CIRCADIAN RHYTHMS IN PLANTS

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Key Words biological clocks, cryptochrome, flowering time, photoperiodism, phytochrome

■ Abstract Circadian rhythms, endogenous rhythms with periods of approximately 24 h, are widespread in nature. Although plants have provided many examples of rhythmic outputs and our understanding of photoreceptors of circadian input pathways is well advanced, studies with plants have lagged in the identification of components of the central circadian oscillator. Nonetheless, genetic and molecular biological studies, primarily in Arabidopsis, have begun to identify the components of plant circadian systems at an accelerating pace. There also is accumulating evidence that plants and other organisms house multiple circadian clocks both in different tissues and, quite probably, within individual cells, providing unanticipated complexity in circadian systems.

CONTENTS

DEDICATION
INTRODUCTION
A BASIC MODEL OF THE PLANT CIRCADIAN SYSTEM
RHYTHMIC OUTPUTS 141
Movement and Growth Rhythms 141
Stomatal Aperture, Gas Exchange and CO ₂ Assimilation
Hormone Production and Responsiveness
Calcium
Rhythms in Gene Expression
ENTRAINMENT (INPUT) 145
Light
Temperature
Imbibition and Others
THE OSCILLATOR: A Negative Feedback Loop
Single Myb Domain DNA-Binding Proteins 148
TOC Genes
WHEN DOES TIMING BEGIN? 151
HOW MANY CLOCKS?

ARE CIRCADIAN CLOCKS OF ADAPTIVE SIGNIFICANCE?	153
CONCLUDING REMARKS	154

DEDICATION

This review is dedicated to the memory of Richard C Crain (1951–1998), Dartmouth Class of 1973, a pioneer in the study of inositol phospholipids in plant signal transduction and an enthusiastic advocate of second messenger and circadian rhythms research.

INTRODUCTION

It is often opined that death and taxes are the only two inescapable aspects of the human existence, but Ernest Hemingway correctly noted that "The Sun Also Rises" (50). Indeed, the daily rotation of the earth on its axis has meant that biological evolution has occurred in an environment that changes drastically every day. It should come as no surprise that, since much of an organism's biochemistry, physiology, and behavior is temporally organized with respect to the environmental oscillation of day and night, most organisms express diurnal rhythms. It is less obvious that many of these rhythms should persist in the absence of environmental time cues (e.g. light:dark or temperature cycles). However, organisms from cyanobacteria to humans endogenously measure time and temporally regulate aspects of their biology. This review focuses on recent advances in our understanding of the molecular bases of plant circadian rhythms.

Circadian rhythms are defined by three fundamental parameters: periodicity, entrainability, and temperature compensation. Although daily environmental changes drive diurnal rhythms, a true circadian rhythm persists in the absence of environmental time cues with a free-running period of approximately 24 h (Figure 1). Environmental time information from the daily rotation of the Earth on its axis, such as light:dark and temperature cycles, entrains the oscillation to precisely 24 h. Experimentally, one can entrain circadian oscillations to non-24 h periods with imposed environmental cycles. An intriguing characteristic of circadian rhythms is that the period of the rhythm is temperaturecompensated and remains relatively constant over a range of physiological temperatures, in sharp contrast to the temperature dependence of most biochemical processes.

The earliest known account of a circadian rhythm dates from the fourth century BC, when Androsthenes, in descriptions of the marches of Alexander the Great, described diurnal leaf movements of the tamarind tree (101). The endogenous nature of leaf movement rhythms was experimentally demonstrated in the eighteenth century (24, 28). The deviation of the endogenous period from exactly 24 h was

first described for the free-running period of leaf movements in the nineteenth century (23). Now, at the dawn of the twenty-first century, we are finally unraveling the molecular details of plant circadian systems.

A BASIC MODEL OF THE PLANT CIRCADIAN SYSTEM

Formally, one can divide the circadian system into three conceptual parts: input pathways that entrain the clock, the central oscillator (clock), and output pathways to generate overt rhythms (Figure 2). I first address the output pathways in order to introduce the assays that feature in the analysis of plant clocks. Then I discuss input pathways and consider the central oscillator and the exciting recent progress in elucidating the oscillator mechanism in plants.

RHYTHMIC OUTPUTS

One of the attractions of plants as model clock systems is the myriad rhythmic outputs, or "hands" of the clock. The clock times (gates) different overt rhythms to distinct times of day (phase angle). I do not attempt an exhaustive survey as plant rhythmic processes have been reviewed in detail (89, 94, 139, 148).

Movement and Growth Rhythms

These include the classic system of pulvinar leaf movements, in which 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 (32). Swelling is driven by volume changes resulting from ion fluxes (69). This provides an excellent system in which to study the roles of second messengers including calcium and phosphoinositides (43, 93).

There are also rhythms that reflect growth rate, chiefly cell elongation. For example, inflorescence stems of Arabidopsis (66) exhibit a circadian oscillation in elongation rate 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. Decapitation of the inflorescence stem abolishes elongation but application of IAA to the decapitated stem restores rhythmic elongation, implicating a rhythm either in polar transport of IAA or in the ability to elongate in response to IAA and excluding rhythmic synthesis of IAA in the shoot apex (65). 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. Arabidopsis also exhibits a circadian rhythm in the rate of hypocotyl elongation (27). Although defective inhibition of hypocotyl elongation has been a staple of screens for photoperception mutants, the hypocotyl elongation defect may also result from a primary dysfunction in the circadian system with a resulting failure to impose a daily period of growth arrest (27).

There is also 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 (32). Leaf movements of individual seedlings are easily monitored by video imaging, providing the basis of a search for natural alleles that contribute quantitatively (quantitative trait loci, or QTLs) to period length in Arabidopsis (147).

Stomatal Aperture, Gas Exchange and CO₂ Assimilation

Circadian rhythms in stomatal aperture are well documented (157) and are correlated with a circadian rearrangement of guard cell cytoskeleton (40). In beans there is circadian control of Calvin cycle reactions in addition to control of stomatal aperture and gas exchange (51). Arabidopsis exhibits a circadian rhythm in the rate of CO₂ assimilation (EV Kearns & CR McClung, unpublished), but circadian regulation of the Calvin cycle has not been investigated. Circadian rhythms of CO₂ assimilation in Crassulacean Acid Metabolism (CAM) have been extremely well studied, and the molecular mechanism is understood in considerable detail (111). There is a rhythm in the transport of malate across the tonoplast (111). In addition, flux through PEP carboxylase (PEPc) is regulated by reversible phosphorylation; at night PEPc is phosphorylated and less sensitive to inhibition by malate. Although second messengers typically regulate kinases, PEPc kinase from Kalanchoë fedtschenkoi is unusual in that it lacks regulatory domains. The circadian oscillation in PEPc kinase activity stems purely from a rhythm in protein abundance that requires de novo protein synthesis, which reflects a circadian oscillation in transcript accumulation (47, 48).

Hormone Production and Responsiveness

In addition to the circadian oscillations in auxin levels and transport/sensitivity described above (65, 66), ethylene production exhibits circadian rhythmicity in a number of species (34, 56). In sorghum there are underlying rhythms in mRNA abundance for the *SbACO2* gene encoding 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase and in ACC oxidase activity (35). It is possible, although not established, that the diurnal oscillation demonstrated in gibberellic acid levels in sorghum is truly circadian (36). It is likely that more hormones will exhibit circadian rhythms in production. More interesting and challenging is the potential rhythmicity of hormonal responsiveness. Components of the biosynthetic machinery, of the perception and signaling mechanisms, or of the response pathways could be targets of circadian regulation.

Calcium

 Ca^{2+} plays a critical role in guard cell signaling (79, 136) and so is suspected in the circadian regulation of stomatal aperture and gas exchange. Because Ca^{2+} is

a ubiquitous second messenger in plant signaling pathways (132) and has been implicated in red and blue light signal transduction (6, 7, 39, 87), it is possible that Ca^{2+} plays a role in the entrainment of the circadian oscillator as well as in the regulation of clock-controlled gene expression. Indeed, external application of either Ca^{2+} or a Ca^{2+} ionophore phase shifts the leaflet movement rhythm of *Robinia pseudoacacia* (43). Intriguingly, free cytosolic and possibly chloroplastic Ca^{2+} levels, monitored by aequorin luminescence, oscillate with a circadian rhythm in tobacco and Arabidopsis (62). The light to dark transition stimulates a spike in chloroplast stromal Ca^{2+} levels (62), although whether this signals the circadian clock is not known.

Rhythms in Gene Expression

The list of plant clock-controlled genes (CCGs; see 33, 94, 139) has expanded considerably since Kloppstech's (71) original observation of a circadian oscillation in mRNA abundance of a chlorophyll a/b binding protein gene (LHCB or CAB). This list continues to grow (75, 95) and it seems likely that microarray analysis should soon identify most genes showing circadian oscillations in mRNA abundance. Initial estimates suggest that from 5% to 6% of Arabidopsis genes are rhythmically expressed (46a). This is a far cry from the apparent universality of circadian regulation of transcription in the cyanobacterium Synechococcus (85). but suggests that there are between \sim 1250 and 1500 Arabidopsis CCGs, based on a current estimate of \sim 25,000 Arabidopsis genes (154). Of course, the biological material used to generate the hybridization probes limits the detection of oscillating transcripts to those that are regulated in those tissues at the developmental stage under the specific growth conditions sampled, and it will take many iterations to exhaustively sample all possible developmental stages and environmental conditions. Nor will these initial experiments identify genes whose induction or repression in response to environmental or biological stimuli is gated by the clock.

Although most genes exhibiting circadian oscillations are nuclear, a number of *Chlamydomonas* plastid transcripts show circadian oscillations (55, 129) that are correlated with a circadian oscillation in DNA supercoiling in the plastid genome (130). The plastid-encoded *psbD* gene oscillates robustly in wheat (107). This oscillation, as well as light regulation, is dependent on an atypical -35 promoter element and it is hypothesized that transcription of this gene requires a plastid-encoded RNA polymerase and a nuclear-encoded sigma factor that itself is a CCG (107). Consistent with this hypothesis, transcription of nuclear-encoded sigma factor genes is circadian in Arabidopsis and wheat (67, 102). This echoes the clock regulation of a *Synechococcus* sigma factor (153) and, moreover, offers a mechanism for temporal coordination between the nuclear and plastid genomes.

Circadian oscillation of *LHCB* mRNA abundance is widespread, if not universal, among angiosperms (33, 116), although not gymnosperms (11). Both nuclear run-on experiments and transcriptional gene fusions establish a transcriptional component to this regulation in several angiosperms (33, 116). Typical reporters are unsuitable for circadian studies. Even though mRNA abundance oscillates in response to clock-gated transcription, the reporter activity (e.g. β -glucuronidase or chloramphenicol acetyltransferase) is too stable to allow turnover within a circadian cycle, and the accumulation of reporter activity obscures the underlying rhythm in transcription. Luciferase (LUC) protein is stable and accumulates over time, but LUC activity (light production) is unstable; activity over time requires translation of new LUC protein and provides a reliable assessment of *LUC* transcription (99). The measurement of LUC activity is nondestructive and quantitative and allows both temporal and spatial resolution of gene expression in real time in vivo.

Minimal nuclear promoters sufficient to confer maximal circadian transcription at a mid-morning phase have been identified for several *LHCB* genes (33, 116), tomato *LHCA* genes (68), and the Arabidopsis *RCA* gene (86). Of course, the rates of maximal transcription of different genes occur at distinct circadian phases (times of day) and a number of different phase angle markers are available (Figure 1). For example, mRNA abundance of the *CAT2* and *CAT3* catalase genes of Arabidopsis peaks at dawn and dusk, respectively (162). We have defined a minimal *CAT3* promoter sufficient to confer evening-specific circadian transcription (TP Michael & CR McClung, unpublished; see Figure 1). Evening-specific promoters have also been defined for the Arabidopsis genes encoding a glycinerich RNA-binding protein (*ATGRP7/CCR2*) and a germin-like protein (*AtGER3*) (142–144). As is discussed below, many genes implicated in the input and central oscillator mechanisms are themselves *CCGs*. It will soon be possible to target the expression of one's favorite gene to a particular time of day with the same precision that sets of tissue- and cell type–specific promoters afford for spatial expression.

In vivo functional analysis of progressively truncated LHCB1*1 (CAB2) promoter fragments fused to luciferase 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 (16, 33). The CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) gene that had been previously implicated in phytochrome regulation (155) encodes a single Myb domain protein that shows circadian binding to an element (consensus AAa/cAATCT) within the functionally defined region of the LHCB1*1 promoter (156). This CCA1-binding element is also found in the functionally defined minimal LHCA and RCA promoters (68, 86), although the functional importance of CCA1 binding to the circadian transcription of LHCA or RCA has not yet been established. Curiously, sequences closely related to the CCA1-binding consensus are also found in the functionally defined minimal evening-specific AtGRP7/CCR2 (142) and CAT3 promoters (TP Michael & CR McClung, unpublished). Again, the functional significance of these elements has not been established, but that CCA1 binding sites are in promoters that are transcribed nearly 180° out of phase suggests 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.

Not all regulation of gene expression is transcriptional. In addition to the oscillation in phosphorylation and dephosphorylation of PEPc in CAM plants (111), sucrose phosphate synthase activity in tomato is regulated circadianly by a protein phosphatase (63). The rhythm in nitrate reductase (NR) mRNA abundance in Arabidopsis reflects posttranscriptional control, as shown by the failure to detect transcriptional oscillations in nuclear run-on experiments (117). In tomato, the circadian oscillation in NR mRNA is blocked by a protein phosphatase inhibitor, although the precise targets of phosphorylation and dephosphorylation remain unknown (64).

ENTRAINMENT (INPUT)

Circadian rhythms persist in the absence of external time cues but are entrainable to the environment. It has long been clear that clock response to environmental stimuli varies over the circadian cycle. A plot of the magnitude of the phase shift resulting from the application of a given stimulus at a series of discrete times spanning a circadian cycle yields the phase response curve (PRC), a powerful tool with which to study the circadian oscillator (59, 60).

Light

Although many environmental parameters provide stimulus to the clock, the most potent and best-characterized entraining stimulus in plants is light. Light perception in plants has been studied and reviewed in detail (17, 26, 82, 105, 108). The Arabidopsis genome includes five phytochrome genes (*PHYA-PHYE*) and two cryptochrome genes (*CRY1* and *CRY2*). There are other blue light receptors, including phototropin (NPH1) and possibly zeaxanthin, thought to be the stomatal blue light receptor (10).

Period length is inversely related to light intensity (parametric, or continuous, entrainment) in plants and animals that are active in the light (3). In Arabidopsis, PHYA and PHYB as well as CRY1 and CRY2 contribute to the establishment of period length (100, 139a). PHYB is important at high intensities of red light whereas PHYA functions at low intensities (139a). CRY1 functions at high intensities of blue light and both PHYA and CRY1 function at low intensities (139a). Double mutant studies also demonstrate a role for CRY2 in the establishment of period, although that role is redundantly specified by CRY1 (PF Devlin & SA Kay, personal communication). PHYA and CRY1 interact at the molecular level and CRY1 can be phosphorylated by PHYA (2). Direct interaction between PHYB and CRY2 in vivo has been established by Fluorescence Resonance Energy Transfer (91a).

Red light pulses (nonparametric, or discrete, entrainment) phase shift clockcontrolled gene expression by a very low fluence PHYA response (73, 104). Far red light pulses phase shift in a PHYA-dependent fashion (160). A bacteriophytochrome, CikA, provides light input to the cyanobacterial clock, and *cikA* mutants show dramatic alterations in phase angle of multiple gene expression rhythms (135). Similarly, novel alleles of Arabidopsis PHYB and CRY1 do not affect the period but instead alter the phase angle of multiple rhythms, indicating that PHYB and CRY1 contribute to the establishment of circadian phase as well as period (PA Salomé & CR McClung, unpublished). Light phase response curves are available for a number of angiosperms (60). Two types of light phase response curves have recently been generated in Arabidopsis. High-intensity red light pulses given upon a dim red background shift the phase of *LHCB::LUC* transcription (S Panda & SA Kay, personal communication). *AtGRP7/CCR2* transcription oscillates in extended dark without damping (144), which has allowed the generation of a phase response curve for pulses of red, blue, or white light over a dark background (S Panda & SA Kay, personal communication).

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. Indeed, *PHYB* expression (both mRNA accumulation and transcription, as monitored with *PHYB*::*LUC* fusions) is rhythmic in tobacco and Arabidopsis, although it is important to note that bulk PHYB protein abundance does not oscillate (9). Recently, this result has been extended to other photoreceptors: In Arabidopsis, expression of *PHYA*, *PHYC*, and *CRY1* shows robust circadian oscillations at both mRNA abundance and transcriptional levels. Expression of *CRY2* is not rhythmic whereas *PHYD* and *PHYE* expression is, at most, weakly rhythmic (L Kozma-Bognár & F Nagy, personal communication). That the clock may regulate its own sensitivity to entraining stimuli complicates use of the PRC to probe the state of the oscillator.

The understanding of the downstream signaling pathways from PHY and CRY is incomplete. Various signaling intermediates (e.g. cGMP and Ca²⁺-calmodulin) and phosphorylation are implicated (10, 26, 82), and a number of signaling components downstream from the photoreceptors have been identified (10, 26, 82). 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 (91), which establishes that light signaling pathways can be unexpectedly short. This is relevant to light input to circadian clocks because the targets of PIF3 include the promoters of CCA1 and LATE ELONGATED HYPOCOTYL (LHY) (91), two putative oscillator components (see below). The plant G box (CACGTG) is related to the animal E box (CANNTG) targeted by heterodimeric transcription factors of Drosophila and mammalian central oscillators (29, 46). However, the binding of PIF3 to G boxes of light and clock-regulated promoters is likely to represent only part of a complicated signaling network entailing multiple pathways and targets. For example, it has recently been established that PHYA and PHYB signaling target distinct regions of the Arabidopsis LHCB1*2 promoter (160). Similarly, phytochrome and circadian regulation target distinct elements of the tomato LHCA3 gene (120).

The timing of flowering in many species is regulated by photoperiod as well as by light quality and vernalization (81, 138). Bünning (14) 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 (81, 138). Conversely, flowering timing mutants constitute a reservoir of potential circadian clock mutants. Null mutations of *FLOWER*-*ING LOCUS C*, in the autonomous flowering pathway, confer early flowering and shorten the circadian period in leaf movement (147). Two mutations in the Arabidopsis 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.

elf3 loss-of-function alleles yield early flowering, hypocotyl elongation, and conditional arrhythmicity in continuous light (53). *ELF3* is a *CCG* encoding a nuclear protein that contains a glutamine-rich motif, suggesting it is a transcription factor; both mRNA and protein abundance oscillate (84). Genetic experiments suggest substantial redundancy in ELF3 and PHYB function (123). Interestingly, ELF3 interacts with PHYB in the yeast two-hybrid assay (21) and plays a key role in the regulation of light input to the clock (95a).

GI is a CCG whose transcript abundance oscillates with a circadian rhythm that is altered in a number of mutants affected in clock function, including *elf3*. gi mutants are altered in leaf movement and gene expression rhythms of GI itself and of other CCGs, including LHCB, LHY, and CCA1 (38, 114). In gi-2, a null allele, the period of leaf movement is shortened but the period of gene expression rhythms gradually lengthens (114). 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. Collectively, these data are consistent with GI acting in light input rather than in a central oscillator (114). gi was independently identified on the basis of a defect in inhibition of hypocotyl elongation in red but not in far red light, which implicates GI in PHYB signaling (54). GI is localized to the nucleoplasm, which is consistent with a role in early PHYB signaling and in the transcriptional regulation of CCGs, although the GI sequence lacks any motifs that might suggest it is a transcription factor (54). However, 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 vet gi mutants are late flowering, which is opposite to the early flowering phenotype of phyB null alleles. This may suggest that GI plays different roles at different developmental stages or may simply indicate our incomplete knowledge of the signaling pathways leading to the hypocotyl and flowering responses (54).

Temperature

Although the circadian oscillator is temperature compensated, temperature pulses or temperature steps are potent entraining stimuli. Temperature pulse PRCs have been generated for several plants (60). Temperature cycles entrain Arabidopsis rhythms in *LHCB* (141) and *CAT3* transcription (TP Michael & CR McClung, unpublished). Curiously, the temperature step associated with release from stratification at 4°C to growth at 22°C was ineffective in phase resetting in Arabidopsis (163), suggesting a refractory period before temperature is capable of entraining the Arabidopsis oscillator. This is quite similar to observations that a light-insensitive circadian oscillator is detected shortly after germination of tobacco and Arabidopsis (72, 73).

Imbibition and Others

Although germinating seedlings are refractory to temperature and light input, the timing of imbibition (hydration) of the dried seed serves as a novel entraining stimulus synchronizing the clocks within populations of Arabidopsis seedlings (163). Other entraining stimuli that have been used to generate PRCs in various plants include abscisic acid, cAMP, and various antimetabolites and amino acid analogs (60).

THE OSCILLATOR: A Negative Feedback Loop

Genetic and molecular biological analyses in a variety of systems suggest that the central oscillator is a negative feedback loop (29, 57, 161) or, as emerging evidence from eukaryotic systems indicates, two interlocked feedback loops (42, 80, 137). Rhythmic transcription of key clock genes is inhibited by the nuclear (in eukaryotes) accumulation of the protein products of these genes (29, 94). For example, in Neurospora, FREQUENCY (FRQ) negatively autoregulates by preventing its own transcriptional activation by the WHITE COLLAR (WC-1/WC-2) heterodimer. However, FRQ also positively regulates rhythmic WC-1 translation from nonoscillating *WC-1* mRNA (80). Protein stability, phosphorylation, ubiquitination, and degradation via the proteasome also play roles in the intertwined negative feedback loops (29, 57, 94).

This leaves the clear expectation that the plant clock will emerge as a negative feedback loop or, more likely, interlocked loops, although this model almost certainly represents an oversimplification (57, 78, 96, 125). There is a great deal of conservation among the components of the fly and mammalian clocks (29) but the PAS domain, a protein-protein interaction domain (149), is the only element that has been found in all clock systems. Happily, a growing number of putative components of plant clocks have recently been identified. No clear picture has yet emerged, but it is apparent that many of the themes of other clock systems are conserved in plants (Figure 3). At present, two myb transcription factors, CCA1 and LHY, and a pseudo response regulator, TOC1, are strong candidates as canonical clock components of interlocked feedback loops, although the molecular details of these loops remain unknown.

Single Myb Domain DNA-Binding Proteins

CCA1 and LHY are closely related single Myb domain DNA-binding proteins (134, 155, 156). Additional members of this family, termed REVEILLE (RVE),

have been identified, and a single Myb domain related to that of CCA1 has been identified in an Arabidopsis pseudo response regulator, APRR2 (90). CCA1, LHY (134, 156), and 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*1 (CAB2) promoter sufficient to confer phytochrome responsiveness and circadian transcription. Overexpression of CCA1 or LHY or several RVEs results in elongated hypocotyls, late flowering, and abolishes several circadian rhythms, including LHCB transcription and leaf movement. Consistent with roles as components of negative feedback loops, both CCA1 and LHY negatively autoregulate, although the mechanism is unknown (134, 156). CCA1 loss of function shortens the circadian period of several CCGs but does not confer arrhythmicity, suggesting that there is redundancy of CCA1specified clock functions (45). 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 (134, 156). That PIF3 binds to the CCA1 and LHY promoters provides a mechanism for phytochrome input into the clock (91).

CCA1 DNA binding is affected by phosphorylation by casein kinase II (CK2) (145), which also phosphorylates LHY in vitro (146). Phosphorylation by casein kinase I is critical in Drosophila and mammalian clocks (29, 88, 161), and autophosphorylation of the cyanobacterial clock protein, KaiC, is essential for rhythmicity (57). Overexpression of the regulatory CKB3 subunit increases CK2 activity, which would be presumed to enhance CCA1 activity. However, CKB3 overexpression results in period shortening and early flowering, similar to that seen in plants with reduced CCA1 activity (146). This apparent inconsistency probably indicates our incomplete understanding of the role of CCA1/LHY/RVE proteins in the circadian system. For example, promoters transcribed at different phases (e.g. LHCB versus CAT3 or AtGRP7/CCR2) contain very similar CCA1 binding targets. The specification of circadian phase may entail differential binding by different members of this family of proteins at distinct circadian phases. Alternatively, phase specification may involve differential modification (quite likely by phosphorylation but other modifications are possible) of family members at distinct circadian phases. There may also be different interacting partners recruited to the promoters that modulate CCA1/LHY/RVE function. Finally, it has long been known that Drosophila Krüppel, for example, can act either as an activator or as a repressor of transcription when present at different concentrations (133). Clearly, a great deal remains to be learned about CCA1, LHY, and their relatives.

TOC Genes

A genetic screen on the basis of alterations in rhythmic expression of a *CAB2* (*LHCB*)::*LUC* transgene in Arabidopsis has identified a series of *timing of CAB* (*toc*) mutations that disrupt clock function (97). *toc1-1* shortens the period of multiple rhythms, including *LHCB* transcription, leaf movement, and stomatal conductance, and results in early flowering (141). Interestingly, the early flowering

phenotype of *toc1* is rescued in 21 h light:dark cycles that mimic the shortened *toc1* period (144). 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; the period of this oscillation is shortened by the *toc1-1* mutation, indicating that TOC1 feeds back to control its own oscillation (144). Collectively, these data suggest that TOC1 is likely to be a component of an oscillator, although it is curious that *TOC1* mRNA oscillations damp rapidly in extended darkness (144), yet a number of gene expression rhythms, including *AtGRP7/CCR2* (76, 144) and *CAT3* (164; TP Michael & CR McClung, unpublished), persist robustly in the dark.

TOC1 encodes an Arabidopsis pseudo-response regulator (APRR1) (90, 144), which implicates signal transduction through two-component systems (128) in clock function. Typically, 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, like other APRRs, lacks the invariant phosphor-accepting Asp residue and is unlikely to function in the conventional His-Asp relay (90, 144). Nonetheless, this suggests a mechanistic link to cyanobacterial clocks (57, 61), in which the sensory histidine kinase, SasA, interacts with the oscillator component KaiC (58).

TOC1 also has a carboxy-terminal motif seen in the CONSTANS family of transcriptional activators (121) and an acidic region often found in transcriptional activators. TOC1/APRR1 interacts with a PIF3-like protein (90), which suggests a mechanism by which the clock might regulate acute induction by light or gate its sensitivity to light input (98, 163). Moreover, TOC1/APRR1 was also identified as an ABSCISIC ACID INSENSITIVE 3-interacting protein (77), which might indicate an interaction of the clock with abscisic acid (ABA) as an input or provide clock regulation of ABA responses.

The gene identified by a second of these *toc* mutants, *ZEITLUPE* (*ZTL*, identified as *toc7*), has recently been cloned (70a, 140) and is a member of a three-gene family including *FKF* (*FLAVIN-BINDING KELCH REPEAT F-BOX*) (109) and *LKP2* (*LOV DOMAIN KELCH PROTEIN*). *ztl* mutants exhibit lengthened period length (140) whereas *fkf* mutants exhibit altered waveform in *CCA1* and *LHCB* mRNA oscillations (109). Both mutants flower late. *FKF* but not *ZTL* mRNA abundance oscillates with an evening-specific maximum. These proteins have an aminoterminal PAS (also called LOV, for light oxygen voltage) domain most similar to those of NPH1, the phototropism blue light receptor (19), an unusual phytochrome from a fern (112) and Neurospora WC-1 (4). In NPH1, this domain binds the flavin chromophore (20), suggesting that these proteins may serve as photoreceptors or on a light input pathway. This is supported by the fluence rate dependence of the *ztl* phenotype (140).

ZTL, FKF, and LKP2 also contain multiple kelch repeats, which define a propeller-like structure that functions in protein-protein interaction (1). A significant clue to the function of these three proteins is that they each contain an F-box, a domain that recruits target proteins to E3 ubiquitination complexes (115).

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; 131) and auxin responses (TIR; 44). That the ZTL/FKF/LKP2 proteins are involved in the light-regulated ubiquitination and degradation of critical clock proteins is an attractive hypothesis. For example, Drosophila TIM is degraded in the light by an ubiquitin-proteasome mechanism; TIM degradation is correlated with changes in phosphorylation (106), which is required for substrate recognition by F-box proteins (22). As described above, Arabidopsis CCA1 is phosphorylated by CK2, a serine-threonine kinase (145, 146). Is phosphorylation and degradation of CCA1, LHY or another key target part of the circadian oscillation (Figure 3) and might this explain the phenotypic results (short period and late flowering) of CKB3 overexpression?

WHEN DOES TIMING BEGIN?

The circadian clock regulates multiple outputs throughout plant growth and development. How early in development can one demonstrate clock activity? In mammals, the circadian clock starts to function during late fetal and early postnatal life and is entrained by maternal signals (124). In zebrafish, mRNA abundance of the clock gene Per3 exhibits circadian oscillations throughout embryonic development (as early as 40 h postfertilization), although rhythmic expression of an output gene, Rev-erb α , exhibits a developmental delay (25). In plants, a circadian rhythm has been detected in the respiration rate of dry onion seeds in continuous dark (13), although we have been unable to detect similar rhythms in Arabidopsis (EV Kearns & CR McClung, unpublished). However, a variety of studies confirm that a circadian clock is functioning upon germination. For example, circadian oscillations have been detected in transcription and mRNA abundance of a number of genes in both etiolated and light-grown seedlings (33), and as little as a single light pulse is sufficient to induce circadian oscillations of LHCB mRNA abundance in etiolated seedlings (33). Moreover, light inducibility of LHCB and CAT2 in etiolated Arabidopsis seedlings is gated by the clock (98, 163). As mentioned above, imbibition entrains Arabidopsis seedlings, although it is not known whether it initiates clock function or synchronizes oscillators that were functioning embryonically (163). Nonetheless, this means that the circadian clock is running from the time of imbibition. Interestingly, temperature steps capable of entraining the clocks of older plants (76, 141) have no effect in these young seedlings (163). It is well established that seed germination of many species is affected by light treatment mediated through phytochrome. Thus, one of the critical sensory transduction systems that provides input to the clock is functional in the seed. However, phytochrome-regulated expression of LHCB genes in Arabidopsis seedlings is preceded by a period in which expression is light independent (12). LHCB genes in very young tobacco seedlings are regulated by two circadian rhythms with distinct phases of maximal transcription and mRNA accumulation, only one of which is phytochrome responsive (72, 73). The light-independent oscillation is expressed from germination, and short light pulses from 12 to 44 h after sowing induce a second oscillation without affecting the first light-independent oscillation. Repeated red light pulses given 60 h after sowing synchronize the two rhythms, but earlier pulses that induced the second oscillation fail to synchronize the two oscillations. Apparently, the light-insensitive clock of very young seedlings either acquires light-responsiveness during development or is supplanted by the light-responsive clock that becomes active after germination (73).

HOW MANY CLOCKS?

Considerable evidence supports the existence of multiple oscillators in multicellular plants. Most of the evidence takes the form of multiple rhythms running with different periods (internal desynchronization), which was demonstrated in Phaseolus coccineus (92) and Chenopodium rubrum (70) in the 1970s. In Phaseolus vulgaris, rhythms in CO₂ assimilation and stomatal aperture exhibit a different period from the rhythm in leaf movement (52). Similarly, in Arabidopsis the free-running periods in leaf movement and LHCB (CAB) expression are different, although both are shortened by the toc1-1 mutation (97). Moreover, the gi-2 mutation shortens the period in leaf movement but lengthens the period in gene expression (114). In extended darkness, the period of LHCB transcription lengthens to \sim 30 h whereas the oscillations in AtGRP7/CCR2 and AtGRP8/CCR1 (15, 144), and CAT3 (164; TP Michael & CR McClung, unpublished) mRNA abundance and transcription retain 24 h periods, again suggesting that they are driven by distinct oscillators. Tobacco seedlings exhibit rhythms in cytosolic Ca²⁺ and LHCB transcription with different periods (127). In each case, 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. Although these data indicate that distinct oscillators drive the rhythms with different periods, they cannot distinguish between the presence of two distinct molecular oscillators within a single cell or a single oscillator that exhibits organ- or cell type-specific differences in period. The demonstration of two oscillators within a single cell is not simple, but has been achieved in the dinoflagellate, Gonyaulax polyedra (103, 126). First, these authors established in long time courses that two distinct rhythms with different periods actually showed phase crossings (126) and second, they showed that the two rhythms could be independently reset by a single stimulus (103).

A recent study has unambiguously demonstrated that explants of different organs retain rhythmicity in *LHCB*, *CHS*, and *PHYB* transcription in culture, establishing firmly the existence of multiple self-sustaining and entrainable circadian oscillators (150). Furthermore, the two cotyledons of intact Arabidopsis and tobacco seedlings could be entrained to novel phases that are antiphase to one another and, in tobacco, distinct from the initial phase retained by the shoot apex, indicating that the clocks were not coupled. Similar results were obtained with two primary tobacco leaves or with roots versus aerial tissues of Arabidopsis. Collectively, these experiments argue compellingly for autonomy of the clocks of different organs and tissues.

The circadian rhythms of cyanobacteria and of unicellular eukaryotes, such as Chlamydomonas and Gonyaulax, make it clear that a circadian clock (or clocks) can exist within a single cell and several mammalian cell types retain a functional circadian oscillator in culture (5, 30, 151). Thus, we can assume that every cell in a multicellular plant potentially contains a clock. Are these clocks coordinated or are they cell-autonomous? Thain et al (150) showed that distal and proximal areas of a single primary tobacco leaf could be entrained to distinct phases! This suggests that clock autonomy at all levels of the circadian system, from photoperception through gene expression output rhythms, exists at a cellular level. However, the authors note that short-range signaling like that induced by phytochrome (8) remains to be addressed. Nonetheless, there quite clearly is no systemic phototransduction signal that coordinates the plant circadian system on an organismal scale. This contrasts sharply with the situation in animals, where a central neural oscillator (e.g. the mammalian suprachiasmatic nucleus or the ventral lateral brain neurons of Drosophila) regulates behavioral rhythms (29). In recent years, it has become clear that peripheral tissues of a variety of animals, including Drosophila, iguanas, and zebrafish, contain multiple additional clocks that can be independently entrained in culture (41, 119, 152, 158). In vivo, these peripheral clocks are probably entrained by coupling pathways from the central neural oscillator (159).

ARE CIRCADIAN CLOCKS OF ADAPTIVE SIGNIFICANCE?

Why are circadian rhythms ubiquitous? Is adaptive fitness enhanced by the synchronization of an organism's internal clock with the diurnal cycle imposed by its environment? The "escape from light" hypothesis (118) posits advantage in phasing sunlight-sensitive cellular events to the night. Cell division in unicellular organisms is frequently gated to the dawn, with DNA replication occurring in the preceding night (31). The adaptive fitness of aspects of circadian biology such as dawn anticipation has been addressed by recent studies with cyanobacteria (113) and green algae (110).

Mutants of the cyanobacterium *Synechococcus elongatus* PCC 7942 with alterations in period length have been identified on the basis of the rhythmic expression of a photosynthetic gene fused to bacterial luciferase (74). 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 light:dark cycles of 22, 24, or 30 h, in each case the strain whose period most closely matches that of the imposed cycle rapidly eliminates the competitor (113). Although the mechanism of this fitness enhancement in *Synechococcus* remains poorly understood (61),

the "escape from light" hypothesis has received strong support from recent studies with the green alga *Chlamydomonas*. *Chlamydomonas* exhibits circadian rhythms in cell division and in sensitivity to UV irradiation (110). Maximal UV sensitivity occurs at the end of the day and beginning of the night, coincident with DNA division. This is entirely consistent with the idea that circadian clocks evolved under selective pressure to time DNA replication to the night. The widespread role of cryptochrome in circadian systems of mammals, flies, and plants is also consistent with this reasoning. Cryptochromes are related to and probably evolved from DNA photolyases, which play a critical role in the repair of UV-induced DNA damage (18). Although it remains speculative, it seems plausible that an ancestral protein that contributed to the daily repair of UV-induced DNA damage was recruited into the circadian system.

CONCLUDING REMARKS

The study of plant circadian clocks has matured in recent years and a great deal of progress has been made, particularly in the identification of molecular components. The next and more difficult phase will be to assemble the components into a coherent molecular model. Obviously, the relatively simple models of the circadian system presented in Figures 2 and 3 are inadequate. Some clock functions are redundantly specified, as shown by the loss-of-function ccal and lhy alleles. In addition, there are likely to be many interconnections among both input and output pathways. We now have good evidence that some genes encoding light input pathway components are themselves CCGs whose abundance and, probably, activity are modulated over the circadian cycle; outputs can feed back to affect input to the clock. Components can play multiple roles on input and output pathways, and perhaps in the central oscillator(s). Moreover, we do not yet have reliable criteria with which to unambiguously assign molecules to roles as input, output, or oscillator components (37, 96, 125). Indeed, even the concept of a single central oscillator is inadequate, as it is certain that a single cell can contain two self-sustaining circadian oscillators (103, 126) as well as non-self-sustaining oscillators (49), and there is good evidence in plants as well as in animals for tissue- and cell-specific oscillators that can run independently (150). We can anticipate this breakneck pace of advancement in our understanding of plant clocks will continue; the timing could not be better.

ACKNOWLEDGMENTS

I thank many colleagues for sharing unpublished information and apologize to those whose work could not be cited due to space limitations. I particularly thank Todd Michael, Patrice Salomé, Tom Jack, and Mary Lou Guerinot for helpful discussion. Work in my lab is supported by grants from the National Science Foundation (MCB 9723482 and IBN-9817603).

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Figure 1 Characteristics of circadian rhythms illustrated using the circadian oscillation in luciferase activity of Arabidopsis seedlings carrying either a *CAB2::LUC* transgene (yellow) or a *CAT3::LUC* transgene (orange). Under entraining conditions (12 h light:12 h dark, indicated by the white and black bars, respectively) the rhythms exhibit 24 h periods. The peak in luciferase activity for each rhythm maintains constant phase relationship with dawn. The peak in *CAB2::LUC* activity occurs ~4–6 h after dawn whereas the peak in *CAT3::LUC* activity occurs ~4–6 h after dawn whereas the peak in *CAT3::LUC* activity occurs ~4–6 h after dawn whereas the peak in *CAT3::LUC* activity occurs ~10–12 hours after dawn. The amplitude of the rhythm is defined as one half of the peak to trough difference. Both rhythms persist when the seedlings are released into continuous conditions (constant dim light), although the period lengthens to reveal the endogenous free-running period of ~25 h. This results in the peaks in luciferase activity shifting with respect to subjective dawn as defined by the entraining 12:12 light:dark cycle (indicated by the gray and white bars, respectively).



Although not indicated, different oscillators may drive separate rhythms with distinct periods. The number of output pathways and the degree of Figure 2 A simple (linear) conceptual 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). 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. 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 h. The double-headed arrows indinteraction among them is not known, although some cross talk among output pathways is possible. Some outputs may be driven by individual cate possible coupling between the oscillators. Multiple output pathways are drawn as each regulating an overt rhythm with a distinct phase. oscillators whereas others may receive input from more than one oscillator.



Figure 3 A speculative model of an Arabidopsis circadian clock. Light input via phytochromes and cryptochromes (PHYA/CRY1 and PHYB/CRY2 complexes are shown, although other configurations are likely to occur) is mediated through ELF3 and GI, or through PIF3. PHYA-PIF3 and PHYB-PIF3 interactions are known to occur. 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. CCA1/LHY/RVE and FKF/LKP2/ZTL are clustered, although there is no evidence that they form molecular complexes. Components on the internal circular arrows oscillate in mRNA or protein abundance. *FKF* but not *ZTL* mRNA oscillates, so FKF is indicated closest to the circular arrows. 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 emanate from the oscillator to input components known to be regulated by the clock at transcriptional, mRNA abundance or protein abundance levels.