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Minireview

Circadian rhythms in plants: a millennial view

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Circadian rhythms are endogenous rhythms with periods of approximately 24 h. These rhythms are widespread both within any given organism and among diverse taxa. As genetic and molecular biological studies, primarily in a subset of model organisms, have begun to identify the components of circadian systems, there is optimism that we will soon achieve a detailed molecular understanding of circadian timing mechanisms. Although plants have provided many examples of rhythmic outputs, and our understanding of photoreceptors of circadian input pathways is well-advanced, plants have lagged behind other groups of organisms in the identification of components of the central circadian oscillator. However, there are now a number of promising candidates for components of plant circadian clocks, and it seems probable that we will soon know the details of a plant central oscillator. Moreover, there is also accumulating evidence that plants and other organisms house multiple circadian clocks, both in different tissues and, quite probably, within individual cells. This provides an unanticipated level of complexity with the potential for interaction among these multiple oscillators.

Introduction

Most organisms exhibit temporal organization of their activities with respect to the environmental oscillation of day and night, and thus express diurnal rhythms. When deprived of environmental time cues, such as light-dark or temperature cycles, many of these rhythms persist, indicating that organisms have the endogenous capacity to measure time and to use this time information to temporally regulate their biology. Circadian rhythms are a subset of endogenous rhythms with periods of approximately 24 h (Pittendrigh 1981b, Edmunds 1988, Johnson et al. 1998). The deviation of the free-running period from exactly 24 h has been termed the "strongest single piece of evidence that the overt rhythm is under the control of an endogenous timing mechanism" (Feldman 1982) because this finding makes it clear that circadian rhythms are truly intrinsic, and do not result from the organism deriving time cues from some subtle geophysical signal originating with the 24 h period of the rotation of the earth. The period of a circadian rhythm remains relatively constant over the range of physiologically relevant temperatures (exhibits temperature compensation), which makes intuitive sense; a reliable clock should not run faster or slower in response to the vagaries of the weather, or in response to the passage of the sun behind a cloud. However, both abrupt temperature changes or temperature cycles as well as pulses or cycles of light act as potent stimuli that can shift the phase of the clock. The primary effect of the environmental time cues associated with the diurnal cycle is to entrain the endogenous timing system to a period of 24 h (Pittendrigh 1981a), which precisely corresponds to the environmental period resulting from the rotation of the earth on its axis.

The ubiquity of circadian rhythmicity across a broad taxonomic spectrum has prompted the speculation that adaptive fitness is enhanced by the synchronization of an organism's internal clock with the diurnal cycle imposed by its environment. The scientific testing of this hypothesis largely has been avoided and one has been forced to rely on the adage "the early bird gets the worm" in any effort to justify the adaptive fitness of aspects of circadian biology, such as dawn anticipation. However, the study of the circadian biology of cyanobacteria (Johnson et al. 1998, Johnson and Golden 1999) has opened the door to rigorous scientific

Abbreviations – CAM, crassulacean acid metabolism; L:D, light:dark; LUC, luciferase; QTL, Quantitative Trait Locus.

testing of the adaptive significance of circadian rhythmicity in studies that recapitulate evolutionary events in the laboratory. Mutants of *Synechococcus* sp. strain PCC7942, with alterations in period length, have been identified on the basis of the rhythmic expression of a photosynthetic gene fused to bacterial luciferase (*luxAB* from *Vibrio harveyi*), a reporter that allows non-invasive measurement of gene expression in real time (Kondo et al. 1994). Strains with wild type (25 h), short (23 h) or long (30 h) period grow at essentially the same rate in pure culture in either continuous light or in light-dark cycles. However, when these strains are mixed and competed against each other in either 22 h (light:dark [L:D] 11:11), 24 h (L:D 12:12), or 30 h (L:D 15:15) cycles, in each case the strain whose period most closely matches that of the environmental L:D cycle eliminates the competitor (Johnson and Golden 1999). The rapidity with which the successful strain eliminates its competitor implies that the selective coefficient is unexpectedly high. The mechanism of this fitness enhancement remains poorly understood (Johnson and Golden 1999). Nonetheless, it is gratifying to the circadian biologist that the circadian system is significant in Darwinian terms.

It is possible, at least formally, to divide the circadian system into 3 conceptual parts: an input pathway or set of pathways that provide temporal information from the environment to the clock, the central oscillator ('clock') mechanism itself, and an output pathway or set of pathways through which the temporal information provided by the clock is used to generate overt rhythms in various processes (Fig. 1). In practice, the assignment of a component gene or gene product to a role in input, oscillator or output is often complicated as input pathways can themselves be regulated by the clock, and outputs often feedback to regulate inputs. Nonetheless, this conceptual view of the circadian system provides a useful framework within which to organize the consideration of circadian timekeeping. In this review, I will first address the central oscillator mechanism as it has been described in *Drosophila* in order to familiarize the reader with a reasonably well-understood molecular oscillator. Second, I will discuss plant clock outputs, as this will introduce the overt rhythms that have been studied. Then I will examine input pathways, and finally I will return to a consideration of the plant oscillator mechanism.

The oscillator: a negative feedback loop? The paradigm in cyanobacteria, fungi, flies and mammals

For many years the Holy Grail of clock research has been the identification of components of the central oscillator. A combination of mutational analyses, chiefly in *Drosophila*, *Neurospora*, and *Synechococcus*, together with molecular analyses both in these systems and mammals, has yielded a model of the central oscillator as a negative feedback loop in which rhythmic transcription of key clock genes is inhibited by the nuclear accumulation of the protein products of these genes. Please note that in the following description (see Fig. 2) of the *Drosophila* version of this negative feedback cycle, I will only cite those primary references that have been published since the excellent recent reviews by Young (1998) and Dunlap (1999). During the subjective day phase, a heterodimer of the transcription factors *Drosophila* CLOCK (dCLK) and CYCLE (CYC) binds to conserved E-boxes (Hao et al. 1999) to transcriptionally activate *PERIOD* (*PER*) and *TIMELESS* (*TIM*). The dCLK/CYC heterodimer also negatively regulates *dCLK* transcription, although this may be indirect (Glossop et al. 1999). PER is unstable in the cytoplasm, but becomes stabilized by heterodimerization with TIM. Upon reaching a threshold concentration, the PER/TIM heterodimer translocates into the nucleus. This process introduces a time delay that is critical to the generation of a 24-h periodicity. The PER/TIM heterodimer is phosphorylated by DOUBLETIME (DBT), a casein kinase type Iε. The phosphorylated PER/TIM heterodimer negatively regulates the dCLK/CYC heterodimer through formation of a larger complex. This both blocks the positive regulation of *PER* and *TIM* transcription and relieves the negative regulation of *dCLK* transcription (Bae et al. 1998, Glossop et al. 1999). At dawn, the *Drosophila* photoreceptor, CRYPTOCHROME (dCRY), undergoes a photochemical change that allows it to interact with TIM,

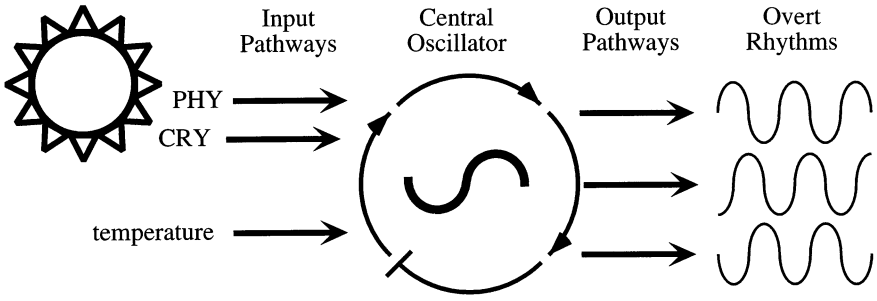

Fig. 1. Conceptual model of a simple circadian system consisting of a set of input (entrainment) pathways, a central oscillator, and a set of output pathways. Entraining stimuli include light, mediated through phytochromes (PHY) and cryptochromes (CRY), and temperature. Although the input pathways are drawn as discrete linear pathways, there are multiple phytochromes and cryptochromes as well as interaction among them and their downstream signaling pathways. The central oscillator is illustrated as a loop, including positive and negative components that yields a self-sustaining oscillation with a period of approximately 24 h. Multiple output pathways are drawn as each regulating an overt rhythm with a distinct phase. The number of output pathways and the degree of interaction among them is not known, although some cross-talk among output pathways is possible.

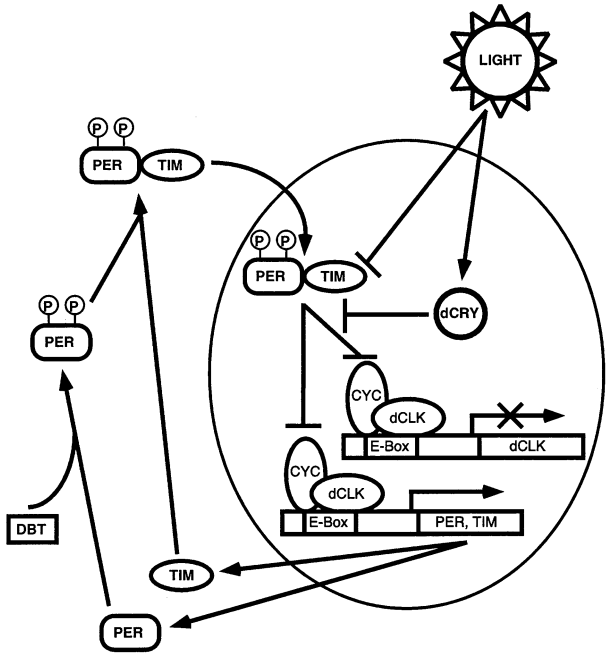

Fig. 2. A model of the *Drosophila* central oscillator. The CYCLE (CYC)/dCLOCK (dCLK) heterodimer binds to E-box promoter elements and is a positive regulator of *PERIOD* (*PER*) and *TIMELESS* (*TIM*) transcription, but is a negative regulator of *dCLK* transcription, indicated by the X through the transcriptional arrow. PER and TIM protein accumulate in the cytoplasm, although PER is destabilized through DOUBLETIME (DBT)-mediated phosphorylation, indicated by the P-containing circles. Heterodimerization of PER with TIM stabilizes PER and allows PER accumulation, although the kinetics of heterodimerization and nuclear localization introduce a lag in the accumulation of PER. The PER/TIM heterodimer complexes with the CYC/dCLK heterodimer, abrogating positive regulation of *PER* and *TIM* transcription and relieving negative regulation of *dCLK* transcription. Light activates CRYPTOCHROME (CRY), which sequesters TIM, which is ubiquitinated and degraded via the 26S proteasome. Removal of TIM from the PER/TIM heterodimers allows renewed transcription through the association of CYC/dCLK heterodimers with E-box containing promoters.

inactivating the PER/TIM complex and relieving the negative regulation of dCLK/CYC transcription of *PER* and *TIM* (Ceriani et al. 1999). TIM is degraded in the light by an ubiquitin-proteasome mechanism (Naidoo et al. 1999).

This affords the view of the *Drosophila* oscillator as comprised of two interlocked feedback loops in which two heterodimers, PER/TIM and dCLK/CYC, are positive regulators of each other and negative regulators of themselves (Glossop et al. 1999). Some key clock components (PER, TIM, dCLK) are regulated by the clock at the level of mRNA accumulation and protein accumulation or activity whereas other key components (CRY, CYC, DBT) do not oscillate. Furthermore, a number of the key components are remarkably well-conserved between *Drosophila* and mammals (Dunlap 1999). In contrast, there is little obvious conservation of these components with those identified to date in the *Neurospora* and cyanobacterial clocks, although *Neurospora* does employ the PAS domain, a protein-protein interaction motif originally identified in PER and also found in dCLK and CYC. Nonetheless, it seems that the basic mechanism of a negative feedback loop inhibiting positive regulators has been conserved and offers a likely paradigm for consideration in our pursuit of the plant central oscillator.

Output rhythms

Growth rhythms

Most studies of plant leaf movements have addressed the pulvinar sleep movements in which cells in the extensor and flexor regions of the pulvinus swell in antiphase (180° out of phase), mediating a circadian oscillation in leaf position (Satter et al. 1990, Engelmann and Johnsson 1998). Swelling is driven by ion (chiefly potassium) fluxes (Kim et al. 1993). These volume changes of flexor and extensor cells persist in protoplasts in constant conditions, providing an excellent system in which to study the roles of second messengers, including calcium and phosphoinositides (Mayer et al. 1997).

It has recently been demonstrated that there are circadian oscillations in cotyledon position in *Arabidopsis* that are thought to arise from oscillations in elongation rates of abaxial and adaxial cells of the petiole (Engelmann et al. 1992, Engelmann and Johnsson 1998). As these oscillations are in elongation rates, I will consider these so-called 'leaf movement' rhythms together with circadian rhythms in hypocotyl and inflorescence stem elongation. Leaf movements of individual seedlings are easily monitored by video imaging (see http://www.dartmouth.edu/ ~ rmcclung/leafmovement.html), allowing a high throughput assay to evaluate the effects of mutations on fundamental clock properties, including period, phase, and temperature compensation. Recently, the rhythm in leaf movement was exploited as the basis for a search for natural alleles that contribute quantitatively (quantitative trait loci, or QTLs) to the circadian period length in *Arabidopsis* (Swarup et al. 1999).

There is a circadian rhythm in the elongation rate of the stem of *Chenopodium rubrum* (Lecharny and Wagner 1984) that is correlated with a rhythm in oleic acid content (Lecharny et al. 1990). More recently, it has been shown that there is a rhythm in floral stem elongation in *Arabidopsis* (Jouve et al. 1998). This oscillation in stem growth rate is correlated with an oscillation in the level of indole-3-acetic acid (IAA) in rosette leaves, but not, curiously enough, in the inflorescence stem itself. Decapitation of the inflorescence stem abolishes elongation; application of IAA, but not the IAA-aspartate conjugate, to the surface of the decapitated stem restores both elongation and the oscillation in the rate of elongation (Jouve et al. 1999). This argues that the rhythm in elongation does not result from rhythmic synthesis of IAA in the shoot apex, but rather reflects a rhythm either in polar transport of IAA or in the ability to elongate in response to IAA. The IAA polar transport inhibitor N-(1-naphthyl)phthalamic acid (NPA) blocks elongation and evidence of a rhythm, indicating that polar transport of auxin from the apex is necessary for elongation, but this does not allow one to distinguish between either rhythmic IAA transport or sensitivity as critical for the overt rhythm in elongation rate.

It has long been known that light signaling leads to a cessation of hypocotyl elongation and this phenotype has been effectively exploited to identify components of the light perception and signal transduction pathways through forward genetic (mutational) analysis. Mutations that reduce photoperception and subsequent signaling yield an elongated hypocotyl phenotype; conversely, overexpression of elements of the phototransduction pathways reduces hypocotyl elongation relative to wild type. An elongation of the hypocotyl also frequently accompanies a disruption of circadian rhythmicity. For example, *early flowering 3* (*elf3*) mutants are conditionally arrhythmic in continuous light and display an elongated hypocotyl (Hicks et al. 1996). It was initially suspected that this reflected alterations in photoperception or transduction of the photic signal. However, mutants overexpressing *LATE ELONGATED HYPOCOTYL* (*LHY*) (Schaffer et al. 1998) or *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) (Wang and Tobin 1998) are arrhythmic and also display elongated hypocotyls, suggesting a more direct link between hypocotyl elongation and the circadian system. Indeed, careful analysis of hypocotyl elongation, using a video imaging system developed to monitor leaf movements, demonstrated that the rate of hypocotyl elongation upon germination is regulated by the circadian clock with an episode of rapid elongation at subjective dusk and a daily growth arrest at subjective dawn (Dowson-Day and Millar 1999). In at least some mutants, the hypocotyl elongation defect may result from a primary dysfunction in the circadian system with a resulting failure to impose a daily period of growth arrest (Dowson-Day and Millar 1999).

Stomatal movements, gas exchange and CO_2 assimilation

Circadian rhythms in stomatal aperture and in the responsiveness of the guard cells to environmental stimuli have been described in a number of systems (Gorton 1990, Webb 1998). In beans, there is circadian control of the underlying biochemical reactions of the Calvin cycle in addition to control of stomatal aperture and gas exchange (Hennessey and Field 1991). Although *Arabidopsis* exhibits a circadian rhythm in the rate of CO_2 fixation (C.R. McClung, unpublished), it is not known if this rhythm includes circadian regulation of the Calvin cycle reactions. Circadian regulation of sucrose metabolism has been demonstrated in tomato (Jones and Ort 1997, Jones et al. 1998).

Rhythms in gene expression: transcriptional rhythms

Circadian control of transcription is widespread (Dunlap 1999). The most extreme example yet described is the cyanobacterium *Synechococcus*, where circadian regulation of transcription may be global (Johnson and Golden 1999, Kondo and Ishiura 1999). It is important to note that the peaks in mRNA abundance of different genes can occur at distinct circadian phases. For example, mRNA abundance of the *CAT2* and *CAT3* catalase genes of *Arabidopsis* peaks at dawn and dusk, respectively (Zhong and McClung 1996). The list of plant genes regulated by the circadian clock is already extensive (Kreps and Kay 1997, Fejes and Nagy 1998). For example, many genes with photosynthetic roles show circadian regulation of transcript abundance (Giuliano et al. 1988, Martino-Catt and Ort 1992, Pilgrim and McClung 1993, Salvador et al. 1993, McClung 1997, Scandalios et al. 1997, Nakahira et al. 1998, Piechulla 1999, McClung et al. 2000). These include genes with roles in light harvesting, oxygen evolution, ATP synthesis, carbon assimilation and photorespiration (Table 1). Although most genes exhibiting circadian oscillations are encoded in the nucleus, there are also oscillations in abundance of chloroplast transcripts in wheat (Nakahira et al. 1998) and in *Chlamydomonas* (Salvador et al. 1993, Hwang et al. 1996). In *Chlamydomonas*, these oscillations are correlated with and may derive from a circadian oscillation in DNA supercoiling in the plastid genome (Salvador et al. 1998). With the imminent completion of the *Arabidopsis* genomic sequence it seems highly probable that a microarray strategy (Kehoe et al. 1999) will soon define the proportion of the *Arabidopsis* genome that is under circadian control at the level of oscillations in mRNA abundance.

Of course, oscillations in mRNA abundance can arise from differential rates of transcription or from differential mRNA stability. To date, studies in plants that have moved beyond the simple description of a circadian oscillation in

Table 1. Photosynthetic genes exhibiting circadian oscillations in transcript abundance.

Role	Gene	Genome	Gene product	Species	Reference
Light harvesting	*psbD*	Nuclear	Photosystem II (PSII) subunit	Wheat	Nakahira et al. 1998
	LHCA	Nuclear	Chlorophyll *a*/*b* binding (PSI)	Tomato	Kellmann et al. 1999
	LHCB	Nuclear	Chlorophyll *a*/*b* binding (PSII)	Many	Piechulla 1999
Oxygen evolution	OEE1	Nuclear	O_2-evolving enzyme protein 1	Tomato	Giuliano et al. 1988
ATP synthesis	*ATPA*	Chloroplast	ATP synthase	*Chlamydomonas*	Salvador et al. 1993
	ATPB	Chloroplast	ATP synthase	*Chlamydomonas*	Salvador et al. 1993
CO_2 assimilation	*RBCS*	Nuclear	Rubisco small subunit	*Arabidopsis*	Pilgrim and McClung 1993
	RBCL	Chloroplast	Rubisco large subunit	*Chlamydomonas*	Salvador et al. 1993
	RCA	Nuclear	Rubisco activase	Tomato	Martino-Catt and Ort 1992
				Arabidopsis	Pilgrim and McClung 1993
Photorespiration	*CAT*	Nuclear	Catalase	Maize	Scandalios et al. 1997
				Arabidopsis	McClung 1997
	SHM	Nuclear	Serine hydroxymethyltransferase	*Arabidopsis*	McClung et al. 2000

mRNA abundance have focused on transcriptional control. The first description of a circadian oscillation in mRNA abundance of a plant gene was of a chlorophyll *a*/*b* binding protein gene (*CAB* or *LHCB*) of pea (Kloppstech 1985). Circadian oscillations in *LHCB* mRNA abundance has proven a general phenomenon (Fejes and Nagy 1998, Piechulla 1999); all 19 of the tested tomato *LHCB* genes showed essentially identical circadian mRNA oscillations (Kellmann et al. 1993). Both nuclear run-on experiments and transcriptional promoter-reporter gene fusions have established a transcriptional component to this regulation in several plant species (Fejes and Nagy 1998, Piechulla 1999). Minimal *LHCB* promoters sufficient to confer circadian transcription have been identified in wheat (Nagy et al. 1988, Fejes et al. 1990), tomato (Piechulla et al. 1998) and *Arabidopsis* (Millar and Kay 1991). These studies were aided enormously by the development of firefly and beetle luciferases (*LUC*) as reporter genes. Typical reporter genes are unsuitable for these studies because the stability of the protein they encode (e.g. β-glucuronidase or chloramphenicol acetyltransferase) is too great to allow turnover within a circadian cycle. Even though mRNA abundance oscillates in response to rhythmic transcription, the reporter activity is stable and accumulates over time, which obscures the underlying rhythm in transcription. Luciferase protein itself is stable and western blot analysis indicates that it accumulates over time. However, luciferase activity (light production) is quite unstable. This renders light production dependent upon de novo translation, thus allowing light production to accurately track the circadian rhythm in *LHCB* transcription (Millar et al. 1992a,b). The measurement of luciferase activity is non-destructive and allows quantitative resolution of gene expression in both temporal and spatial terms in real time in individual seedlings. This has proven a boon to both the definition of *cis*-acting elements and to the identification of mutants with defects in circadian timing.

In vivo analysis of progressively truncated *LHCB1*1* (*CAB2*) promoter fragments fused to the luciferase gene defined a 36-bp region sufficient to confer circadian transcription. In vitro analysis of DNA-binding by electrophoretic mobility shift assays and DNA footprinting identified binding sites for multiple complexes in this short fragment (Anderson et al. 1994, Anderson and Kay 1995, Carré and Kay 1995). A single Myb domain transcriptional activator encoded by the *CCA1* gene that had been previously implicated in phytochrome regulation (Wang et al. 1997) shows circadian binding to an element (consensus AAa/cAATCT) within the functionally defined region of the *LHCB1*1* promoter (Wang and Tobin 1998). CCA and the closely related LHY (Schaffer et al. 1998) define a growing family of single Myb domain proteins that figure prominently in the *Arabidopsis* circadian system, as will be discussed in greater detail below.

Minimal promoter fragments (278 and 119 bp) have also been defined for two tomato *LHCA* genes (Kellmann et al. 1999). A minimal (330 bp) clock-responsive promoter has been defined for the *Arabidopsis RCA* gene, whose expression oscillates in phase with the *LHC* genes (Liu et al. 1996). Each of these promoter fragments includes a consensus CCA1 binding site, although the functional importance of CCA1 binding to the circadian transcription of *LHCA* or *RCA* has not yet been experimentally demonstrated.

In addition to the *LHCB* and *RCA* promoters, which confer morning-specific transcription, 3 dusk-specific promoters have also been defined. To confer circadian transcription with a dusk-specific phase, 490 bp of the *Arabidopsis GER3* promoter are sufficient (Staiger et al. 1999); 265 bp of the *Arabidopsis GRP7* (*CCR2*) promoter confer a robust high amplitude rhythm with dusk-specific phase, and as few as 56 bp are sufficient to confer a low amplitude (about twofold) oscillation (Staiger and Apel 1999). This smallest promoter fragment retains a CCA1 binding site, as does a minimal promoter (230 bp) of the *CAT3* catalase gene that is sufficient to confer dusk-specific transcription (T.P. Michael and C.R. McClung, unpublished). The functional significance of the CCA1 sites in the dusk-specific promoters has not been directly tested, but it is intriguing that the same CCA1 binding site can be found in promoters that are transcribed nearly 180° out of phase with one another. The elucidation of the mechanism by which the phase of transcription is determined is an interesting puzzle that will undoubtedly be the focus of considerable experimental effort.

Rhythms in gene expression: post-transcriptional rhythms

One of the best-defined systems of post-transcriptional regulation is the activation state of phosphoenolpyruvate (PEP) carboxylase. In crassulacean acid metabolism (CAM) plants, PEP carboxylase activity is driven by a circadian rhythm in the phosphorylation state of the enzyme that, in turn, reflects the activity of a protein kinase (Nimmo 1998). PEP carboxylase kinase itself is regulated at the level of translatable mRNA (Hartwell et al. 1996). Phosphorylation is increasingly prominent as a mechanism of circadian post-translational regulation: recent studies demonstrate circadian regulation of sucrose phosphate synthase activity in tomato by protein phosphatase activity (Jones and Ort 1997).

Post-transcriptional regulation can either obscure or contribute to oscillations in mRNA abundance. For example, despite circadian transcription of *LHCB1*3* (*CAB1*), a post-transcriptional mechanism of mRNA stabilization obscures a rhythm in mRNA abundance (Millar and Kay 1991). In contrast, the lack of circadian transcription of the *Arabidopsis NIA2* nitrate reductase gene, as measured by nuclear run-on experiments, suggests that the observed rhythm in mRNA abundance reflects post-transcriptional control (Pilgrim et al. 1993). The chloroplast-encoded *tufA* gene of *Chlamydomonas* exhibits a pronounced circadian oscillation in mRNA abundance (Hwang et al. 1996). There is a transcriptional component to this oscillation seen in light-dark (diurnal) cycles that persists into continuous light and continuous dark. Additionally, there is an oscillation in the stability of *tufA* mRNA in light-dark cycles. Although this oscillation in post-transcriptional regulation does not persist in continuous light and so is apparently not regulated by an endogenous circadian oscillator, this represents a well-documented example of post-transcriptional regulation contributing to an oscillation in gene expression.

Rhythmic synthesis of both luciferase (Mittag et al. 1998) and the luciferin binding protein (LBP) (Mittag et al. 1994) of the dinoflagellate, *Gonyaulax polyedra*, results from circadian regulation of translation of mRNAs that do not oscillate in abundance (Morse et al. 1989, Mittag et al. 1998). Interaction of an RNA-binding protein with the 3′ untranslated region (UTR) is correlated with the translation of *LBP* mRNA (Mittag et al. 1994). Excitingly, a protein capable of binding to this 3′ UTR element is conserved in *Chlamydomonas*, as is the circadian oscillation in abundance of this binding activity (Mittag 1996). This should greatly facilitate the genetic analysis of this post-transcriptional mode of circadian regulation.

In *Arabidopsis*, overexpression of *GRP7* blocks accumulation of the endogenous *GRP7* and *GRP8* transcripts, but does not affect the rhythmic expression of several other genes (Heintzen et al. 1997). This negative effect of GRP7, an RNA-binding protein, on its own transcript abundance is presumably through a mechanism of transcript destabilization because circadian transcription of the *GRP7* promoter is unaffected by overexpression of an ectopic copy of *GRP7* (Staiger and Apel 1999).

Hormone production and responsiveness

The study of plant growth regulators has been complicated for a number of reasons, and now several studies have indicated that the additional complication of circadian regulation can no longer be ignored. Ethylene production shows a circadian rhythm in barley, wheat and rye (Ievinsh and Kreicbergs 1992), *Chenopodium rubrum* (Machácková et al. 1997) and sorghum (Morgan et al. 1997, Finlayson et al. 1998). In sorghum, it has been shown that this rhythm in ethylene production reflects underlying rhythms in mRNA abundance for the *ACO2* gene encoding 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase and in ACC oxidase activity (Finlayson et al. 1999). It was earlier mentioned that there is a circadian oscillation in rosette leaf auxin levels in *Arabidopsis* (Jouve et al. 1999). Through heroic effort, a diurnal rhythm in gibberellin (GA) biosynthesis was demonstrated in sorghum (Foster and Morgan 1995); although this study did not address continuous conditions, it seems quite reasonable to suspect that there may be circadian control over production of GA. Thus, circadian control has been demonstrated over production of certainly two and probably 3 of the major plant hormones, and examples with other hormones may be forthcoming. It is well established that there is circadian control over the sensitivity of plants to light, for example, at the level of stomatal aperture (Webb 1998) and in the induction of *LHCB* and *CAT* gene expression (Millar and Kay 1996, Zhong et al. 1998). It seems quite possible that plants will exhibit circadian rhythmicity in their responsiveness to hormones. This might contribute to the rhythm in stomatal aperture, even in the absence of a rhythm in abscisic acid levels. Similarly, the rhythm in inflorescence stem elongation in *Arabidopsis* in the absence of rhythmic IAA levels (Jouve et al. 1999) might reflect rhythmic responsiveness to auxin.

Calcium

Free Ca^{2+} levels, monitored by aequorin luminescence in transgenic tobacco and *Arabidopsis*, oscillate with a circadian rhythm in both the cytosolic and chloroplastic compartments (Johnson et al. 1995). This has significant implications for all 3 aspects of circadian regulation, including both input and output pathways and the central oscillator itself. Ca^{2+} is a ubiquitous second messenger in plant signaling pathways and has been implicated in phytochrome-mediated light signal transduction (Barnes et al. 1997) as well as in UV-B and UV-A/blue light-mediated signaling (Christie and Jenkins 1996, Frohnmeyer et al. 1998, Long and Jenkins 1998). Pulses of blue light induce transient spikes in cytosolic, but not in chloroplastic Ca^{2+}, and these transients are implicated in signaling downstream of NON-PHOTOTROPIC HYPOCOTYL 1 (NPH1) in the phototropic response (Baum et al. 1999). Ca^{2+} also plays a critical role in guard cell signaling and so is implicated in the circadian regulation of stomatal aperture and gas exchange (McAinsh et al. 1997, Leckie et al. 1998).

Photoperiodism

The shift from vegetative to reproductive growth is a critical and tightly regulated decision. Flowering time in many species, including *Arabidopsis*, is environmentally-regulated by photoperiod, light quality and vernalization (Koornneef et al. 1998, Levy and Dean 1998, Lumsden and Millar 1998, Piñeiro and Coupland 1998). *Arabidopsis* is a quantitative long day plant because flowers are initiated more rapidly under long than under short days. Mutations at many loci affect the timing of flower initiation. These mutants can be broadly grouped as early flowering or late flowering. Late flowering mutants are more numerous, which may reflect the preponderance of genetic studies in the common lab ecotypes Columbia (Col) and Landsberg *erecta* (Ler), both of which are relatively early flowering. Genetic analyses suggest 3 different pathways promoting flowering: one constitutive, one environmentally-responsive to photoperiod and to light quality, and one responding to vernalization. Each pathway negatively regulates a default state of floral repression.

In 1936, Bünning hypothesized that the circadian clock was required for photoperiod sensitivity in flowering (Bünning 1936), and physiological and more recent genetic data from *Arabidopsis* have corroborated his insight (Lumsden and Millar 1998). Mutations that affect circadian rhythmicity frequently also affect flowering time. For example, *timing of CAB 1* (*toc1*) and *elf3* show defects in flowering time, circadian gene expression and circadian leaf movement (Millar et al. 1995, Hicks et al. 1996, Somers et al. 1998b), and *early in short days 4* (*esd4*) shows defects in both flowering time and leaf movement (Carré 1998). Both LHY and CCA1 overexpressing mutants are late flowering (Schaffer et al. 1998, Wang and Tobin 1998). Conversely, this suggests that mutations affecting the photoperiodic pathway of flowering induction are candidates for also conferring circadian defects. At least two examples of the utility of this approach have been recently described. Mutations at the *GIGANTEA* (*GI*) locus confer late flowering. *GI* has been recently cloned

(Fowler et al. 1999, Park et al. 1999) and *GI* transcript abundance oscillates with a circadian rhythm that is altered in *elf3*, *lhy* and *CCA1* overexpressing lines. Mutations in *gi* affect rhythmicity of *CCA1* and *LHY* transcripts (Fowler et al. 1999, Park et al. 1999). A second example is seen with null mutations of *FLOWERING LOCUS C* (*FLC*, also known as *FLF*), a repressor of flowering that encodes a MADS domain protein (Michaels and Amasino 1999, Sheldon et al. 1999), which confer early flowering and also shorten the circadian period in leaf movement (Swarup et al. 1999). Collectively, these analyses suggest that there is an intimate interaction between the circadian clock and photoperiodic timing and that the many mutations identified on the basis of flowering time defects will also contribute additional components of the circadian system.

Input to the clock

Light

By definition, circadian rhythms persist in the absence of external time cues, but are entrainable to the environment. The best-characterized input to the circadian clock is light. Light pulses shift clock phase (Pittendrigh 1981a, Johnson 1992), although the resetting mechanisms vary among different organisms. For example, light resets the *Neurospora* clock through an induction of *FREQUENCY* (*FRQ*) transcription (Crosthwaite et al. 1995, 1997), whereas light resets the *Drosophila* clock through increased TIM protein degradation (Young 1998, Naidoo et al. 1999) and through a sequestration of TIM by CRY (Ceriani et al. 1999).

A great deal is known about light perception in plants (Chory 1997, Batschauer 1998, Casal et al. 1998, Whitelam and Devlin 1998), although little is specifically known about phototransduction to the clock, beyond the identity of key photoreceptors. Light pulses phase shift clock-controlled gene expression in wheat by a very low fluence phytochrome response (Nagy et al. 1993). In addition to the acute effects of pulses of light upon circadian phase, continuous applications of light of specific intensities and spectral qualities can influence period length (Pittendrigh 1981a). A painstaking analysis of the effects of either red or blue light of various fluence rates on the period length of the circadian rhythm in *LHCB*(*CAB2*)::*LUC* expression has established that phytochrome B (PHYB) is the high-intensity red light photoreceptor for the clock whereas PHYA is the low-intensity red light photoreceptor (Somers et al. 1998a). CRY1 is the high-intensity blue light photoreceptor for the clock, and both PHYA and CRY1 serve as low-intensity blue light photoreceptors (Somers et al. 1998a). CRY2 (initially defined genetically as the *fha* late flowering mutant) is specifically implicated in the timing of flowering (Guo et al. 1998) and also contributes to the establishment of circadian period (Somers et al. 1998a).

The understanding of the downstream signaling pathways from PHY and CRY is far from complete (Deng and Quail 1999). Nucleoside diphosphate kinase as well as G-proteins that function through cGMP or Ca^{2+} and Ca^{2+}-calmodulin pathways are implicated in red light signaling (Barnes et al. 1997, Choi et al. 1999), and phosphorylation and Ca^{2+} are implicated in blue light signaling (Jenkins 1997, Lasceve et al. 1999). Recent studies have established a direct molecular interaction between PHYA and CRY1 in vitro (Ahmad et al. 1998), offering a rationalization for co-action or synergism between these signaling molecules (Mohr 1994). Both PHYA and PHYB bind directly to PHYTCHROME INTERACTING FACTOR 3 (PIF3), a PAS-domain putative bHLH transcription factor (Ni et al. 1998, Halliday et al. 1999) and, critically, binding to PHYB is light-regulated with the phytochrome signature of induction in response to red light and reversibility by far red light (Ni et al. 1999). It is intriguing that both phytochrome and PIF3 are PAS-domain proteins, given the prominence of PAS-domain proteins in circadian systems of fungi, flies and mammals (Dunlap 1999). PHYTOCHROME KINASE SUBSTRATE 1 (PKS1) is a substrate for light-regulated phosphorylation by PHYA and PHYB (Fankhauser et al. 1999). Other downstream signaling components are specific to PHYA or to PHYB (reviewed by Deng and Quail 1999, von Arnim 1999). However, little is known of the signal transduction cascade(s) leading specifically to the circadian clock (Kreps and Kay 1997, Somers 1999). *elf3* was identified genetically on the basis of a daylength-insensitive early flowering phenotype. *elf3* exhibits conditional arrhythmicity of both leaf movement and *LHCB* gene expression in continuous light, but shows normal clock function in continuous dark (Hicks et al. 1996). Thus, *ELF3* is interpreted as encoding a component of a light input pathway as opposed to a component of a central oscillator.

Temperature

It is well established that temperature is as effective an entraining stimulus as light (Bünning 1973), and recent data have established that temperature is a stronger entraining stimulus than light in *Neurospora* (Liu et al. 1998). Temperature provides a strong entraining input to rhythms in CO_2 assimilation in CAM plants (Wilkins 1992). The mechanism of temperature resetting in CAM plants is complex and incompletely understood, but may involve alterations in compartmentalization of malate (Grams et al. 1997). Cyclic heat shocks can entrain mRNA oscillations in barley seedlings (Kloppstech et al. 1991) and, more recently, temperature cycles have been shown to entrain *LHCB* transcription in *Arabidopsis* (Somers et al. 1998b). Cold pulses reset the phase of the oscillations in *LHCB* and *CCR2* (*AtGRP7*) mRNA (Kreps and Simon 1997). However, the temperature step from 4 to 22°C, associated with release from stratification, did not reset the circadian clock in *Arabidopsis*, suggesting that very young seedlings are refractory to this temperature step (Zhong et al. 1998). In chilling sensitive plants, such as tomato, it seems that cold pulses actually stop the clock (Martino-Catt and Ort 1992, Jones et al. 1998).

Other inputs to the central oscillator

A circadian oscillator is running in etiolated seedlings that have not been exposed to light or temperature cycles or

pulses. For example, circadian oscillations have been detected in *LHCB* mRNA abundance in etiolated wheat (Nagy et al. 1993) and tobacco (Kolar et al. 1995), and in *Arabidopsis LHCB1*1* (*CAB2*) transcription monitored either in etiolated transgenic tobacco seedlings (Millar et al. 1992a, Anderson et al. 1994) or in *Arabidopsis* (Millar and Kay 1996). The amplitude of the acute induction of both *LHCB* and *CAT2* mRNA abundance by light varies strikingly according to the timing (phase) of the onset of illumination, which indicates that a circadian clock running in etiolated seedlings regulates (gates) the induction of *LHCB* and *CAT2* by light (Millar and Kay 1996, Zhong et al. 1998). The acute induction of *CAT2* mRNA varies with time after imbibition, indicating that imbibition provides a signal capable of resetting the circadian clock, although the nature of this signal remains unknown (Zhong et al. 1998).

The oscillator in plants

Genetic and molecular approaches

The forward genetic approach (the hunt for mutants that affect key clock properties, such as period length) has been effective in the identification of clock components in other systems (Dunlap 1999, Johnson and Golden 1999). In plants, this genetic approach has emphasized *Arabidopsis*. A genetic screen based on alterations in rhythmic expression of a luciferase (*LUC*) transgene driven by regulatory elements of the *LHCB1*1* (*CAB2*) gene identified a series of *timing of CAB* (*toc*) mutations that disrupt clock function (Millar et al. 1995). The best-described of these mutants is *toc1-1*, which shortens the period of multiple rhythms, including *LHCB* transcription (Millar et al. 1995), *GRP7* (*CCR2*) mRNA accumulation (Kreps and Simon 1997), leaf movement, and stomatal conductance (Somers et al. 1998b). In addition, *toc1-1* affects the photoperiodic flowering response, although this effect depends strongly on ecotype. The most consistent aspect of the *toc1-1* flowering phenotype is a reduction of the difference between flowering times in long versus short days (Somers et al. 1998b). Additional *toc1* alleles as well as other non-allelic *toc* loci have been identified, but none of the genes identified by these mutations has yet been cloned. To date, TOC1 protein is probably the strongest known candidate for a component of the central circadian oscillator, although the rigorous testing of this hypothesis will require molecular manipulation that can only be attempted once the *TOC1* gene is cloned. A QTL strategy, based on segregation of natural variation in the circadian period length of leaf movement, has identified a number of oscillator candidates in *Arabidopsis*, including *NON TROPPO*, *ANDANTE* (possibly *FLC*), and *ESPRESSO* and *RALENTANDO*, one of which may be *GI* (Swarup et al. 1999).

late elongated hypocotyl (*lhy*) was identified in a screen for late flowering mutations among an *Arabidopsis* population carrying the maize Ac/Ds transposon system and was cloned by virtue of the molecular Ds tag (Schaffer et al. 1998). The Ds-tagged *lhy* mutants are functionally LHY overexpressors in which the Ds element has inserted upstream of *LHY*, resulting in constitutive high level expression (Schaffer et al. 1998). The most prominent feature of the LHY deduced amino acid sequence is a single 47 residue region related to the helix-turn-helix DNA-binding domain of the Myb family (Schaffer et al. 1998). This region is 87% identical to the corresponding region of CCA1, which was identified by virtue of its ability to bind to the DNA element of the *LHCB1*1* promoter shown to be sufficient to confer robust circadian oscillations on reporter genes (Carré and Kay 1995, Wang et al. 1997). The similarity between LHY and CCA1 extends beyond the Myb domain; they share 3 other regions, each of 20–25 amino acids, that have at least 80% identity (Schaffer et al. 1998, Wang and Tobin 1998). CCA1 and LHY are the founding members of a sub-family of plant Myb proteins that have only single copies of the Myb motif, in contrast to the vast majority of the numerous plant Myb proteins [*Arabidopsis* has more than 80 (Romero et al. 1998)] in which the Myb domain is repeated.

The phenotype of plants carrying the dominant *lhy* allele is pleiotropic and similar to the phenotype of plants overexpressing CCA1 (CCA1-ox) (Schaffer et al. 1998, Wang and Tobin 1998). Overexpression of either LHY or CCA1 delays flowering, consistent with disruption of the rhythmic expression of the late flowering gene, *GI* (Fowler et al. 1999), and results in elongated hypocotyls. *CCA1* and *LHY* mRNA abundance oscillates with a circadian rhythm in wild type plants, but becomes arrhythmic in overexpressing mutants. Constitutive overexpression of either CCA1 or LHY results in arrhythmicity of multiple clock outputs, including mRNA abundance of all clock-regulated genes tested to date and leaf movement (Schaffer et al. 1998, Wang and Tobin 1998). These results, together with the sequence similarity between LHY and CCA1, suggest that they might redundantly specify central oscillator functions. However, loss of CCA1 function in a T-DNA disruptant line shortens the period of mRNA oscillation in at least 3 clock-controlled genes (*LHCB*, *LHY* and *CAT2*) and indicates that CCA1 and LHY cannot be fully redundant (Green and Tobin 1999). Moreover, CCA1 function cannot be required for oscillator function, because the *cca1* null plants are rhythmic (Green and Tobin 1999).

Are either CCA1 or LHY components of a central oscillator? A set of criteria to define components of the circadian oscillator have been proposed (Aronson et al. 1994) and provide a helpful, if imperfect set of guidelines (see Foster and Lucas 1999): (1) the activity (possibly, but not necessarily, the simple abundance) of a component should oscillate with appropriate periodicity in the absence of external time cues; (2) blocking the oscillation in the activity of a clock component should abolish normal rhythmicity; (3) mutations in a clock component should affect canonical clock properties, such as period length or temperature compensation, and null mutations should eliminate normal rhythmicity; (4) induced changes in a component's activity should, by feedback, change the component's activity; (5) the overt rhythm must be reset by induced changes in the activity of a clock component; (6) the phase of component's oscillation should be reset by shifts in the light-dark regimen that reset the clock.

For example, the mRNA and protein abundances of *GRP7* oscillate, satisfying criterion 1 (Heintzen et al. 1997).

However, overexpression of GRP7 in transgenic *Arabidopsis* blocks the mRNA oscillation of *GRP7* and *GRP8*, but does not affect other circadian oscillations, satisfying criterion 5, but failing criterion 2. Thus, GRP7 is interpreted to be a key component of a slave (non-self-sustaining) oscillator, but not of a central oscillator (Heintzen et al. 1997).

What then of *CCA1* and *LHY*? *CCA1* clearly plays a role as an output component regulating circadian rhythmicity as well as phytochrome responsiveness of *LHCB* transcription (Wang et al. 1997). If *CCA1* were an oscillator component, then the output pathway from the oscillator to the *LHCB* hand of the clock would be exceedingly short! It is also possible that *CCA1* is playing roles on both output and input pathways (McClung 1998). If one systematically rates *CCA1* and *LHY* by the criteria outlined above, both oscillate at mRNA and protein levels and so both satisfy criterion 1. Overexpression studies indicate that both *CCA1* and *LHY* satisfy criterion 2. In each case, overexpression feeds back to repress expression of the endogenous gene, which satisfies criterion 4. The *cca1* null mutation retains rhythmicity and, thus, *CCA1* fails criterion 3. However, criterion 1 assumes that oscillator functions cannot be redundantly specified, which seems unnecessarily restrictive. The remaining criteria have not been tested. The use of inducible transgenes to provide pulses of CCA1 and LHY should address criterion 5. Criterion 6 should also be testable through phase shifting experiments.

However, it is important to recall that these criteria are imperfect (Foster and Lucas 1999). Although the *Neurospora FRQ* locus satisfies these criteria (Aronson et al. 1994), recent experiments have established the presence of a temperature entrainable circadian oscillator persisting in *frq* null mutants and it is possible to reinterpret the *FRQ* oscillator as participating in an input pathway (Roenneberg and Merrow 1998, Merrow et al. 1999). Nonetheless, it seems highly unlikely that anyone would argue that *FRQ* is unimportant to the *Neurospora* circadian system. It may be that, at least in the short term, one may have to be content with the determination that any particular component is important in the circadian system of an organism and defer a decision on residence in input, oscillator or output compartments until one has a fuller understanding of the system in question. Clearly *CCA1* and *LHY* (as well as *TOC1* and *ELF3*) play important roles in the circadian system of *Arabidopsis*.

As discussed earlier, the flowering time genes *GI* and *FLC* also contribute to circadian timing. *flc* null mutations shorten the period length in leaf movement (Swarup et al. 1999), but at this time there has been insufficient experimental manipulation of the expression of *FLC* to allow one to draw conclusions about potential roles of *FLC* in the circadian system. *gi* mutants are altered in leaf movement and gene expression rhythms of *GI* itself, and of *LHCB*, *LHY* and *CCA1* (Fowler et al. 1999, Park et al. 1999). In *gi-2*, a null allele, the period of leaf movement is shortened, but the period of gene expression rhythms gradually lengthens (Park et al. 1999). Thus, it seems unlikely that GI represents an oscillator component. The period shortening effect of *gi-1* on gene expression rhythms is less severe in extended dark than in continuous light. In addition, the extension of period length seen in light of decreasing fluence is less pronounced in *gi-1* than in wild type (Park et al. 1999). Collectively, these data suggest that GI acts on a light input pathway. However, *GI* mRNA oscillates with a circadian rhythm, making *GI* a clock output. Thus, one might best interpret GI as part of an outer feedback loop necessary to sustain both amplitude and period length of a central oscillator (Park et al. 1999).

Phosphorylation

Differential phosphorylation of key clock components (PER, TIM and FRQ) occurs throughout the circadian cycle in *Drosophila* and *Neurospora* (Young 1998, Dunlap 1999). In *Drosophila*, *DBT* encodes a casein kinase Iε activity responsible for the PER phosphorylation, and *dbt* null mutants are arrhythmic (Kloss et al. 1998, Price et al. 1998). Is there a role for phosphorylation in the activity of CCA1 or LHY? A yeast two-hybrid screen for *Arabidopsis* proteins that interact with CCA1 identified a regulatory β subunit of casein kinase II, CKB3 (Sugano et al. 1998). In vitro experiments showed that CCA1 interacts with two other casein kinase β subunits, CKB1 and CKB2, as well as with two casein kinase catalytic (α) subunits, CKA1 and CKA2. CK, as well as a CK2-like activity from *Arabidopsis* whole cell extracts, phosphorylates CCA1 in vitro, although this phosphorylation does not affect the ability of recombinant CCA1 to bind to its DNA target in electrophoretic mobility shift assays. Treatment of plant extracts with λ protein phosphatase abolished the formation of the major CCA1-DNA complex, as did treatment of the extracts with CK2 inhibitors (Sugano et al. 1998). Overexpression of the CKB3 regulatory subunit in transgenic *Arabidopsis* increases CK2 activity, resulting in early flowering and shortening of the period of the oscillations in mRNA abundance of *CCA1* and *LHY*, as well as of 4 clock-controlled genes (*LHCB1*1*, *CCR2*, *CAT2* and *CAT3*) (Sugano et al. 1999). Clearly the serine-threonine kinase activity of CK2 affects clock function, presumably via regulation of CCA1 activity, although there may be other key targets (Sugano et al. 1999).

More than one clock?

A single cell, *Gonyaulax polyedra*, can house two distinct circadian oscillators (Roenneberg and Morse 1993, Morse et al. 1994). Have multiple oscillators been demonstrated in multicellular plants? The rhythms in CO_2 assimilation and stomatal aperture exhibit a different period from the rhythm in leaf movement in *Phaseolus vulgaris*, which indicates that they are being driven by different oscillators, although this may represent a single oscillator mechanism exhibiting a difference in period between different organs (Hennessey and Field 1992). The *toc1-1* mutation in *Arabidopsis* shortens the periods in *LHCB* transcription and leaf movement to different degrees (Millar et al. 1995). Similarly, the *gi-2* mutation shortens the period in leaf movement, but lengthens the period in gene expression (Park et al. 1999). In neither example have the two rhythms been simultaneously measured in the same plant, but the data suggest that distinct oscillators, presumably in the distinct tissues, are driving the two rhythms (Millar 1998). In extended dark-

ness, the period of *LHCB* transcription lengthens to about 30 h whereas the oscillations in mRNA abundance of *CAT3* (Zhong et al. 1997), *CCR1* (*GRP8*) and *CCR2* (*GRP7*) (Carpenter et al. 1994) retain 24 h periods. Here the genes are expressed in a single organ, the leaf, but it remains to be established that the genes are being expressed within the same cell. It has been recently shown that, in tobacco seedlings in continuous red light, rhythms in cytosolic Ca^{2+} and *LHCB* transcription exhibit different periods (Sai and Johnson 1999). Again, it is difficult to establish that these 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). It is also apparent that the rhythm in Ca^{2+}, as measured by Sai and Johnson, cannot be responsible for the rhythmic transcription of *LHCB* (Sai and Johnson 1999), even though Ca^{2+} has been implicated in phytochrome-mediated transcriptional control (Barnes et al. 1997).

Conclusions and perspectives

It is apparent that the relatively simple model of the circadian system presented in Fig. 1 is inadequate. Input pathways may change in sensitivity over the circadian cycle. Outputs can feedback to provide input to the clock. Components can play multiple roles on input and output pathways, and perhaps in the central oscillator itself. Moreover, we do not yet have reliable criteria with which to unambiguously assign molecules to roles as input, output or oscillator components (Roenneberg and Merrow 1998, Merrow et al. 1999). Indeed, even the concept of a single central oscillator has been revealed as inaccurate, as it is certain that a single cell can contain two self-sustaining circadian oscillators (Roenneberg and Morse 1993) as well as non-self-sustaining slave oscillators (Heintzen et al. 1997). Whether there are commonly multiple oscillators in a single cell or whether a single molecular oscillator will behave differently in distinct cell populations, tissues or organs remains to be determined in most organisms. Nonetheless, it is evident that circadian systems are complicated. Although this will be disappointing to those who had hoped that the cloning of one or two key 'clock genes' would solve the puzzle, the rest of us are left with the exhilarating challenge of unraveling a reticulate network of output feedback loops providing input to interlocked oscillators.

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