A COMPARATIVE EXPRESSIONAL ANALYSIS OF A FAMILY OF CCA-LIKE MYB TRANSCRIPTION FACTORS IN TWO HIGHER PLANT SPECIES

by

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A THESIS PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

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To my parents with love and respect

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Circadian clocks are ubiquitous among most living species. Since life on Earth originated in the presence of light/dark cycles, organisms had to evolve mechanisms to cope with such environmental fluctuations. An accurate timekeeping apparatus affords an organism with temporal organization of crucial molecular and cellular processes. The components that constitute the central oscillator of the clock vary greatly among plants and animals but the basic architecture appears similar. This resemblance serves as a foundation on which evolutionary-based investigations on the conservation of such machinery can be conducted. In the plant *Arabidopsis thaliana*, two genes involved in circadian regulation were identified and characterized as members of a family of Myb transcription factors characterized by only one Myb repeat sequence. These genes, *CCA1* and *LHY*, are necessary to maintain rhythmicity in the plant and have been shown to have a role in floral induction. Using the Floral Genome Project (FGP) database of known flowering genes, two EST homologs of CCA1 and LHY were identified in *Eschscholzia californica*, eca_4_183384 and eca_4_184056 (E1 and E2), based on sequence similarity. These orthologs demonstrate transcript oscillations over a 24hr period with peak levels of expression occurring just prior to dawn. *In situ* analyses revealed similar patterns of expression in young and older floral tissue of both plant species. In this paper I present

molecular evidence that the transcription- translation- based feedback loop mechanism of the circadian oscillator is conserved in these higher plant species and suggest that this mechanism can also be observe din other higher plants.

CHAPTER 1
LITERATURE REVIEW

Biological Rhythms

The daily rotation of the earth leads to periodic fluctuations in environmental conditions. Because life on Earth originated in the presence of a cyclical environment, many organisms have evolved timing mechanisms to organize important events. In order to adapt to the changes presented by the environment, organisms must modulate their behaviors with the daily cycles of light and temperature variation, and establish an effective method for tracking time. Since the environment imposes a period of approximately 24 hours, organisms with rhythmic behavior that matches these oscillations have higher fitness than those that do not.

The first noted rhythmic behavior occurred in the fourth century BC when the sleep movements of the tamarind tree were noted by Androsthenes, a Greek philosopher (Chandrashekaran, 1998). In the mid-1700s, a French astronomer named Jean- Jacques d'Ortous de Mairan recorded the daily leaf movements of the *Mimosa pudica* plant and demonstrated that the rhythms persisted for several days when the plant was subjected to complete darkness (Golden and Strayer, 2001; Sweeney, 1987). This was the first evidence that the rhythmic behavior must be endogenous and that the rhythmicity, once established, continues in the absence of environmental cues. Erwin Bunning's work during the 1930s, in which he demonstrated a rhythm in the eclosion behavior of the fruit fly *Drosophila*, further supported the idea of an endogenous timekeeping mechanism. Since the rhythms occurred with a 24 hour period, the term *circadian* was coined from the Latin words *circa* and *dias*, meaning "about a day" (Chandrashekaran, 1998; Halberg, 1959).

Circadian rhythms are variations in physiological and behavioral activities that occur over a period of about 24 hours (Hardin, 2000). In the context of a biological process, the time

interval between two successive events is described as a biological ‘rhythm’ (Kumar, 2002). Biological rhythms are found in almost all living organisms. They have been described extensively in mammals, insects, fungi, plants and bacteria (Dunlap et al., 1999; McClung, 2001; Ouyang et al., 1998). These rhythms occur over a large range of time scales: from millisecond oscillations to seasonal changes. The ubiquity of circadian rhythmicity across a broad taxonomic spectrum suggests that adaptive fitness is enhanced by the synchronization of certain events with the diurnal cycle imposed by the environment (McClung 2000).

In a number of different species, a plethora of activities are regulated by circadian rhythms. Cyanobacteria demonstrate daily oscillations in nitrogenase activity, photosynthesis and metabolic activities (Kondo and Ishiura, 1999). In vertebrates, a number of behavioral and life processes, ranging from the molecular level to the cellular and systemic levels, are driven by circadian rhythms including eating, sleeping, seasonal migration and cell proliferation (Gillette and Sejnowski, 2005). Similarly, a variety of events such as leaf movement, stomatal opening and regulation of flowering time and fragrance emission are tightly regulated by the plant timekeeper. In other organisms, such as *Neurospora* and *Drosophila*, development, cell signaling and stress responses with self-sustaining rhythms can be regulated by the circadian clock. The role that diurnal rhythms play in a number of different activities suggest that an endogenous timekeeping system provides an adaptive advantage, enabling the anticipation of environmental change and the coordination of crucial events to occur at specific phase relationships with the environment (Más, 2005).

The Circadian Clock

Circadian or biological rhythms, although they parallel the environmental cycles of light and dark, are generated within an organism by a complex timekeeping system (Hardin, 2000). Many organisms have evolved an endogenous ‘chronometer’, the circadian clock, to temporally

coordinate important processes with the daily variations in the environment. Circadian rhythm of gene expression has been shown to function as the underlying mechanism of the clock (Wang and Tobin, 1998).

Two important attributes of the circadian clock, entrainment and temperature compensation, ensure synchrony between important rhythmic activities and the surroundings of an organism. The first, entrainment, is the manner in which the clocks are set to 'local' time by environmental cues such as the light / dark cycles. The second, temperature compensation, describes the ability of the clock to run at the same rate independent of temperature changes (Hardin 2000).

The notion that biological rhythms are created within an organism stimulated interest in the mechanism that maintains these oscillatory patterns. In theory, numerous regulatory schemes could achieve such fluctuations. The most common method involves a regulatory circuit with a positive and negative product (Fig. 1-2). The negative element feeds back to slow down the rate of the process itself and creates a delay in the execution of the feedback. A positive element is required to activate the clock and prevent it from winding down (Dunlap et al., 1999). The generally conserved clock mechanism consists of an autoregulatory feedback loop in which positive factors act on genes encoding negative factors that in turn feedback to inhibit their own expression (Strayer et al., 2000; Dunlap, 1999). A circadian system often consists of one or more interconnected feedback loops.

The circadian system is divided into three main components: input pathways, a central oscillator and output pathways (Fig. 1-1). Input pathways, also known as entrainment pathways, transmit environmental signals to the central timekeeping apparatus. In plants, this signal transduction pathway adjusts the clock in response to external cues most frequently through the

action of cryptochromes and phytochromes. The entrainment of circadian clocks to the light/dark cycles is usually mediated by light-induced changes in the level of a component of the oscillatory feedback loop. The ability to re-entrain the clock ensures synchrony with the environment and allows the anticipation of dawn and dusk (Devlin, 2002).

The core timekeeping component of the circadian clock is the central oscillator (Hardin, 2000). It serves as the system's pacemaker and is responsible for generating circadian rhythms. In order for the oscillator to function in the absence of environmental cues it must be synchronized with the external time via the input pathways. Environmental transitions between dawn and dusk help to adjust the endogenous period created by the oscillator to precisely match the 24- hour period found in nature (Más, 2005).

Completing the circadian clock model are the output pathways which provide a link between the central oscillator and the rhythmic physiological responses they control. These pathways are activated at specific times of the circadian cycle and the outputs are in phase with the oscillation of light cycles (Dodd et al., 2005). In both animals and plants, there are a substantial number of physiological and metabolic processes that are regulated by the circadian outputs. These events include such varied responses such as olfactory responses in Drosophila, leaf movements and hypocotyl growth in plants, and enzyme activity. The rhythmic behaviors that are generated in response to day length are known as photoperiodic responses (Schultz and Kay, 2003).

The discovery of components of the circadian oscillator has enabled scientists to concentrate on the mechanisms that interconnect the three components to form an effective timekeeping system. Recent studies of several model organisms including *Drosophila*, *Neurospora* and mice have revealed a common molecular mechanism at the center of the

circadian oscillator (Barak et al., 2000). Clock proteins, encoded by clock genes, serve as negative elements that repress their own expression by blocking their transcriptional activators, or positive elements (Fig. 1-2). A decrease in clock transcripts and proteins results in the de-repression of the transcriptional activators and thus reinitiates the cycle.

Clock- Controlled Genes

The identification of genes which function at the center of the clock machinery has rapidly increased over the last decades. These clock-controlled genes, or ccgs, encode pieces of the central oscillator. Their products produce and maintain the oscillations that drive other circadian rhythms. A substantial number of clock-associated genes whose expression relies on the rhythms generated by the oscillator have been identified in the genomes of many organisms. Recent microarray analyses of the model plant Arabidopsis indicated that up to 6% of the genes are rhythmically expressed (Harmer et al., 2000; Schaffer et al., 2001).

Components of the biochemical feedback loop whose rhythmic activity is required for oscillator function are referred to as state variables. A number of criteria have been defined to identify and characterize state variables of the circadian system (Aronson et al., 1994; Kay and Millar, 1995). First, the component itself must demonstrate circadian oscillations in its activity and expression. The second criterion requires the component to control its own levels by feedback inhibition of its synthesis. Additionally, clamping of the amount of the putative oscillator component at any level from null to high stops the clock and thus rhythmicity. The final criterion states that induced transient perturbations in the abundance of the component should cause a phase shift in the clock output. These criteria are applied in the investigations of putative oscillatory components to assign positive and negative roles to the elements. Knowledge of interconnected loops is obtained from the identification through classical genetics of genes that are within one of the core loops of the oscillator (Roenneberg and Merrow, 1998).

The molecular basis of circadian clocks is best understood in *Neurospora* and *Drosophila*. The genetics of rhythms originated in these two organisms and much of what scientists have learned about how mammalian clocks operate closely parallels the behavior of one or both of these species. In each of these model systems, genetic screens for rhythm mutants that affect the period length of the clock or abolish its activities were found (Hardin and Siwicki, 1995; Dunlap, 1996).

The first clock mutations to be discovered were the *period* (*per*) mutant from *Drosophila* and the *frequency* (*.frq*) mutant from *Neurospora* (Dunlap, 1993; Konopka and Benzer, 1971; Feldman and Hoyle, 1973). The *PER* gene product, as well as its transcript, oscillates with a circadian rhythm and the PER protein was required for a feedback regulation of its own gene products (Williams and Sehgal, 2001). Oscillations of PER transcripts and the protein they encode persisted in continuous dark conditions, suggesting that the gene must be under the control of the circadian clock. The phases of the *per* messenger RNA (mRNA) and protein rhythms were noticed to be quite distinct; PER protein level peaked in abundance approximately six hours after the peak in *PER* mRNA levels. This difference accounts for the delay or lag time in oscillations required by the central timekeeper. In addition, mutant *per* fruit flies exhibited altered rhythmic behavior, which suggests a role for this gene in the oscillator. The *tim* mutant was identified in a screen for recessive mutants that affected the eclosion behavior of the fly. Similar to the *per* mutant, the *tim* mutants exhibited arrhythmic behaviors, periods that were shortened or lengthened, and in some mutant alleles, PER expression was dampened (Allada et al., 2001).

PER contains a protein interaction domain known as a PAS domain, which enables the protein to interact with the second *Drosophila* circadian gene identified, *timeless* (*tim*). PER and

TIM form a heterodimer that serves as part of a feedback loop to inhibit *per* and *tim* transcription. In return, the PER-TIM heterodimer activates a gene called *Drosophila Clock* (dClk), a transcription factor that is also rhythmically expressed (Scully and Kay, 2000). dCLK interacts with another transcription factor, CYCLE (CYC) and together the two form a heterodimer complex that is required for the activation of *PER* and *TIM* transcription. The dCLK-CYC complex binds to a specific sequence in *PER* and *TIM* promoters known as the *E-box* motif, a consensus hexanucleotide sequence found in basic helix-loop-helix (bHLH) transcriptional factors (Fairman et al., 1993). Binding of the complex to the *E-box* motif allows transcription of *PER* and *TIM* and results in a increase in PER-TIM heterodimers in the cytoplasm. When expression is high, usually in the evening, these complexes move into the nucleus where PER binds to the dCLK-CYC complexes. This releases the dCLK and CYC proteins from the promoters and results in the shutting off of *PER* and *TIM* transcription. As the levels of PER and TIM decline, the dCLK-CYC dimers are released and thus the dCLK-CYC-dependent repression of *dClk* expression is lifted. As the levels of dCLK begin to rise, usually in the morning, an increased number of dCLK-CYC complexes are formed and *per* and *tim* transcription is re-activated (Glossop et al., 1999). Since the products of the *per* gene and the *dClk* gene inhibit their own synthesis it appears that the *Drosophila* oscillator consists of two interconnected negative feedback loops: a *per-tim* loop and a *dClk* loop (Glossop et al., 1999). In this model PER and TIM serve as the negative elements while dCLK and CYC serve as the positive elements (Dunlap et al., 1999).

The second timekeeping component identified through forward genetic screens for clock mutants was the *FREQUENCY* (*FRQ*) gene in the fungus *Neurospora crassa*. Mutations of this gene resulted in arrhythmic expressions, altered periodicity and deficiencies in temperature

compensation (Aronson et al., 1994). The *FRQ* RNA and protein levels cycle with a circadian rhythm and the protein negatively regulates its own transcript resulting in a feedback loop similar to observed in *Drosophila* (Dunlap, 1996). The expression of *FRQ* is activated by two PAS-domain-containing transcription factors, WHITE COLLAR 1 (WC-1) and WHITE COLLAR-2 (WC-2) that form the white collar complex (WCC), which bind to circadian photoreceptor connecting light signals and the oscillator (He et al., 2002). After the accumulation of FRQ, the proteins begin to dimerize, enter the nucleus and interact with the WCC diminishing its activity and dampening *FRQ* expression (Froelich et al., 2003). FRQ also promotes the synthesis of WC-1 increasing the level of WCC. This results in a mass of WCC that is held inactive by FRQ until the protein is phosphorylated and targeted for ubiquitination, accounting for the delay that is required by the circadian oscillator. Therefore *Neurospora* has a transcriptional/translational negative feedback loop at the core of its oscillator with WC-1 and WC-2 acting as the positive elements and FRQ as the negative (Dunlap et al., 1999). Recent evidence suggests that this FRQ-based oscillator might work in cooperation with other oscillators within the organism as well (Correa et al., 2003).

Over recent decades, numerous advances in understanding the mechanisms underlying the biological oscillator in mammals have been made. The first cloned mammalian clock component identified by forward genetics was the *CLOCK* (*CLK*) gene of *Mus musculus*, the mouse (Antoch et al., 1997). Like transcription factor proteins that are central to the clock in other organisms, the mouse *Clock* gene contains 1) a PAS domain, 2) its levels of mRNA and proteins oscillate, and 3) in *clk* mutant mice, the cyclic expression the *mPeriod* (*mPer*) homolog is reduced. These data suggest that CLOCK, as a member of the oscillator, controls the transcription of circadian genes (Gekakis et al., 1998). It was shown that CLOCK binds to another transcription factor, BMAL1

(Brain and Muscle Aryl Hydrocarbon Receptor Nuclear Translocator (ARNT)-Like1) and this complex activates the transcription of the *PER* and *CRYPTOCHROME* (*CRY*) genes. The mPER and CRY proteins form heterodimers and homodimers and upon translocation to the nucleus, they inhibit the activity of the CLOCK-BMAL1 complex, which in turn suppresses *PER* and *CRY* transcription (Panda et al., 2002). Once the mPER and CRY proteins are phosphorylated, they are targeted for degradation and transcriptional repression is relieved. Although other oscillators might be present in the mouse, central to its core oscillator is the negative feedback loop in which the negative elements, the PER homologs repress the activation of the positive elements CLOCK and BMAL1 (Dunlap, 1999).

Circadian rhythms, once only thought to be a feature of eukaryotic organisms, have recently been identified in some prokaryotes. The cyanobacteria *Synechococcus elongatus* serves as the model system for molecular investigations of this group. Approximately one hundred clock mutants identified from an ethylmethansulfonate (EMS) mutagenesis screen that were characterized by arrhythmia, atypical periods and some mutants that could be rescued by the introduction of wild-type DNA from a *Synechococcus* genomic library (Lorne et al., 2000). One cluster of DNA fragments that could be rescued represented the *kai* cluster of genes: *kaiA*, *kaiB* and *kaiC*. Transcribed from two different promoters, *PkaiA* and *PkaiBC*, a monocistronic *kaiA* mRNA and a dicistronic *kaiBC* mRNA are produced and both transcripts cycle in abundance. Overexpression studies revealed that KaiC represses the activation of the *PkaiBC* promoter while *KaiA* enhances *PKaiBC* transcription (Ishiura et al., 1998). Since KaiC represses its own transcription it functions as the negative element of the negative feedback loop at the core of the *Synechococcus elongatus* oscillator while KaiA, which helps drive expression from *PkaiBC*, functions as the positive element. A role for KaiB has yet to be determined.

Although circadian rhythms were first observed in photosynthetic organisms, the molecular mechanisms underlying the circadian oscillator in plants have been difficult to elucidate. Much of what we know about circadian rhythms has come from the studies of animal systems. Over the last decade there has been substantial effort put into identifying and understanding the roles of oscillator genes in the model plant *Arabidopsis* and in a few other plant species. Plant researchers are working to determine if a mechanism similar to those observed in other organisms is conserved among members of the plant family.

The Arabidopsis Central Oscillator

The molecular basis of circadian rhythms has been thoroughly studied in Drosophila, Neurospora, mice and cyanobacteria model systems. The common denominator among these organisms is a biological clock based on a central oscillator that uses transcriptional feedback loops to generate a circadian oscillator with a 24 hour period that regulates circadian outputs (Dunlap, 1999). The oscillator responds to environmental signals through input pathways, which entrain the clock, and controls output pathways that generate a rhythm in phenotype or biochemical pathway Mizoguchi et al., 2002). Although some of the first recorded circadian rhythms were identified in plants, the molecular mechanisms underlying these rhythms have remained unclear until the last decade. Advances in the identification and characterization of plant circadian components have been made primarily through genetic studies of *Arabidopsis thaliana*.

The first Arabidopsis clock mutant was identified by fusing a luciferase marker gene to the *CAB2* (*chloropyhll a/b binding*) promoter to investigate clock-regulated gene expression in different populations. The result was the identification of a mutant, *timing of CAB* (*toc*) that altered the period of the clock (Millar et al., 1995). The TOC1 protein was shown to contain a pseudo-response regulator motif, similar to those in two-component signal transduction

pathways, at its amino terminus and a CONSTANS (CO)-like motif at its carboxyl terminus (Strayer et al., 2000). Interestingly the CO family represents a group of plant transcription factors that are involved in flowering response (Putterill et al., 1995). Since the TOC1 protein shows similarity to the CO family, *TOC1* might play a role in flowering as well. The levels of *TOC*1 mRNA cycled in light-dark conditions and peak levels of transcript were observed late in the day while minimal levels were observed at dawn. In addition, since the *toc1-1* mutant was characterized by a circadian oscillator with a shortened period which demonstrated that *TOC1* products reduce their own expression (Strayer et al., 2000). Based on the observed data, *TOC1* appears to be a component of the central circadian oscillator of Arabidopsis.

A second potential Arabidopsis clock component was identified as a result of the identification of a day length-insensitive flowering mutant. The mutant *late elongated hypocotyl* (*lhy*) caused an elongated hypocotyl and reduced chlorophyll, as well as an altered flowering phenotype (Schaffer, 1997). *lhy* mutants were also arrhythmic for leaf movements and for the expression of several other clock-regulated genes. Rhythmic expression of *LHY* was observed with levels of the transcript peaking at dawn.The sequence of the LHY protein was used to screen the GenBank database using the TBLASTN program to identify any potential homologs in the plant. The *Arabidopsis* DNA-binding protein CIRCADIAN CLOCK ASSOCIATED-1 (CCA1) was most closely related to LHY (Schaffer et al., 1998) CCA1 was first identified as a factor that binds to the promoter of the *Chlorophyll a/b-binding light-harvesting complex* (*LHCB*) gene in Arabidopsis and functions in the phytochrome signaling pathway to induce the transcription of *LHCB* (Wang et al., 1997). Later studies revealed that *cca1* mutants display a shorter period of circadian rhythms. Overexpression of this protein disrupted rhythmicity in several clock outputs including hypocotyl elongation, leaf movements and circadian gene

expression (Green and Tobin, 1999; Wang and Tobin, 1998). *CCA1* transcripts also oscillate with peak levels of expression early in the morning and in constant conditions the rhythms persist suggesting this gene is under circadian control (Wang and Tobin, 1998). In *lhy cca1* double mutants circadian rhythms were observed with an abnormal phase and oscillations of transcripts were dampened. Early expression of *LHY* and *CCA1* (morning genes) and some evening genes were also observed in the double mutants suggesting that these two genes function as components of a negative feedback loop (Schaffer et al., 1998).

LHY and *CCA1* genes function redundantly and are required for the maintenance of circadian rhythms in Arabidopsis (Alabadí et al., 2002). Both genes are closely related MYB-like transcription factors but are unique in that they only possess a single MYB repeat sequence whereas other myb transcription factors usually contain two to three of the motifs. LHY and CCA1 are also related outside of the MYB domain sharing other regions that exhibit at least 80% identity (Fig. 1-3). Overall, the two genes are 46% identical to one another (Schaffer et al., 1998). This sequence analysis suggests that LHY and CCA1 encode related DNA-binding proteins with a single MYB repeat that function as transcription factors. This notion is supported by evidence that shows LHY and CCA1 bind specifically to a sequence known as the 'evening element' (EE) in the promoter of many genes whose expression peaks nears dusk (Alabadí et al., 2001; Harmer et al., 2000).

In *Arabidopsis*, the model for the plant clock components is based on the regulation of the three plant genes described above, *CCA1*, *LHY* and *TOC1*. These components appear to operate in a transcriptional/translational-based negative feedback loop similar to that observed in other studied systems. Light activation of *LHY* and *CCA1* expression results in transcript levels that peak at dawn followed by a peak in proteins approximately two hours later. Both of the proteins

bind to the EE motif located in the promoter of the *TOC1* gene, a positive element, and repress TOC1 expression during the day (Alabadí et al., 2001). A drop in TOC1 protein results in the reduction of *LHY* and *CCA1* transcript and protein levels. The low levels of expression of the two genes results in the derepression of *TOC1* transcription. In return, levels of TOC1 protein peak during the late evening resulting in the activation of *LHY* and *CCA1* transcription just prior to dawn (Carré and Kim, 2002). This cross-regulation between LHY, CCA1 and TOC1 is proposed to function as the central oscillator of the *Arabidopsis* clockwork where LHY and CCA1 function as the negative elements and TOC1 serves as the positive element (Fig. 1-4). This central clockwork regulates numerous genes in Arabidopsis responsible for photosynthesis, nitrogen assimilation, biosynthesis of photo-protective pigments, lipid modification, hypocotyl elongation and flowering (Harmer et al., 2000; Schaffer et al., 2001; Más, 2005).

Evolution of Core Clock Components

The circadian clock has been well characterized in organisms from cyanobacteria to fungi, mice and plants. In these organisms, the central oscillator measures time with a molecular feedback loop or loops that cycle with a 24-hr period (Dunlap, 1999). The central timekeeper generates rhythms by controlling transcription of numerous clock genes. Regulation of the feedback loop is based on negative elements, which repress their own expression and positive elements that stimulate transcription. The negative feedback along with a delay is sufficient to produce oscillations. Although the basic architecture of the circadian oscillator appears conserved among different species, the mechanisms at the core of the feedback loop differ. Biological clocks have either evolved multiple times to perform similar tasks thus they are an example of convergent evolution.

The use of positive and negative elements to regulate transcriptional and translational activity in a feedback loop is common among the well-studied circadian systems (Fig. 1-6). The

positive elements serve as the transcriptional activators in the loop and they have been found in *Synecohcoccus* (kaiA), *Neurospora* (WC-1 and WC-2), *Drosophila* (CLK and CYCLE), mammals (CLOCK and BMAL1) and *Arabidopsis* (TOC1). Similarly, negative elements also compose a portion of the feedback mechanism by inhibiting the action of the positive elements and these include kaiC, FRQ, PER and TIM, PER and CRY and CCA1 and LHY in cyanobacteria, fungi, fruit flies, mice and plants, respectively (Dunlap et al., 1999). Yet despite their similarities, the time at which these elements are expressed, late in the evening or early morning, differs among the organisms.

Transcription factor proteins serve important roles in the circadian oscillatory system. The type of transcriptional inducer varies among model systems. In mammals and *Drosophila*, the activators are basic helix loop helix (bHLH) proteins which contain a specific region which binds to DNA (Gekakis et al., 1998; Darlington et al., 1998). The *Neurospora* positive elements are similar to the *Drosophila* complex but they contain an additional zinc finger binding domain thus they are categorized as zinc finger factors (Loros and Dunlap, 2001). In the plant circadian oscillator, the activation of transcription is induced by MYB-like transcription factors (Carré and Kim, 2002). The oscillatory mechanism of cyanobacteria is still under investigation.

Another difference between the circadian machinery of different organisms is the number and location of oscillators. Mammals have a master circadian pacemaker that is localized to the suprachiasmatic nucleus (SCN) located within the hypothalamus of the brain. The SCN entrains multiple clocks that are located in the periphery of the organism (Yamazaki et al., 2000). In contrast, plants contain at least one oscillator in each cell and these oscillators function autonomously and independently of any central pacemaker (Thain et al., 2000; Barak et al.,

2000). This organization makes it is possible to set different rhythms of gene expression to different phases in varying parts of a single plant or organ.

The general organization of the circadian apparatus suggests a selective advantage in the rhythmic control of physiological and behavioral processes. The ubiquity of the system implies that the endogenous circadian programs enhance fitness. Evidence from cyanobacteria suggests that an organism with a circadian rhythm close to that of its external environment is favored under competition as a result of soft selection (Futuyama, 1998). Circadian clocks are also important since they provide a timing mechanism required for the response of organisms to daily and seasonal changes in light. Temporal organization of processes such as those involving photo-labile enzymes in plants is crucial for the optimization of important endogenous events. The close connection between the clock and light signaling pathways allow an organism to predict environmental changes even in their absence. Thus, the circadian clock provides an adaptive advantage by enabling the anticipation of the external transitions and the temporal synchronization of physiological events with specific phases of the environment (Johnson, 2001).

Since a number of discrepancies exist between the circadian components of different systems, the next phase of chronobiology concentrates on elucidating the molecular mechanisms that underlie the oscillator in other species. The identification of similar elements allows insight into the evolutionary lineage of the clock apparatus as well as the resulting rhythmic outputs while the differences between systems provide relevant information on species-specific adaptations. From this data, it is possible to create evolutionary relationships between specific clock-controlled genes across numerous taxa. One such project, the Floral Genome Project (FGP) examines gene families in a number of different plant species that play a role in the

evolution of flowering (Albert et al., 2005). One identified family of genes involved in floral initiation was shown to be similar to the LHY and CCA1 transcription factors of the *Arabidopsis* central oscillator.

Genetic and molecular analyses have proven valuable tools in the elucidating the central oscillatory mechanism of circadian clocks. In this study, two EST homologs to LHY and CCA1, the MYB-like family of transcription factors in Arabidopsis, are identified in *Eschscholzia californica*, the California poppy plant. Expressional analyses suggest that these genes are rhythmic components of the circadian oscillator that participate in the initiation of flowering. This suggests that the feedback loop mechanism of the plant circadian oscillator is conserved in these two species.

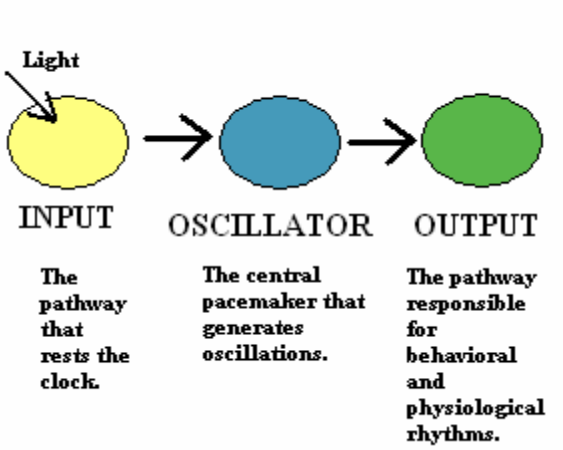

Figure 1-1. The three basic components of the circadian clock. Numerous input and output pathways function within the system.

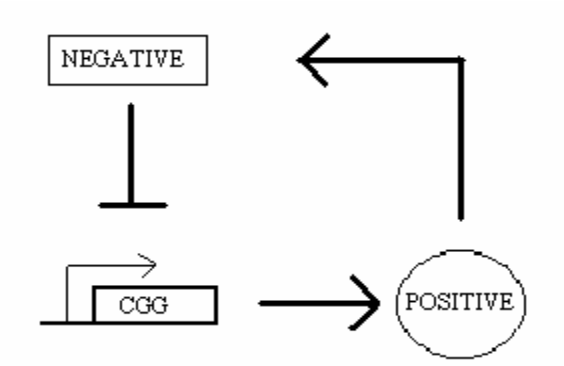

Figure 1-2. Positive and negative factors act upon the circadian clock. Negative elements block the activation of the positive elements (blunted arrow) which in turn promotes the expression of the negative element (pointed arrow).

Figure 1-3. Comparisons of three conserved regions of LHY and CCA1. The number at the end of each row corresponds to the last amino acid shown within the original protein. Conserved amino acids are highlighted.

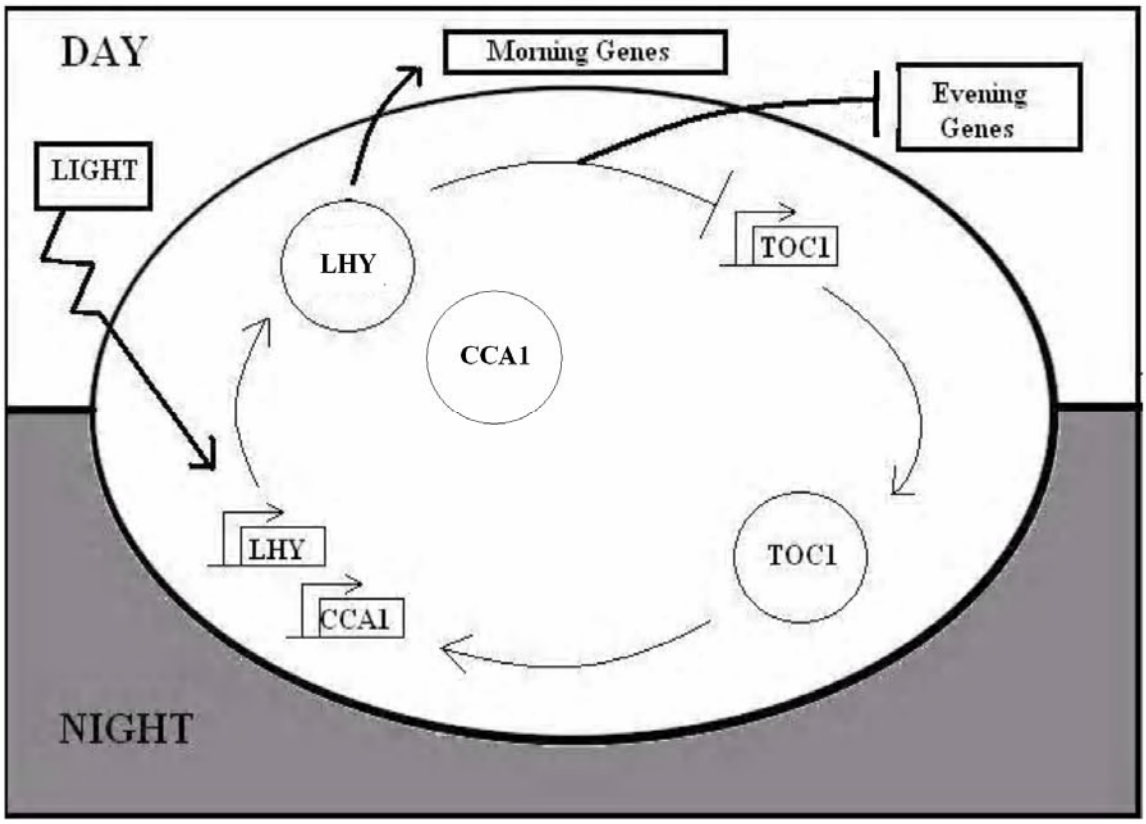

Figure 1-4. The Arabidopsis circadian oscillator. Light activates expression of *LHY* and *CCA1* inducing their transcription near dawn. Their gene product binds to the *TOC1* promoter and inhibits its expression during the early day. As LHY and CCA1 expression dwindles, the repression of *TOC1* is lifted and TOC1 protein accumulates in the evening. TOC1 then induces the expression of *LHY* and *CCA1* starting the cycle over again.

CHAPTER 2
TWO CCA-LIKE MYB TRANSCRIPTION FACTORS ARE PRESENT IN CALIFORNIA POPPY

Summary

Biological clocks play an important role in the lives of many organisms. This machinery allows species to coordinate important activities or behaviors in relation to their environment. The clock generates rhythms over which these events operate with a period of approximately twenty- four hours, thus the term circadian rhythms. The clock is primarily composed of three main components: input pathways, a central oscillator and output pathways.

An analysis was conducted to determine if two circadian clock genes found in the California poppy plant, RISE and SHINE, are related to two transcription factors involved in maintaining circadian rhythms in Arabidopsis, LHY and CCA1. In Arabidopsis, these two genes function in a feedback loop of the central circadian oscillator and are crucial for maintaining rhythms within the organism. A comparison of the genomic sequences revealed that there was an acceptable degree of homology between the two sequences. In addition, an expressional analysis revealed that the levels of messenger RNA (mRNA) of the genes oscillated over a twenty- four hour period which suggests circadian control. The location of expression was also similar between the two plant species. In young tissue, the transcripts were localized to the meristematic regions as well as the premature leaves. In older tissue, expression was highest in the reproductive organs and pollen grains. Since the circadian clock plays a role in promoting flowering and the release of pollen, it is no surprise the transcripts were localized to these various regions.

Due to these similarities this paper proposes that homologs of LHY and CCA1 exist in the California poppy plant and function as critical components of the negative feedback loop at the center of the circadian oscillator. Additional potential homologs of LHY and CCA1 have been

identified in other species based on sequence similarity. This discovery suggests that the circadian machinery is conserved among higher plants. In addition, temporal organization of important events seems to confer a selective advantage for the organisms.

Introduction

Biological clocks are important in maintaining the rhythmicity of crucial events in different species. In *Arabidopsis thaliana*, two genes, *LHY* and *CCA1* play important roles in generating and maintaining the rhythms within the organism (Schaffer, 1997; Schaffer et al., 1998; Wang et al., 1997). They have been identified as components of the central oscillator of the clock, one of its three core components. LHY and CCA1 function in a feedback loop, along with the *TOC1* gene, in which they negatively regulate their own expression (Alabadí et al., 2001; Carré and Kim, 2002). The pauses that occur as a result of this feedback loop are efficient for generating the observed oscillations. These oscillations, in turn, are conveyed as changes in the organisms' physiological or behavioral changes via the output pathway.

Two potential homologs of the *LHY* and *CCA1* genes were identified in *Eschscholzia californica* on the FGP database (Albert et al., 2005). These two EST sequences could potentially serve as components of a central oscillator in California poppy. Designated as *RISE* and *SHINE*, these genes might be the functional equivalents of *LHY* and *CCA1*. This paper reveals that these genes show an acceptable degree of sequence homology and share similar expressional patterns suggesting they are components of the central oscillator in *Eschscholzia californica*.

Materials and Methods

Plant Growth Conditions

Arabidopsis thaliana and *Eschscholzia californica* plants were grown under ideal temperate conditions in the University of Florida Department of Botany greenhouse in

Gainesville, Florida between March and July 2004 and June through September of 2005. California poppy was grown on sterile soil under normal light conditions with a period that matched the exogenous environment. Arabidopsis was sowed on autoclaved soil at an irradiance of 100 µmol m^{-2} s^{-1} as recommended by Kranz and Kirchheim (1987), and the day length was set so that it matched the period of the environment. Tissue for *in situ* hybridizations was harvested mid-morning and included floral meristems and floral buds. The collected material was placed in 4% paraformaldehyde to prepare for fixation. For RT-PCR analysis, plant tissue consisting of small leaves, floral meristems and buds was collected from each species every four hours for three days. Each sample was placed in liquid nitrogen and stored in a -80°C freezer.

Sequence Analysis and DNA Isolation

The *CCA1* and *LHY* genomic sequences, identified as genes At2g46830 and At1g01060, were identified in the Floral Genome Project (FGP) database (Albert et al, 2005; Carlson et al., 2006) and recognized as a distinct family of transcription genes associated with the circadian clock. The sequences were used to search for homologs in other plant species associated with the FGP database using the site's BLAST program and a number of candidate genes were identified. In *Eschscholzia californica* (California poppy) two ESTs or expressed sequence tags, which are small fragments of genes that have been cloned, demonstrated a notable level of similarity to the Arabidopsis genes. The FGP identification numbers for these two sequences are eca_4_183384 and eca_4_184056 which I will refer to as RISE and SHINE respectively. Alignments of the cDNA and protein sequences of CCA1, LHY, RISE and SHINE were constructed using the GenomeNet database program CLUSTALW (Thompson et al., 1994)

Clones of the RISE and SHINE sequences were received from Penn State University, a participant in the FGP grant. Their preparation has been described previously (Carlson et al.,

2006). Luria-Bertani (LB) media was prepared and 2.5 mL cultures were prepared with ampicillin at a concentration of 50µg/mL. The cultures were grown for 16 hrs in a shaking incubator at 37°C. It was noted that the cultures grew slowly due to their low turn-over rate. The alkaline lysis mini-prep protocol (Morelle, 1989) was used to purify plasmid DNA from 1 mL of culture.

Arabidopsis genetic material was obtained from wild type plants as previously has been described (Edwards et al., 1991).

***In situ* Hybridizations**

Except for the modifications noted below, previously described methods were used for *in situ* hybridization (Jackson, 1991; Drews et al., 1991). To generate templates for probe synthesis, DNA from plasmids containing the RISE and SHINE EST sequences as well as genomic DNA isolated from Arabidopsis was PCR amplified. The T7 RNA polymerase promoter sequence (TAATACGAGTCACTATAGGG) was placed in front of each reverse primer which allowed direct synthesis of digoxigenin-labeled antisense probes from PCR products. The sense control probes were designed with the T7 promoter sequence in front of the forward primers. In addition, probes were designed within the exons of the genomic sequences of each species in order to hybridize to corresponding messenger RNAs (mRNAs) *in situ*. Since Myb transcription factors contain a similar conserved motif in their amino terminus or 5' region, it was important to design primers in the carboxyl or 3' region that would be unique to each sequence. The following primers were used to amplify RISE templates for probe synthesis: TCTCTTTCGCCTCTACCGAACA and TAATACGACTCACTATAGGGAAGCACTCTTCAGGGAACCTCA. The primers used to amplify SHINE DNA were ACCACCACCAACTGCAACTCCTAT and TAATACGACTCACTATAGGGTGTACGGCGATTACTGAAGGGT. Amplification of LHY

DNA used the following primers: CAGTTCCAACTCCAGCAATGAC and TAATACGACTCACTATAGGGCTGAAACGCTATACGACCCTCT. The primers for CCA1 (TCTGGTTATTAAGACTCGGAAGCCAT and TAATACGACTCACTATAGGGTTCATTGGCCATCTCAGGATGC) were used to amplify its PCR product. RNA probes were synthesized using the Dig-RNA labeling kit (Roche Applied Science, Indianapolis, IN). The cRNA products, 263bp for RISE, 266bp for SHINE, 476bp for LHY and 361bp for CCA1, were synthesized and added to the hybridization buffer a so the final concentration was 500 ng mL^{-1}. Slides were hybridized at 45°C overnight and washed at 50°C. For signal detection, a few grains of tetramisole hyrochloride (Sigma, St. Louis, MO) was added to the Western Blue substrate (Promega, Madison, WI). Slides were evaluated using a Zeiss Axiostar Plus Microscope (Carl Zeiss, Inc, Thornwood, NY) and images were photographed with an Axiocam MRc5 camera (Carl Zeiss, Inc., Thornwood, NY).

Quantitative RT-PCR

For each plant species, fresh tissue including leaves, floral buds and meristematic tissue was collected every four hours for three days and placed immediately in liquid nitrogen and stored at -80°C. Total RNA was isolated from the tissues using the RNeasy plant RNA isolation kit (Qiagen, Valencia, CA). RNA concentration was measured using Ribogreen dye (Molecular Probes, Eugene, OR) and a TBS-380 Mini Fluorometer (Turner BioSystems, Sunnyvale, CA). The RNA templates were transcribed using the Reverse Transcriptase product protocol (Roche , Applied Science, Indianapolis, IN). PCR was used in order to determine the differences in transcript expression using the same primers described previously for probe construction. Differences in the 18S ribosomal RNA (rRNA) positive control transcripts were determined by using the following primers: TTGTGTTGGCTTCGGGATCGGAGTAAT and

TGCACCACCACCCATAGAATCAAGAA (Cho and Cosgrove, 2000). PCR products were separated by size on agarose gels stained with ethidium bromide and visualized under a UV light. Images of the gels were captured using a ChemImager 4400 (Alpha Innotech Corp., San Leandro, CA) and the relative sizes of bands were determined by comparison to a standard 1kb plus DNA ladder (Invitrogen, Carlsbad, CA).

Results

RISE and SHINE Encode CCA1-like Myb Transcription Factors

Sequence similarity among genes of different species can provide relevant information about the evolution of particular gene families and the conservation of important mechanisms. *RISE* and *SHINE* share significant sequence identity with the *CCA1* and *LHY* genes of Arabidopsis. In particular, RISE was shown to be comparable to LHY with over 40% identity in a region at the C-terminus. SHINE displayed similarity to the CCA1 sequence with 30% identity in the C-terminal region (Fig. 2-1). These regions located at the carboxyl or 5' end of the genes and ESTs corresponds to a DNA-binding domain that is found in plant Myb transcription factors. This motif is highly conserved among the Myb gene family and provides evidence that RISE and SHINE are indeed part of the family (Fig. 2-2). Additionally, RISE and SHINE share 40% identity in the region investigated which demonstrates the redundancy between the two components (Fig. 2-3).

RISE and SHINE Expression is Under Circadian Control

Circadian clock genes (ccgs) show a rhythmic pattern of transcript and protein expression. In *Eschscholzia californica*, this is no exception. The RISE and SHINE transcripts oscillate over a 24 hr period. Both RISE and SHINE oscillate in a pattern analogous to CCA1 and LHY in Arabidopsis (Fig. 2-4). The transcripts abundance varies during the day. Peak levels of transcription occur just prior to dawn and decrease throughout the day. By evening, the levels of

RISE and SHINE transcripts are greatly reduced but begin to rise in the early hours of the morning. This evidence supports the notion that RISE and SHINE are activated by a light signal similar to the mechanisms of CCA1 and LHY (Fig. 2-5). The transcripts also oscillate with a period of approximately 24 hrs, which matches the external environment of the organism, further supporting their role in the circadian clock.

Similar Expression Patterns in a CCA1- like Family of Myb Transcription Factors

In situ hybridizations are ideal for determining the location of transcript expression within an organism. This method was used to analyze the expression pattern of CCA1 and LHY in *Arabidopsis thaliana* and RISE and SHINE in *Eschscholzia californica*. For both species, two stages of development were analyzed: a younger stage characterized by premature inflorescence meristems and a later stage which is exemplified by floral buds.

In the young Arabidopsis tissue, a strong LHY signal is detected in the meristematic region and in the stamen and carpel primordia (Fig 2-6A). In older tissue, LHY is expressed in the gynoecium, ovules, and anthers and to a lesser degree in pollen grains (Fig. 2-6B). The expression of the LHY homolog, CCA1, is similar to its counterpart. High transcript levels are detected in the young developing floral meristem and include the premature reproductive organs (Fig. 2-6C). Expression of CCA1 in older tissue is limited to the reproductive tissues (Fig. 2-6D). The sense probe does not have a signal (Fig. 2-6E).

The expression patterns of the CCA-like Myb transcription factors in *Eschscholzia californica* is similar to the patterns observed in Arabidopsis. No signal could be detected on the sense probe control (Fig. 3-7E). High levels of RISE transcript were detected in the young developing tissue in the meristematic region, premature leaves and axillary buds (Fig. 2-7A). In older tissue, expression is highest in the carpel, ovules, anthers and pollen grains but is still

detected in the developing petals (Fig. 2-7B). Like its Arabidopsis counterpart, SHINE transcripts are detected in both the young and older stages of the poppy plant. Transcripts are detected in the sepal primordia, cauline leaves, and the floral meristem (Fig. 2-7C). SHINE expression in older tissue is confined to the reproductive organs and petals (Fig. 2-7D). However, it should be noted that a lower expression level can be detected for both genes throughout the specimen (Fig. 2-7A-D), showing that RISE and SHINE transcripts are located within a variety of tissue types..

Discussion

Feedback Loop Mechanism as the Basis of the Circadian Oscillator

An autoregulatory feedback loop involving both positive and negative elements is central to the circadian oscillator. Circadian systems are often composed of one or more interconnected loops. Knowledge of these interlocked loops results from the identification of genes that function within the core loop of the oscillator. In *Arabidopsis thaliana*, three genes with required roles in maintaining rhythmicity have been identified: *LHY, CCA1* and *TOC1* (Schaffer, 1997; Wang et al., 1997; Millar et al., 1995). The two Myb transcription factors LHY and CCA1 serve as the negative elements of the core loop and function to block the activation of the positive element TOC1. The positive regulator, TOC1 activates expression of LHY and CCA1. In this study, we have identified two potential homologs to LHY and CCA1 in *Eschscholzia californica*. These genes are hypothesized to serve similar roles in the core oscillator of the poppy plant, thus providing a conserved mechanism for maintaining rhythmicity in higher plants.

The *RISE* and *SHINE* Genes Encode MYB Transcription Factors That Are Similar to LHY and CCA1

Sequence analyses revealed that two California poppy EST sequences located in the FGP database (Albert et al, 2005; Carlson et al., 2006) share sequence identity with known

components of the central circadian oscillator from Arabidopsis (Fig. 2-1). The Myb domain, which functions as a DNA-binding domain, shares the most sequence similarity with RISE and SHINE. Research shows that LHY and CCA1 bind to an evening element (EE) located within the promoter of TOC1, an evening gene (Alabadí et al., 2001; Harmer et al., 2000). Because of the similarity between the components of these two circadian systems, I wanted to determine if RISE and SHINE function in a similar manner to CCA1 and LHY in the Arabidopsis central oscillator.

A prerequisite for a protein to function as a negative element in the circadian clock is that its expression and activity must oscillate in synchrony with the environmental oscillations. In addition, this component regulates its own transcription by negative feedback which creates a delay in the rhythmic cycle. In the Arabidopsis model plant, *LHY* and *CCA* transcripts were shown to oscillate over a 24 hr period with peak levels accumulating just prior to dawn (Mizoguchi et al., 2002). Similar results for this species were obtained (Fig. 2-5). The *RISE* and *SHINE* transcripts displayed a similar pattern of expression with minimal levels of mRNA detected in the evening (Fig.2-4). The accumulation of transcripts just prior to dawn shows that LHY and CCA1, as well as RISE and SHINE, are regulated by a light signal and are entrained to anticipate dawn.

The genes at the center of the Arabidopsis circadian oscillator serve as either positive or negative factors to influence the rate of transcription. The activation and inhibition of ccgs occurs at particular points within the circadian cycle and when coupled, form a loop in which the components serve crucial roles in generating and maintaining rhythmicity within an organism. The *LHY* and *CCA1*gene products in Arabidopsis function in a manner that is antagonistic to TOC1. In *Eschscholzia californica*, putative homologs for LHY and CCA1 have been identified

but other components of the oscillator remain unknown. Based on the previously described similarities between the two systems, it is reasonable to hypothesize that a *TOC1*- like gene also functions in the poppy oscillator (Fig. 2-8.).

The spatial expression patterns of the Myb transcription factors in Arabidopsis and California poppy provide relative information about their functional similarities. In both species, the young tissue contained a high level of expression in the meristem and sepal primordia (Fig. 2-6A, C; Fig. 2-7A, C). The older tissues were characterized by high levels of transcript in the reproductive organs and petals (Fig. 2-6B, D; Fig. 2-7B, D). The similarity in the expression pattern suggests that these genes might be true orthologs stemming from a common ancestor. In addition, the location of expression provides relevant information on the processes regulated in that particular region. The high level of transcript expression in pollen grains in the older tissue of Arabidopsis and poppy could control the timed release of pollen, a mechanism that evolved for maximizing reproductive success (Subba et al., 1998). The fact that the circadian clock regulates expression of floral pathway genes that in turn activate floral meristem identity genes (Vijayraghavan et al., 2005) seems logical to explain the high level of expression of the Myb transcription factors in the meristematic regions of the young tissue.

Together these sequence comparison and mRNA expression data suggest that *RISE* and *SHINE* encode Myb transcription factors that could function as the negative elements in the oscillator of the California poppy plant similar to the manner of LHY and CCA1 in Arabidopsis.

An Evolutionary Conserved Clock Mechanism in Higher Plants

Although the above data suggests similarities exist among the circadian systems of *Arabidopsis thaliana* and *Eschscholzia californica*, little is known about the elements and mechanisms underlying the clocks of other higher plants. It is possible that the molecular

components that form the clock machinery are unique to higher plants. In this case it is important to determine whether other plant species have homologs for each of the Arabidopsis clock components and whether they share similar functions. This paper demonstrates that homologs for two Arabidopsis clock genes exist in the California poppy plant and that they appear to be expressed in a similar manner to their counterparts. In other plant species, components of the central oscillator remain unknown however, recent evidence has identified several clock-associated genes that are involved in the input pathway to the clock. Studies of *Pisum sativum*, peas, have revealed circadian clock gene homologs of *TOC1*, *CCA* and *LHY* referred to as *TOC1* and *MYB1* (a *CCA1*/*LHY* homolog) respectively (Hecht et al., 2007). Two additional Arabidopsis orthologs, *EARLY FLOWERING4* (*ELF4*) and *LATE BLOOMER1* (*LATRISE*), were characterized in pea plants and their diurnal rhythm expression conformed closely to those associated with their counterparts, *ELF4* and *GIGANTEA* (*GI)*. In Arabidopsis, ELF4 promotes clock entrainment and is required for sustained rhythms in the absence of environmental cues (McWatters et al., 2007). The *GI* gene regulates flowering in long day (LD) conditions in a clock-controlled pathway, where it acts as an intermediate between the central oscillator and the *FLOWERING LOCUS T* (*FLT*) gene (Mizoguchi et al., 2005). Investigations in the clock components in other species continue, including *Oryza sativa* (rice), *Medicago trunculata*(a legume) and *Lycopersicon esculentum* (tomato). In addition, sequence analysis of the Myb family of transcription factors using the FGP database (Albert et al, 2005; Carlson et al., 2006) revealed one CCA1/LHY homolog in *Cucumis sativus* (cucmber), *Asparagus officinalis, Liriodendron tulipifera* (tuliptree) and *Saruma henryi* (standing ginger) and two homologs in *Acorus americanus* (the American Sweet Flag) and *Nuphar advena* (water lily). The presence of similar sequences across a wide variety of species suggests that the oscillator mechanism that

involves CCA1 and LHY in Arabidopsis is conserved in higher plants. The conservation of this mechanism and its components implies that such organization is beneficial for the organism.

The ubiquity of the feedback loop mechanism of plant circadian oscillators suggests that an adaptive advantage results from the spatial and temporal organization of important rhythmic activities. A recent experiment compared the performance of wild type Arabidopsis plants with lines having mutations that alter period length in a range of environmental period lengths that were either matched or mismatched to the endogenous clock. The results showed that a photosynthetic advantage was conferred by matching the endogenous clock period with the light/dark period (Dodd et al., 2005). Incorrect matching of the periods resulted in reduced leaf chlorophyll, reduced assimilation, reduced growth and increased mortality (Dodd et al., 2005). Optimization of physiological parameters by the circadian clock probably has been selected during plant evolution. Similar results have been described in the cyanobacteria *Synechococcus* as well (Ouyang et al., 1998)

The Biological Importance of Circadian Clock Genes

Although this paper addresses the circadian oscillator and its key mechanisms in higher plants, the importance of the circadian machinery also resonates throughout the animal kingdom. In addition, elucidating the components underlying the feedback loops of the oscillator in either plants or animals provides relative information on the general architecture of the mechanisms. Both plants and animals use circadian clocks to temporally organize important processes involving reproduction and development which are crucial in the evolution of every species. In humans, many behaviors are regulated by the circadian clock including the sleep/wake cycle, feeding patterns, hormone production and cell regeneration (Edgar et al., 1993; Stokkan et al., 2001; Czeisler and Klerman, 1999; Shibata, 2004).

A number of human illnesses are attributed to a dysrhythmia in a behavioral or physiological process. Abnormal circadian rhythms have been associated with affective disorders like A number of human illnesses are attributed to a dysrhythmia in a behavioral or physiological process. and the existing therapy drugs used to treat these disorders such as lithium act upon the circadian cycle (Hallonquist et al., 1986). Insomnia and sleep problems also result from abnormal circadian rhythmicity and usually are characterized by an endogenous clock that runs faster or slower than the norm (Zisapel, 2001). Individuals that suffer from attention-deficit hyperactivity disorder (ADHD) are often plagued by sleep disturbances which result from a dysrhythmic clock (Owens, 2005). In women who suffer from menopause, hot flashes disrupt the clock's rhythm resulting in a clock that is misentrained. This abnormal entrainment results in sudden awakenings during the sleep cycle (Freedman et al., 1995). Recently a role for the circadian clock has been identified in cancer studies. Research suggests that at least eight central clock genes coordinate many basic functions, including cell proliferation, tumor growth and apoptosis in circadian time. This work indicates that circadian clock genes and their products potentially represent novel targets for the control of cancer growth (Wood et al., 2006).

Elucidating the mechanisms that lie beneath the circadian oscillator has become the primary focus of chronobiologists. A wealth of knowledge stands to be gained since nearly all processes crucial for species survival involve rhythmicity of one or more elements. The availability of technologies to analyze global gene expression should become a powerful tool in clock research. This advancement should aid in the identification of new genes affected by the timekeeping apparatus and help characterize the interactions of those clock proteins that have been previously identified. However, the question of how the genes involved in the clocks are regulated is just starting to be addressed.

In this study, two homologs of the Arabidopsis Myb transcription factors CCA1 and LHY were identified in *Eschscholzia californica*. Sequence analyses suggest that these genes are true orthologs and are similar in their temporal and spatial expression. This information provides evidence that there is a conserved transcriptional- translational feedback loop at the center of the circadian oscillator in higher plants. Based on this congruence, other circadian clock genes involved in the maintenance of rhythms in California poppy should resemble those described in Arabidopsis (Fig. 2-8).

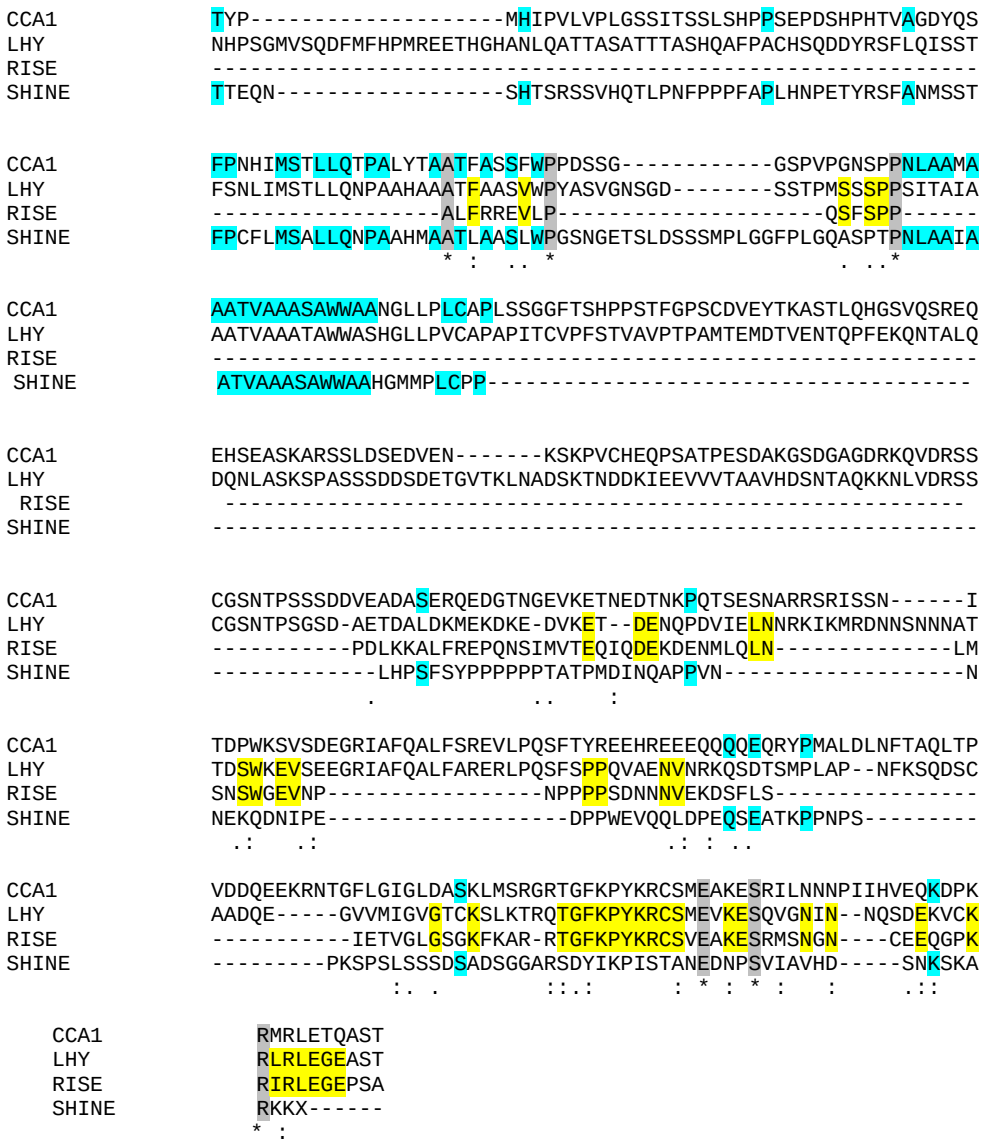

Figure 2-1. Alignment of the LHY/CCA1 genes in *Arabidopsis thaliana* and *Eschscholzia californica*. The nucleotide sequences of LHY and CCA1 were used to search the FGP database (Albert et al., 2005; Carlson et al., 2006) using BLASTN in order to form an alignment with the SHINE and RISE ESTs. The residues shaded in gray represent the bases that are conserved among all four genes. Those highlighted in yellow represent the nucleotides that are conserved between LHY and RISE. The residues in blue illustrate the conserved bases among CCA1 and SHINE.

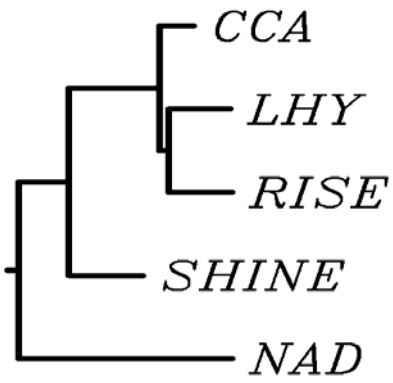

Figure 2-2. A phylogenetic analysis of CCA-like family of genes. This tree illustrates the relationships among the CCA-like family of Myb transcription factors in *Arabidopsis thaliana* (CCA, LHY), *Eschscholzia californica* (SHINE, RISE) and *Nuphar advena* (NAD), a basal angiosperm. Here, the *NAD* gene was identified in the FGP EST database and serves as the outgroup for this analysis. Multiple sequence alignment and tree construction were produced using the MAFFT program (Katoh et al., 2002; Katoh et al., 2005).

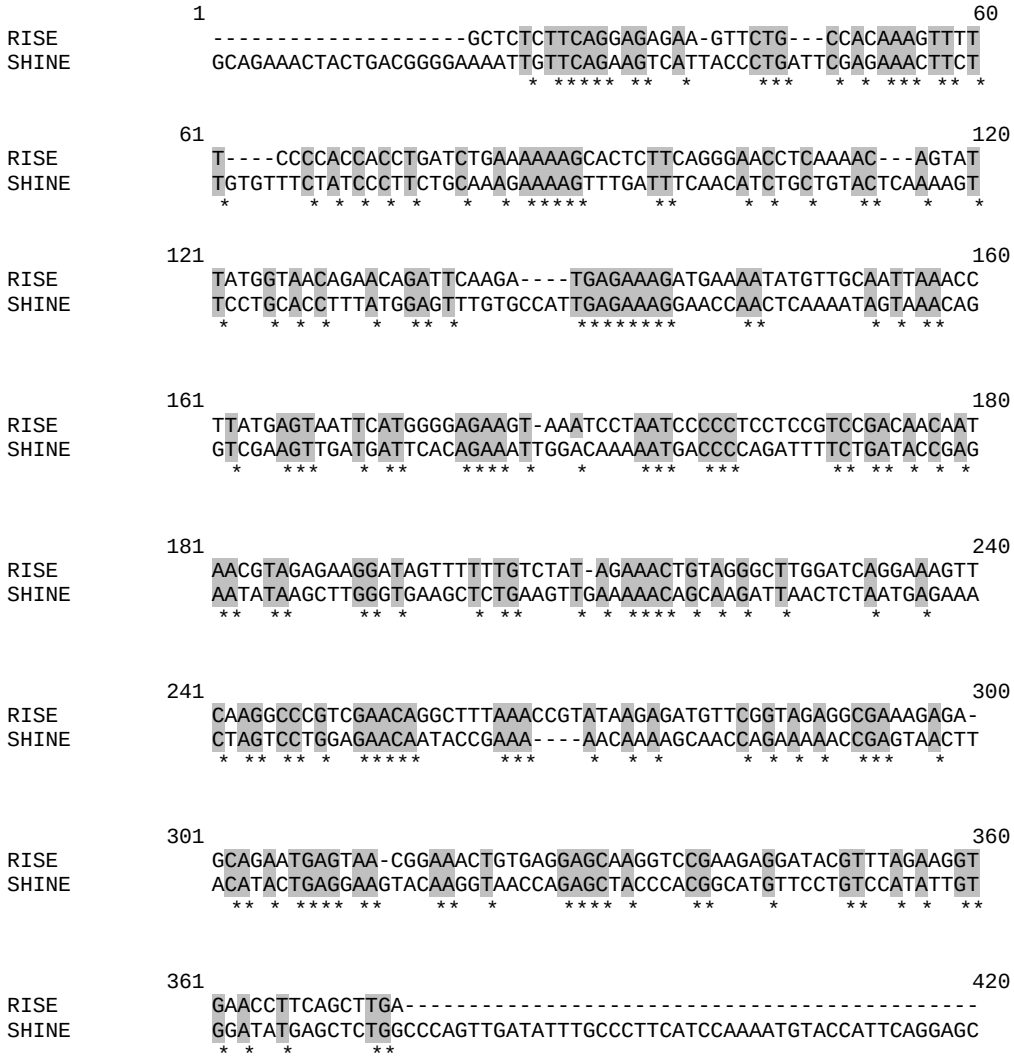

Figure 2-3. Alignment of the RISE (eca_4_183384) and SHINE (eca_4_184056) EST sequences using the BLASTN program from the FGP database (Albert et al, 2005; Carlson et al., 2006). Conserved residues are shaded in gray.

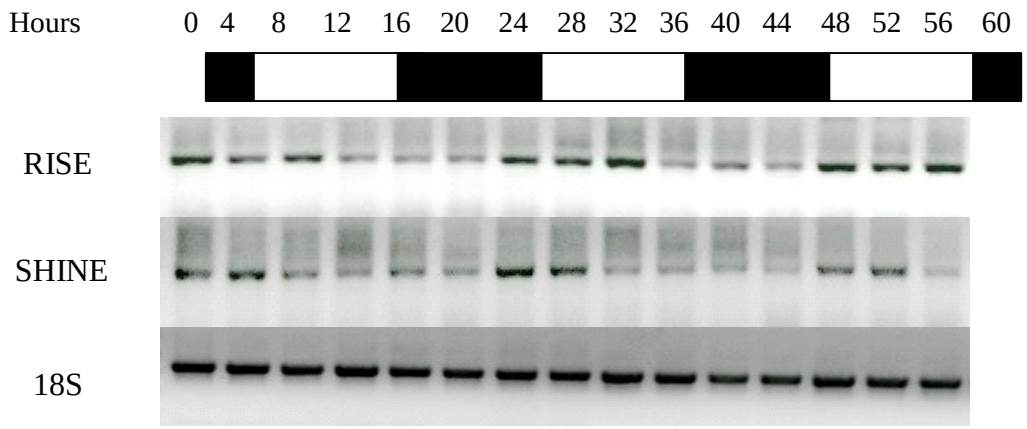

Figure 2-4. Analysis of *RISE* and *SHINE* mRNA expression. Peak *RISE* and *SHINE* transcript levels occur just prior dawn and dwindle throughout the day. The 18S r RNA transcripts demonstrate a constant expression level throughout the day. The bar above reflects the light/dark cycles to which the plants were exposed for this experiment. The black boxes correspond to periods of darkness.

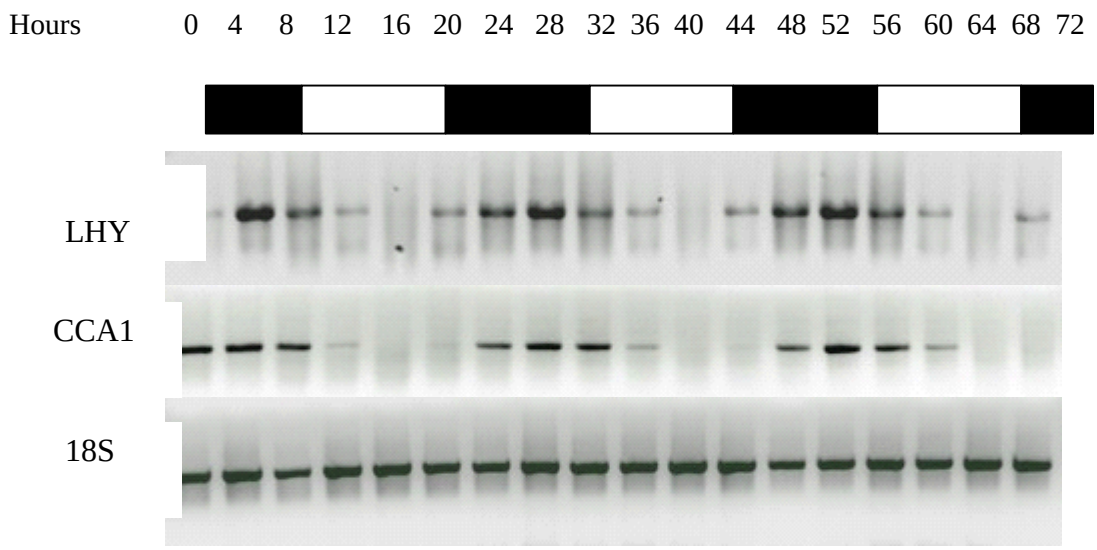

Figure 2-5. Analysis of *LHY* and *CCA1* mRNA expression. Peak transcript levels of the transcription factors occur just prior dawn and become greatly reduced by evening. The 18S rRNA transcripts demonstrate a constant expression level throughout the day. The bar above reflects the light/dark cycles to which the plants were exposed for this experiment. The black portions represent the evening hours.

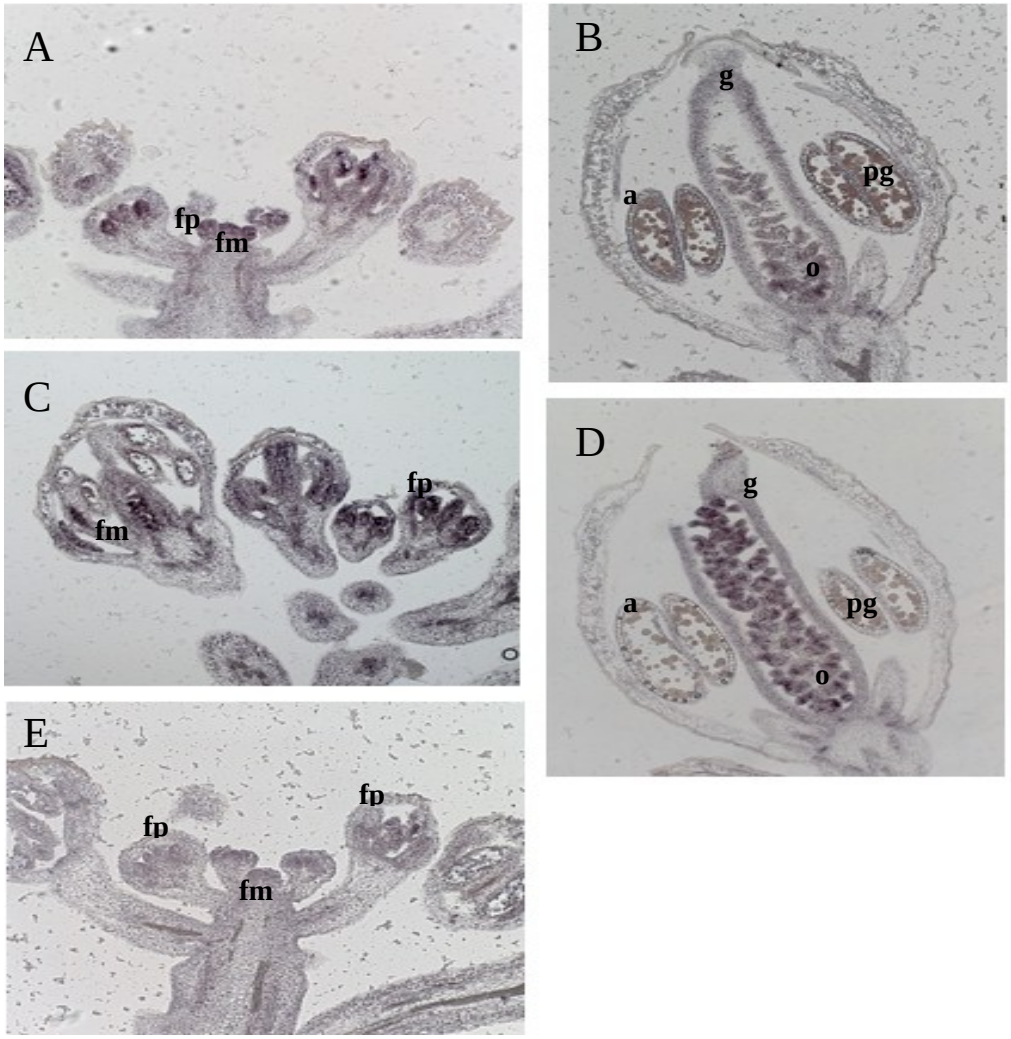

Figure 2-6. Expression of *LHY* and *CCA1* in Arabidopsis tissue. *LHY* and *CCA1* transcripts were located in both young and mature Arabidopsis floral tissue. The localization of the transcripts was determined by *in situ* hybridizations. *LHY* transcripts were present in A) the floral meristem (fm) and the floral primordia (fp) of the young tissue and in B) the gynoecium (g), ovules (o), anthers (a) and pollen grains (pg) of the mature floral tissue. *CCA1* transcripts were detected in C) younger tissue specifically in the fm and fp and in D) the older tissue in the same location as observed with LHY: g, o, a and pg. E) The sense probe control shows no signal.

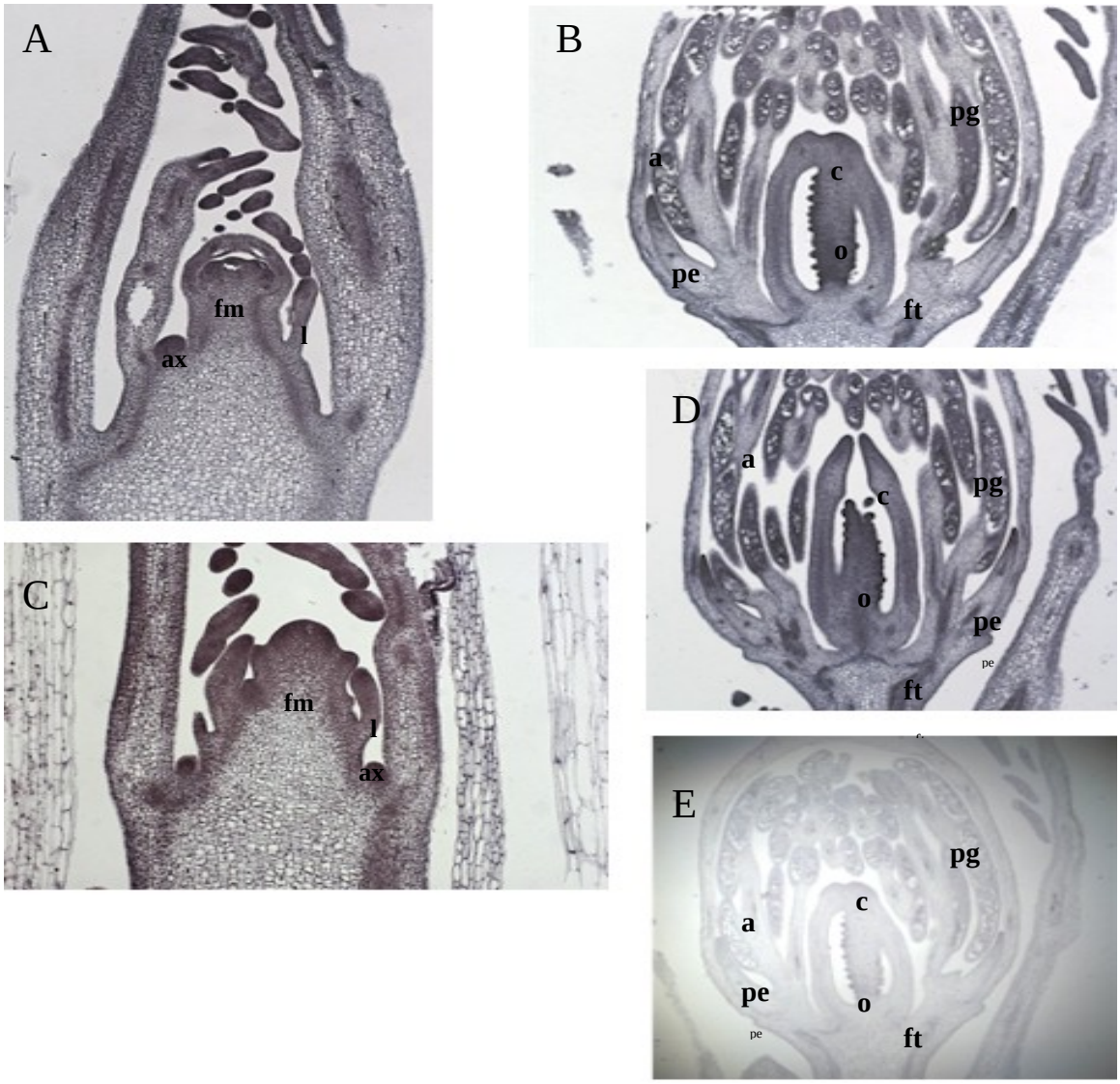

Figure 2-7. *RISE* and *SHINE* transcripts are expressed in both young and mature floral tissue of the California poppy plant. The localization of the transcripts was determined by *in situ* hybridizations. *RISE* transcripts were present in A) the floral meristematic region (fm), premature leaves (l) and in the axillary buds (ax) of the younger tissue while in B) the older tissue, high expression was detected in the carpel(c), ovules (o), anthers (a) and pollen grains (pg) while lower expression was observed in the floral tube (ft) and developing petals (pe). *SHINE* transcripts were detected in C) younger tissue in a pattern identical to RISE: fm, l and ax. *SHINE* transcripts in D) older tissue was restricted to the c, o, a and pg as well as the ft and pe. E) The sense probe control shows no signal.

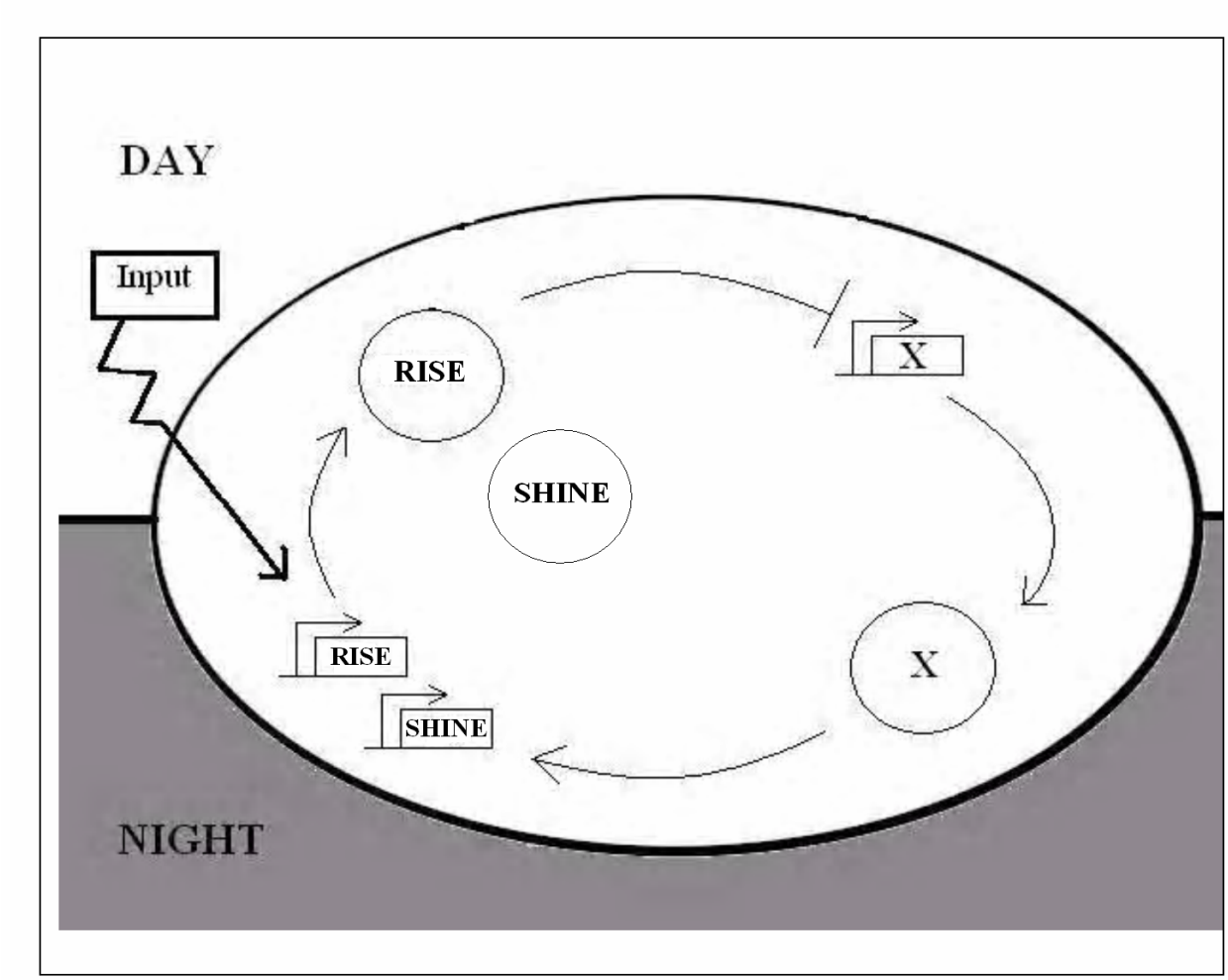

Figure 2-8. Proposed mechanism of the central oscillator of *Eschscholzia californica*. RISE and SHINE serve as negative regulators of a gene 'X' (designated by a blunted arrow), a TOC1 homolog. That gene in turn should induce expression of RISE and SHINE (pointed arrows). Candidate genes for component 'X' have yet to be identified.

CHAPTER 3
CONCLUSION

Elucidating the mechanisms that lie beneath the circadian oscillator has become the primary focus of chronobiologists. A wealth of knowledge stands to be gained since nearly all processes important for species survival involve rhythmicity of one or more elements. Research on the crucial components that are required to maintain rhythmicity could provide insight into possible therapies and treatments for diseases that target the clock system. In addition, by analyzing the mechanisms and proteins of the central oscillator and comparing them among different species, the evolutionary history of the clock can be examined.

In this study, it was proposed that a common clock ancestor exists among higher plant species. In *Eschscholzia californica*, two homologs of Arabidopsis Myb transcription factors were identified as potential components of the biological clock. Other probable homologs to components of the Arabidopsis central oscillator LHY and CCA1 have been identified in other species including *Cucumis sativus* (cucumber), *Asparagus officinalis*, *Liriodendron tulipifera* (tuliptree), *Saruma henryi* (standing ginger), *Acorus americanus* (the American Sweet Flag) and *Nuphar advena* (water lily). Further investigation of these species should provide relevant information on the clock mechanism and its conservation. Genetic screens for clock mutants aids in the assignment of potential components to roles within the circadian clockwork. Altered expression, either increased or greatly reduced, of these constituents should result in aberrant clock phenotypes. Although it is proposed that the *RISE* and *SHINE* genes of *Eschscholzia californica* play a role in the central oscillator of the plant's clock, further studies similar to those mentioned above are required for verification.

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BIOGRAPHICAL SKETCH

Meredith Lynn Sullivan was born on January 13, 1979 in Tuscaloosa, Alabama. The younger of two children, she graduated from Central High School in 1997. Following graduation, Meredith enrolled at Shelton State Community College where she was a member of the women's varsity soccer team. She subsequently enrolled at the University of Alabama (UA) where she earned her B.S. in Biology in 2002.

Upon receiving her B.S. degree, Meredith enrolled in graduate school at the University of Florida (UF) in 2003. As a graduate student in the Department of Botany, she pursued molecular biology- based studies to elucidate the components and mechanisms involved in flower regulation. This information allowed her to analyze the evolutionary significance of circadian clocks in development.

Upon completion of her M.S. degree, Meredith will pursue a career in medical research. She plans to utilize the knowledge she has obtained throughout her education to aid in the identification of therapeutic drugs for certain illnesses.