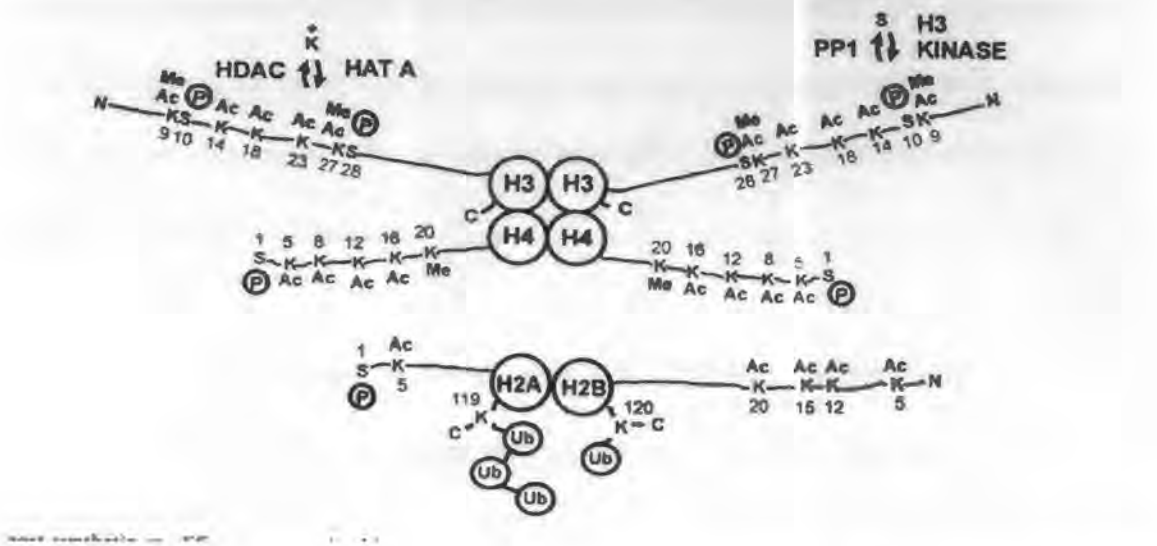

Figure 5. Sites of post-synthetic modifications on the core histones. The structure of the (H3-H4)2 tetramer, H2A-H2B dimers and sites of modifications are shown. The modifications shown are acetylation (Ac), methylation (Me), phosphorylation (P) and ubiquitination (Ub) (From: Spencer and Davie, 1999).

1.3.1.1 Histone acetylation and gene activation

Histone acetylation was the first histone modification to be studied (Zhang and Reinberg, 2001). The core histones are reversibly acetylated at specific lysine residues located in the N-terminal tail domain. Acetylation occurs at four to five sites on the core histones, with the exception of H2A (Spencer and Davie, 1999). In particular, H3 is acetylated at the -amino group of lysine 9 (lys9), 14, 18 and 23 and H4 is acetylated at lys5, 8, 12 and 16 (Roth *et al.*, 2001). Acetylation of the histone tails promotes the solubility of chromatin at physiological ionic strength, disrupts higher order chromatin folding, i.e. promotes an "open" conformation and therefore promotes transcription (Garcia-Ramirez *et al.*, 1995, Walia *et al.*, 1998, Tse *et*

al., 1998). Acetylation also causes neutralisation of the core histone which results in the reduction of their affinity for DNA and this alters histone-histone interaction between adjacent nucleosomes as well as the interactions of histones with other regulatory molecules (Grant and Berger, 1999; Grant, 2001). It has also been suggested that histone acetylation may alter the structure of histone tails by increasing their alpha-helical content (Wang *et al.*, 2000; Rice and Allis, 2001).

Acetylation of histones and their subsequent deacetylation are dynamic processes occurring at more than one rate. In mammals, one population of core histones is characterized by rapid hyperacetylation and deacetylation (Davie, 1997). This process occurs in only 15% of the core histones.

1.3.1.2 Histone acetyltransferases (HATs) and their role in gene activation

Histone acetyltransferases (HATs) are enzymes which catalyse histone acetylation. The A. *thaliana* genome has 12 putative HATs distributed among all five chromosomes (*Arabidopsis* Genome Initiative 2000). Like other eukaryotes, plants have two major classes of HATs: Type A and Type B (Lusser *et al.*, 2001). Type B HATs are cytoplasmic complexes involved in the acetylation of histone H4 at position 5 and 12 before its incorporation into nucleosomes. Type A HATs are nuclear proteins and fall into four different classes with different specificities: the GCN5, CPB/p300, TAF_{II} 250, and the MYST classes (Reyes *et al.*, 2002). Benhamed *et al.*, (2006) demonstrated that HATs such as GCN5 and TAF1, and the histone deacetylase (HDAC) HD1 regulate histone acetylation and deacetylation of light-responsive genes in *A. thaliana*. Recently Perales and Más, 2006 demonstrated rhythmic

histone acetylation in *TOC1* promoter. These findings have led us to investigate potential HATs involved in the functioning of the plant circadian clock.

1.3.1.2.1 GCN5/ HAG1

Yeast Gcn5, a member of two transcriptional regulatory complexes, SAGA and ADA, was among the first HATs to be characterized (Spencer and Davie, 1999). Gcn5 has been shown to acetylate histone H3 in the nucleosome (Grant *et al.*, 1997; Saleh *et al.*, 1997). Studies done by Sterner and Berger (2000) also revealed that yeast and animal Gcn5 also acetylate non-histone proteins such as transcription factors. In yeast, 4% of genes were shown to be both up- and down-regulated in *gcn5* deletion mutants (Lee *et al.*, 2000). Most HATs have a bromodomain which is an acetyl-lysine binding motif found in many chromatin related proteins and the *A. thaliana* GCN5 (AtGCN5) protein shows significant similarities to the HAT catalytic domains and bromodomains of other Gcn5 homologues (Stockinger *et al.*, 2001). Vlachonasios *et al* (2003) tested 8200 genes in vegetative tissues and demonstrated that a T-DNA insertion in *AtGCN5* caused pleiotropic effects on plant growth and development in about 5% of these genes, suggesting that AtGCN5 is involved gene regulation as proposed for the yeast and mammalian Gcn5 family members.

1.3.1.2.2 TAF1 (TATA-binding protein associated factor 1)

TAF1 also called TAF_{II} 250 is one of the 10-12 TATA- binding protein (TBP)-associated factors (TAF_{II}) that form with TBP, the TFIID complex in eukaryotic cells (Bertrand *et al.*, 2005). Using chromatin immunoprecipitation (ChIP) assays, Bertrand *et al.*, (2005), demonstrated that the mutation of *HAF2*, which encodes TAF1, reduced acetylation of histone H3 in light-responsive genes in *A. thaliana*.

It has been suggested that the acetylation of histones in nucleosomes destabilizes the higher order of the chromatin structure (Spencer and Davie, 1999). Studies with yeast and animals provided evidence that HATs of the CBP/ p300 and GCN5 families are recruited to promoters by specific transcription factors (Stockinger *et al.*, 2001). *A. thaliana* CBF1, a transcription activator involved in cold-regulated gene expression, interestingly interacts with GCN5 in vitro, which suggests that a similar recruitment of HATs to promoter regions can occur in plants (Stockinger *et al.*, 2001). Different mechanisms are implicated in how the recruited HATs stimulate transcription from a chromatin template: a HAT(s) recruited by a promoter-bound activator might result in localized acetylation of histone and non histone chromosomal proteins. The other mechanism involves a HAT being recruited by an enhancer- or locus control region-bound activator resulting in widespread acetylation (Spencer and Davie, 1999). NuA4, a multiprotein complex that acetylates nucleosomal H2A and H4, has been shown to stimulate transcription by acetylation of H4 (Ikeda *et al.*, 1999). It has been suggested that further components of the HAT complex might facilitate the formation of the pre-initiation complex, thus stimulating transcriptional initiation and/ or elongation (Ikeda *et al.*, 1999).

1.3.1.2 Histone deacetylases (HDACs) and gene repression

Histone deacetylases (HDACs) are enzymes which catalyse histone deacetylation. The *A. thaliana* genome consists of 18 putative HDACs distributed among all five chromosomes (*Arabidopsis* Genome Initiative 2000). In plants, HDACs can be divided into four classes based on their sequence homology, substrate specificity and cofactor requirements (Lusser *et al.*, 2001). Class I and class II are homologues to the yeast RPD3 and HDA1 proteins,

respectively. Class III HDACs are related to the yeast SIR2 protein. A fourth class of HDAC first discovered in maize, is the HD2 protein and occurs only in plants (Lusser *et al.*, 2001).

In contrast to HATs, recruitment of HDACs to promoter regions can lead to gene repression. It is also important to note that both HATs and HDACs are recruited to these regions. Since acetylation of histones and their subsequent deacetylation are dynamic processes occurring at more than one rate, the chromatin will take on the repressive state when deacetylation becomes favoured (Spencer and Davie, 1999).

1.3.1.2.1 *A. thaliana* HD1 (Histone deacetylase 1) role in transcription

AtHD1, also known as HDA19 or AtRPD3A, belongs to the class I HDACs. AtHD1 is expressed at high levels in leaves, stem, flowers, and young siliques (Reyes *et al.*, 2002). Direct evidence that HDACs are involved in transcription in plants was provided by Wu *et al.*, (2000) who demonstrated that the AtHD1 fusion protein can repress transcription when recruited to a promoter through interaction with a DNA-binding domain. *AtHD1* antisense RNA mutants showed a hyperacetylated histone H4 and various phenotypic and developmental defects including the delay of flowering and male and female sterility (Tian and Chen, 2001). This suggests that histone deacetylation directly or indirectly affects the expression of many genes in the regulatory network (Finnegan, 2001). DNA microarray analysis showed that down regulation of *AtHD1* induced up- and down-regulation of >7% of the transcriptome, including genes which are involved in cellular biogenesis, protein synthesis, and plant hormonal regulation (Tian *et al., 2005*).

1.3.1.3 Histone methylation

Although histone methylation was first described in 1964, the link between histone methylation and transcription was only discovered about 35 years later when then the histone H3 arginine-specific histone methyltransferase (HMT) CARM1 was shown to interact with the steroid-hormone-receptor coactivator CRIP1 in transcriptional activation (Grant, 2001; Rice and Allis, 2001). The core histones H2B, H3 and H4 are modified by methylation, but the site of methylation varies in different species.

There are two types of histone methylation, targeting either arginine or lysine residues (Stallcup *et al.*, 2000; Koh *et al.*, 2001). Methylation of histone arginine residues is done by (histone methyltransferases) HMTs which are recruited to promoters. These are CARM1 and PRMT, and they predominantly target either H3 or H4, respectively (Chen *et al.*, 1999; Wang *et al.*, 2001). Histone arginine methylation is involved in gene activation (Stallcup *et al.*, 2001; Berger, 2002).

Lysine methylation can have different consequences according to which lysine residue is modified and how many methyl groups are added lysine residues can be mono-, di- or trimethylated (Bannister *et al*, 2002). Methylation of H3K9 and H3K27 have generally been associated with transcriptional repression while methylation of H3K4, H3K36 and H3K79 has been associated with transcriptional activation (Jenuwein and Allis, 2001; Peters et al, 2003; Fischle et al., 2003; Ringrose *et al.*, 2004). The level of methylation has also been shown to have different effects in animals. For example tri-methylation of H3K9 (H3K9me3) shows a different distribution from H3K9me1 and H3K9me2 in mammals (Peters *et al*,

2003). Like acetylation, methylation has been shown to be a reversible process (Shi *et al*, 2004; Tsukada *et al*, 2006).

1.3.1.3.1 CLF (CURLY LEAF)

In both animals and plants, Trithorax (Trx) and Polycomb group (PcG) proteins carry out the stable propagation of established gene expression states during mitotic cell divisions (Makarevich et al., 2006). The mechanism by which this is achieved still remains unknown. The PcG complexes consist of three separate protein complexes (PRC1, PRC2 and PhoRC).These complexes assemble on chromatin and coordinate ubiquitination of H2A lysine 119 (H2AK119) and methylation of H3K27 (Schwartz and Pirrotta, 2007). In plants, only the PRC2 is structurally conserved (Reyes and Grossniklaus, 2003). Four core members of Drosophila PRC2 have been identified: SUPPRESSOR OF ZESTE 12 (SU(Z)12), P55, EXTRA SEX COMBS (ESC) and ENHANCER OF ZESTE (E(Z)) (Ringrose et al, 2004). (E(Z) carries a histone methyltransferase domain, SET for SU[VAR]3–9, Enhancer of Zeste (E(Z)), TRX). The SET domain is conserved across species, suggesting that the maintenance of cellular memory involves histones methylation (Cao and Zhang, 2004). The SET domain can trimethylate lysine 9 and 27 of histone H3 but requires other PRC2 members to accomplish its catalytic activity (Cao et al, 2002; Czermin et al, 2002; Kuzmichev et al, 2002; Muller et al, 2002). In *A. thaliana*, there are three E(z) homologues: MEDEA (MEA), CURLY LEAF (CLF) and SWINGER (SWN, also known as EZA1). MEA predominantly acts during seed development, whereas CLF and SWN are expressed more generally in plants. CLF is required to repress floral homeotic genes such as AGAMOUS (AG), a gene that plays a central role in reproductive organ (stamen and carpel) development, in whorls 1

and 2 of flowers, and in vegetative organs and also the homeobox gene SHOOTMERISTEMLESS (STM) (Goodrich et al, 1997; (Kim et al., 1998, Katz et al, 2004).

1.4 The role of histone modifications in other circadian systems

In mammals, circadian clocks are governed and maintained by a "master clock" called the suprachiasmatic nuclei (SCN) which contains about 20,000 nerve cells and located at the base of the hypothalamus in the brain. The SCN are entrained and to the 24 hour day by the daily light/dark cycles acting through the retina-to-SCN neural pathways (Akhtar *et al.*, 2001). To date, various core circadian clock genes have been identified in mammals: *Clock, Bmal, casein kinase I epsilon* (*CKIε*), *cryptochromes 1 and 2* (*Cry1, Cry2*), *Period1, 2 and 3* (*Per1, Per2 and Per3*) *and Rev-erb-α. Clock* and *Bmal1* genes encode basic helix-loop-helix-PAS transcription activators that heterodimerise and induces the expression of *Per* and *Cry* genes via the binding to E-box elements (CAGGTG) which are present in their promoters (Reppert and Weaver, 2002).

CLOCK-BMAL heterodimer has been shown to regulate many clock controlled genes (CCGs), which in turn have in turn influence a wide variety of physiological functions external to the oscillatory mechanism (Duffield *et al.*, 2002; Panda *et al.*, 2002). Studies have revealed that the activation of CCGs by CLOCK-BMAL1 is coupled to circadian changes in histone acetylation which indicates that transcription-permissive chromatin states are dynamically established in a circadian time specific manner (Reviewed in Nakahata *et al.*, 2007). The coupled modification of Ser-10 phosphorylation and Lys-14 acetylation on H3 has been shown to be an excellent place for activation of transcription (Cheung *et al.*, 2000b;

Lo *et al.*, 2000) supporting a notion that HATs are involved in circadian gene expression (Doi *et al.*, 2006; Ripperger and Schibler, 2006).

It is important to note that the effect of gene expression does not only depend on the type of histone modification present, but also on the spatial distribution of a given modification across a gene region and the combination of other modifications present (Bernstein *et al.*, 2007; Kouzarides, 2007; Pfluger and Wagner, 2007). For example, tri-methylation of histone H3 on lys 9 and 36 (H3K9me3 and H3K36me3) is repressive when found in the promoter of a gene but activating when found in within the gene (Kouzarides, 2007).

1.5 Aims

Although rhythmic histone acetylation has been demonstrated in the promoter of *TOC1* in *A. thaliana* (Perales and Más, 2007), the enzymes responsible have not been identified. I therefore aimed to identify enzymes involved in rhythmic histone modification by investigating *TOC1* expression of in *A. thaliana* in plants with mutations in genes encoding histone acetyl transferases or histone deacetylases (*gcn5, taf1, hd1*) and a histone methyltransferase (*clf*). My hypothesis was that if any of these enzymes are involved in the rhythmic modification of histones at the *TOC1* locus, the expression of *TOC1* would be affected by loss-of-function mutation of their genes. Furthermore, the overall functioning of the circadian oscillator may be affected by loss-of-function mutations in these genes and this could be detected by measuring their circadian leaf movement and delayed fluorescence rhythms.

SECTION 2: Material and Methods

All chemicals were of the best available quality, AR or molecular biology grade where available. Biochemicals, enzymes and kits were from Sigma (St Louis, MI, USA), Merck (Wadeville, South Africa), Promega (Madison, WI, USA), and Kapa Biosystems (Cape Town, South Africa) except where specifically mentioned. Murashige and Skoog medium was purchased in powdered form from Highveld Biological (Lyndhurst, South Africa).

2.1 Plant Material

A. thaliana seeds of the Wassilewskija (Ws-2) and Landsberg (Ler-0) accessions were acquired from the Nottingham Arabidopsis Stock Centre (NASC) and used as wild-type controls in these studies. A comprehensive list of all mutants used in this study is given in Table 2.1.

2.1.1 Sterilisation of *A. thaliana* seeds

The desired amount of seeds was transferred to a sterile 1.5 ml Eppendorf tube. The seeds were surface sterilised with sodium hypochlorite /Tween-20 solution (± 2 % chlorine and 0.025 % (v/v) Tween-20) for five minutes with occasional agitation. The sterilising solution was decanted by pipetting. Thereafter seeds were washed three times with sterile H_2O water and resuspended in either appropriate volume of sterile 0.15 % (w/v) agarose or sterile H_2O.

Table 2.1. List of mutants used in this study

Mutant	Histone modification	Type of mutation	Ecotype	Reference	Source
clf-2	Methylation	Transposon insertion	Ler-0	Goodrich, et al., 1997	NASC code N8853
gcn5	Acetylation	SALK T-DNA exon Insertion	Ws-2	Benhamed *et al.*, 2005	NASC code N662010
Hd1	Deacetylation	T-DNA Insertion	Ws-2	Fong *et al.*, 2006	Tian Lu (Texas A&M University, USA)
taf1	Acetylation	T-DNA Insertion	Ws-2	Bertrand *et al.*, 2005	Dao Xiu Zhou (University of Paris, France)

2.1.2 Growth on Nutrient Media

Sterilised *A. thaliana* seeds were sown on plates of Murashige and Skoog (Merck, South Africa) medium solidified with 8 g L^{-1} agar supplemented with 30 g L^{-1} sucrose. This was done by either plating the seeds in a lawn or by transferring a single seedling with a sterile Pasteur pipette in a laminar flow. After three days of stratification at 4°C in the dark, seeds were entrained under 12-h-light/12-h-dark cycles under 56.0 $mol.m^{-2}.s^{-1}$ of white light (cool white fluorescent) at 23°C for 16 days. Plates were then transferred to constant light (LL) of the same quality and intensity, or constant dark (DD) conditions. Seedlings were then harvested four hourly, at circadian times (CT) 1, 5, 9, 13, 17, 21, 25 and 29 for LL samples and at CT 25, 29, 33, 37, 41, 45, 49 and 53 for DD samples. Samples were wrapped in foil and frozen in liquid nitrogen and stored at -70 °C until use.

2.1.3 PCR based genotyping

PCR assays to distinguish between wild-type and mutant DNA sequences were carried out for *gcn5, hd1* and *taf1* to confirm their mutant genotype. DNA was extracted from Ws2 wild type plants and mutant plants using a method developed by Edwards *et al.*, 1991.

Table 2.2 Primer sets used for genotyping *A. thaliana* mutants

Mutant	Primer sequence (5' to 3')	Reference
gcn5	GGTATCGGGGAGTTGTAAGTTCTAC (*GCN5* specific) CTACAAATTGCCTTTTATCGAC (T-DNA specific)	Benhamed *et al.*, 2006
hd-1	ATGCTGGAGGATCTGTTGG (T-DNA specific) CCAGACAATGAATCAGCACC (*HD1* specific)	Benhamed *et al.*, 2006
taf-1	ATGGGAGCAATGATGAAGAG AAAGGCTCGAGCATGTTGTT	Bertrand *et al.*, 2006

Genomic DNA was isolated from 16 progeny of self-fertilised mutant lines. A 2.5 µL aliquot of genomic DNA from each sample was used in the appropriate PCR reaction. PCR reactions were carried out in a 96 well Gene Amp PCR System 2700 (Applied Biosystems, Forster City, USA). All PCR amplifications were performed with GoTaq DNA Polymerase from Promega (Madison WI, USA) in 50 µL reactions containing 1 X CG Buffer (Promega), 0.3 mM dNTPs and 0.3 M primer.

PCR steps for HD1 amplification: DNA was denatured at 95 °C for 2 min. This was followed by 35 cycles of 95 °C for 30 sec, annealing at 53 °C for 30 sec and extension at 72 °C for 45

sec. This was followed by a final extension at 72 °C for 5 min. The results of the PCR are displayed in Figure 6.

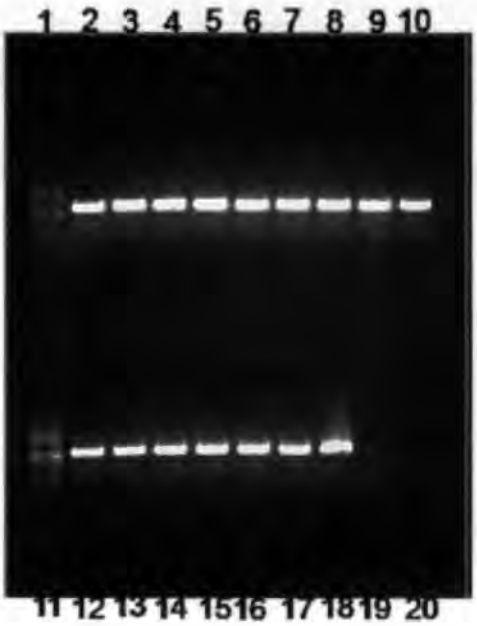

Figure 6. Genotyping of *hd1* T-DNA insertion mutants by PCR with primer set *HD1* and T-DNA specific primer. Lanes 1 and 11 contain 5µg of 100bp DNA ladder from New England Biolabs (Ipswich, MA, USA), lanes 2 to 10 and lanes 12 to 18 contain *hd1* mutant DNA, Lane 19 contains Ws2 DNA and lane 20 contains the negative control. This indicates that all the progeny of the *hd1* mutant plants carried the T-DNA, and the parental lines were thus homozygous.

GCN5 was amplified by denaturing DNA at 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 sec, annealing at 48 °C for 30 sec and extension at 72 °C for 45 sec. This was followed by a final extension at 72 °C for 5 min. The results are shown in Figure 7.

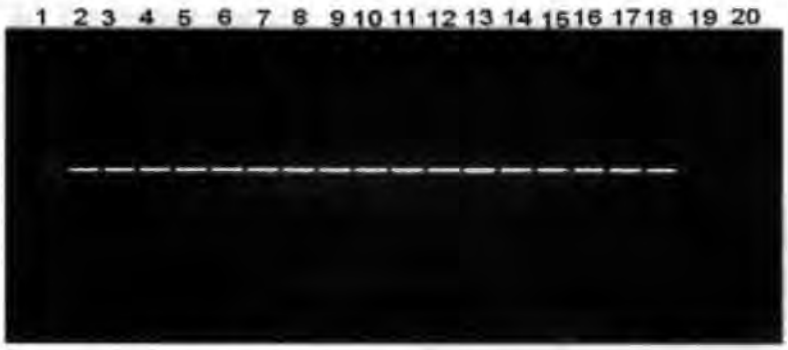

Figure 7. Genotyping of *gcn5* T-DNA insertion mutants by PCR with primer set GCN5 and T-DNA specific primer. Lane 1 contains 5µg of 100bp DNA ladder from New England Biolabs (NEB), lanes 2 to 18 contain *gcn5* mutant DNA, lane 19 contain Ws2 DNA and lane 20 contains the negative control. This indicates that all the *gcn5* mutant plants tested were homozygous.

The *TAF1* locus was amplified using forward primer 5'-CTACAAATTGCCTTTTCTTATCGA-3' and reverse primer 5'-AAAGGCTCGAGCATGTTGTT-3' both which are specific for *TAF1* (Bertrand *et al.*, 2005). The wild-type locus produces an amplification product while the insertion mutant does not. The results are shown in Figure 8.

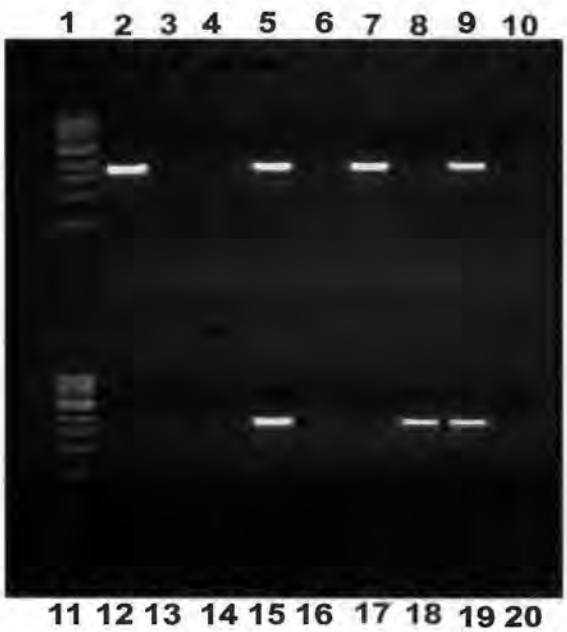

Figure 8. Genotyping of *taf1* T-DNA insertion mutants by PCR with primer set taf1_1 and taf1_2 (which are specific for the wild type plants) primers. Lanes 1 and 2 contain 5µg of 100bp DNA ladder from NEB, lane 2 contains Ws2 DNA, lanes 3 to 10 and lanes12 to 19 contain *taf1* mutant DNA. Lane 20 contains the negative control. This indicates that the *taf-1* seeds represented a segregating population where10 out of 16 plants were homozygous mutants, and six were heterozygous or wild-type.

2.2 Transcript analysis

2.2.1 RNA extraction

RNA extraction was carried out according to Smart and Roden (2010). The RNA concentration was estimated by measuring the absorbance at 260 nm (Nanodrop ND-100 spectrophotometer, NanoDrop Technologies, Wilmington, USA).

2.2.2 Electrophoresis of RNA

In order to determine RNA integrity, each RNA sample (4 L) in 6X loading buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 40% (w/v) sucrose) was analysed after electrophoresis through a 1% (w/v) agarose gel. The gels were electrophoresed at 100 V in 1 X Tris Acetate EDTA (TAE; 40 mM Tris, 1 mM EDTA, 0.11% (v/v) glacial acetic acid) and 0.16 g/ mL ethidium bromide for approximately 45 minutes. The ethidium bromide stained RNA gel was viewed using the Quality One 1-D Discovery Series Analysis software on the BioRad™ gel doc system (Biorad™).

2.2.3 cDNA Synthesis

RNA template (1 g) was added to 0.5 g random primers (Promega, Madison, WI, USA) in thin-walled pre-chilled PCR tubes, in a total volume of 5 L. The tube contents were mixed by gently vortexing and immediately incubated at 70 °C for 5 minutes to denature the RNA. Denatured RNA was then transferred to ice for at least 5 minutes and 15 L reaction cocktail (1X ImProm-II™ reaction buffer, 3 mM $MgCl_2$, 0.5 mM deoxy nucleotide triphosphate (dNTP) mix and ImProm-II™ reverse transcriptase (Promega, USA) was added. The samples were gently vortexed, incubated at 25 °C for 5 minutes followed by heating for 60 minutes at

42 °C. ImProm-II™ reverse transcriptase was inactivated by heating the samples at 70 °C for at least 15 minutes.

2.2.4 Semi-quantitative Reverse-Transcription Polymerase Chain Reaction (semi-quantitative RT-PCR)

Primers were designed within the *A. thaliana TOC1* (Locus: AF272039) from1029 bp to 1521 bp and *TUBULIN* (*TUB*- Atg44340): from 484 bp to 1011 bp. PCR reactions were carried out in a 96 well Gene Amp PCR System 2700 (Applied Biosystems, Forster City, USA). PCR amplification was performed with KAPATaq™ DNA polymerase (KAPA Biosystems Cape Town, SA). cDNA that had been diluted 3-fold was used as template for PCR. PCR reactions were carried out in a 20 L reaction volumes containing 1 X KAPA Buffer A, 2 mM $MgCl_2$ 0.3 mM dNTPs and 0.3 M primers. *TUB* was amplified using forward primer 5'-ACAGCGATTGTCTTCAAGGTTAG–3'and reverse primer 5'-ATGAGATGGTTAAGATCACCAAC- 3'. PCR reaction: DNA was denatured at 95 °C for 5 min. This was followed by 28 cycles of 95 °C for 30 sec, annealing at 49 °C for 30 sec and extension at 72 °C for 45 sec. This was followed by a final extension at 72 °C for 5 min. *TOC1* was amplified using forward primer 5'- ATGGTGGTCTTGGTGCTGATG -3' and 5'-GTCCCTCTACTTCTGTGTGCT- 3'. PCR reaction: DNA was initially dentatured by one cycle of 95 °C for 5 min. This was followed by 28 cycles of 95 °C for 30 sec, annealing at 52 °C for 30 sec and extension at 72 °C for 45 sec. This was followed by a final extension at 72 °C for 2 min. The PCR products were electrophoresed on a 1% (w/v) agarose in 1x TAE gel at 60V for approximately 45 minutes. The gels were stained with 0.1% v/v ethidium bromide and destained with distilled water. Visualisation was with a Bio-Rad gel doc system and quantification was performed with Quality One 1-D Discovery Series Analysis software

(Bio-Rad). Optimal cycle numbers for amplification of each target amplicon were determined using 3 L of 3x diluted cDNA in PCR followed by quantification using Quality One 1-D Discovery Series Analysis software (Bio-Rad). Cycle numbers used for each amplicon were chosen based on the results on Figures 9 and 10, where amplification was in exponential phase of the curve. The quantitative nature of the assay using these cycle numbers was confirmed by a linear relationship between the amount of cDNA in the PCR reaction and the intensity of the bands determined.

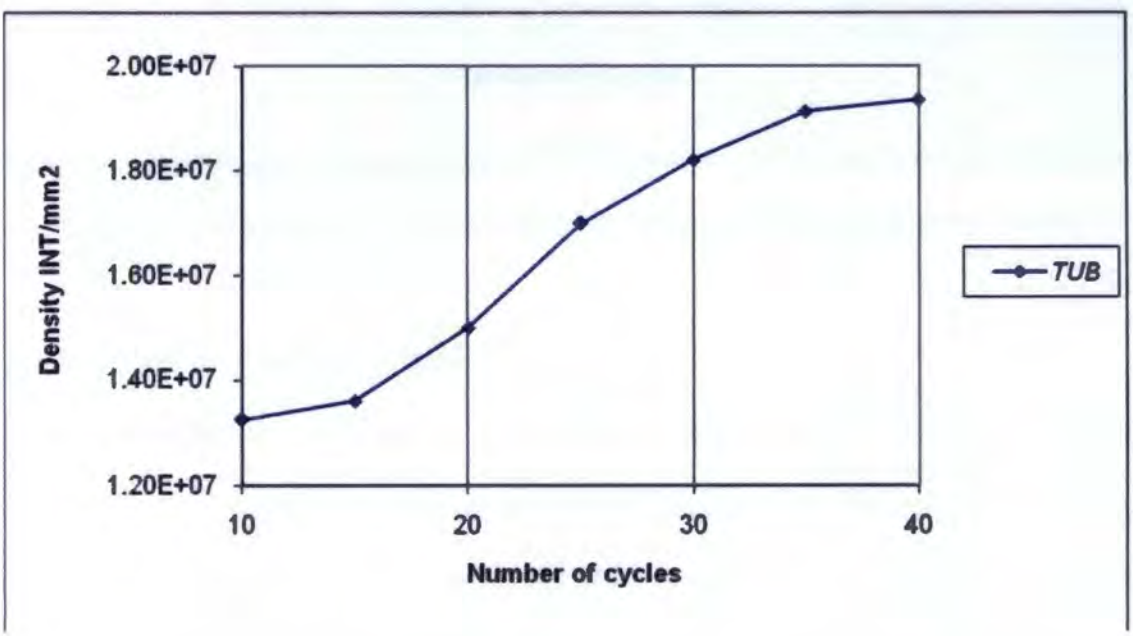

Figure 9. Determination of semi-quantitative RT-PCR cycle numbers for use in the amplification of housekeeping gene, *TUBULIN* (*TUB*). The number of cycles chosen was 28 cycles as this cycle number lies within the exponential phase of the curve.

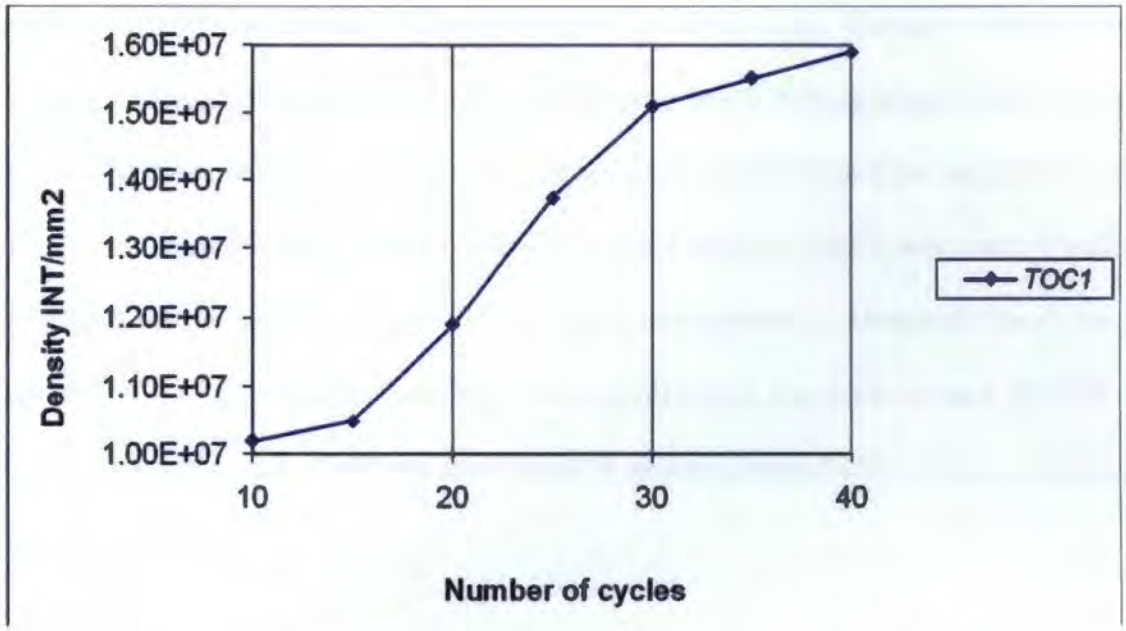

Figure 10. Determination of semi-quantitative RT-PCR cycle numbers for use in the amplification of *TOC1*. The number of cycles chosen was 28 cycles as this cycle number lies within the exponential phase of the curve.

2.3 Rhythmic leaf movement analyses

Leaf movements were measured as a simple and reliable indicator of the overall functioning of the circadian clock, according to the method described by Edwards and Millar (2007). Sterilised *A. thaliana* seeds were sown on MS medium solidified with 8 gL^{-1} agar (Merck, South Africa) supplemented with 30 gL^{-1} sucrose. After three days of stratification, seeds were entrained under 12-h-light/12-h-dark cycles under 56.0 $mol.m^{-2}.s^{-1}$ of cool white light at 23°C for seven to nine days. Individual seedlings were then cut out of MS plates and placed one per well into sterile 25 well 100 X 100 mm plates for imaging. Plates were transferred to LL (56.0 $mol.m^{-2}.s^{-1}$ of cool white light at 23°C) conditions and the movements of the leaves of seedlings were imaged and recorded every 20 minutes over several days (4-5 days) using time lapse photography. Two systems were used for image acquisition; the Digital Video Recording System (DVRS) which relied on four Panasonic WV-CP254E Colour CCTV cameras (Panasonic System Solutions, Suzhou China) under the

control of the AVerDiGi NV5000 software (AVerMedia®, Taiwan), and the VGA IP Meerkat system (Hennie Roos IT&S, Stellenbosch, South Africa) which used a Linux server to control the time-lapse image capture of seedling growth from four cameras.). Image-Pro Plus® 6.2 software from Media Cybernetics, Inc Bethesda, USA), was used to track the Y (vertical) position of the leaves and the data was exported to Microsoft Excel for rhythm analysis. BRASS (available from http://www.amillar.org) was used to carry the FFT –NLLS analysis (Plautz *et al.*, 1997) on each series to generate period and Relative amplitude error (RAE) estimates.

2.4 Delayed Fluorescence (DF)

DF has recently been demonstrated to oscillate with a circadian rhythm and can thus also be used an output to measure circadian clock function (Gould *et al.*, 2009). Following seeds sterilisation (see section 3.1.1), *A.thaliana* seedlings were grown on MS medium solidified with 8 gL^{-1} agar, containing 30 gL^{-1} sucrose. Seeds were sown in clusters ranging from 8-36 seeds. Seeds were kept at 4 °C in the dark for three days and entrained under 12-h-light/12-h-dark cycles under 56.0 $mol.m^{-2}.s^{-1}$ of cool white light at 23°C for nine days.

On dawn of the ninth day, the seedlings were placed in the Xenogen-Lumina In vivo imaging system (IVIS) by Caliper Life Sciences (MA, USA) chamber for imaging. Every hour, plants were illuminated with red light (filter) at 640 nm 60s, followed by a 1s luminescent image to allow the lights to go off, and chlorophyll delayed fluorescence was captured at 695-770nm for 60 s. DF measurements were collected every hour for four to five days in DD. The acquired data was analysed using Living Image ® 3.2 software (Caliper Life Sciences). The luminescence was normalised by subtracting the Y value of the best straight line from the raw

Y value (Gould et al. 2009). BRASS was used to carry the FFT –NNLS analysis (Plautz et al., 1997) on each DF time series to generate period and RAE estimates.

SECTION 3: Results and Discussion

3. 1 Investigating rhythmic histone modifications in *cca1lhy* mutants

3.1.1 *TOC1* in *cca1lhy* mutants

The circadian clock coordinates most biochemical processes and in turn physiological processes collectively known as the circadian clock-output pathways (Harmer *et al.*, 2000). In *A. thaliana,* TOC1, CCA1 and LHY form part of the central oscillator and are therefore very important in the functioning of the circadian clock (Strayer *et al.,* 2000; Schaffer *et al.*, 1998; Wang and Tobin 1998). Furthermore, CCA1 and LHY have been demonstrated to bind at the *TOC1* promoter (Alabadí *et al.*, 2001) where rhythmic acetylation of histones has been demonstrated (Perales and Más 2007). We therefore used semi-quantitative RT-PCR to investigate the circadian oscillation of *TOC1* in *cca1lhy* double mutants in both DD (Figure 11) and LL conditions (Figure 12). We wanted to ensure that our assay was suitable for the measurement of aberrant circadian rhythms in plants, before we applied it to our test mutants.

As expected the wild type plants maintained robust rhythms in both DD and LL conditions (Figure 11 and 12 respectively). In DD the *TOC1* expression was dampened after 25 hours in the dark in the *cca1lhy* double mutant plants while the wild type plants exhibited robust rhythms of *TOC1* (Figure 11).The *cca1lhy* double mutant plants however exhibited a shorter *TOC1* period the first cycle in LL conditions (Figure 12). This data is in agreement with that of Ding *et al.*, (2007) which indicated that double mutant lines including *cca1lhy*, *cca1toc1*

and *lhytoc1* displayed short periodicity in LL (free-running conditions). This suggests that in the absence of both CCA1 and LHY, and in the presence of fully functional TOC1, the normal function of the *A. thaliana* circadian clock is disturbed. This is in agreement with data shown by Ding *et al.*, (2007) and confirmed that our assay was suitable for these measurements.

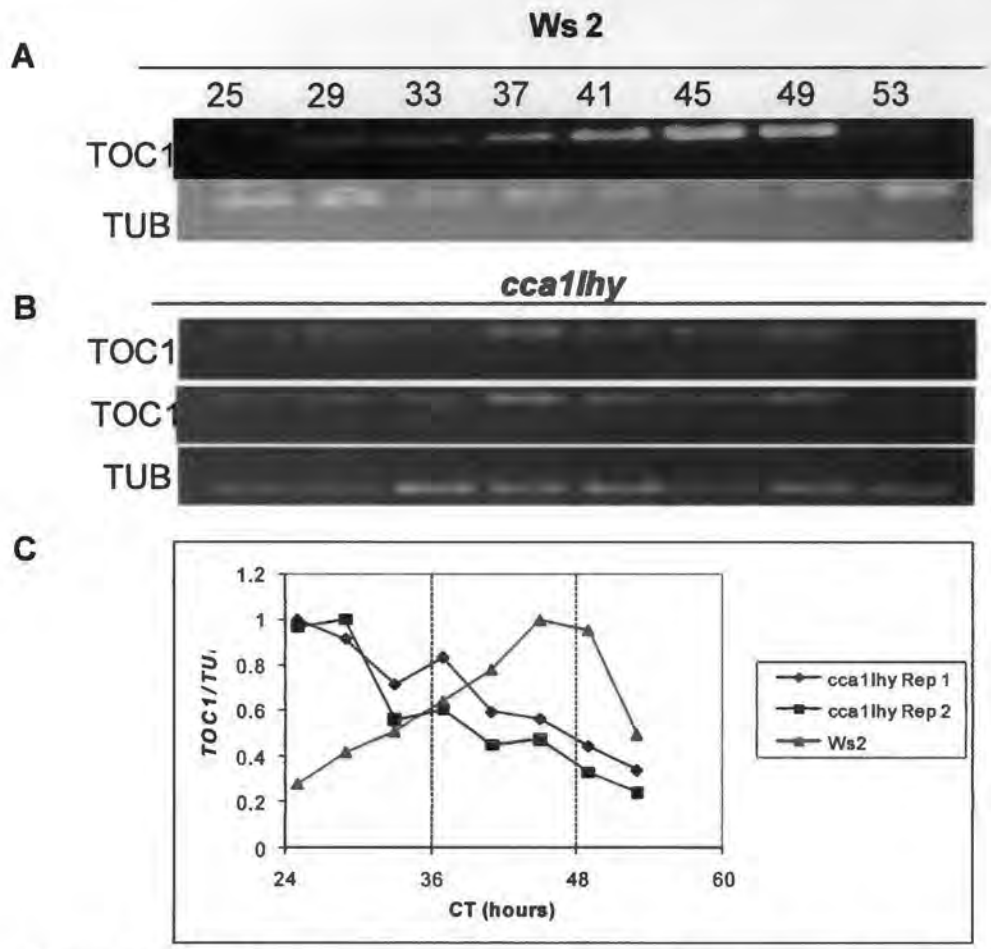

Figure 11. Relative levels of *TOC1* transcript in Ws2 and *cca1lhy* double mutant plants in DD conditions as determined by semi-quantitative RT-PCR. **A** and **B** are representative PCR bands from semi-quantitative RT-PCR. Seedlings were entrained under 12-h-light/12-h-dark cycles and subsequently released to DD conditions. Samples were collected four hourly for a period of 28 hours: CT 25, 29, 33, 37, 41, 45, 49 and 53. **C**. Relative changes in *TOC1* transcript abundance normalised to *TUBULIN*. Experiments were carried out in duplicate (Rep 1 and 2). Only one replicate of Ws2 is shown for simplicity.

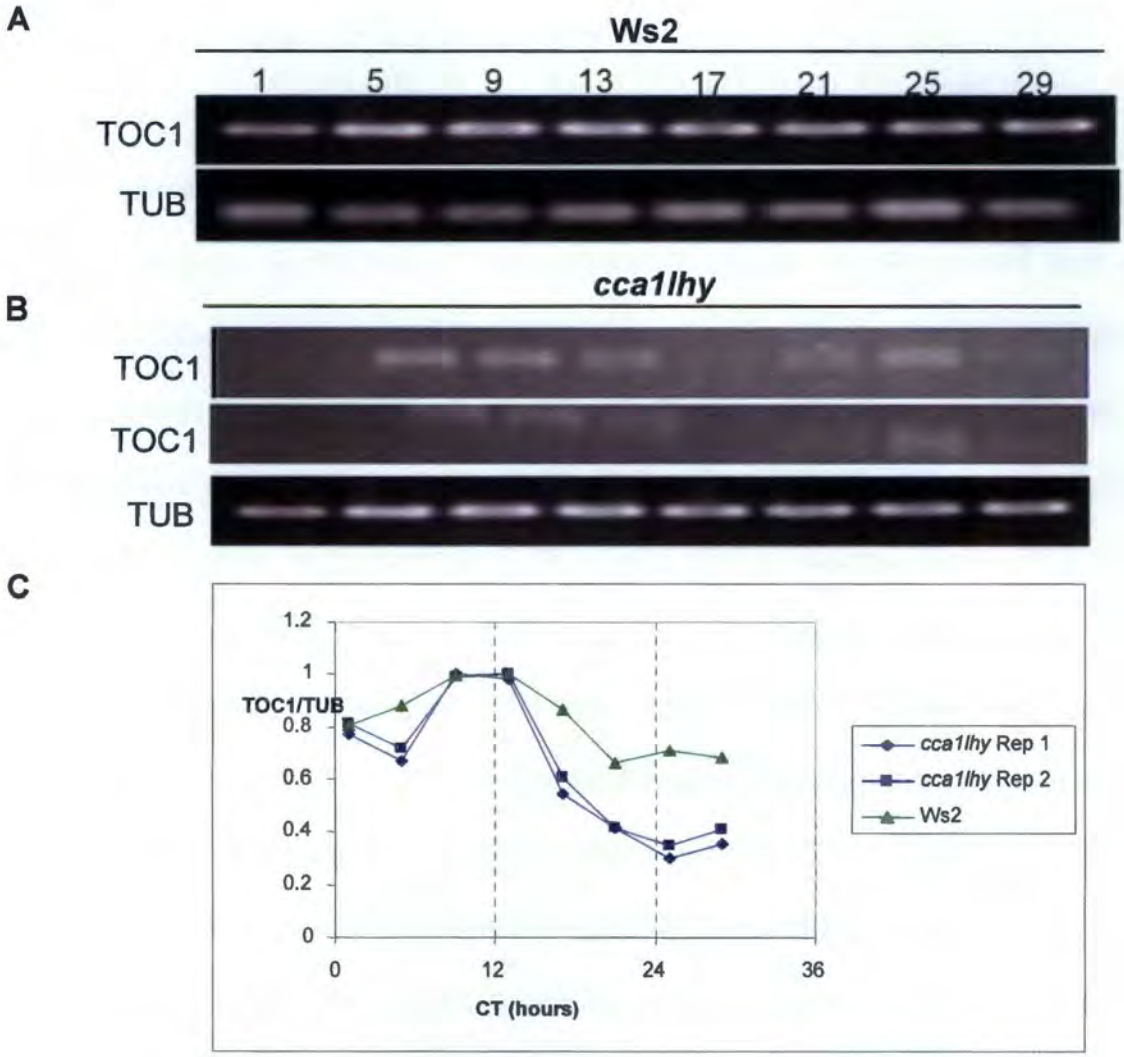

Figure 12. Relative levels of *TOC1* transcript in Ws2 and *cca1lhy* double mutant plants in LL conditions as determined by semi-quantitative RT-PCR. **A** and **B** are representative PCR bands from semi-quantitative RT-PCR. Seedlings were entrained under 12-h-light/12-h-dark cycles and subsequently released to LL conditions. Samples were collected four hourly for a period of 28 hours: CT 1, 5, 9, 13, 17, 21, 25 and 29. **C.** Relative changes in *TOC1* transcript abundance normalised to *TUBULIN*. Experiments were carried out in duplicate (Rep 1 and 2). Only one replicate of Ws2 is shown for simplicity.

3.1.2 Delayed Fluorescence in *cca1lhy* mutants

The profound disruption of *TOC1* circadian rhythms in 12-h-light/12-h-dark cycles -entrained *cca1lhy* seedlings regardless of free-running condition led us to evaluate the extent of the

clock defect in these mutants. Recent studies demonstrated that DF could be used as a tool to measure circadian rhythms (Gould et *al.*, 2009).

To test the circadian rhythms of wild type and mutants, DF was assayed as described with 1 hour time resolution for 91 hours. A physiologically relevant circadian period must range between 15-30 hours. RAE is a measure of rhythm robustness varying from 0 (a perfect fit to the cosine wave) to 1 (not statistically significant). Therefore, the closer the RAE is to 0 the more robust the rhythm (Plautz *et al.*, 1997). The data clearly demonstrated that the clock drives robust rhythms in the amount of DF in wild type *A. thaliana* (Figure 13 A and C). These seedlings exhibited a period of 23.8, 22.6 and 21.8 hours (average of 22.7 hours) accompanied by RAE $\leq$0.6 (Figure 13 C). These period estimates match closely with those given by Gould *et al.*, (2009). DF was also used to investigate the extent of clock defect in *cca1lhy* mutants. The data demonstrates a clear defect in the circadian clock of *cca1lhy* seedlings (Figures 13 B and C). One group of seedlings exhibited a short period of 16.4 hours accompanied by RAE > 0.6. Interestingly, this was the group with the least number of seedlings (10 seedlings).By monitoring *CAB::LUC* bioluminescence rhythms in *cca1-11 lhy-21*, previous studies demonstrated that this double mutant exhibited a shorter period when compared to the Ws seedlings (Ding *et al.*, 2007). Therefore, this could indicate that for the purpose of DF assaying, it is better to use small numbers of seedlings than a large group.

Using chromatin immunoprecipitation (ChIP) assays to investigate circadian histone acetylation in the promoters of light and circadian regulated genes in *A. thaliana*, my own preliminary unpublished results (L. Motleleng, Honours dissertation UCT, 2007) demonstrated that there is rhythmic histone acetylation of H4K27 in *LHY* and

CHLOROPHYLL A/B BINDING PROTEIN (*CAB*), promoters. Misregulation of *LHY* or *CCA1* expression due to aberrant acetylation could also have an effect on *TOC1* expression as these proteins are known to repress *TOC1* expression.

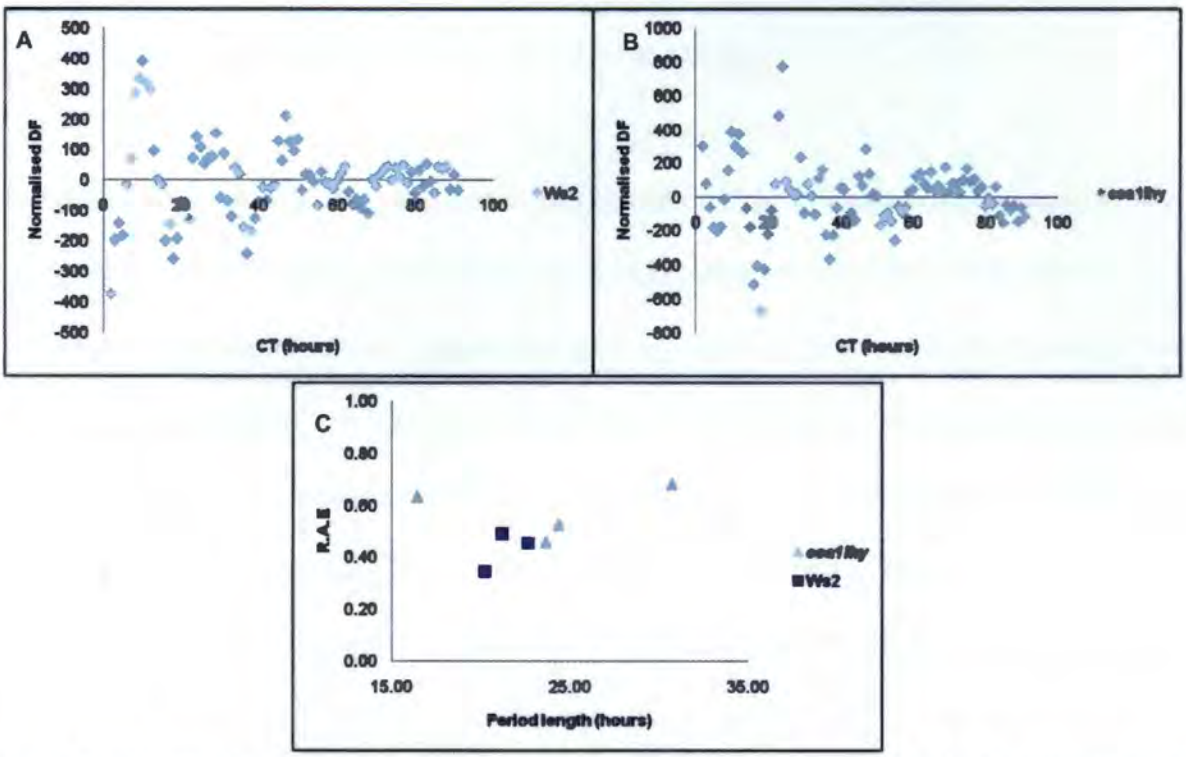

Figure 13. Delayed fluorescence rhythms in Ws2 and *cca1lhy* double mutant. **A** and **B** are representative plots of normalised DF of three to four groups of seedlings of Ws2 and *cca1lhy* respectively. Seedlings were entrained under 12-h-light/12-h-dark cycles for nine days and subsequently released to DD conditions. DF was assayed with a one hour time resolution for 91 hours. **C**. Period estimates for groups of seedlings plotted against their R.A.E. Ws2 n=3 (closed squares) and *cca1lhy* n= 4 (closed triangles).

3.2 Investigating rhythmic histone modifications in *gcn5* mutants

3.2.1 TOC1 in *gcn5* mutants

In yeast, Gcn5 has been shown to acetylate histone H3 in the nucleosome (Grant *et al.*, 1997; Saleh *et al.*, 1997). *A. thaliana* GCN5 is involved in acetylation of histones at promoters of light controlled genes (Benhamed et al., 2006). In *A. thaliana*, Perales and Más (2007)

demonstrated rhythmic histone acetylation in the promoter of *TOC1*. We therefore investigated whether GCN5 was a potential candidate involved in the rhythmic histone acetylation in the promoter of *TOC1* in *A. thaliana*. This was done using semi-quantitative RT-PCR in order to determine the circadian expression profile of *TOC1* in *gcn5* mutants in both DD and LL conditions Figure 14 and 15, respectively.

From the results, it was observed that in DD conditions, the circadian oscillation of *TOC1* in the *gcn5* mutant plants appeared to have been maintained to some extent (Figure 14). In LL conditions however, the *TOC1* expression was very erratic in the *gcn5* mutant plants. In fact, the circadian oscillation of *TOC1* seemed to have been completely abolished in *gcn5* mutant plants placed in LL conditions (Figure 15). Interestingly, by investigating the effects of mutation of *GCN5* on acetylation of light inducible promoters, Benhamed *et al.*, (2006) demonstrated that *gcn5* mutants have reduced gene expression of light inducible promoters and that this reduced gene expression was more exacerbated in the light. They also demonstrated that *gcn5* mutants had an elongated hypocotyl phenotype suggesting that acetylation of specific histone Lys residues, regulated by *GCN5* is required for light-regulated gene expression.

Since acetylation of histones is associated with gene activation (Garcia-Ramirez *et al.*, 1995, Walia *et al.*, 1998, Tse *et al.*, 1998) and LL *gcn5* mutant plants showed non-robust rhythms, the results implicate GCN5 as a potential HAT involved in histone acetylation affecting circadian clock gene expression. However, the fact that this effect was only be observed in LL and given the previous involvement of GCN5 in histone acetylation in the promoters of

light-controlled genes (Benhamed *et al.*, (2006) raises an interesting potential dual role for GCN5 in light and circadian regulation of gene expression.

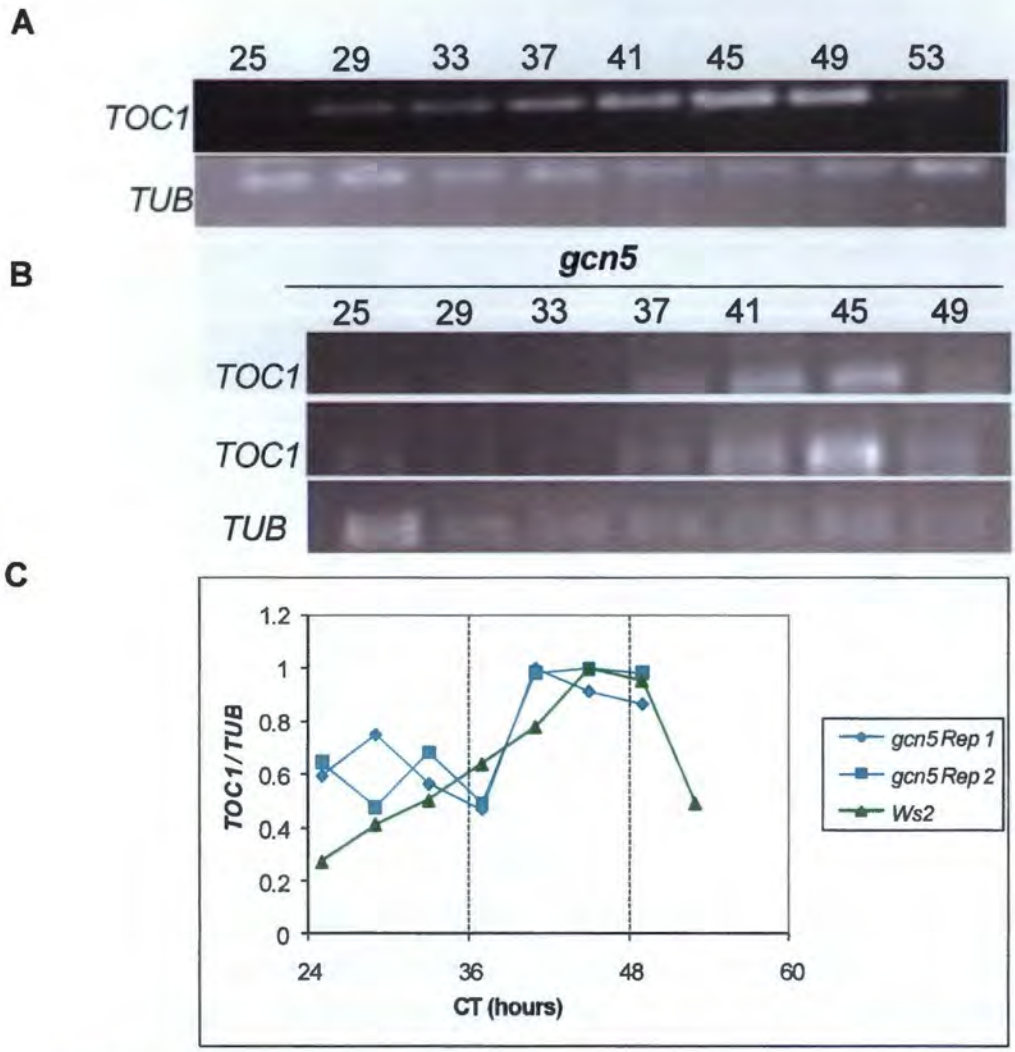

Figure 14. Relative levels of *TOC1* transcript in Ws2 and *gcn5* mutant plants in DD conditions as determined by semi-quantitative RT-PCR. **A** and **B** are representative PCR bands from semi-quantitative RT-PCR. Seedlings were entrained under 12-h-light/12-h-dark cycles and subsequently released to DD conditions. Samples were collected four hourly for a period of 28 hours: CT 25, 29, 33, 37, 41, 45 and 49. **C.** Relative changes in *TOC1* transcript abundance normalised to *TUBULIN*. Experiments were carried out in duplicate (Rep 1 and 2). Only one replicate of Ws2 is shown for simplicity.

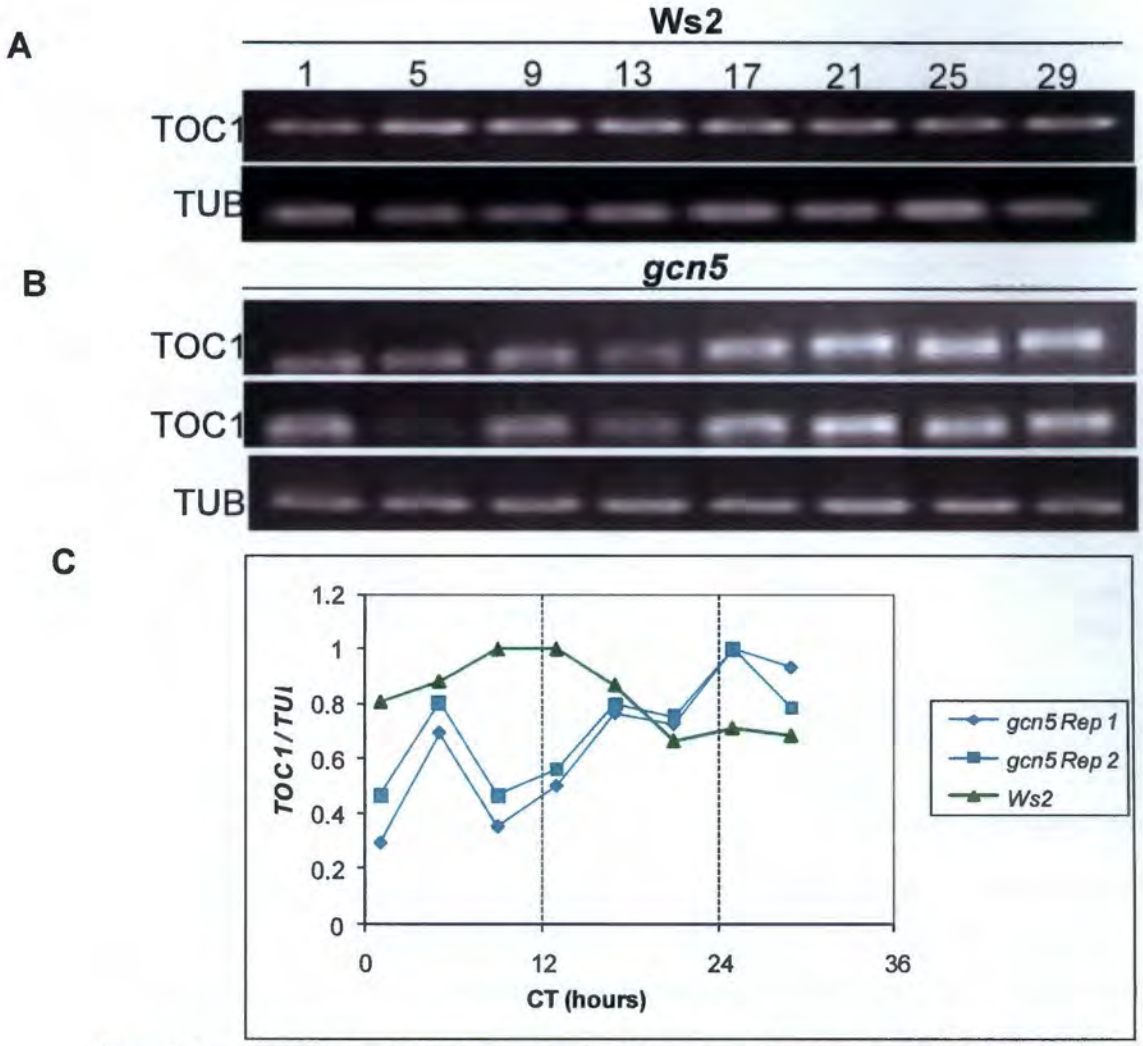

Figure 15. Relative levels of *TOC1* transcript in Ws2 and *gcn5* mutant plants in LL conditions as determined by semi-quantitative RT-PCR. **A** and **B** are representative PCR bands from semi-quantitative RT-PCR. Seedlings were entrained under 12-h-light/12-h-dark cycles and subsequently released to LL conditions. Samples were collected four hourly for a period of 28 hours: CT 1, 5, 9, 13, 17, 21, 25 and 29. **C.** Relative changes in *TOC1* transcript abundance normalised to *TUBULIN*. Experiments were carried out in duplicate (Rep 1 and 2). Only one replicate of Ws2 is shown for simplicity.

3.2.2 Delayed Fluorescence in *gcn5* mutants

From semi-quantitative RT-PCR results *TOC1* exhibited erratic rhythms in LL while maintained circadian rhythms to some extent in DD conditions (Figures 14 and 15).We therefore analysed the circadian regulation of DF in *gcn5* mutants to investigate the extent of

the clock defect in *gcn5* mutants. *gcn5* seedlings were grown in groups of ~13-38. From the results, it can be observed that the circadian rhythms of DF in *gcn5* seedlings were impaired (Figure 16). Of the tested groups, three out of four groups of seedling exhibited periods of 23.4, 19.6 and 18.0 hours (average of 20.3 hours) with RAE values > 0.6 (0.81, 0.95 and 0.98, respectively). The impaired DF rhythms indicate an impaired circadian clock.

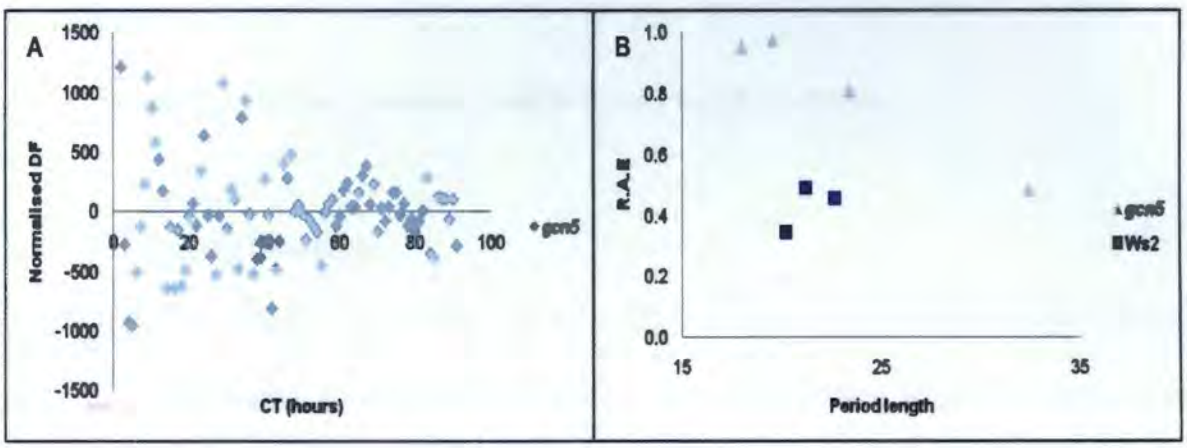

Figure 16. Delayed fluorescence rhythms in *gcn5* mutant plants. **A** is a representative plots of normalised DF of one of four groups of seedlings of *gcn5*. Three out of four plots looked similar. Seedlings were entrained under 12-h-light/12-h-dark cycles for nine days and subsequently released to DD conditions. DF was assayed with a one hour time resolution for 91 hours. **B**. Period estimates for groups of seedlings plotted against their R.A.E. Ws2 n=3 (closed squares) and *gcn5* n= 4 (closed triangles).

In *A.thaliana*, C-repeat/drought-responsive element binding factor (CBF) proteins bind to promoter regions of many cold regulated genes via the C-repeat/ dehydration-responsive elements (CRT/DRE) and regulate their transcription (Baker *et al.*, 1994; Yamaguchi-Shinozaki and Shinozaki, 1994). Stockinger *et al.*, (2001) demonstrated that in yeast, CBF1 requires Ada2, Ada3 and Gcn5 to activate transcription. They also demonstrated that CBF1 interacts with GCN5 in *A. thaliana*. This indicates that GCN5 is involved in cold-regulated gene expression. Since *TOC1* is an evening phased gene, and many cold-response genes have

evening elements in their promoters (Mikkelsen and Thomashow 2009) we might expect GCN5 to similarly regulate *TOC1* expression. The correlation between semi-quantitative RT-PCR results in LL conditions and DF results support the conclusion that acetylation by GCN5 maybe important in the functioning of the circadian clock. We can also hypothesise that acetylation by GCN5 may be important not just in *TOC1* regulation but also in global gene regulation.

3.3 Investigating rhythmic histone modifications in *taf1* mutants

3.3.1 TOC1 in *taf1* mutants

TAF1 has been shown to acetylate histone H3K9, H3K27 and H4K12 of light inducible promoters (Benhamed *et al.*, 2006). We used semi-quantitative RT-PCR to investigate whether TAF1 was involved in the rhythmic histone acetylation in the promoter of *TOC1* in *A. thaliana*. *TOC1* expression demonstrated robust rhythms in *taf1* mutants under both DD and LL conditions (Figures 17 and 18, respectively), however with an altered waveform to the wild-type.

The fact that *taf1* mutants exhibited an altered waveform suggests that TAF1 may also play a role in the rhythmic histone acetylation affecting *TOC1* expression. Interestingly, unlike *gcn5* mutants, which exhibited elongated hypocotyls (Benhamed *et al.*, 2006) the *taf1* (*haf2*) single mutants did not show any significant change in their hypocotyl phenotype in any tested conditions (Bertrand *et al.*, 2005). This suggested that TAF1 and GCN5 might also play different roles in the rhythmic histone acetylation.

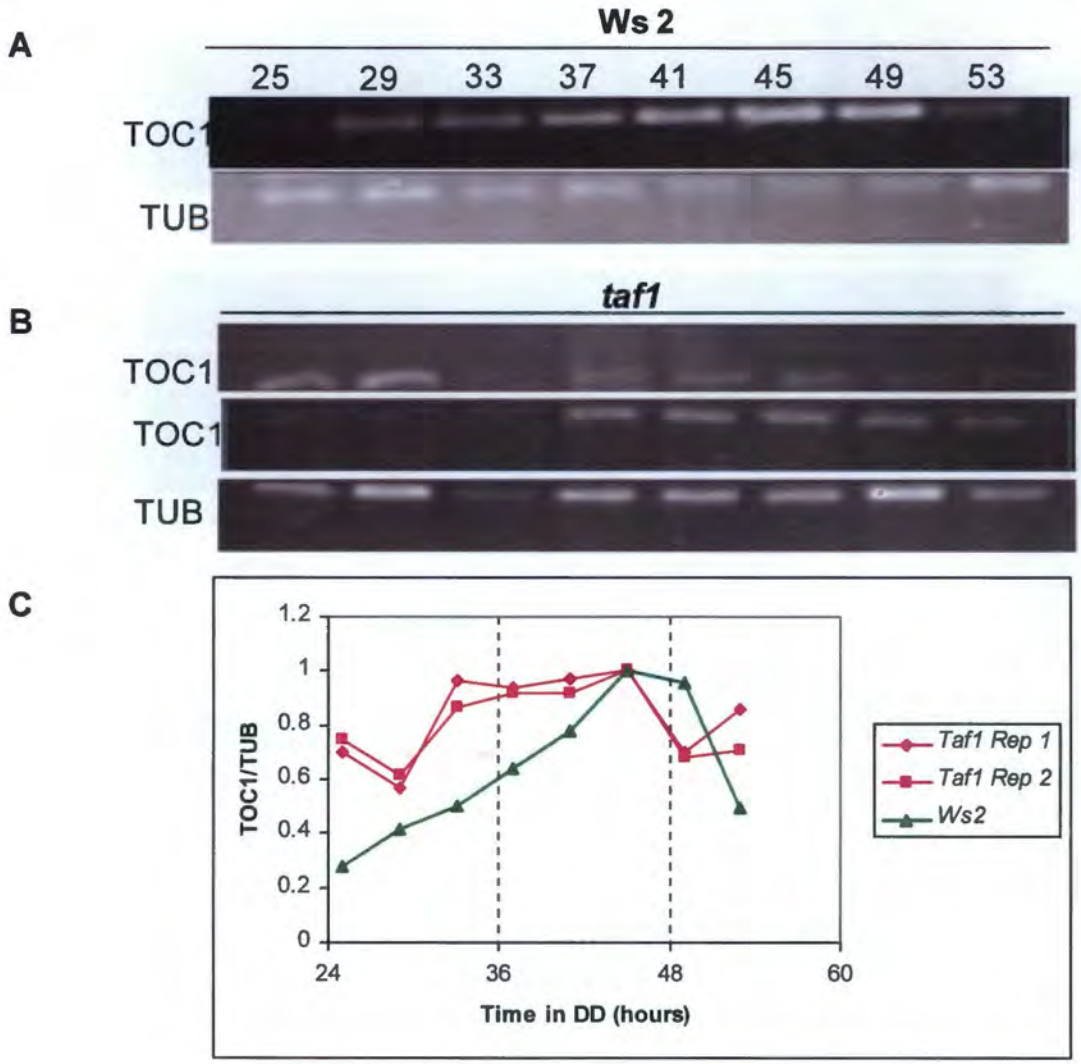

Figure 17. Relative levels of *TOC1* transcript in Ws2 and *taf1* mutant plants in DD conditions as determined by semi-quantitative RT-PCR. **A** and **B** are representative PCR bands from semi-quantitative RT-PCR. Seedlings were entrained under 12-h-light/12-h-dark cycles and subsequently released to DD conditions. Samples were collected four hourly for a period of 28 hours: CT 25, 29, 33, 37, 41, 45, 49 and 53. **C**. Relative changes in *TOC1* transcript abundance normalised to *TUBULIN*. Experiments were carried out in duplicate (Rep 1 and 2). Only one replicate of Ws2 is shown for simplicity.

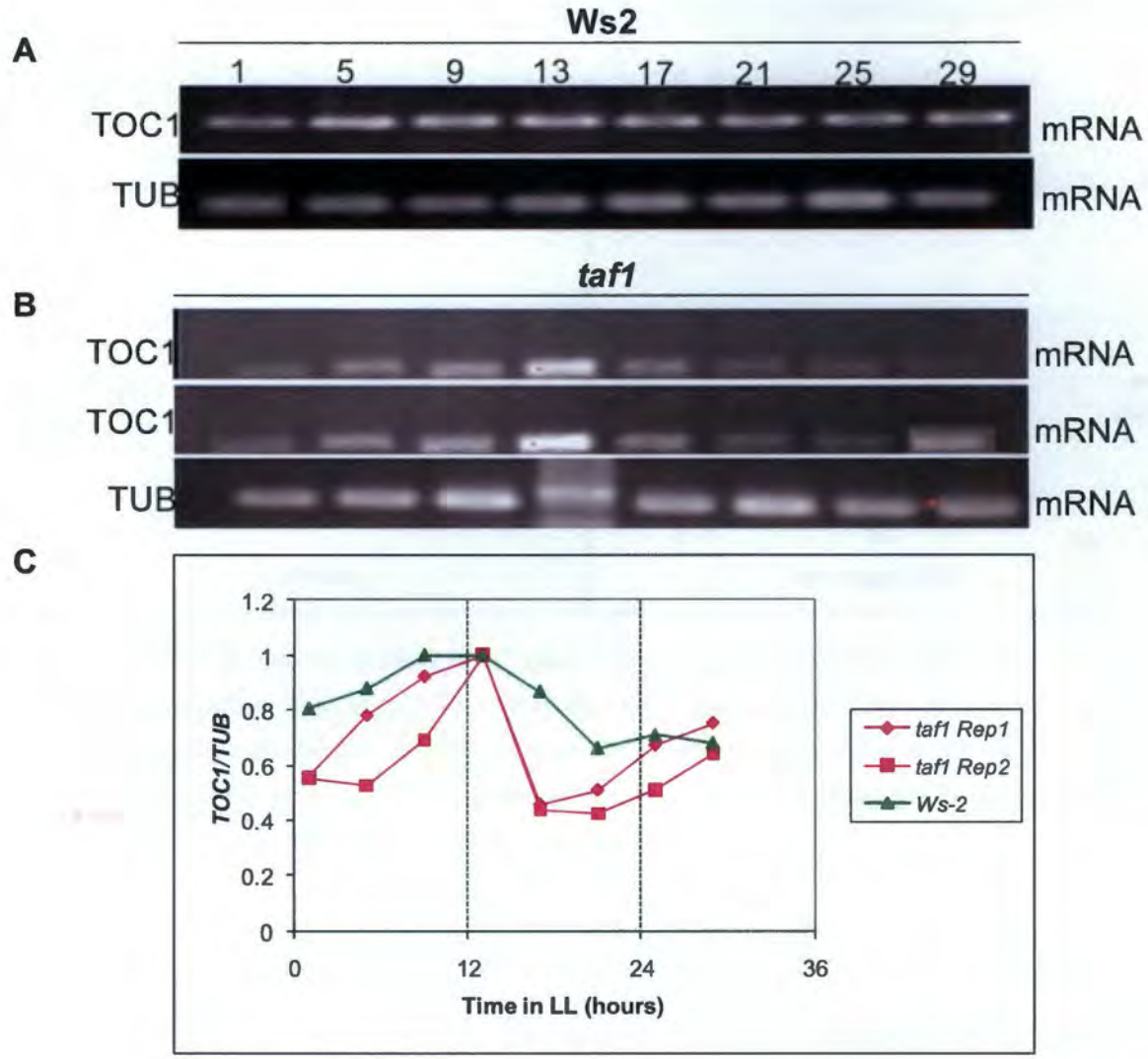

Figure 18. Relative levels of *TOC1* transcript in Ws2 and *taf1* plants in LL conditions as determined by semi-quantitative RT-PCR. **A** and **B** are representative PCR bands from semi-quantitative RT-PCR. Seedlings were entrained under 12-h-light/12-h-dark cycles and subsequently released to LL conditions. Samples were collected four hourly for a period of 28 hours: CT 1, 5, 9, 13, 17, 21, 25 and 29. **C.** Relative changes in *TOC1* transcript abundance normalised to *TUBULIN*. Experiments were carried out in duplicate (Rep 1 and 2). Only one replicate of Ws2 is shown for simplicity.

3.3.2 Delayed Fluorescence in *taf1* mutants

Semi-quantitative RT-PCR of TOC1 expression suggested that TAF1 was in some way involved in the histone acetylation at the *TOC1*promoter (Figures 17 and 18). We also wanted

to investigate whether histone acetylation by TAF1 played an important role in the overall functioning of the circadian clock by measuring the DF rhythms in the *taf1* mutant.

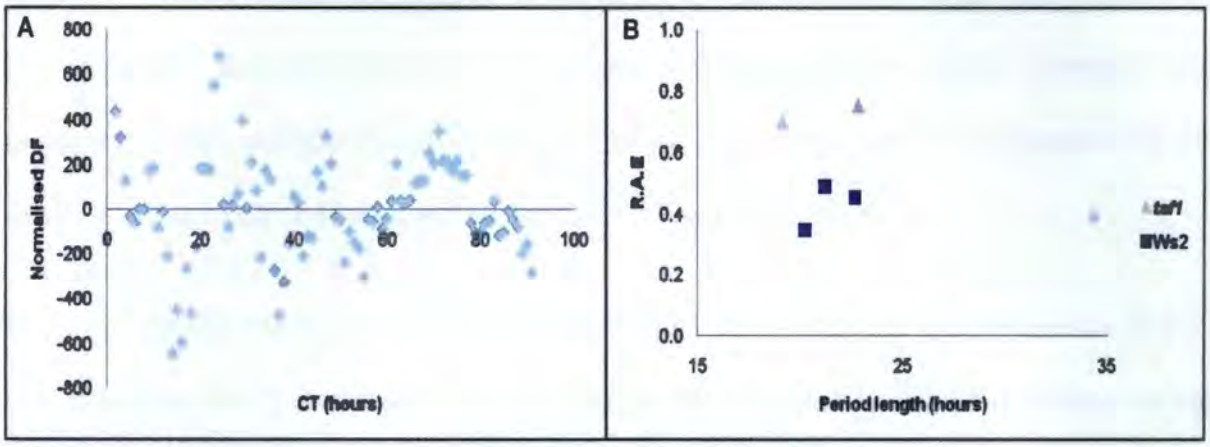

Figure 19. Delayed fluorescence rhythms in *taf1* mutant plants. **A** is a representative plots of normalised DF of one of four groups of seedlings of *taf1*. Two out of three plots looked similar. Seedlings were entrained under 12-h-light/12-h-dark cycles for nine days and subsequently released to DD conditions. DF was assayed with a one hour time resolution for 91 hours. **B**. Period estimates for groups of seedlings plotted against their R.A.E. Ws2 n=3 (closed squares) and *taf1* n= 4 (closed triangles).

From Figure 19, it can be observed that the rhythm of DF in *taf1* seedlings was not maintained. Only three out of four of the analysed groups of seedlings returned estimated period values. The returned periods were accompanied by RAE > 0.6 (0.70 and 0.76), except one with a long period of 34.4 hours. One group did not return a period at all. In this case, the precise period of this mutant could not be determined. As a result, genotyping was carried out in order to determine whether these groups contained a mixed (heterozygous and homozygous mutants) population (Figure 8). Although these data suggest that histone acetylation by TAF1 may play an important role in the functioning of the *A. thaliana* circadian clock, further investigation with individual seedlings was required before I could be sure. The *taf1* line I was using was segregating and the methods I had employed thus far used groups of seedlings which may have masked the mutant phenotype.

3.3.3 Leaf movement *of taf1* mutants

The diurnal change in the position of cotyledons or young leaves in plants can reveal insight in the functioning of their endogenous circadian clock (Edwards and Millar, 2007). Like DF, leaf movement assays require no prior genetic manipulation of the plant. Therefore leaf movement assays provide scientists with a simple and reliable method in the analysis of the plant circadian clock (Edwards and Millar, 2007; Gould *et al.*, 2009).

Since *taf1* clearly contained a mixed population, leaf movement assays were used to further verify the DF data. For analysis, 10 individual young Ws2 leaves and 12 individual young *taf1* leaves were tracked for 4-5 days. Image-Pro 6.2 software allowed us to track the vertical movement of individual young leaf tips. FFT-NLLS returned period values (average of 22.9 hours, which is very similar to that given for DF of 22.7 hours) for all Ws2 leaves. Only 10 out of 12 *taf1* leaves returned period values (Figure 20).

The heterozygous lines exhibited a period similar to the Ws2 (average of 23.1 hours). These were accompanied by RAE values ≤0.6 (Figure 20C). For two homozygous lines, FFT-NLLS did not return a period. One homozygous lines exhibited arrhythmia (RAE of 0.86) while the rest exhibited long period values (average 30.0 hours). These data suggest that histone acetylation by TAF1 may not only be important in the rhythmic histone acetylation of *TOC1* expression but also in the overall functioning of the circadian clock. This implicates TAF1 as a candidate HAT in rhythmic histone acetylation of *TOC1* and other components of the *A. thaliana* circadian clock.

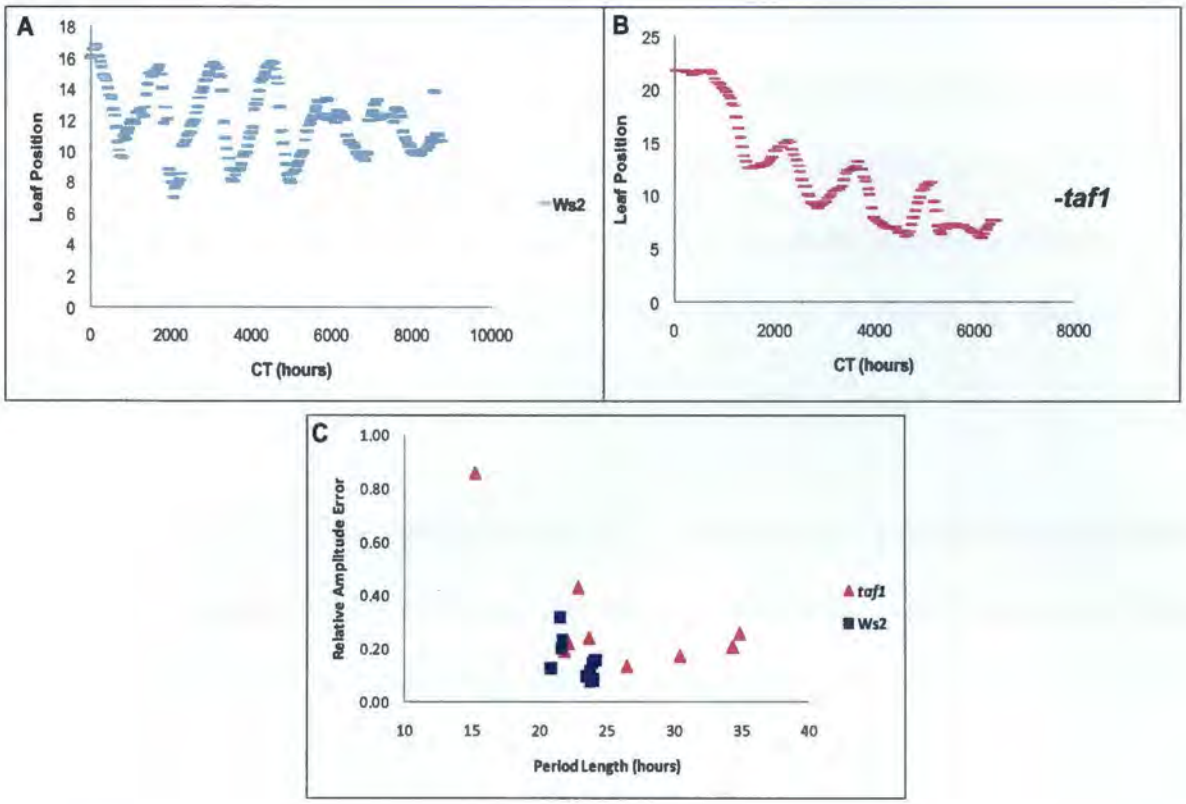

Figure 20. Leaf movement rhythms in Ws2 and *taf1* mutants. **A** is a representative plots of leaf movement rhythms of one of ten seedling of Ws2. 7 out 10 plots looked similar. **B** is a representative plots of leaf movement rhythms of one of 12 seedling of *taf1* mutant plants. Five out of 10 plots looked similar. Seedlings were entrained under 12-h-light/12-h-dark cycles for eight days and subsequently released to LL conditions. Seedlings were imaged and recorded every 20 minutes over several days (4-5 days) using time lapse photography. Image-Pro Plus® 6.2 software was used to track the Y (vertical) position of the leaves and the data was exported to Microsoft Excel for rhythm analysis. BRASS was used to carry the FFT –NLLS analysis **C.** Period estimates for leaves plotted against their R.A.E. Ws2 n=10 (closed squares) and *taf1* n= 12 (closed triangles).

3.4 Investigating rhythmic histone modifications in *hd1* mutants

3.4.1 Semi-quantitative RT-PCR of *TOC1* in *hd1* mutants

In *A. thaliana,* mutations in the *HD1* gene has been shown to cause pleiotropic developmental abnormalities including defective shoot apical meristems, aborted seeds, late flowering and short hypocotyls (Tian *et al.*, 2003; Benhamed *et al.*, 2006). Benhamed *et al.*, (2006)

demonstrated that mutations in *HD1* resulted in induced increased acetylation on H3K9, H3K27, H4K5 and H4K8 at light inducible promoters. My aim was to investigate whether *HD1* was a potential candidate in the rhythmic histone deacetylation. Since the balance between acetylation and deacetylation are necessary in the proper regulation of transcription (Davie, 1997) if histone acetylation in *TOC1* promoter is rhythmic, so should be its subsequent deacetylation.

To test this hypothesis, we examined the circadian expression profile of *TOC1* in *hd1* mutants using semi-quantitative RT-PCR under DD and LL conditions. In DD conditions, *TOC1* expression appeared to be completetely arrhythmic in *hd1* mutants (Figure 21) and appeared to have erratic rhythms in LL conditions (Figure 22). This suggests that HD1 may be involved in the rhythmic histone deacetylation affecting circadian clock gene expression. Interestingly, previous studies with genes that showed distinct changes in their mRNA abundance in DD and LL grown *A. thaliana* seedlings demonstrated that acetylation of H3K9 level at specific loci, was higher in *hd1* mutant plants compared with the wild type in both LL and DD grown seedling (Gou *et al.*, 2008), suggesting HD1 involvement in the maintenance of H3K9 acetylation in a light dependent manner.

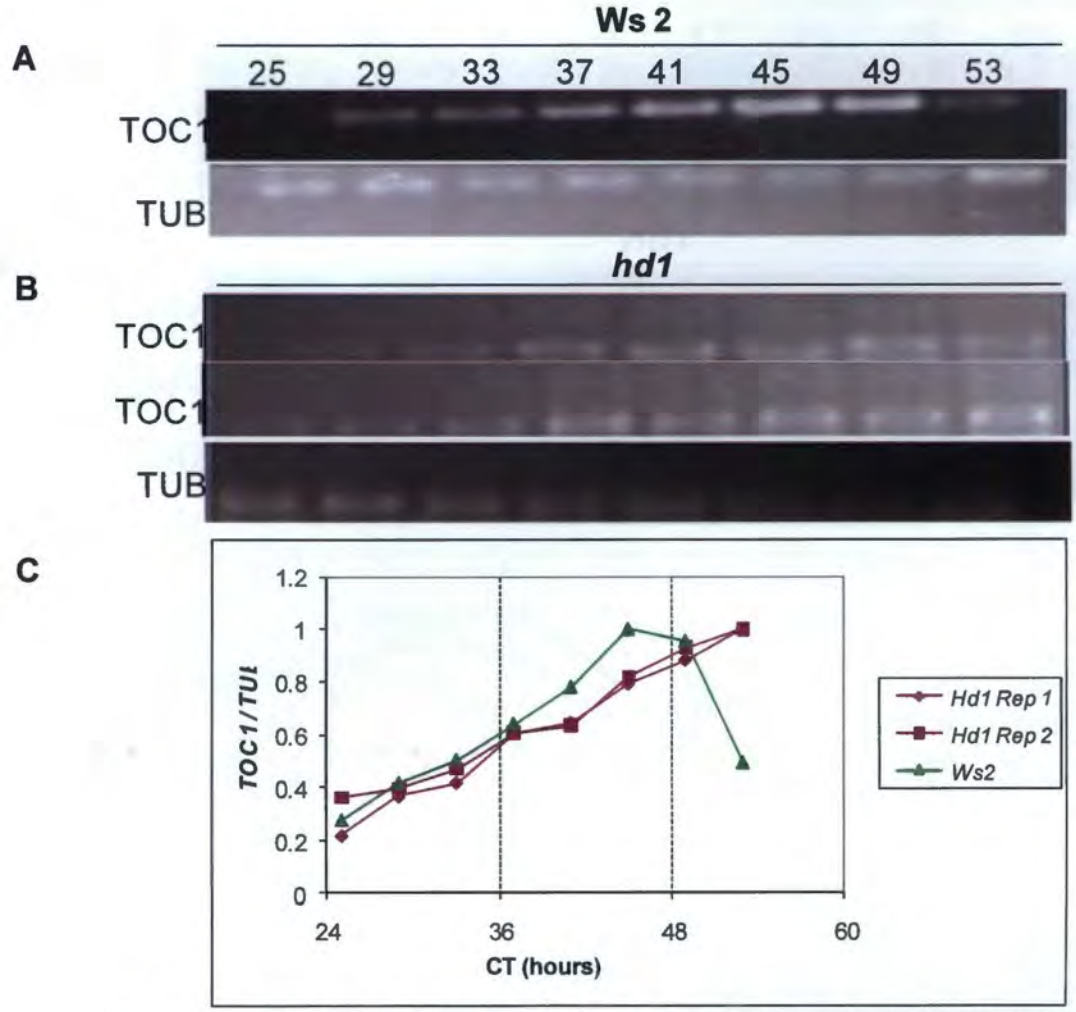

Figure 21. Relative levels of *TOC1* transcript in Ws2 and *hd1* mutant plants in DD conditions as determined by semi-quantitative RT-PCR. **A** and **B** are representative PCR bands from semi-quantitative RT-PCR. Seedlings were entrained under 12-h-light/12-h-dark cycles and subsequently released to DD conditions. Samples were collected four hourly for a period of 28 hours: CT 25, 29, 33, 37, 41, 45, 49 and 53. **C.** Relative changes in *TOC1* transcript abundance normalised to *TUBULIN*. Experiments were carried out in duplicate (Rep 1 and 2). Only one replicate of Ws2 is shown for simplicity.

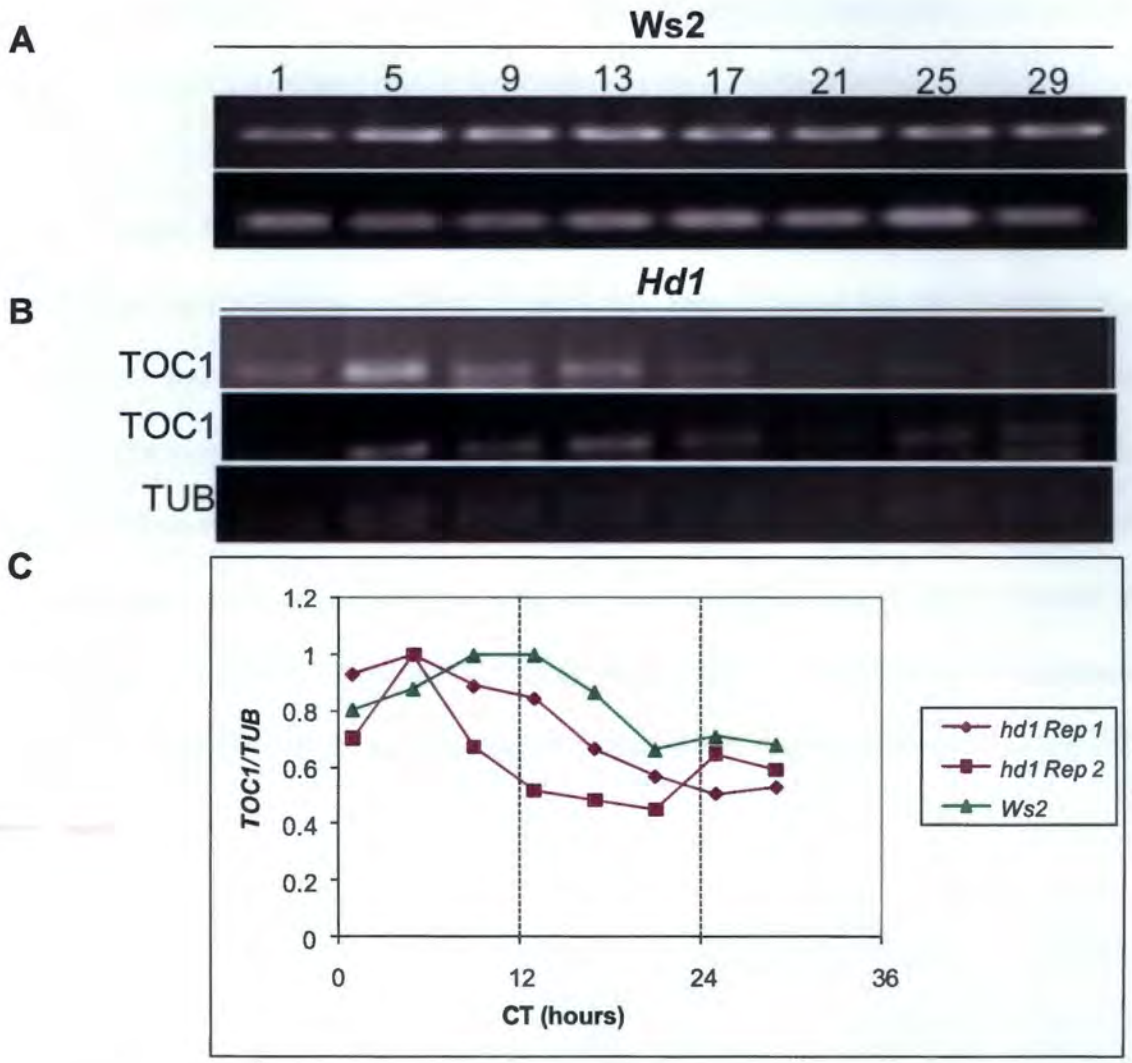

Figure 22. Relative levels of *TOC1* transcript in Ws2 and *hd1* plants in LL conditions as determined by semi-quantitative RT-PCR. **A** and **B** are representative PCR bands from semi-quantitative RT-PCR. Seedlings were entrained under 12-h-light/12-h-dark cycles and subsequently released to LL conditions. Samples were collected four hourly for a period of 28 hours: CT 1, 5, 9, 13, 17, 21, 25 and 29. **C**. Relative changes in *TOC1* transcript abundance normalised to *TUBULIN*. Experiments were carried out in duplicate (Rep 1 and 2). Only one replicate of Ws2 is shown for simplicity.

3.4.2 Delayed Fluorescence in *hd1* mutants

Data given by semi-quantitative RT-PCR suggested that HD1 may be involved in the rhythmic histone deacetylation affecting *TOC1* transcription (Figures 21 and 22). We

therefore monitored DF accumulation in *hd1* mutants to investigate whether histone deacetylation by HD1 plays a role in functioning of the *A.thaliana* circadian clock.

For two groups, FFT-NLLS analysis returned long period values (31.4 and 32.6). These were accompanied by RAE values > 0.6 (0.83 and 0.65). This indicated that the circadian clock of these groups was perturbed. The third group exhibited an RAE value of 0.61, indicating the loss of rhythmicity in these groups (Figure 23B). Together with semi-quantitative RT-PCR data, these data suggests that HD1 may be involved in the histone deacetylation that affects circadian clock gene expression as well as in the functioning of the circadian clock. Interestingly, publically available microarray data (GEO datasets GSE3416) demonstrated that *HD1* is diurnally expressed, peaking just around dawn, slightly before *CCA1* and *LHY*.

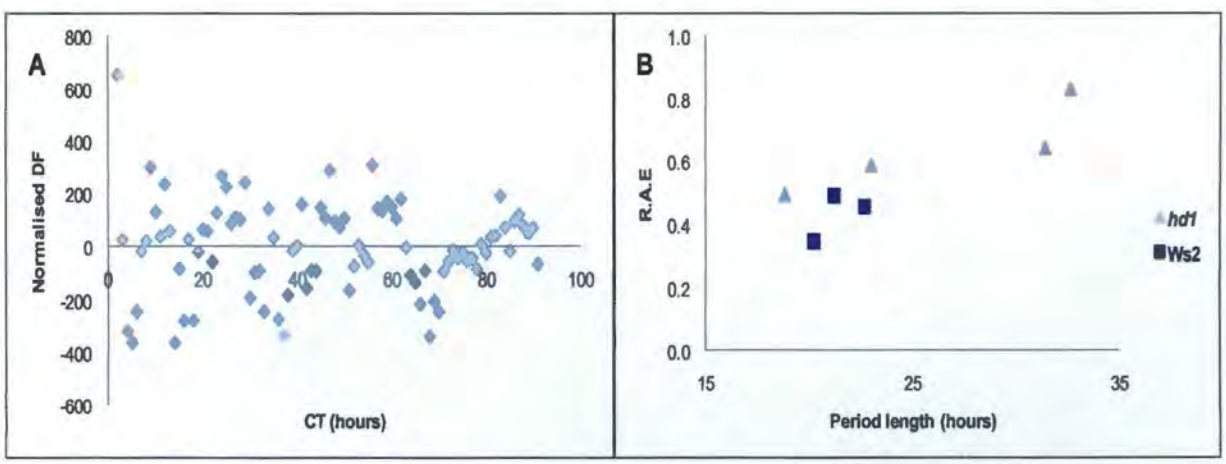

Figure 23. Delayed fluorescence rhythms in *hd1* mutant plants. **A** is a representative plots of normalised DF of one of four groups of seedlings of *hd1*. Two out of four plots looked similar. Seedlings were entrained under 12-h-light/12-h-dark cycles for nine days and subsequently released to DD conditions. DF was assayed with a one hour time resolution for 91 hours. **B**. Period estimates for groups of seedlings plotted against their R.A.E. Ws2 n=3 (closed squares) and *hd1* n= 3 (closed triangles).

3.5 Investigating rhythmic histone modifications in *clf* mutants

3.5.1 Semi-quantitative RT-PCR of *TOC1* in *clf* mutants

Both acetylation and methylation of histones are reversible reactions (Berger, 2002). Histone acetylation is important in the rhythmic expression of *TOC1* but histone methylation may also have an important role. Previous studies indicated that *CLF* is required to repress floral homeotic genes such as AGAMOUS (AG) and also the homeobox gene SHOOTMERISTEMLESS (*STM*) (Goodrich *et al*, 1997; Katz *et al*, 2004). We therefore investigated whether methylation by *CLF* may play a role in the circadian clock by investigating the rhythmic expression of *TOC1 in clf*. *clf* mutants showed an arrhythmic *TOC1* expression in both DD and LL conditions which indicated the perturbation of the biological clock (Figures 24 and 25). These data suggest that *CLF* may methylate histones in the promoter of *TOC1*.

We did not carry out leaf movement analyses on these mutants as the "curly leaf" phenotype interferes in the analysis.

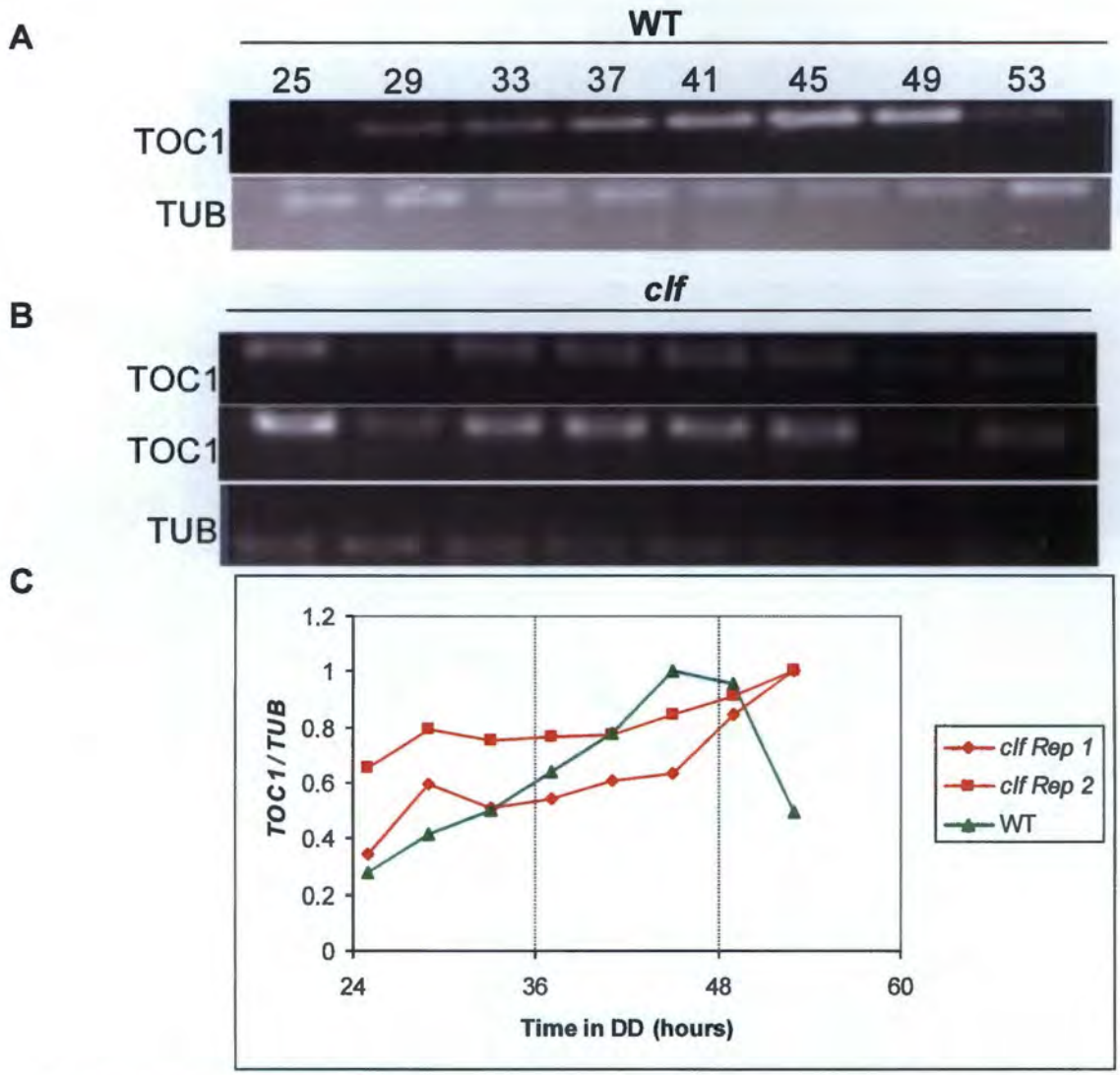

Figure 24. Relative levels of *TOC1* mRNA in WT and *clf* mutant plants in DD conditions as determined by semi-quantitative RT-PCR. **A** and **B** are representative PCR bands from semi-quantitative RT-PCR. Seedlings were entrained under 12-h-light/12-h-dark cycles and subsequently released to DD conditions. Samples were collected four hourly for a period of 28 hours: CT 25, 29, 33, 37, 41, 45, 49 and 59. **C**. Relative changes in *TOC1* transcript abundance normalised to *TUBULIN*. Experiments were done in duplicates (Rep 1 and 2). Only one replicate of the WT is shown for simplicity.

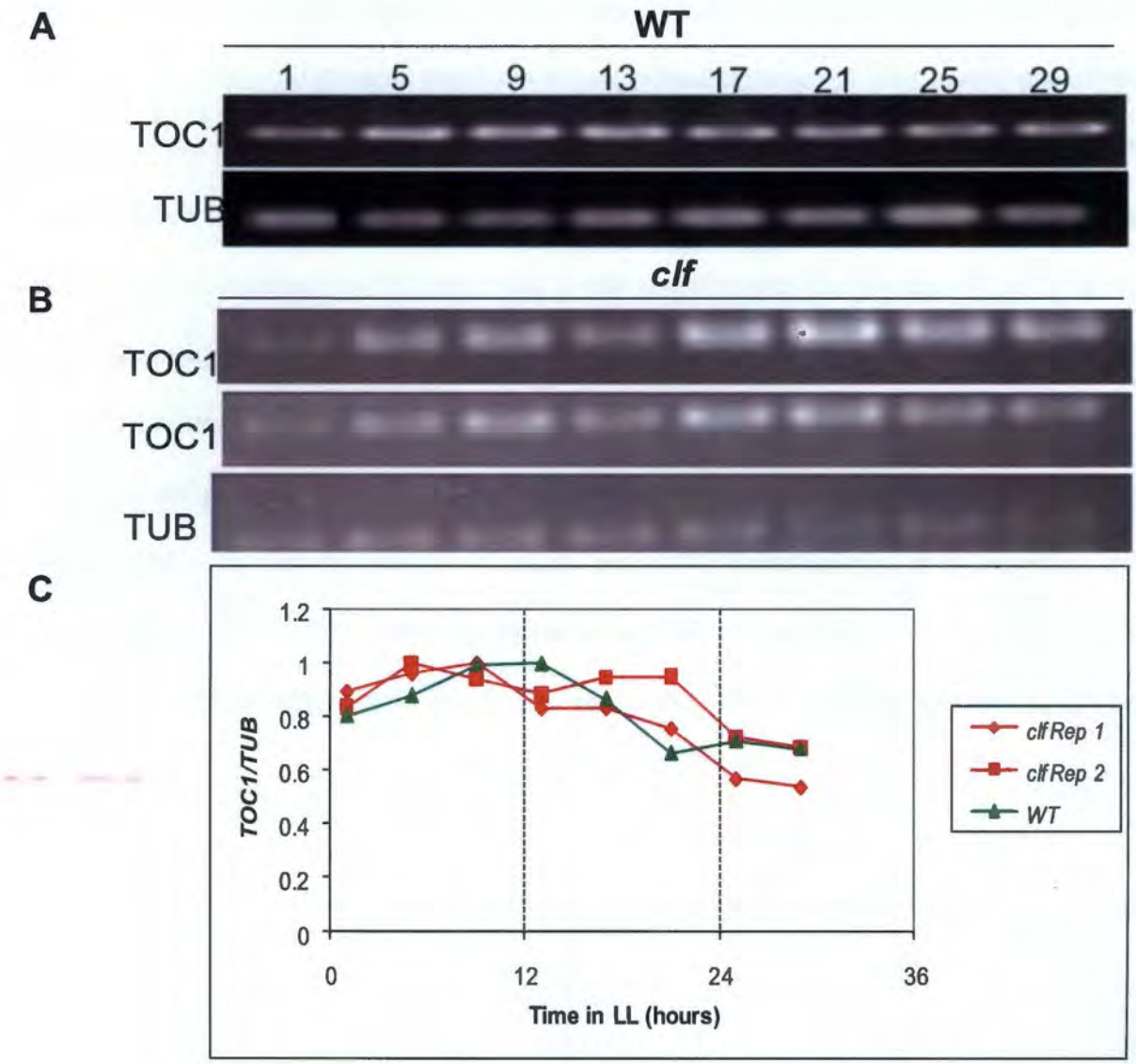

Figure 25. Relative levels of *TOC1* mRNA in Ler-0 and *clf* mutant plants in LL conditions as determined by semi-quantitative RT-PCR. **A** and **B** are representative PCR bands from semi-quantitative RT-PCR. Seedlings were entrained under 12-h-light/12-h-dark cycles and subsequently released to LL conditions. Samples were collected four hourly for a period of 28 hours: CT 1, 5, 9, 13, 17, 21, 25 and 29. **C.** Relative changes in *TOC1* transcript abundance normalised to *TUBULIN* mRNA. Experiments were carried out in duplicates (Rep 1 and 2). Only one replicate of the WT is shown for simplicity.

3.5.2 Delayed Fluorescence in *clf*

A. thaliana clf seems to play an important role in maintaining rhythmic histone methylation in *TOC1* (Figures 24 and 25). We therefore investigated whether CLF was an important

histone methylase in the functioning of the circadian clock by measuring the DF rhythms. Through FFT-NLLS, *clf* mutants exhibited a long period (average of 32.3 hours) phenotype compared to the wild type plants (average of 23.6 hours) (Figure 26). This indicated that the circadian clock of the *clf* mutants was disrupted. Therefore, this strongly suggests that methylation in *A.thaliana* by CLF may play a role in the proper functioning of the circadian clock. This assay was very important in determining the overall functioning of the circadian clock in these mutants as leaf movement measurements could not be adequately taken due to the altered leaf phenotype of *clf* mutant. Measuring period length of DF in Ws2, Col-0 and C24, previous work demonstrated the circadian periods of these ecotypes to be similar to the period length in CAB::LUC rhythms (Edwards *et al.*, 2005; Gould *et al.*, 2009). We conclude that the DF data is sufficient to verify the importance of CLF in the functioning of the circadian clock.

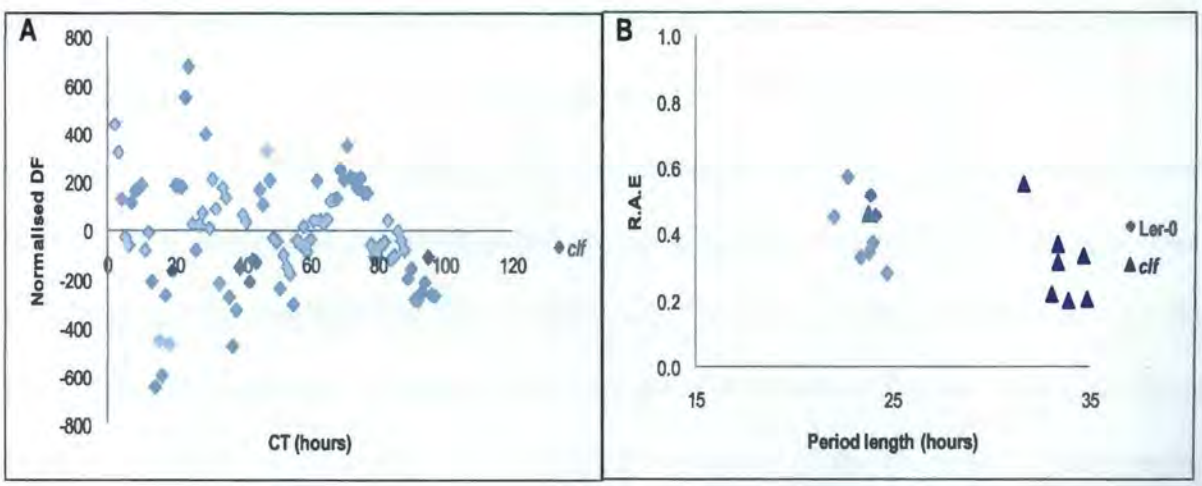

Figure 26. Delayed fluorescence rhythms in Ler-0 and *clf* mutant plants. **A** is a representative plots of normalised DF of one of seven groups of seedlings of *clf* (one group was dissimilar). All plots for Ler-0 looked similar. Seedlings were entrained under 12-h-light/12-h-dark cycles for nine days and subsequently released to DD conditions. DF was assayed with a one hour time resolution for 91 hours. **B.** Period estimates for groups of seedlings plotted against their R.A.E. Ler-0 n=8 (closed squares) and *taf1* n= 8 (closed triangles).

The SET (SU(VAR) 3-9, E(Z) and Trithorax) domain of *CLF* is very similar to E(Z) proteins' SET domains, which is the domain required for histone methylation (Schubert *et al.*, 2006). Schubert *et al.*, (2006) demonstrated that *A. thaliana CLF* is required for dispersed trimethylation of H3K27 (H3K27met3) which represses the expression of *AGAMOUS (AG)* and *SHOOTMERISTEMLESS (STM).* It will be interesting to investigate whether H3K27met3 is also implicated in the rhythmic expression of *TOC1.* In *Saccharomyces cerevisiae,* the methylation of H3K36 by RNA polymerase II-associated Set2 methyltransferase creates marks on nucleosomes which targets them for H3 and H4 deacetylation by the *Rpd3S* deacetylase complex after the passage of RNA polymerase (Lee and Shilatifard, 2007).

The EZH2-containing PRC2 of mammals is involved in X-chromosome inactivation, genomic imprinting during germ line development, the regulation of stem cell pluripotency, and the promotion of cancer metastasis (Lund and van Lohuizen, 2004; Peters and Schubeler, *2005*). Endogenous EZH2, a polycomb group enzyme, has been shown to methylate lysine 27 on histone H3. In 2006, Etchegaray *et al.* demonstrated that EZH2 co-immunoprecipitates with CLOCK and BMAL1 throughout the circadian cycle in liver nuclear extracts in mice. ChIP experiments revealed that EZH2 binds and di- and trimethylase H3K27 on both the *Per1* and *Per2* promoters. Overexpression and RNA interference studies supported role of EZH2 in cryptochrome-mediated transcriptional repression of the clockwork. These results indicated that EZH2 is important for the maintenance of circadian rhythms and extend the activity of the polycomb group proteins to the core clockwork mechanism of mammals (Etchegaray *et al.*, 2006).

Therefore, it is possible that in *clf* mutants the nucleosomes cannot be marked for H3 and H4 deacetylation, thereby preventing transcription of *TOC1* and hence impairing the circadian clock function. Interestingly, Stacey Harmer has identified a new circadian clock component called pst1 which is a jumonjiC domain protein which may be a histone demethylase or a protein hydroxylase (Unpublished data, presented at Society for Resaerch on Biological Rhythms 2010 conference, May 2010 Sandestin FL).

SECTION 4: Conclusion

Using semi-quantitative RT-PCR in conjunction with DF and leaf movement assays, we have demonstrated that histone acetylation by GCN5 and TAF1, which has already been implicated in the regulation of cold response genes, could be involved in both the rhythmic expression of a central oscillator gene, *TOC1*, and in overall plant clock function. However, these two HATs could play different roles in rhythmic histone acetylation. We also identified HD1 as a potential candidate in the rhythmic histone deacetylation of of *TOC1* and overall functioning of the circadian clock. Furthermore, CLF methyltransferase was identified as a potential candidate in the rhythmic histone methylation of *TOC1* and also implicated in the proper functioning of the *A. thaliana* circadian clock. Histone methylation has not been reported to be involved in the rhythmic expression of genes, to date. We also propose that the effect of mis-acetylation/ methylation has a profound effect on all gene expression and so it is difficult to put one or the other in the "clock" alone. However, we do not discredit the fact that more studies with more gene loci, and greater numbers of replicates, still need to be carried out in order to make valid conclusions about the specificities of the involvement of these histone modifiers in the circadian clock. It will be interesting to do *in vivo* studies such as ChIP assay experiments to determine which positions of histones are acetylated and

methylated in *TOC1* promoter and compare these results to what happens in the acetylation and methylation mutant lines.

SECTION 5: References

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