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The Arabidopsis Circadian System

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ABSTRACT

Rhythms with periods of approximately 24 hr are widespread in nature. Those that persist in constant conditions are termed circadian rhythms and reflect the activity of an endogenous biological clock. Plants, including Arabidopsis, are richly rhythmic. Expression analysis, most recently on a genomic scale, indicates that the Arabidopsis circadian clock regulates a number of key metabolic pathways and stress responses. A number of sensitive and high-throughput assays have been developed to monitor the Arabidopsis clock. These assays have facilitated the identification of components of plant circadian systems through genetic and molecular biological studies. Although much remains to be learned, the framework of the Arabidopsis circadian system is coming into focus.

Dedication

This review is dedicated to the memory of DeLill Nasser, a wonderful mentor and an unwavering advocate of both Arabidopsis and circadian rhythms research.

INTRODUCTION

The evolution of life within the biosphere has taken place in a context that has changed continuously over time. It is common to think of this temporal change as linear. Cyanobacteria appeared 3.5 billion years ago in the Pre-Cambrian, land plants appeared 425 million years ago in the Silurian, and flowering plants appeared 135 million years ago in the Cretaceous. Undoubtedly, environmental changes played key roles in many of the stages in the evolution of modern species, including *Arabidopsis thaliana*. For example, the number and density of stomatal complexes have responded to changing atmospheric CO_2 concentration, contributing to the appearance of the planate leaf form 360 million years ago in the Late Paleozoic (Beerling et al., 2001).

Often neglected in this linear view of temporal environmental change is the periodic change in the immediate environment that results from the rotation of the earth on its axis every 24 hours. This alternation of day and night produces profound environmental changes that must be faced by any organism. Thus, life has evolved in an environment that shows short term (~24 hr) recurrent change that is drastic but that is entirely predictable. Time may fly like an arrow in the long term, but in the short term each dawn is followed by a successive dawn and time can more appropriately be thought of as having the trajectory of a boomerang rather than that of an arrow. Given this repetitive perspective, it seems intuitive that any organism would accrue advantage from the temporal organization of

biochemistry, physiology and behavior with respect to the environmental oscillation of day and night. Not surprisingly, most organisms express diurnal rhythms. It is less obvious that many of these rhythms should persist in the absence of environmental time cues. However, if one accepts the adage that "the early bird gets the worm," it would seem to be to the advantage of the worm to anticipate the dawn and hide so as not to serve as the early bird's breakfast. No less accomplished a naturalist than William Shakespeare described such a case of dawn anticipation in Hamlet, Act I, scene v: "*The glowworm shows the matin to be near And gins to pale his uneffectual fire*." More recently, a fitness advantage conferred by the circadian clock has been demonstrated in cyanobacteria and Chlamydomonas (Johnson, 2001; McClung, 2001).

Organisms from cyanobacteria to humans endogenously measure time and temporally regulate aspects of their biology. There is no more obvious significance to the synchronization of the organism to local time than in the case of photosynthetic organisms that depend on sunlight for energy. Indeed, the endogenous nature of circadian rhythms was first established for the leaf movements of the "sensitive" heliotrope (probably *Mimosa pudica*) (de Mairan, 1729). Prior to 1900, plants were the primary system for the study of circadian rhythms and plant studies yielded the historical milestones of circadian rhythm research (summarized by McClung and Kay, 1994). Over the last decade, Arabidopsis has become the premiere system for the analysis of plant circadian rhythms and this review will focus on recent advances in our understanding of the molecular bases of Arabidopsis circadian rhythms.

Circadian (from the Latin *circa*, approximately, and *dies*, day) rhythms are endogenous rhythms with periods of approximately 24 hr. When placed in constant conditions and, thus, deprived of external time cues, circadian rhythms persist and "free-run" with an endogenous period that is close to but not exactly 24 hr. This establishes that these rhythms are truly endogenous and self-sustaining. In the real world, of course, organisms are exposed to environmental time cues such as light and temperature cycles, and these cues serve to synchronize or "entrain" the endogenous organismal clock with local solar time. The period of a circadian rhythm remains relatively constant over the range of physiologically relevant temperatures, which is referred to as temperature compensation. This means that the circadian clock maintains its pace over a range of temperatures, but does not imply that temperature changes or cycles cannot serve as potent stimuli that can entrain the clock. These three properties: persistence in constant conditions with an approximately 24-hr period, entrainment and temperature compensation, are the diagnostic criteria of a circadian rhythm (Johnson et al., 1998; Sweeney, 1987).

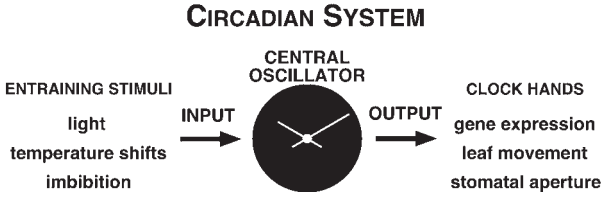

Figure 1. Conceptual scheme illustrating simple linear information flow from input (entrainment) pathways through the central oscillator to output pathways. Modified from Eskin (Eskin, 1979)

AN OVERVIEW OF THE CIRCADIAN SYSTEM

Commonly, one considers the circadian system as consisting of three components: an input pathway (usually described as a photoreceptor) that entrains the clock, the central oscillator (clock), and output pathways to generate overt rhythms. These three components are arranged in a linear array with information flowing unidirectionally from input through the oscillator to the output (Figure 1). This review will deviate from the linear order of Figure 1 in its consideration of the Arabidopsis circadian system. In order to introduce the assays that feature in the analysis of plant clocks, output pathways will be considered first. Input pathways will be addressed second. Finally, the exciting recent progress in elucidating the oscillator mechanism in plants will be considered. We are frequently being reminded that the formal dissection of any circadian system into input, oscillator and output components is arbitrary and the lines between components are blurred. This will become particularly evident in the attempt to identify components of the central oscillator.

It has become increasingly clear that the simple linear of Figure 1 is inadequate and a more complex model has emerged (Figure 2). There are multiple photoreceptors that provide input to the clock (Devlin and Kay, 2001). In addition, there must be sensors for temperature and for other environmental stimuli that entrain the clock. Accumulating evidence suggests that there are multiple oscillators. These can represent central master oscillators as well as non-self-sustaining slave oscillators (Pittendrigh, 1960; Pittendrigh, 1981). In animals there are organ and tissue-specific oscillators (Plautz et al., 1997; Tosini and Menaker, 1998; Yamazaki et al., 2000). In the unicellular dinoflagellate, *Gonyaulax polyedra*, it has been established that there are at least two circadian oscillators within a single cell (Morse et al., 1994; Roenneberg and Morse, 1993).

Finally, some components of input pathways are themselves outputs and the circadian clock regulates its own sensitivity to environmental input (Devlin and Kay, 2001; Merrow et al., 1999; Merrow et al., 2001).

Rhythmic Outputs

Plants are richly rhythmic, presumably because of their dependence upon sunlight for energy and because they are sessile. Arabidopsis is no exception and displays myriad rhythmic outputs, or "hands" of the clock. Plant rhythmic processes have been reviewed in detail (Kreps and Kay, 1997; Lumsden and Millar, 1998; McClung, 2000; McClung, 2001; Millar, 1999; Somers, 1999; Sweeney, 1987). Here I will focus on those rhythms that have proven most useful in the study of the Arabidopsis clock.

Rhythms in mRNA Abundance

Differential gene expression underlies many overt rhythms in biochemistry, physiology and behavior. The cataloging of plant clock-controlled genes (*CCG*) began with Kloppstech's (Kloppstech, 1985) observation of a circadian oscillation in mRNA abundance of a chlorophyll *a/b* binding protein gene (*LHCB* or *CAB*). *LHCB* remains the best-studied clock-regulated plant gene, and circadian oscillation of *LHCB* mRNA abundance is widespread, if not universal, among angiosperms (Fejes and Nagy, 1998; Piechulla, 1999). Curiously, this does not extend into the gymnosperms (Piechulla, 1999).

The list of plant *CCGs* has grown to considerable length (Fejes and Nagy, 1998; Kreps et al., 2000; McClung, 2000; McClung, 2001; Somers, 1999). Happily, the genomic age has greatly facilitated this analysis with the application of both oligo-based and cDNA microarrays (Harmer et al., 2000; Schaffer et al., 2001). The cDNA microarray experiment suggested that ~11% of the 7800 genes sampled showed diurnal oscillations in mRNA abundance in a light–dark cycle and that oscillations for 2% persisted in continuous light and so were truly circadian (Schaffer et al., 2001). The oligo-based microarray, with better time resolution (4 hr versus 6 hr sampling intervals, and encompassing two versus a single circadian cycle), allowed the detection of statistically significant circadian (in continuous light) oscillations in mRNA abundance of 5-6% of the 8200 genes examined (Harmer et al., 2000). This latter measurement suggests that there are at least 1275-1530 Arabidopsis *CCGs*, based on a current estimate of ~25,500 Arabidopsis (The Arabidopsis Genome Initiative, 2000). More recent cDNA microarray work, in which hybridization results were normalized by hybridization against genomic DNA, is more consistent with this higher estimate (Schaffer and Wisman, 2001). This is likely to represent a minimum estimate, limited to the detection of oscillating transcripts in those tissues, at that developmental stage, and under those specific growth conditions employed. It will take some time and many chips to exhaustively sample all possible developmental stages and environmental conditions. In addition, one must recall that transcript abundance per se does not distinguish between changes in transcription rates and mRNA stability. Nor will these experiments identify genes whose induction or repression in response to environmental or biological stimuli is gated by the clock. Nonetheless, 5-6% is considerably less than the situation encountered in the cyanobacterium *Synechococcus elongatus,* in which essentially the entire transcriptome is clock-regulated (Liu et al., 1995).

What genes are clock-regulated? Not unexpectedly, microarray analysis confirms that many genes associated with photosynthetic light harvesting oscillate synchronously with peaks in mRNA abundance after subjective dawn (Harmer et al., 2000; Schaffer et al., 2001). These include genes encoding LHCA and LHCB proteins as well as photosystem I and II reaction center proteins. A number of Arabidopsis genes involved in photosynthetic carbon assimilation (Pilgrim and McClung, 1993) as well as in photorespiration (McClung et al., 2000; Zhong and McClung, 1996; Zhong et al., 1994) are also clock-regulated. Microarray experiments have extended the list of clock-regulated genes involved in carbon metabolism. For example, mRNA abundance for a number of genes encoding enzymes of the glycolytic and oxidative pentose phosphate pathways, as well as genes encoding hexose transporters peak in the subjective afternoon. Genes encoding enzymes of starch mobilization, including starch kinase, ß-amylase, fructose-bisphosphate aldolase, and sugar transporters peak at night (Harmer et al., 2000).

It has long been known that nitrogen assimilation is clock-regulated (Cohen and Cumming, 1974). Circadian oscillation in mRNA abundance of genes encoding nitrate reductase has been demonstrated, first in tobacco (Deng et al., 1990; Deng et al., 1989) and also in Arabidopsis (Cheng et al., 1991; Pilgrim et al., 1993). Microarray analysis has verified these observations (Schaffer et al., 2001) and extended them to include other genes with roles in nitrogen assimilation, including putative carboxypeptidases, amidase, glutamate dehydrogenase, and asparagine synthetase, as well as nitrate and ammonium transporters (Harmer et al., 2000). There is also a clock-regulated

group of genes involved in sulfur metabolism (Harmer et al., 2000).

Plant responses to biotic and abiotic stress responses are often gated by the circadian clock (Rikin, 1992; Rikin et al., 1993; Rikin et al., 1984). Both microarray experiments identified oscillations in mRNA abundance of a number of genes involved in responses to stresses, including cold and pathogens (Harmer et al., 2000; Schaffer et al., 2001). Particularly noteworthy is the oscillation in mRNA abundance of the *DREB1a/CBF3* gene encoding a transcription

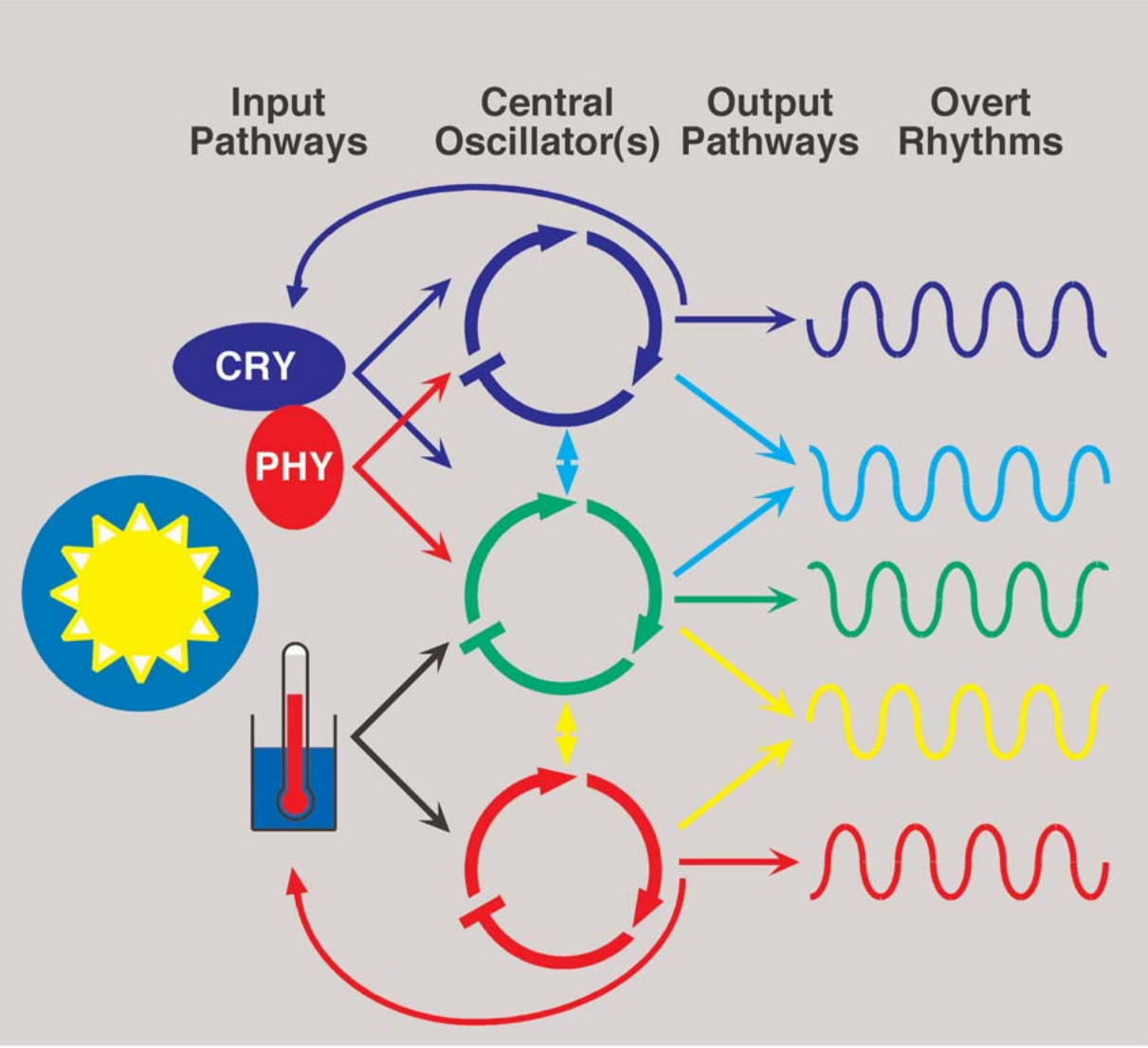

Figure 2. A more realistic model of a simple circadian system consisting of a set of input (entrainment) pathways, multiple central oscillators, and sets of output pathways. Entraining stimuli include light, mediated through phytochromes (PHY) and cryptochromes (CRY), temperature, and imbibition (not shown). Complexity in input pathways arises from multiple phytochromes and cryptochromes as well as interaction among them and their downstream signaling pathways. Each central oscillator is illustrated as a loop including positive and negative components that yields a self-sustaining oscillation with a period of approximately 24 hr. Coupling between oscillators is suggested by double-headed arrows. Multiple output pathways are drawn as each regulating an overt rhythm with a distinct phase. Some outputs may be driven by individual oscillators whereas others may receive input from more than one oscillator. Additionally, different oscillators may drive separate rhythms with distinct periods.

factor that plays a key role in cold tolerance (Thomashow et al., 2001). Rhythmic expression of this transcription factor could underlie a circadian rhythm in cold tolerance (Harmer et al., 2000). The downstream effector genes themselves would not necessarily exhibit circadian oscillations in mRNA abundance, but their induction might be gated by the clock through circadian regulation of key sensors or transcription factors.

One of the most interesting observations stemming from microarray analysis was that 23 genes encoding enzymes of phenylpropanoid biosynthesis are coordinately regulated, oscillating with mRNA peaks about 4 hr before subjective dawn (Harmer et al., 2000). Oscillating together with these genes is *PRODUCTION OF ANTHOCYANIN PIGMENT 1* (*PAP1*), which encodes a Myb domain transcription factor. PAP1 overexpression up-regulates several enzymes of the pathway (Borevitz et al., 2000). This offers the likely scenario that the clock regulates PAP1 expression which in turn regulates the entire phenylpropanoid biosynthetic pathway. This is a remarkable illustration of the power of genome-wide expression analysis to show not only that an entire pathway is co-regulated, but also to suggest the key transcription factor that mediates this regulation.

Although most genes exhibiting circadian oscillations are nuclear, there is accumulating evidence of circadian regulation of plastid-encoded genes of *Chlamydomonas* (Hwang et al., 1996; Salvador et al., 1993) and wheat (Nakahira et al., 1998). This oscillation of the wheat *psbD* gene is dependent on an atypical –35 promoter element and it was hypothesized that transcription of this gene requires a plastid-encoded RNA polymerase and a sigma factor encoded by a nuclear *CCG* (Nakahira et al., 1998). Indeed, circadian transcription of nuclear-encoded sigma factor genes has been shown in Arabidopsis and wheat (Kanamaru et al., 1999; Morikawa et al., 1999). This provides a novel mechanism for temporal coordination between the nuclear and plastid genomes.

Circadian Transcription

Circadian regulation of transcription underlies many of the rhythms in mRNA abundance described above. For example, both nuclear run-on experiments and transcriptional gene fusions have established a transcriptional component to the circadian regulation of *LHCB* genes in several angiosperms (Fejes and Nagy, 1998; Piechulla, 1999). The instability of luciferase (LUC) activity *in planta* means that light production requires translation of new LUC protein and provides a reliable assessment of *LUC* transcription (Millar et al., 1992a). Moreover, the measurement of LUC activity is non-destructive and quantitative and allows both temporal and spatial resolution of gene expression in real time *in vivo.* Thus, LUC has become the reporter of choice for monitoring circadian gene expression (Figure 3; Millar et al., 1992a; Millar et al., 1992b).

Minimal nuclear promoters sufficient to confer maximal circadian transcription at a mid-morning phase have been identified for several Arabidopsis genes, including *LHCB* (Fejes and Nagy, 1998; Piechulla, 1999) and *RCA* (Liu et al., 1996). In both cases, transcription is well correlated with the mid-morning phase of peak mRNA abundance. The microarray experiments emphasize that the mRNA abundance of different genes occurs at distinct circadian phases (times of day) and a number of different phase angle markers are available (Harmer et al., 2000; Schaffer et al., 2001). For example, mRNA abundance of the *CAT2* and *CAT3* catalase genes of Arabidopsis peaks at dawn and dusk, respectively (Zhong and McClung, 1996) and we have defined minimal promoter sequences sufficient to confer dawn- and evening-specific circadian transcription on a *LUC* reporter (TP Michael, PA Salomé & CR McClung, unpublished data; see Figure 3). Evening-specific promoters have also been defined for the Arabidopsis genes encoding a glycine-rich RNA-binding protein (*ATGRP7*/*CCR2*) and a germin-like protein (*AtGER3*) (Staiger and Apel, 1999; Staiger et al., 1999; Strayer et al., 2000). Both mRNA abundance and transcription of *EARLY FOWERING 3* (*ELF3*) peak about 4 hr after subjective dusk (Covington et al., 2001; Hicks et al., 2001). It is now pos-

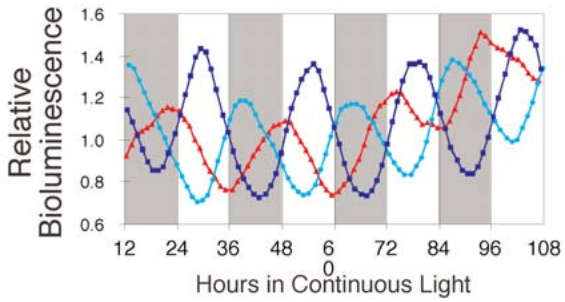

Figure 3. Rhythmic gene transcription, monitored as LUC activity in seedlings carrying *promoter::LUC* transgenes to illustrate phase-specific transcription. Seedlings (Col ecotype) carrying either *CAT2::LUC* (red triangles), *LHCB*::*LUC* transgene (blue squares) or a *CAT3*::*LUC* transgene (cyan circles) were entrained to a light-dark (12:12) cycle and released into continuous light at T=0. The peak in *CAT2*::*LUC* activity occurs at subjective dawn, in *CAB2::LUC* activity at mid-day, and in *CAT3*::*LUC* activity at subjective dusk. Gray boxes indicate subjective night.

sible to target the expression of one's favorite gene to any particular time of day (Table 1).

The definition of minimal promoters both necessary and sufficient to confer phase-specific transcription represents a key step in the analysis of the mechanisms by which genes are transcribed at different times of day. The experiments have chiefly begun with progressive 5' deletion analysis, followed by site-directed mutagenesis to test the functional relevance of potential clock responsive elements. For example, *in vivo* functional analysis of progressively truncated *LHCB1*1* (*CAB2*) promoter fragments fused to *LUC* defined a 36 bp region sufficient to confer circadian transcription that was bound, *in vitro*, by multiple complexes (Carré and Kay, 1995). The *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) gene encodes a single Myb domain protein that shows circadian binding to an element (consensus AAAa/cAATCT) within the functionally defined region of the *LHCB1*3* (*CAB1*) promoter (Wang and Tobin, 1998). This CCA1-binding element is also found in the minimal *RCA* promoter (Liu et al., 1996), although the functional importance of CCA1 binding to the *RCA* promoter has not been established. A related consensus, AAAATATCT, is also found in the functionally defined minimal evening-specific *AtGRP7*/*CCR2* (Staiger and Apel, 1999) and *CAT3* promoters (TP Michael & CR McClung, unpublished data), as well as in the putative promoters of 31 evening-specific genes identified by microarray analysis (Harmer et al., 2000). Mutation of this element eliminates evening specific transcription of *CCR2* (Harmer et al., 2000) and *CAT3* (TP Michael & CR McClung, unpublished data). It seems surprising that as little as a single nucleotide change in a consensus element might result in transcription nearly 180° out of phase, although the functional significance of this T/A substitution has not yet been established. It seems quite likely that flanking sequences, perhaps themselves the targets of other DNA-binding proteins, may contribute to the establishment of phase. Certainly, it seems that the mechanism by which the phase of transcription is determined will not necessarily be the simple solution of a series of phase-specific transcriptional activators, as implied by the apparent co-regulation of the phenylpropanoid pathway by PAP1 (Borevitz et al., 2000; Harmer et al., 2000).

Movement and growth rhythms

The initial written description of a plant circadian rhythm, by Androsthenes in the 4th century BC, described the diur-

Table 1. Clock regulated promoters drive circadian transcription at phases that span the circadian day. Shown are those promoters that have been experimentally shown to drive rhythmic transcription in transgenic plants. Phases are defined in Circadian Time (CT), which is a formalism to allow comparison of the circadian phasing of rhythms for which the period varies from 24 hr. CT = 24 x phase/period.

Phase	Gene(s)	Reference
CT0	*CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*)	(Covington et al., 2001)
	CATALASE 2 (*CAT2*)	PA Salomé & CR McClung, unpublished
CT4	*LHCB*	(Millar et al., 1992a)
	RUBISCO ACTIVASE (*RCA*)	(Liu et al., 1996)
	PHYTOCHROME B (*PHYB*)	(Bognár et al., 1999)
CT8	*COLD AND CIRCADIAN REGULATED 2* (*CCR2*)	(Strayer et al., 2000)
CT12	*TIMING OF CAB 1* (*TOC1*)	(Golden and Strayer, 2001)
	CATALASE 3 (*CAT3*)	TP Michael & CR McClung, unpublished
CT16	*EARLY FLOWERING 3* (*ELF3*)	(Covington et al., 2001)
CT20	*CHALCONE SYNTHASE* (*CHS*)	(Thain et al., 2000)

nal leaf movements of the tamarind (*Tamarindus indicus*) observed during the marches of Alexander the Great (Moore-Ede et al., 1982). In this classic system of pulvinar leaf movements, cells in the extensor and flexor regions of the pulvinus swell in antiphase (180° out of phase) to drive a circadian oscillation in leaf position (Engelmann and Johnsson, 1998). These leaf movements may have adaptive value in regulating perception of photoperiodic light signals (Bünning, 1971).

Although Arabidopsis lacks the pulvinus, it displays rhythms in cell elongation and, thus, in growth rate. For example, there is a circadian rhythm in the elongation rate of the abaxial and adaxial cells of the petiole that confers an oscillation in position of cotyledons and leaves (Engelmann and Johnsson, 1998). Leaf movements of individual seedlings are easily monitored by video imaging (Engelmann et al., 1992; Millar et al., 1995a). Figure 4 illustrates the use of this leaf (more properly, cotyledon) movement assay to compare period lengths in wild type (ecotype C24) versus *timing of CAB expression 1* (*toc1*) mutant seedlings, which exhibit a shortened period. The still images of a single C24 seedling presented in Figure 4B were derived from a time lapse video.

In addition to the growth rhythm in petioles, Arabidopsis also exhibits circadian rhythms in the rate of hypocotyl elongation (Dowson-Day and Millar, 1999). There is also a circadian rhythm in the elongation rate of inflorescence stem that is correlated with the level of indole-3-acetic acid (IAA) in rosette leaves, although IAA levels in the inflorescence stem do not oscillate (Jouve et al., 1998). There does not seem to be rhythmic synthesis of IAA in the shoot apex. Rather, there is either a rhythm either in polar transport of IAA or in the ability to elongate in response to IAA, because decapitation abolishes elongation that is restored by application of IAA (Jouve et al., 1999). Inhibition of IAA polar transport blocks elongation, but this does not distinguish between either rhythmic IAA transport or sensitivity as critical for the overt rhythm in elongation rate. Microarray analysis has shown circadian rhythms in mRNA abundance of two auxin transporter genes, *PIN3* and *PIN7* (Harmer et al., 2000), which would be consistent with rhythmic auxin transport. Additionally, transcript abundance for a putative expansin, a putative polygalacturonase and an aquaporin also oscillate, peaking towards the end of subjective day, with wall-synthesis genes coordinately peaking towards the end of the subjective night (Harmer et al., 2000). Collectively, these observations suggest that the movement of the hormone that signals elongation as well as the ability of the plant cell to expand in response to that signal may be regulated by the clock. However, this remains to be directly established.

The issue of possible circadian regulation of phytohormone production and responsiveness has been largely ignored. Circadian regulation of auxin transport is implied. In addition, *AUXIN RESPONSE TRANSCRIPTION FACTOR 3* (*ETTIN*/*ARF3*) exhibits mRNA oscillations in continuous light (Harmer et al., 2000), which suggests that the ability to respond to auxin will also prove to be gated by the clock. Ethylene production exhibits circadian rhythmicity in a number of species (Finlayson et al., 1998; Ievinsh and Kreicbergs, 1992). In sorghum, 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase mRNA abundance and activity are rhythmic (Finlayson et al., 1999). mRNA abundance oscillations have been established by microarray analysis for two Arabidopsis ACC oxidase genes (Harmer et al., 2000). There is also a diurnal oscillation in gibberellic acid levels in sorghum, although it has not been established that this rhythm persists in continuous conditions (Foster and Morgan, 1995).

Stomatal aperture, gas exchange and CO2 assimilation

Like many plants, Arabidopsis exhibits a circadian rhythm in stomatal aperture (Webb, 1998). This is correlated with a circadian rhythm in the rate of CO_2 assimilation (P.A. Salomé, E.V. Kearns & C.R. McClung, unpublished). In beans there is circadian control of Calvin cycle reactions in addition to control of stomatal aperture and gas exchange (Hennessey and Field, 1991), but circadian regulation of the Calvin cycle has not been investigated in Arabidopsis. However, microarray analysis has indicated circadian oscillations in mRNA levels for a number of genes encoding Calvin cycle enzymes (Harmer et al., 2000). The molecular basis of the circadian rhythm of CO_2 assimilation in Crassulacean Acid Metabolism (CAM) plants is understood in considerable detail (Nimmo, 2000). Flux through PEP carboxylase (PEPc) is regulated by reversible phosphorylation in which the night (phosphorylated) form of PEPc is less sensitive to inhibition by malate. PEPc kinase from *Kalanchoë fedtschenkoi* is atypical in that it lacks regulatory domains and thus is not regulated by second messengers. Rather, rhythmic PEPc kinase activity reflects rhythmic transcript accumulation and *de novo* protein synthesis (Hartwell et al., 1999; Hartwell et al., 1996). The CAM CO_2 assimilation rhythm also entails metabolite compartmentalization in that there is a rhythm in the transport of malate across the tonoplast (Nimmo, 2000).

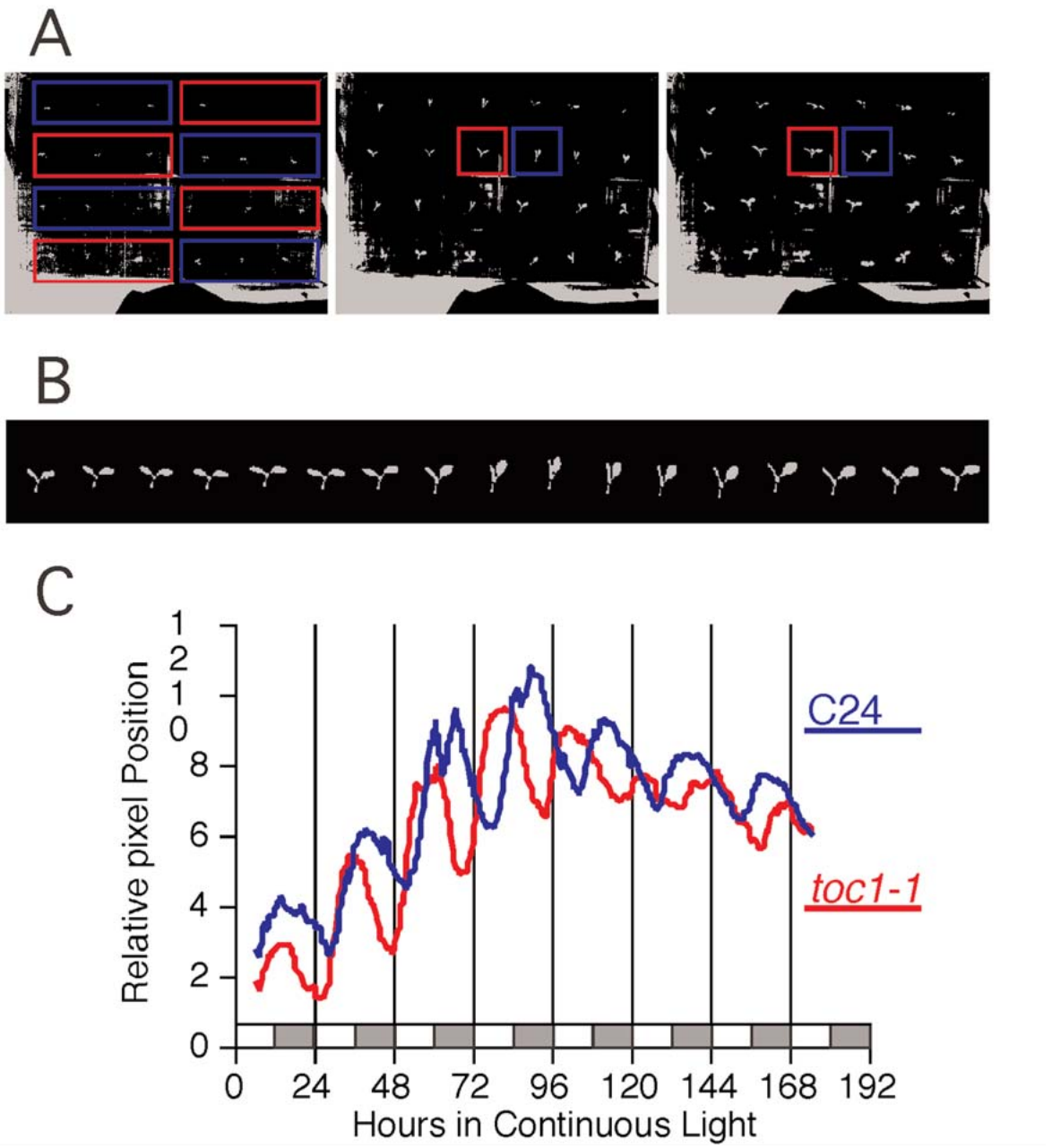

Figure 4. *toc1-1* shows a short period in leaf (cotyledon) movement. **(A)** Three images (initial, middle and last) of a video clip that can be reached by double-clicking on the image. Twelve *toc1-1* (red) and twelve isogenic wild type C24 ecotype (blue) seedlings were imaged for seven circadian cycles. **(B)** Images of a single C24 seedling [outlined by the blue box in the middle and right panels of **(A)**] captured at 90 min intervals across a single circadian cycle. **(C)** Quantification of cotyledon position from the images shown in **(A)** for *toc1-1* (red) and C24 (blue). The data represent the average for the two cotyledons of the seedlings indicated by the boxes in the middle and right panels of **(A)**. The shortened period of *toc1-1* is evident. Seedlings were entrained to a light-dark (12:12) cycle and released into continuous light at T=0. Gray boxes indicate subjective night as defined by the entraining cycle.

Calcium

Ca^{2+} is a ubiquitous second messenger in plant signaling pathways (Sanders et al., 1999). The relevance of Ca^{2+} signaling to circadian rhythmicity has been experimentally established; for example, application of either Ca^{2+} or a Ca^{2+} ionophore phase shifts the leaflet movement rhythm of *Robinia pseudoacacia* (Gómez and Simón, 1995). As monitored by aequorin luminescence in both tobacco and Arabidopsis, free cytosolic but not nuclear Ca^{2+} levels oscillate with a circadian rhythm (Johnson et al., 1995; Wood et al., 2001). Wood et al. (2001) looked at Ca^{2+} levels in different tissues by expression of apoaequorin from several tissue-specific promoters. Interestingly, the peaks of the Ca^{2+} oscillations occur at different phases in different tissues (Wood et al., 2001).

Microarray analysis shows circadian transcript oscillations for a number of genes associated with Ca^{2+} signaling, including genes encoding Calmodulin and a Calmodulin-like Ca^{2+}-binding protein, as well as a putative Ca^{2+}-binding EF-hand protein and a Ca^{2+}-transporting ATPase (Harmer et al., 2000). Ca^{2+} is important in guard cell signaling (Leckie et al., 1998; Schroeder et al., 2001) and is likely to be involved in the circadian regulation of stomatal aperture and gas exchange. Ca^{2+} is also implicated in red and blue light signal transduction (Barnes et al., 1997; Baum et al., 1999; Frohnmeyer et al., 1998; Long and Jenkins, 1998; Guo et al., 2001) and may play a role in the entrainment of the circadian oscillator. The light to dark transition stimulates a spike in chloroplast stromal Ca^{2+} levels (Johnson et al., 1995), although whether this signals the circadian clock is not known. Thus, Ca^{2+} is likely to play multiple roles in the circadian system, but none of these roles are yet well defined.

Photoperiodism

The timing of flowering in many species is photoperiodic (Lin, 2000a; Simpson et al., 1999). Bünning (Bünning, 1936) hypothesized that circadian timekeeping was essential for photoperiodic time measurement and many mutations that affect circadian rhythms in gene expression and leaf movement also affect flowering timing (Lin, 2000a; Simpson et al., 1999). Alteration of the activity of a number of components of the Arabidopsis circadian system through overexpression or through loss of function (whether by antisense or mutation), often alters flowering time (Table 2). For example, mutations in two components of the photoperiodic pathway, *early flowering 3* (*elf3*) and the late flowering *gigantea* (*gi*), confer defects in the circadian timing and define components of the light input pathway. Overexpression of *LATE ELONGATED HYPOCOTYL* (*LHY*), a third component of the photoperiodic pathway, results in arrhythmicity as well as late flowering (Schaffer et al., 1998). However, overexpression of *CONSTANS* (*CO*), a *CCG* that accelerates flowering in response to long days, does not disrupt circadian rhythmicity (Súarez-López et al., 2001). Presumably *CO* lies distal to the circadian oscillator in the photoperiodic pathway. Nonetheless, flowering timing mutants constitute a reservoir of potential circadian clock mutants. For example, null alleles of *FLOWERING LOCUS C*, in the autonomous flowering pathway, confer early flowering and shorten the circadian period in leaf movement (Swarup et al., 1999).

Although CO may not be a component of the circadian oscillator, it plays a key role in the integration of circadian timekeeping and daylength perception (Súarez-López et al., 2001). *CO* mRNA abundance oscillates and the phase of the peak in *CO* mRNA varies with photoperiod. Only in long days does *CO* mRNA abundance peak in the light. CO encodes a Zn finger transcription factor (Putterill et al., 1995), and post-transcriptional regulation of CO activity by light is proposed to be critical to the transcriptional activation of *FLOWERING LOCUS T* (*FT*), a CO target gene (Súarez-López et al., 2001). This provides a molecular explanation of the external coincidence model by which long days promote flowering (Bünning, 1936).

Entrainment (Input)

Circadian rhythms persist in the absence of external time cues but are entrainable to the environment. This entrainment is essential to allow the organism to use its circadian clock to anticipate environmental changes associated with dawn and dusk. The changes in light and temperature encountered at dawn and dusk serve as the chief environmental time cues used by most organisms in clock entrainment.

Light

Circadian clocks, without exception, respond to light (Roenneberg and Foster, 1997) and light is the most potent and best-characterized entraining stimulus in plants

Table 2. Putative components of the Arabidopsis clock. Perturbations of clock function are often accompanied by alterations in flowering timing and by altered inhibition of hypocotyl elongation.

Locus	GenBank (MIPS)	Expression Change	Clock Phenotype[b]	Clock Role	Flowering Time[c]	Hypocotyl Length[d]	Reference[a]
TOC1/APRR1	AF272039	loss of function	short	oscillator	early (SD)	WT	(Somers et al., 1998)
	(At5g61380)	overexpression	nd[e]				
CCA1	U79156	loss of function	short	oscillator			(Green and Tobin, 1999)
	(At2g46830)	overexpression	arrh		late	long	(Wang and Tobin, 1998)
LHY	AJ006404	loss of function	short	oscillator?	nd	nd	(Song and Carré, 2001)
	(At1g01060)	overexpression	arrh		late	long	(Schaffer et al., 1998)
CKB3	AF068318	loss of function	nd	?			
	(At3g60250)	overexpression	short		early	WT	(Sugano et al., 1999)
ZTL	AF254413	loss of function	long/arrh (R)	input?	late (LD)	short	(Somers et al., 2000)[f]
	(At5g57360)	overexpression	nd		la te	long	(Nelson et al., 2000)
FKF	AF216523	loss of function	waveform	input?	late (LD)	short	(Nelson et al., 2000)
	(At1g68050)	overexpression	nd				
LKP2	AF252295	loss of function	nd	oscillator?			
	(At2g18910)	overexpression	arrh		late (LD)	long	(Schultz et al., 2001)
ELF3	AC004747.2[g]	loss of function	arrh (LL)	input	early	long	(Hicks et al., 1996)
	(At2g25930)	overexpression	fluence		nd	short	(Liu et al., 2001)
GI	AF076686	loss of function	period	input?	late	long	(Park et al., 1999)[g]
	(At1g22770)	overexpression	nd				
FLC	AF116527	loss of function	short	?	early	nd	(Swarup et al., 1999)
	(At5g10140)	overexpression	nd		late	nd	
PHYB	X17342	loss of function	fluence (R)	input	early	long	(Devlin and Kay, 2001)
	(At2g18790)	phase					(Salomé et al., 2001)
		overexpression	fluence (R)		la te	short	(Devlin and Kay, 2001)

[a] A representative reference is listed for each locus, although more may be available. For more details, see text.

[b] Clock phenotypes include arrhythmic (arrh), short period (short), long period (long), and altered fluence response in period length (fluence). R indicates phenotype seen in red light. LL indicates phenotype seen in continuous light.

[c] SD, short days; LD, long days

[d] WT, wild type

[e] nd = not determined

[f] see also (Jarillo et al., 2001)

[g] protein_id="AAC31242.1

[h] see also (Fowler et al., 1999; Huq et al., 2000)

(Devlin and Kay, 2001). Light perception in plants has been studied and reviewed in detail (see articles in this compilation by Briggs, Deng, and Liscum). The Arabidopsis genome encodes five red/far red-perceiving phytochromes (PHYA-PHYE) and two blue/UV-A-perceiving cryptochromes (CRY1 and CRY2) as well as several other blue light receptors, including the phototropins (PHOT1/NPH1 and PHOT2/NPL1) and zeaxanthin (Casal, 2000; Christie and Briggs, 2001; Lin, 2000b; Nagy and Schäfer, 2000). There is considerable experimental evidence demonstrating the roles of phytochromes and cryptochromes in providing light input to the clock (Devlin and Kay, 2001).

In plants and in light-active animals, period length is inversely related to light intensity; the circadian period shortens as light intensity increases (Aschoff, 1960). This mode of light input is called parametric entrainment, and can be quantified as a fluence response curve (Devlin and Kay, 2000; Somers et al., 1998). Genetic experiments with Arabidopsis mutants have established roles for PHYA, PHYB, PHYD, PHYE, CRY1 and CRY2 in the establishment of period length (Devlin and Kay, 2000; Millar et al., 1995b; Somers et al., 1998). Light-labile PHYA is the predominant photoreceptor for the clock at low intensities of red or blue light, whereas PHYB and CRY1 dominate at high intensities of red and blue light, respectively (Somers et al., 1998). Double mutant studies demonstrate a role for CRY2 in the establishment of period at intermediate intensities of blue light, although that role is redundantly specified by CRY1 (Devlin and Kay, 2000). *cry1 cry2* double mutants retain rhythmicity (Devlin and Kay, 2000), which eliminates the CRY proteins as non-redundantly-specified critical components of the plant circadian oscillator, unlike the case with the mouse clock (van der Horst et al., 1999). Moreover, the quadruple *phyA phyB cry1 cry2* mutant retains both rhythmicity (leaf movement) and the ability to be entrained to a light-dark cycle, making it clear that other photoreceptors (PHYC-PHYE, or others), can provide light input to the clock (Yanovsky et al., 2000b). Roles for PHYD and PHYE in clock input under high intensity red light are supported by period lengthening observed in triple *phyA phyB phyD* and *phyA phyB phyE* mutants versus the *phyA phyB* double mutant (Devlin and Kay, 2000). More triple and quadruple mutant combinations will be required to assess the role of PHYC.

Loss of PHYB or CRY1 (PA Salomé & CR McClung, unpublished data) function does not affect the period length in white light, but instead alters the phase angle of multiple rhythms, indicating that PHYB and CRY1 contribute to the establishment of circadian phase as well as period. PHYB and CRY1 signaling might establish phase through the induction of a critical clock component at dawn. For example, loss of Neurospora *vivid* function alters the phase of the rhythm in *FREQUENCY* (*FRQ*) expression (Heintzen et al., 2001). In addition, mutants lacking the cyanobacterial phytochrome, CikA, show dramatic alterations in phase angle of multiple gene expression rhythms (Schmitz et al., 2000). CikA is thought to mediate light signaling to the clock proteins KaiA and KaiB (Schmitz et al., 2000).

PHOTOTROPIN 1 (PHOT1/NPH1; DE Somers, unpublished observations) mutants are unaffected in period length; neither PHOT2 (NPL1) nor zeaxanthin have been examined as components of the light input pathways to the clock. However, a novel family of putative photoreceptors, ZEITLUPE (ZTL/LKP1/ADO1) and FLAVIN-BINDING KELCH REPEAT F-BOX (FKF/ADO3) has recently been identified by the mutant phenotype of altered circadian rhythms (Jarillo et al., 2001; Nelson et al., 2000; Somers et al., 2000). A third family member, LOV DOMAIN KELCH PROTEIN 2 (LKP2/ADO2), was identified on the basis of the similarity among the PAS domains (Jarillo et al., 2001; Kiyosue and Wada, 2000). *FKF* mRNA abundance oscillates with an evening-specific maximum, but neither *ZTL* nor *LKP2* mRNAs oscillate (Nelson et al., 2000; Schultz et al., 2001; Somers et al., 2000). Recently, LKP2 overexpressing plants were shown to be arrhythmic by leaf movement and gene expression in constant conditions, although a rhythm could still be driven by a light-dark cycle (Schultz et al., 2001). *ztl* mutants show long periods in multiple rhythms and the severity of the period lengthening displays fluence rate dependence (Somers et al., 2000), whereas *fkf* mutants exhibit altered waveform in *CCA1* and *LHCB* mRNA oscillations (Nelson et al., 2000). A T-DNA insertion allele of *ztl*, (called *adagio1* [*ado1*]), is arrhythmic in leaf movement in red light (Jarillo et al., 2001). Both *ztl* and *fkf* mutants are late flowering (Nelson et al., 2000; Somers et al., 2000).

These ZTL/FKF/LKP2 proteins possess novel combinations of known functional domains. Family members have a single PAS domain. PAS domains, which function in protein-protein interactions, are found in many clock proteins (Loros and Dunlap, 2001; Reppert and Weaver, 2001; Williams and Sehgal, 2001). The PAS domains of ZTL/FKF/LKP2 are most similar to those of PHOT1 (NPH1), the Neurospora White Collar clock proteins, and an unusual phytochrome from a fern (Christie and Briggs, 2001; Loros and Dunlap, 2001; Nozue et al., 1998). The PAS (also called LOV, for light oxygen voltage) domain of PHOT1 (NPH1) binds flavin and may be involved in blue light sensing (Christie et al., 1999), which suggests that ZTL/FKF/LKP2 may also be photoreceptors. ZTL, FKF and LKP2 each also contain Kelch repeats that function in protein-protein interactions (Adams et al., 2000). In addition, each contains an F-box, a domain that recruits target proteins to E3 ubiquitination complexes (Patton et al., 1998). Although a role in ubiquitination has not been functionally established for ZTL, FKF or LKP2, such evidence

has been collected for other plant F-box proteins that function in flower development (UFO; Samach et al., 1999) and auxin responses (TIR; Gray and Estelle, 2000). Proteolytic degradation has been shown to play important roles in other clock systems. For example, Drosophila TIM is degraded in the light by an ubiquitin-proteasome mechanism; TIM degradation is correlated with changes in phosphorylation (Naidoo et al., 1999), which is required for substrate recognition by F-box proteins (Craig and Tyers, 1999). As described below, Arabidopsis CCA1 is phosphorylated by casein kinase 2 (CK2) (Sugano et al., 1998; Sugano et al., 1999). Are phosphorylation and degradation of CCA1, LHY or another key target part of the circadian oscillator mechanism (Figure 5)?

There is considerable interaction among photoreceptors. PHYA and CRY1 directly interact at the molecular level, with CRY1 serving as a phosphorylation substrate for PHYA *in vitro* (Ahmad et al., 1998). *In vivo*, CRY1 is phos-

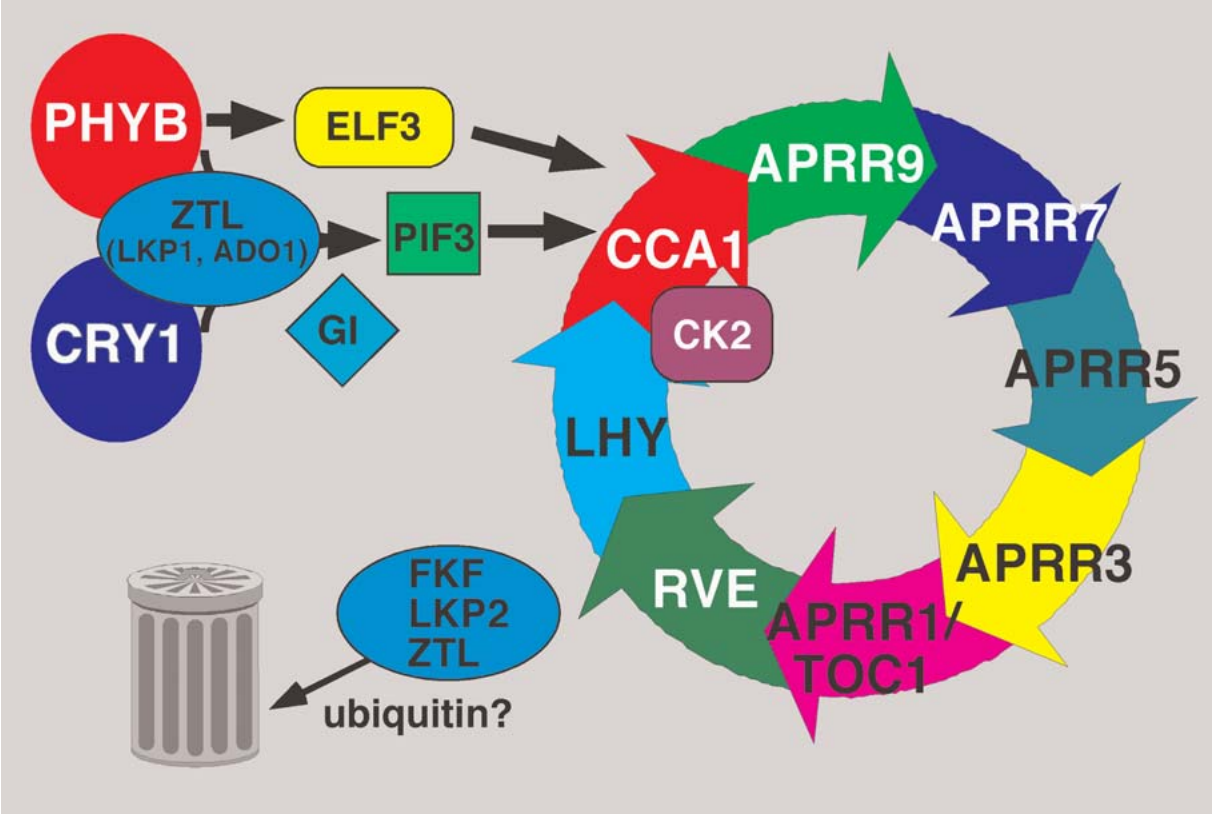

Figure 5. A speculative model of an Arabidopsis circadian clock. Light input via phytochromes and cryptochromes (for simplicity, only PHYB and CRY1 are shown, although others are certainly involved) is mediated through ZTL, ELF3 and GI, or through PIF3. ZTL/ADO1 is known to bind to PHYB and CRY1. PIF3 binds to CCA1 and LHY promoters and possibly to other targets in the clock. The pathway downstream of GI is not known. Although the input pathways are drawn as discrete linear pathways, there may be interaction among them. For simplicity, a single central oscillator is illustrated with a number of putative oscillator components indicated. Components on the circular arrows oscillate in mRNA or protein abundance. One should not infer causal relationships among putative components from the relative order of their placement on the circle as experimental proof is lacking. FKF/LKP2/ZTL are clustered, although there is no evidence that they form molecular complexes. Moreover, *LKP2* mRNA oscillates and overexpression results in arrhythmicity, making LKP2 a strong candidate as an oscillator component. CCA1 and LHY are phosphorylated by CK2, which may make them substrates for the F-box proteins (ZTL, FKF and LKP2) and target them for ubiquitination and degradation by the proteasome (trash can). Output pathways may emanate from any of the putative oscillator components. CCA1, LHY, RVEs and TOC1/APRR1 are DNA-binding proteins, and CCA1 is know to bind to *LHCB* promoters. Other outputs from the oscillator feed back to input components, such as PHYA, PHYB and CRY1, which are regulated by the clock at transcriptional and mRNA abundance levels

phorylated in response to red light in a far-red reversible manner (Ahmad et al., 1998). A *cry1* null mutant shows lengthened period in low intensity red or white light and there is no additivity seen in the double *phyA cry1* mutant (Devlin and Kay, 2000). This suggests that CRY1 acts as a signal transduction component downstream from PHYA in the low intensity light input pathway to the clock (Devlin and Kay, 2001). ZTL has also been shown in the yeast two-hybrid assay to interact physically with the photoreceptors PHYB and CRY1 (Jarillo et al., 2001). However, it is important to recall that the compartmentalization of these photoreceptors and their downstream signaling components is regulated (Nagy et al., 2001), so it is important to confirm the putative interaction *in vivo*. For example, PHYB and CRY2 have been shown to interact *in vivo* by Fluorescence Resonance Energy Transfer (Más et al., 2000).

Light pulses (non-parametric entrainment) can also shift the phase of the clock. Red light pulses phase shift the clock by a very low fluence PHY response (Kolar et al., 1998; Nagy et al., 1993) and far red light pulses phase shift in a PHYA-dependent fashion (Yanovsky et al., 2000a). It has long been clear that clock response to environmental stimuli varies over the circadian cycle. For example, a pulse of light administered prior to dawn advances the phase of the clock whereas the identical pulse applied after dusk delays clock phase. The phase response curve (PRC) is plot of the amplitude of the phase shift resulting from a stimulus applied at discrete phases spanning a circadian cycle (Johnson, 1990). Light phase response curves are available for a number of angiosperms, and recently phase response curves for pulses of red or blue light over a dark background were generated in Arabidopsis (Covington et al., 2001).

One mechanism by which the sensitivity of the oscillator to light might vary over the circadian cycle would be clock regulation of components of the light input pathway; input pathway components may themselves be encoded by *CCGs*. Microarray experiments indicate that *PHYB*, *CRY1*, *CRY2*, and *PHOT1* (*NPH1*) mRNAs oscillate (Harmer et al., 2000; Schaffer et al. 2001). *PHYB* transcription, as monitored with *PHYB*::*LUC* gene fusions, is rhythmic in tobacco and Arabidopsis, although bulk PHYB protein abundance does not oscillate (Bognár et al., 1999). *PHYA*, *PHYD*, *PHYE*, *CRY1* and *CRY2* show circadian oscillations both at mRNA abundance and transcriptional levels (Tóth et al., 2001). *PHYC* mRNA oscillates robustly, although transcription of a *PHYC::LUC* fusion is only weakly rhythmic. It seems that the light responsiveness of the *PHYC* promoter obscures any transcriptional rhythm and that the pronounced circadian oscillation in *PHYC* mRNA abundance reflects an important role of the clock in regulating *PHYC* mRNA stability (Tóth et al., 2001). The clear interpretation of these data is that the clock regulates its own sensitivity to entraining stimuli through regulated expression of photoreceptors.

Signaling pathways downstream from PHY and CRY are under active study. Various signaling intermediates, such as cGMP and Ca^{2+}-calmodulin, as well as phosphorylation, are implicated in light signaling (Briggs and Huala, 1999; Casal, 2000; Christie and Briggs, 2001; Deng and Quail, 1999; Guo et al., 2001; Lin, 2000b). Many signaling components downstream from the photoreceptors have been identified. In particular, red-illuminated PHYB (PfrB) interacts with PIF3, a bHLH protein that binds directly to the G box in a number of phytochrome regulated promoters, including those of *CCA1* and *LATE ELONGATED HYPOCOTYL* (*LHY*) (Martínez-García et al., 2000), two putative oscillator components (see below). This G box (CACGTG) is notably similar to the animal E box (CANNTG) that is targeted by heterodimeric transcription factors of Drosophila and mammalian central oscillators (Reppert and Weaver, 2001; Williams and Sehgal, 2001). However, the G box has not been directly implicated in circadian transcription in plants.

Genetic studies have implicated two novel genes, *EARLY FLOWERING 3* (*ELF3*) and *GIGANTEA* (*GI*), in light signaling to the clock. *elf3* loss-of-function alleles yield early flowering, hypocotyl elongation, and conditional arrhythmicity in continuous light (Covington et al., 2001; Hicks et al., 1996; McWatters et al., 2000). *ELF3* is a *CCG* encoding a nuclear protein; both transcript and protein accumulation in the nucleus peak at dusk (Covington et al., 2001; Hicks et al., 2001; Liu et al., 2001). Genetic experiments suggest substantial redundancy in ELF3 and PHYB function (Reed et al., 2000). ELF3 interacts with PHYB and seems to act as a negative modulator of PHYB signaling to the clock, as ELF3 overexpression both lengthens the circadian period and attenuates the resetting effects of red light pulses whereas loss of *ELF3* function renders the plant hypersensitive to light signals (Covington et al., 2001; Liu et al., 2001; McWatters et al., 2000).

GI is another *CCG* implicated in light input. *gi* mutants are altered in leaf movement and gene expression rhythms of multiple CCGs, including *GI* itself (Fowler et al., 1999; Park et al., 1999). The period shortening effect of *gi-1* on gene expression rhythms is less severe in extended dark than in continuous light and the extension of period length seen in light of decreasing fluence is less pronounced in *gi-1* than in wild type, which indicates that GI acts in light input (Park et al., 1999). *gi* exhibits a defect in inhibition of hypocotyl elongation in red but not in far red light, which implicates GI in PHYB signaling (Huq et al., 2000). GI is nuclear, which is consistent with a role in early PHYB signaling (Huq et al., 2000). However, *gi* phenotypes are complicated. In the null *gi-2* allele, the period of leaf movement is shortened but the period of gene expression rhythms gradually lengthens (Park et al., 1999). In addi-

tion, the effects of loss of GI function on hypocotyl elongation are the same as seen in *phyB* loss of function, which suggests that GI is a positive mediator of PHYB signaling. However, *gi* mutants are late flowering, which is opposite to the early flowering phenotype of *phyB* null alleles. Liu et al. (Liu et al., 2001) showed that the effects of ELF3 on flowering time are independent of PHYB. Perhaps GI and ELF3 mediate PHYB signaling to the clock but that their effects on flowering time are mediated through another signaling pathway (Huq et al., 2000; Liu et al., 2001). Alternatively, GI and ELF3 may play different roles at different developmental stages.

Earlier we discussed circadian regulation of photoreceptor expression as a mechanism to regulate sensitivity of the plant to light stimuli. There are other potential mechanisms to regulate sensitivity to light stimuli. For example, ELF3 is circadian regulated and serves as a negative regulator of PHYB signaling (Covington et al., 2001; Hicks et al., 1996; Liu et al., 2001; McWatters et al., 2000). *SUPPRESSOR OF PHYA* (*SPA1*) is a negative regulator of PHYA signaling, and microarray analysis indicates that *SPA1* mRNA is clock regulated (Harmer et al., 2000). Recent studies may have identified a circadian-regulated enhancer of light signaling. When plants are transferred into the dark, the circadian oscillations in light-inducible genes, such as *LHCB*, rapidly damp to low levels. This has been attributed to the depletion of the positive regulator, Pfr (Kay and Millar, 1993). A tobacco gene, *ZGT* (abbreviated from the Chinese phrase *zhong guang tiaokong*, meaning clock and light controlled) has been isolated (Xu and Johnson, 2001). As the name indicates, *ZGT* is light inducible and is rhythmically expressed with an mRNA abundance peak at mid-day. Although the Arabidopsis *ZGT* homolog has not been directly studied, overexpression of tobacco *ZGT* in Arabidopsis allows the oscillations in *LHCB::LUC* expression to persist in extended dark. In addition, *ZGT* overexpression renders the plant more sensitive to the phase-shifting effects of light pulses. Thus, it seems that ZGT enhances the sensitivity of the plant to light in a circadian pattern (Xu and Johnson, 2001), making it a positively acting counterpart to ELF3 and SPA1.

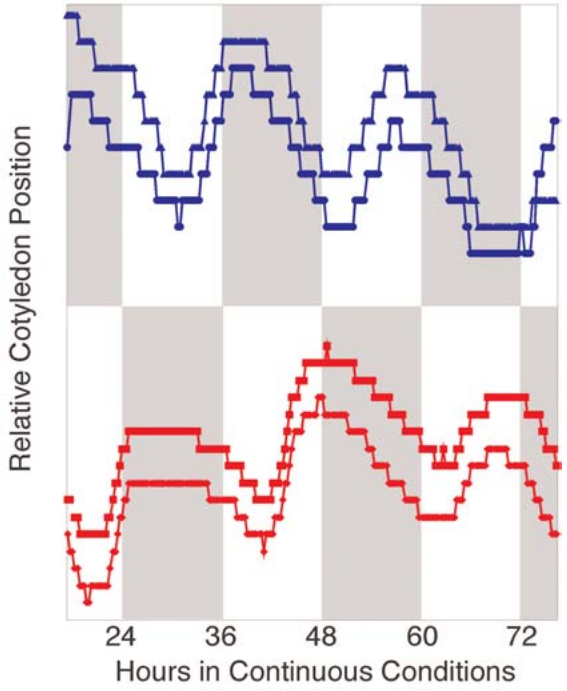

Figure 6. Entrainment of the circadian rhythm in leaf (cotyledon) movement by temperature cycles. Upper panel shows individual traces (blue) of the relative cotyledon positions for the two cotyledons of a representative seedling entrained under continuous light to a temperature cycle of 12 hr at 22°C and 12 hr at 18°C and then released into continuous conditions of constant light at 22°C at T=0. The lower panel shows traces (red) for the two cotyledons of a seedling entrained to the antiphase (180° out-of-phase) temperature cycle of 12 hr at 18°C and 12 hr at 22°C. Gray bars indicate subjective night as defined by the cold period of the entraining cycle.

Temperature and Imbibition

The circadian oscillator is temperature compensated, which means that the pace of the oscillator is relatively constant over a range of temperatures. However, temperature pulses or temperature steps are potent entraining stimuli. Little is known of the mechanisms of temperature sensing by plants and even less is known about the specific pathways that convey temperature information to the clock. However, temperature pulse PRCs have been generated for several plants (Johnson, 1990). Temperature cycles entrain Arabidopsis rhythms in *LHCB* (Somers et al., 1998) and *CAT3* transcription (TP Michael & CR McClung, unpublished data). Figure 6 illustrates an experiment in which we used out-of-phase temperature cycles of 12 hr at 22°C and 12 hr at 18°C to entrain seedlings 180° out of phase, as measured by leaf movement. The ability of temperature to entrain the clock led us to hypothesize that the temperature step associated with release from stratification at 4°C to growth at 22°C would either initiate clock function or synchronize the clocks among a population of seedlings. Surprisingly, release from stratification was ineffective in resetting the phase of the rhythm in *CAT2* induction by light (Zhong et al., 1998), suggesting

a refractory period before temperature is capable of entraining the Arabidopsis oscillator. Similarly, a light-insensitive circadian oscillator is detected shortly after germination of tobacco and Arabidopsis (Kolar et al., 1995; Kolar et al., 1998). Although germinating seedlings are refractory to temperature and light input, the timing of imbibition (hydration) of the dried seed entrains the clocks within populations of seedlings (Zhong et al., 1998). This makes it clear that circadian clock is running from the time of imbibition.

THE OSCILLATOR: INTERLOCKED NEGATIVE AND POSITIVE FEEDBACK LOOPS

Genetic and molecular biological studies have identified a number of the components of circadian systems in cyanobacteria, Neurospora, Drosophila, mice and humans (Johnson, 2001; Loros and Dunlap, 2001; Reppert and Weaver, 2001; Williams and Sehgal, 2001). There is a great deal of conservation among the components of the fly and mammalian clocks but the PAS domain, a protein-protein interaction domain (Taylor and Zhulin, 1999), is the only element that has been found in all clock systems. With the determination of the complete sequence of the Arabidopsis genome (The Arabidopsis Genome Initiative, 2000), no obvious orthologs to most known clock proteins can be found, demonstrating that at least part of the Arabidopsis clock mechanism is novel. Nonetheless, studies in the other model systems have indicated that circadian oscillators are comprised of two interconnected feedback loops, one positive and one negative (Figure 7; Loros and Dunlap, 2001; Reppert and Weaver, 2001; Williams and Sehgal, 2001).

A number of loci have been implicated in the Arabidopsis circadian system and many putative clock genes have now been cloned (Table 2). Current efforts are directed towards testing the relevance of the interconnected feedback loop model and identifying the components of the Arabidopsis oscillator. Mutations in a central oscillator component should affect clock properties, such as period length or temperature compensation. The activity, although not necessarily the simple abundance, of an oscillator component should oscillate and interference with this oscillation, commonly through overexpression, should abolish rhythmicity. Loss of function of key clock components should abolish rhythmicity, but functional redundancy often complicates the interpretation of such experiments. In addition, it can be difficult to distinguish between components of input pathways versus of the central oscillator itself (Merrow et al., 1999; Merrow et al., 2001). At present, a pseudo response regulator, TOC1/APRR1, and two myb transcription factors, CCA1 and LHY, are strong candidates as clock components.

A genetic screen based on alterations in rhythmic transcription of a *LUC* transgene driven by regulatory elements of *LHCB1*1* (*CAB2*) identified a series of *timing of CAB* (*toc*) mutations that disrupt clock function (Millar et al., 1995a). Mutations in *toc1* shorten the period of multiple rhythms, including transcription of a number of genes at distinct circadian phases (Kreps and Simon, 1997; Millar et al., 1995a), leaf movement, and stomatal conductance (Somers et al., 1998). The period-shortening effect of *toc1-1* is independent of light intensity and is seen in extended darkness, which argues that TOC1 does not act in light input. *TOC1* mRNA abundance oscillates in continuous light, peaking late in the day. TOC1 feeds back to control its own oscillation because the period of this oscillation is shortened in *toc1-1* seedlings (Strayer et al., 2000). Collectively, these data suggest that TOC1 is likely to be a component of an oscillator. However, *TOC1* mRNA oscillations damp rapidly in extended darkness (Strayer et al., 2000), yet the clock, as monitored by gene expression

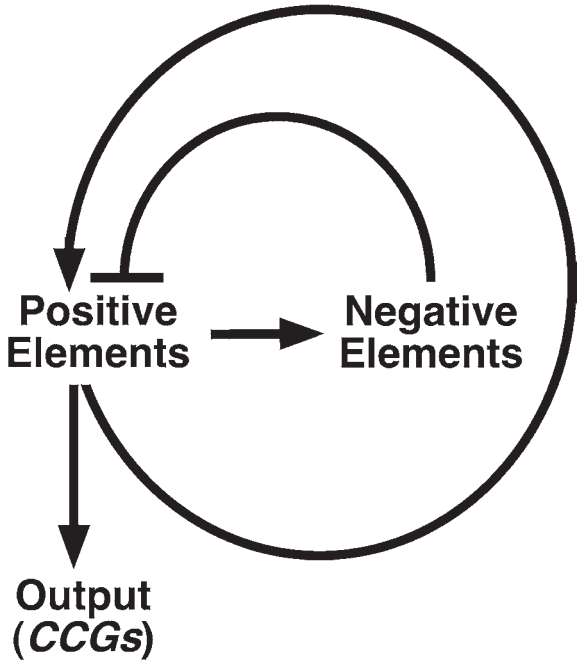

Figure 7. A simple model of a circadian oscillator to illustrate interlocked positive and negative feedback loops. For detailed models of Neurospora, Drosophila and murine clocks, see (Heintzen et al., 2001; Reppert and Weaver, 2001; Williams and Sehgal, 2001).

(Kreps and Simon, 1997; Strayer et al., 2000; Zhong et al., 1997), persists robustly in the dark.

TOC1 encodes a pseudo-response regulator (Strayer et al., 2000) and was independently identified as *ARABIDOPSIS PSEUDO-RESPONSE-REGULATOR 1* (*APRR1*) (Makino et al., 2000). *TOC1/APRR1* is the founding member of a 6-gene *APRR* family. mRNA abundance of 5 *APRR* family members (but not *APRR2*) oscillates with circadian period (Matsushika et al., 2000; Strayer et al., 2000). APRR2 further differs from the other 5 APRRs in that it includes a single Myb domain related to that of CCA1 instead of the CONSTANS-like domain found in the other 5 APRRs (Makino et al., 2000). Each of the 5 cycling *APRRs* shows a peak in mRNA abundance at a distinct time of the day, which suggests that the expression of each *APRR* in turn regulates the accumulation of the subsequent family members (Makino et al., 2001; Matsushika et al., 2000). However, this remains to be established experimentally. We eagerly await the analysis of loss-of-function alleles and of overexpression of the members of this family.

The identification of *TOC1/APRR1* implicates signal transduction through two-component systems (Sakakibara et al., 2000) in clock function. In typical bacterial two-component systems, a sensor histidine kinase responds to an environmental stimulus, autophosphorylates and transfers the phosphate to a response regulator which then effects a response. However, TOC1 and the 5 other APRRs each lack the invariant phosphor-accepting Asp residue, as well as a second of three invariant residues, and are unlikely to function in a conventional His-Asp relay (Makino et al., 2000; Strayer et al., 2000). Might TOC1 or another of the APRRs represent targets of atypical sensor kinases, perhaps PHY? Nonetheless, this suggests a mechanistic link to cyanobacterial clocks (Iwasaki and Kondo, 2000; Johnson, 2001), in which the sensory histidine kinase, SasA, interacts with the oscillator component KaiC (Iwasaki et al., 2000).

TOC1 also has a carboxy-terminal motif seen in the CONSTANS family of transcriptional activators (Putterill et al., 1995) as well as an acidic region often found in transcriptional activators. TOC1/APRR1 interacts with a PIF3-like protein (Makino et al., 2000), which suggests a mechanism by which the clock might regulate acute induction by light or gate its sensitivity to light input (Millar and Kay, 1996; Zhong et al., 1998). Moreover, TOC1/APRR1 was also identified as an ABSCISIC ACID INSENSITIVE 3-interacting protein (Kurup et al., 2000), which might indicate an interaction of the clock with abscisic acid (ABA) as an input or provide clock regulation of ABA responses.

Two *CONSTANS-LIKE* genes, *COL1* and *COL2*, are each clock-regulated and overexpression of *COL1* shortens the periods of leaf movement and of *LHCB::LUC* transcription (Ledger et al., 2001). However, these genes are more likely to encode transcription factors necessary for clock output than to represent true oscillator components, as the plants retain rhythmicity. Two clock-regulated glycine-rich RNA binding protein genes, *AtGRP7* (*CCR2*), and the closely related *AtGRP8 (CCR1)*, exhibit evening-peaking oscillations in transcription and mRNA abundance (Heintzen et al., 1997). *AtGRP7* mRNA abundance declines as protein accumulates, suggesting negative autoregulation which was confirmed by overexpression in transgenic Arabidopsis. Although *AtGRP7* overexpression depresses endogenous *AtGRP7* expression and blocks the oscillations in mRNA abundance of *AtGRP7* and *AtGRP8,* it does not affect circadian oscillations in *LHCB* and *CAT* mRNAs. This suggests that AtGRP7, and presumably AtGRP8, are key components of a slave (non-self-sustaining) oscillator but not of a central oscillator (Heintzen et al., 1997).

LHY and CCA1 are two single Myb domain transcription factors implicated in the circadian oscillator mechanism (Schaffer et al., 1998; Wang and Tobin, 1998). *CCA1* and *LHY* are both *CCGs* and both negatively autoregulate, consistent with roles as clock components (Covington et al., 2001; Schaffer et al., 1998; Wang and Tobin, 1998). Overexpression of either *LHY* or *CCA1* results in arrhythmicity of multiple clock outputs, including mRNA abundance of all clock-regulated genes tested to date (Fowler et al., 1999; Schaffer et al., 1998; Wang and Tobin, 1998), leaf movement (Strayer and Kay, 1999), and CO_2 assimilation (CR McClung & EM Tobin, unpublished observations). Loss of CCA1 function in a T-DNA disruptant line shortens the period of mRNA oscillation in multiple clock-controlled genes, but the plants retain rhythmicity (Green and Tobin, 1999). Recently it has been reported that loss of LHY function results in a similar short period phenotype (Song and Carré, 2001). Possibly CCA1 and LHY play redundant roles in the oscillator. Excitingly, the double *cca1 lhy* mutant is arrhythmic in leaf movement, although rapidly damping gene expression rhythms with abnormal phase could be detected with LUC activity (Song and Carré, 2001). This indicates that CCA1 and LHY are necessary for sustained oscillator function.

The model of the oscillator as a feedback loop, or as interconnected loops (Figure 7; Loros and Dunlap, 2001; Reppert and Weaver, 2001; Williams and Sehgal, 2001) makes testable predictions, most obviously reciprocal regulation of negative feedback loop components. Recently, Alabadí and collaborators (Alabadí et al., 2001) have shown that TOC1/APRR1 and CCA1/LHY comprise a feedback loop in which LHY and CCA1 are negative regulators of TOC1 and in which TOC1 acts as a positive regulator of CCA1 and LHY. Overexpression of either LHY or CCA1 results in non-oscillating low-level accumulation of *TOC1* mRNA. Consistent with their roles as negative regulators of *TOC1* expression, CCA1/LHY binding sites have

been identified in the *TOC1* promoter. Conversely, in plants carrying a strong loss-of-function allele of *TOC1* (*toc1-2*), oscillations of *LHY* and *CCA1* mRNA exhibit both the short period characteristic of *toc1* mutations (Millar et al., 1995a; Somers et al., 1998) and greatly reduced mRNA abundance, consistent with a role of *TOC1* as a positive regulator. However, TOC1 has not yet been shown to bind to either *CCA1* or *LHY* promoters, so it remains to be established that this positive regulation is direct. Nonetheless, the clear conclusion to these data is that CCA1, LHY and TOC1/APRR1 participate in a negative feedback loop and represent true components of an Arabidopsis clock.

Additional members of the *CCA1/LHY* family, termed *REVEILLE* (*RVE*), have been identified (CR Andersson & SA Kay, personal communication). Like *CCA1* and *LHY*, at least some *RVEs* (CR Andersson & SA Kay, personal communication) are *CCGs* and oscillate at both mRNA and protein levels. CCA1 binds in circadian fashion to a short element of the *LHCB1*3* (*CAB1*) promoter sufficient to confer phytochrome responsiveness and circadian transcription (Wang and Tobin, 1998). Thus CCA1/LHY/RVE may represent components of the central oscillator as well as components of the output pathway by which the clock regulates transcription (Schaffer et al., 1998; Wang and Tobin, 1998).

Phosphorylation by casein kinase I is critical in Drosophila and mammalian clocks (Lowrey et al., 2000; Reppert and Weaver, 2001; Williams and Sehgal, 2001). Phosphorylation of the FREQUENCY clock component is essential for Neurospora clock function (Loros and Dunlap, 2001) and autophosphorylation of KaiC is essential for cyanobacterial rhythmicity (Iwasaki and Kondo, 2000). Consistent with a critical role of phosphorylation in the Arabidopsis clock, CCA1 binding to DNA is affected by phosphorylation by casein kinase II (CK2) (Sugano et al., 1998), which also phosphorylates LHY *in vitro* (Sugano et al., 1999). Overexpression of the regulatory CKB3 subunit increases CK2 activity, which would be presumed to enhance CCA1 activity. However, CKB3 overexpression results in period shortening, similar to that seen in plants with reduced CCA1 activity (Sugano et al., 1999). This apparent inconsistency probably indicates our incomplete understanding of the role of CCA1/LHY/RVE proteins in the circadian system.

Other genetic approaches have also identified loci with potential roles in the circadian system. There is considerable natural variation in period length, and Quantitative Trait analysis of lines derived from a cross of Cvi to Ler has identified a number of oscillator candidates in Arabidopsis (Swarup et al., 1999). Null mutations of *FLOWERING LOCUS C*, in the autonomous flowering pathway, confer early flowering and shorten the circadian period in leaf movement (Swarup et al., 1999). It is unlikely that we have as yet identified all the potential components of the Arabidopsis oscillator, and further genetic analyses are likely to identify new candidates as well as new and informative alleles of current candidates.

ARE THERE MULTIPLE CIRCADIAN OSCILLATORS?

The unicellular dinoflagellate, *Gonyaulax polyedra* expresses two distinct circadian oscillators. First, it was established in long time courses that two distinct rhythms with different periods actually showed phase crossings (Roenneberg and Morse, 1993) and second, they showed that the two rhythms could be independently reset by a single stimulus (Morse et al., 1994). This has prompted efforts to demonstrate two or more clocks in multicellular organisms. However, one must be careful to distinguish between the existence of distinct clocks in different organs, tissues, or cell types from the existence of two molecular clocks within a single cell. There are a number of observations of multiple rhythms running with different periods within a plant (internal desynchronization). One recent example is in *Phaseolus vulgaris*, where rhythms in CO_2 assimilation and stomatal aperture exhibit a different period from the rhythm in leaf movement (Hennessey and Field, 1992). In Arabidopsis, the free-running periods in leaf movement and *LHCB* (*CAB*) expression are different, although both are shortened by the *toc1-1* mutation (Millar et al., 1995a). However, this type of data can readily be explained by slight differences in clock function in different tissues, as the leaf movement rhythm is expressed in cells of the petiole whereas the *LHCB* transcription rhythm is measured in leaf mesophyll cells. Explants of different organs retain rhythmicity in *LHCB*, *CHS*, and *PHYB* transcription in culture, which confirms that there are multiple self-sustaining and entrainable circadian oscillators (Thain et al., 2000). Do these clocks communicate to mutually entrain one another? Apparently not. The two cotyledons of intact Arabidopsis and tobacco seedlings can be entrained antiphase to one another. In tobacco, the cotyledons could be entrained to phases distinct from the initial phase retained by the shoot apex. Similar results were obtained with two primary tobacco leaves or with roots versus aerial tissues of Arabidopsis. Collectively, these experiments argue compellingly that there are autonomous clocks in different organs and tissues. Moreover, distal and proximal areas of a single primary tobacco leaf could be entrained to distinct phases (Thain et al., 2000). Thus, there are autonomous cellular clocks and these clocks can exhibit heterogeneity at the level of a single organ.

More suggestive of two distinct clocks within a single cell is the observation that, in extended darkness, the period of *LHCB* transcription lengthens to ~30 h whereas the oscillations in *AtGRP7/CCR2* and *AtGRP8/CCR1* (Carpenter et al., 1994; Strayer et al., 2000), and *CAT3* (Zhong et al., 1997; TP Michael and CR McClung, unpublished data) mRNA abundance and transcription retain 24 hr periods. In tobacco seedlings, cytosolic Ca^{2+} and *LHCB* transcription oscillate with different periods (Sai and Johnson, 1999). In each case, it remains to be established that the two rhythms are expressed in the same cells, but it is nonetheless clear that the rhythms are responding to distinct circadian oscillators (Sai and Johnson, 1999). The issue of one or more clocks at a cellular level represents more than an issue of clock esoterica. If we are to make sense of the complex input pathways, the profusion of putative oscillator components, and the myriad clock outputs described above, we must know if all the pieces fit into a single puzzle.

CONCLUSIONS

The preceding Arabidopsis monograph was published only seven years (~2500 circadian cycles) ago. At that time, the chief accomplishment of the Arabidopsis rhythms community had been to establish that Arabidopsis expressed circadian rhythms. We hope that this review demonstrates the great progress that has been made towards the understanding of the Arabidopsis circadian system. Like the rest of the Arabidopsis research community, circadian rhythms have embraced (or been embraced by) the genomics age. We have a real appreciation for the enormous influence of the circadian clock on the biology of the plant. There remains a great deal to be accomplished. Although we certainly have candidates for the central oscillator, we most certainly do not have a detailed molecular understanding of the mechanism of the Arabidopsis clock. We can be optimistic that this is coming. It is just a matter of time.

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