The Circadian System in Higher Plants

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Abstract

The circadian clock regulates diverse aspects of plant growth and development and promotes plant fitness. Molecular identification of clock components, primarily in *Arabidopsis*, has led to recent rapid progress in our understanding of the clock mechanism in higher plants. Using mathematical modeling and experimental approaches, workers in the field have developed a model of the clock that incorporates both transcriptional and posttranscriptional regulation of clock genes. This cell-autonomous clock, or oscillator, generates rhythmic outputs that can be monitored at the cellular and whole-organism level. The clock not only confers daily rhythms in growth and metabolism, but also interacts with signaling pathways involved in plant responses to the environment. Future work will lead to a better understanding of how the clock and other signaling networks are integrated to provide plants with an adaptive advantage.

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INTRODUCTION

As one adage has it, the only constant is change. A striking example is the regular alterations in the environment caused by the daily rotation of the earth on its axis. Along with the obvious diurnal changes in light and temperature, other important environmental variables such as humidity also change on a daily basis. This periodicity in the geophysical world is mirrored by daily periodicity in the behavior and physiology of most organisms. Examples include sleep/wake cycles in animals, developmental transitions in filamentous fungi, the in-

cidence of heart attacks in humans, and changes in organ position in plants. Many of these daily biological rhythms are controlled by the circadian clock, an internal timer or oscillator that keeps approximately 24-hour time. Less obviously, the circadian clock is also important for processes that occur seasonally, including flowering in plants, hibernation in mammals, and long-distance migration in butterflies. In fact, circadian clocks have been found in most organisms that have been appropriately investigated, ranging from photosynthetic bacteria to trees.

Circadian Rhythms Defined

Circadian rhythms are generated by circadian clocks. Examples of such rhythms can be seen at the cellular level, such as changes in gene expression, and at the whole-organism level, such as changes in activity. Such processes are defined as outputs of the circadian clock rather than mere responses to environmental cues if they meet the following criteria. First, circadian rhythms persist with approximately (but never exactly) 24-hour periodicity after an organism is transferred from an environment that varies according to the time of day (entraining conditions) to an unchanging environment (free-running conditions). Second, the time of onset of these rhythms can be reset by appropriate environmental cues, such as changes in light or temperature levels. Finally, circadian rhythms are temperature compensated; that is, they occur with approximately the same periodicity across a wide range of temperatures. This final characteristic allows the circadian system to keep accurate time even when ambient conditions are cold or hot.

Circadian rhythms often take the form of sinusoidal waves that can be described by mathematical terms such as period, phase, and amplitude (**Figure 1**). When assayed under entraining conditions, these rhythms usually assume the same period as the changing environmental cues. When assayed in free-running conditions, the non–24-hour periodicity of the endogenous circadian clock is revealed (**Figure 1**). Environmental cues, such as light, can reset the clock

and may also affect the rhythmic amplitude of clock outputs (**Figure 1**).

By circadian convention, the time of onset of a signal that resets the clock is defined as zeitgeber ("time giver") time 0, abbreviated ZT0. After the last transition to lights on in **Figure 1**, ZT0–ZT12 represents subjective day, the time when the organism was exposed to light during entrainment cycles, whereas ZT12–ZT24 represents subjective night.

Circadian Physiology is Similar in Diverse Organisms

Circadian rhythms have been studied intensively in plants since the 18th century (animals were not recognized to have circadian clocks until the 20th century; see Reference 72 for an excellent summary of the history of clock research in plants). Physiological experiments performed in a variety of model organisms, including plants, animals, fungi, and cyanonobacteria, revealed fundamental commonalities between the circadian systems of these diverse species. Not only do rhythms in dissimilar organisms persist in constant environmental conditions and show temperature compensation, but they also respond similarly to clock resetting stimuli. As is familiar to all who have experienced and recovered from jet lag, the phase of the circadian clock can (eventually) be reset by cues such as light. A less obvious characteristic is that the sensitivity of the clock to resetting cues varies according to the time of day. Depending on what point during the subjective day or night the stimulus occurs, light can cause a phase delay, a phase advance, or no resetting at all. Thus the ability of the environment to reset the clock is itself under circadian control. This variable resetting response to stimuli given at different circadian times can be depicted in phase response curves.

In addition to setting the phase of the clock, light signals can influence the pace at which the clock runs in constant conditions. In many light-active organisms (including plants), exposure to higher intensities of continuous light shortens the free-running period (2). Fluence
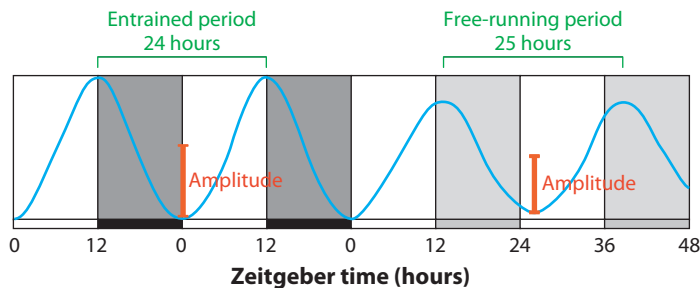

Figure 1

An idealized clock output is depicted in light/dark cycles (entraining conditions) and constant light (free-running conditions). The period of this output is exactly 24 hours in light/dark cycles because of clock entrainment by light. However, in constant environmental conditions the free-running period of 25 hours is revealed.

rate response curves display the relationship between light intensity and free-running period. Phase and fluence rate response curves are both useful tools for investigating light signaling to the circadian clock.

Another point of similarity between clocks in diverse organisms is their cell autonomy. In both cyanobacteria and isolated mammalian fibroblast cells, persistent and robust circadian rhythms in transcription are observed in single cells, with little or no coupling of clock period or phase between adjacent cells (79, 85, 141). The gradual damping of circadian rhythms observed in fibroblast cultures can be attributed to a loss of rhythmic synchrony between cells rather than to the clock "winding down" in individual cells (85, 141). Although similar experiments have not yet been performed at the single-cell level in intact plants, circadian rhythms in gene expression are observed in plant suspension cell cultures and calli (55, 87, 90, 110) and in isolated plant organs (133, 134). Elegant entrainment experiments examining gene expression rhythms suggest that little or no coupling exists between the clocks of cells located within the same organ (133). A subsequent biophysical study using a similar experimental design found weak coupling between cells within the same leaf, with resynchronization between phase-inverted portions of a leaf estimated to require approximately 200 days (26). Examination of a metabolic

process in leaves also provided evidence for weak coupling of circadian rhythms between plant cells (104). Thus in both multicellular and unicellular organisms, circadian rhythms occur at the level of the single cell and do not require strong intercellular interactions.

Why Are Circadian Clocks Widespread in Nature?

Circadian clocks appear almost ubiquitous in higher organisms. Why are they so prevalent? One possible reason is that they allow organisms to anticipate regular changes in the environment and synchronize different physiological processes with each other. Evidence for the importance of circadian clocks in optimizing growth performance has been steadily accumulating. In both cyanobacteria and higher plants, correct matching of the periodicity of the endogenous circadian clock with external light/dark cycles confers a fitness advantage (16, 95). In *Arabidopsis*, the short-period mutant *timing of cab expression 1* (*toc1*) outcompetes the long-period mutant *zeitlupe* (*ztl*) when both genotypes are grown in short day/night cycles. Along with enhanced survival, the *toc1* plants produce more chlorophyll, fix more carbon,

and accumulate more biomass. Conversely, the *ztl* mutants outcompete *toc1* when these plants are both grown in long day/night cycles (16) (**Figure 2a**).

Because *Arabidopsis* can entrain to day lengths very different from 24 hours (108, 122, 146), the growth advantage seen when plants' endogenous rhythms are coordinated with environmental cycles is likely due to optimal phasing of clock outputs (95). In *toc1* mutants grown in artificially short days, a given output would occur at the same environmental time as in a wild-type plant grown in a 24-hour day; in contrast, that output would occur inappropriately late in the long-period *ztl* mutants (**Figure 2b**). Similar arguments can explain the growth advantage of the long-period *ztl* mutants over *toc1* plants when they are maintained in artificially long days (**Figure 2c**).

Therefore, clocks likely provide an adaptive advantage by allowing proper timing of physiology with respect to the environment. However, at first glance it seems that an hourglass-type timer, one that counts down at a constant rate from some environmental transition such as dawn, might serve this function just as well as a circadian clock. So why are self-sustaining circadian clocks so prevalent? One advantage
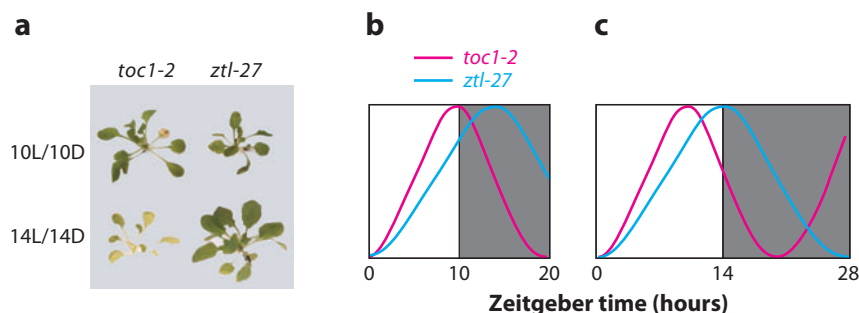

Figure 2

Plants grow best when the timing of their internal clock matches the periodicity of changes in the environment. (*a*) The short-period mutant *timing of cab expression 1-2* (*toc1-2*) and the long-period mutant *zeitlupe-27* (*ztl-27*) were grown in competition in either 20-hour days or 28-hour days. In both cases, the plant with a free-running period closest to the environmental cycles showed enhanced fitness. This result is likely due to incorrect phasing of clock outputs when the internal periodicity does not match environmental periodicity. (*b* and *c*) A hypothetical clock output with a dusk phase in wild type is correctly phased in *toc1-2* in 20-hour days (*b*) and in *ztl-27* in 28-hour days (*c*). Panel (*a*) from Reference 16, reprinted with permission from AAAS.

of circadian clocks over hourglass timers is that their outputs can be differentially regulated such that their peak phase always occurs at the correct time of day, even as day length changes with the seasons. For example, expression of some genes such as *CHLOROPHYLL A/B BINDING PROTEIN 2* (*CAB2*) peaks during the middle of the day regardless of the day length (81), whereas peak expression of others such as *TOC1* closely tracks lights off (100). This flexible regulation of the phases of various outputs is likely an important reason that circadian clocks are found throughout the natural world.

ORGANIZATION OF CIRCADIAN SYSTEMS

In its simplest form, the circadian system can be depicted as a central clock or oscillator that generates rhythmic outputs via specific signaling pathways; this oscillator can be reset by environmental signals such as light or temperature (**Figure 3a**). However, we have already seen that this depiction is an oversimplification because the circadian system influences the ability of light to reset clock phase; that is, clock outputs regulate the light input pathway to the

oscillator. As will be discussed in more detail below, there are other difficulties with this simple model: The central clock is likely composed of multiple interlocked feedback loops, clock outputs may also be directly regulated by clock input signaling pathways, and clock components may act both within the central clock and in input and output signaling pathways (**Figure 3b**). Thus it is more appropriate to consider the circadian system as a complex network rather than try to separate it into discrete input, central clock, and output components.

A long-standing question in the field has been whether biological rhythms are controlled by one or multiple clocks within a single organism. Because circadian rhythms are cell autonomous, multicellular organisms indeed contain multiple clocks. In animals, clocks in different tissues may carry out separate functions. Circadian clocks that control activity rhythms are found in the brain, whereas clocks in peripheral tissues are implicated in control of metabolic processes (4). However, there are no convincing data showing multiple clocks within single cells in animals. Conversely, there is evidence that unicellular organisms have multiple oscillators (necessarily within the same cell). In the alga *Lingulodinium polyedrum* (formerly
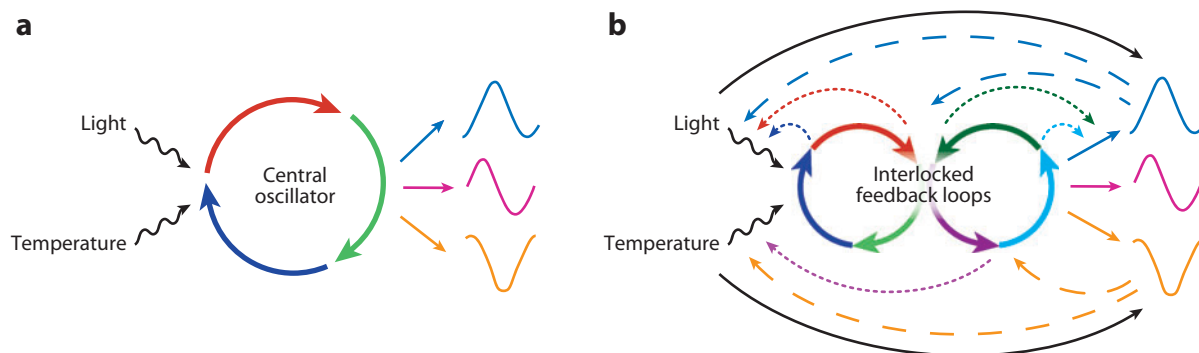

Figure 3

Models of the clock as a linear signaling pathway and as a signaling network. (*a*) Early models of the circadian system suggest it is made of three discrete components: a central oscillator or clock, resetting pathways that change the phase of the clock in response to environmental stimuli such as light and temperature, and a variety of rhythmic outputs. (*b*) Accumulating data suggest instead that the circadian system is a complex network. The oscillator consists of multiple coupled feedback loops (*solid colored lines*). Clock genes often have multiple functions, acting both within the oscillator and in clock input and output signaling pathways (*dotted lines*). Clock outputs can feed back to regulate clock components and input signaling pathways (*dashed lines*). Likewise, input pathways can regulate multiple clock genes and directly affect clock outputs (*solid black lines*).

Gonyaulax polyedra), circadian rhythms in bioluminescence and phototaxis have different free-running periods and respond differently to light (109). Genetic analysis in *Neurospora* suggests that this fungus contains multiple circadian oscillators that display differential degrees of coupling with each other (4). The evidence for multiple oscillators in plants will be considered below, after the molecular components of the plant circadian system have been introduced.

MONITORING THE HANDS OF THE CLOCK IN PLANTS

Investigating the mechanisms underlying circadian rhythms requires a reliable way to monitor the state of the clock. In plants, the most obvious daily rhythm is that of leaf movement position; in fact, the study of leaf movement rhythms led to the fundamental insights that circadian rhythms persist in constant environmental conditions, have a non-24-hour periodicity, and can be reset by light. Later, the study of the control of flowering led to insights into the roles of photoperiod, or day length, and the circadian clock in the regulation of seasonal responses (72). Many other physiological processes such as growth, enzyme activity, photosynthesis, control of stomatal aperture, and release of scent were also recognized as being clock regulated (143). Another important discovery was that the circadian clock controls the abundance and transcription of nuclear-encoded transcripts (57). Although these data made it clear that the circadian clock controls many aspects of plant physiology, the above circadian outputs did not lend themselves well to high-throughput studies.

In contrast, the circadian clock controls easily observed rhythms in many model organisms, including emergence from the pupal case (eclosion) in *Drosophila* and spore formation (conidiation) in *Neurospora*. A pioneering mutant screen by Konopka & Benzer (59) led to the discovery of a single-gene mutation that affected circadian rhythms in fruit flies, and subsequent studies led to the identification of clock mutants in algae and fungi. More than a decade later, the first clock genes were cloned in *Drosophila* and *Neurospora* (148). Forward genetics thus proved to be a powerful way to investigate the molecular mechanisms underlying clock function. However, application of this method to circadian research in plants had to await both an appropriate model species and an easily assayed clock output.

Arabidopsis thaliana proved to be a tractable organism for genetic dissection of complex processes such as circadian rhythms (105). A convenient, nondestructive, and relatively high-throughput assay for circadian clock function arrived in the form of a reporter gene, firefly luciferase, expressed under the control of a clock-controlled reporter. Although a highly sensitive camera is required to detect luciferase activity, luciferase has many traits that make it well suited for circadian studies. Detection of luciferase activity does not require excitation by light (which can perturb the clock) and light emission by the luciferase enzyme closely tracks the activity of the promoter driving its expression. Luciferase-based genetic screens in *Arabidopsis* have been a powerful tool for the discovery of clock mutants (68, 80, 94, 121, 126).

Other assays also have important roles in the study of clock function. Studies of natural variation in circadian clock function often make use of medium-throughput assays monitoring leaf movement rhythms (19, 78, 111, 129). Quantitative reverse transcriptase–polymerase chain reaction assays allow the expression of many genes in diverse genetic backgrounds to be rapidly determined without the need for transgenics. DNA microarrays are used to survey genome-wide circadian regulation of gene expression, leading to important insights into clock function (8, 9, 18, 37, 76, 117). Other platforms allow the systematic assessment of enzyme activities, metabolite levels, and protein levels over circadian time scales (28, 29, 139). Together, these assays have set the stage for molecular dissection of the mechanisms underlying the plant clock and have led to recent rapid progress in the field.

MOLECULAR BASIS OF CIRCADIAN RHYTHMS

Transcriptional Feedback Loops

Recent experimental and mathematical studies have suggested that the plant circadian clock consists of three interlocked transcriptional feedback loops. The first-identified loop consists of three components. *TOC1*, also known as *PSEUDO-RESPONSE REGULATOR 1* (*PRR1*), is an evening-phased, clock-regulated gene of unknown molecular function. The nuclear-localized TOC1 protein indirectly promotes the expression of two dawn-phased, Myb-related transcription factors, *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*). The CCA1 and LHY proteins have partially redundant functions; they bind directly to the *TOC1* promoter and inhibit its expression (1). This negative feedback loop therefore consists of CCA1, LHY, TOC1, and an unknown component X thought to act between TOC1 and the *CCA1* and *LHY* promoters (**Figure 4**, loop A). However, substantial experimental data cannot be
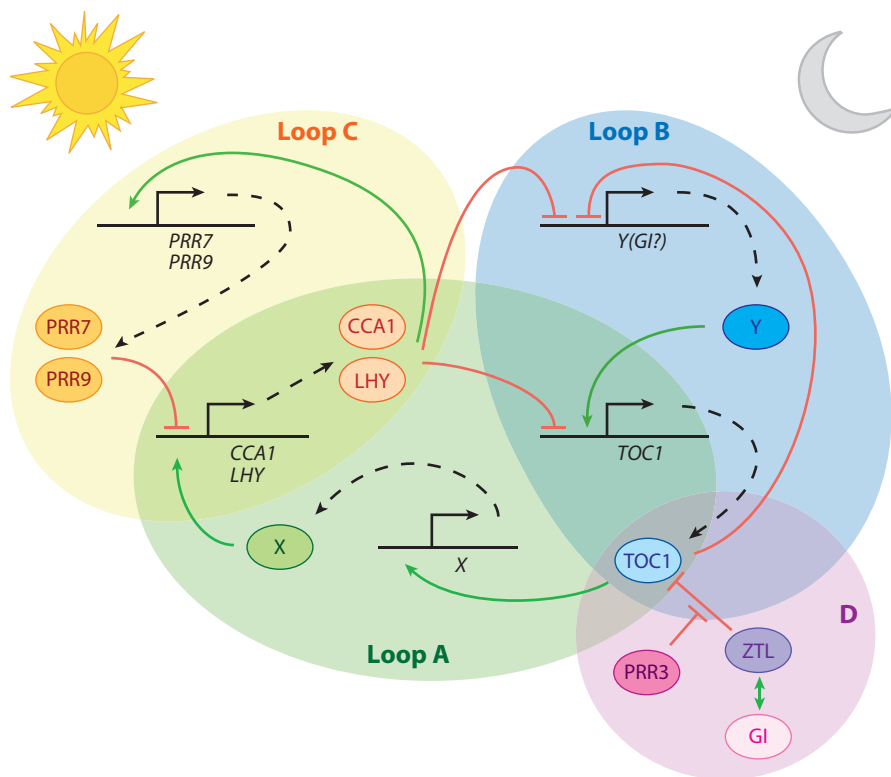

Figure 4

Model of the plant clock. The first-identified transcriptional feedback loop (*loop A*) consists of the dawn-phased Myb-like factors CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), which negatively regulate expression of *TIMING OF CAB EXPRESSION 1* (*TOC1*). TOC1 is postulated to directly or indirectly activate component X, an as-yet-unidentified factor that induces expression of *CCA1* and *LHY*. The second loop (*loop B*) is thought to be composed of two or more evening-phased genes, an unknown factor designated Y, and TOC1 [note GIGANTEA (GI) may provide a portion of Y activity]. The third loop (*loop C*), consists of the morning-phased genes *PSEUDO-RESPONSE REGULATOR 7* (*PRR7*), *PRR9*, *CCA1*, and *LHY*. Posttranscriptional modifications are also very important for clock function (*D*). ZTL negatively regulates TOC1 protein abundance; its activity is regulated by GI and PRR3. Other genes implicated in clock function have been omitted for clarity.

explained by this single loop model of the clock. Mathematical modeling suggests there is an evening-phased negative feedback loop coupled to this one, with an unknown component Y that positively regulates *TOC1* expression. The expression of Y is in turn predicted to be negatively regulated by TOC1, CCA1, and LHY (65) (**Figure 4**, loop B). Some data suggest that a portion of Y activity is provided by the protein GIGANTEA (GI) (64, 65); however, other experiments indicate that GI may only indirectly contribute to Y activity (54, 68, 116).

The *Arabidopsis* genome contains four genes encoding proteins with similarity to TOC1. The PRR3, 5, 7, 9 proteins and TOC1 all contain a domain similar to bacterial response regulator receiver domains but lack the conserved aspartate residue that is phosphorylated in canonical two-component signaling pathways (83). These proteins also share a conserved CCT motif (named for the proteins in which it was first identified: CONSTANS, CONSTANS-LIKE, and TOC1), which contains a nuclear-localization signal (66, 126). Reverse genetic studies revealed that these *PRR* genes all play a role in the plant clock, although the single mutant phenotypes are subtle (20, 21, 48, 78, 88, 112). Higher-order mutants generally have stronger phenotypes; in an extreme case, the *prr5 prr7 prr9* triple mutants are essentially arrhythmic (89). A combination of experimental and modeling studies suggests that CCA1 and LHY promote the expression of *PRR7* and *PRR9*, both morning-phased genes. PRR7 and PRR9 somehow inhibit the expression of *CCA1* and *LHY*. Thus *CCA1*, *LHY*, *PRR7*, and *PRR9* are thought to form one or two morning-phased feedback loops (**Figure 4**, loop C) (64, 149). Together, the three intertwined transcriptional loops depicted in **Figure 4** form an important part of the clock regulatory mechanism. This molecular model of the plant clock was achieved through collaborations between experimental and computational biologists, a beautiful example of the power of systems biology approaches.

The interlocked feedback loops in the plant clock bear marked similarities to the transcriptional mechanisms implicated in circadian function in mammals, fruit flies, and *Neurospora* (148). However, clock components are not conserved between plants, animals, and *Neurospora*. Why then do clock networks in these diverse organisms share similar "wiring diagrams"? Mathematical modeling suggests that interlinked feedback loops enhance the robustness of a network against perturbation (61, 136), which perhaps explains these commonalities.

Posttranscriptional Regulation

The transcriptional feedback loops described above are clearly not the whole story. Multiple types of posttranscriptional regulation play a critical role in regulation of the circadian network. The stability and translation of some mRNAs are influenced by the circadian clock and light signaling (32, 53, 63), and the abundance of many clock proteins is under posttranslational control. One of the first clock mutants to be molecularly identified encodes ZTL, an F-box–containing protein that is part of a Skp/Cullin/F-box (SCF) E3 ubiquitin ligase complex (34, 40, 121, 147). ZTL also contains a LOV domain, a flavin mononucleotide–binding region that confers the ability to sense blue light (44, 54). ZTL interacts with both TOC1 and PRR5, leading to their degradation via the proteosome pathway (25, 51, 70). This degradation is regulated by other protein-protein interactions and by light.

Studies of ZTL have shed light on the biochemical function of GI, a protein with no recognizable domains. GI and ZTL physically interact in a blue light–stimulated manner. This interaction stabilizes both ZTL and GI and may prevent ZTL from interacting with its substrates, leading to more rapid degradation of ZTL, GI, and the substrates PRR5 and TOC1 in the dark than in the light (12, 25, 51, 54, 70) (**Figure 4**, D). GI also interacts with the ZTL homolog FLAVIN-BINDING, KELCH REPEAT, F-BOX 1 (FKF1) in a blue

light–dependent manner, in this case affecting the stability of a transcription factor involved in flowering-time regulation (116). A recent study adds an additional wrinkle: TOC1 binds directly to PRR3 in a manner that interferes with TOC1 binding to ZTL (98). Thus PRR3 appears to stabilize TOC1 by preventing its recruitment to the SCF complex and its subsequent degradation by the proteosome (**Figure 4**, D).

There are many additional examples of regulated degradation of clock proteins. Intriguingly, PRR3, PRR7, and PRR9 levels are modulated by the circadian clock, but in a ZTL-independent manner (22, 25, 45). Other clock-associated proteins whose abundance is regulated by light and/or the clock include LHY, a casein kinase 2 regulatory subunit (CASEIN KINASE 2B4; CKB4), and XAP5 CIRCADIAN TIMEKEEPER (XCT), a novel nuclear protein (55, 67, 101, 123). Phosphorylation of at least some of these proteins likely plays an important role in their regulated degradation (22, 25, 101). Phosphorylation affects the clock in other ways as well, as exemplified by the requirement of CCA1 phosphorylation for normal protein function (11) and the shortening of circadian period upon overexpression of CK2 regulatory subunits (102, 127). Posttranscriptional regulation, specifically phosphorylation, is also of great importance in animal, fungal, and cyanobacterial clocks (27, 86).

Challenges to the Transcriptional Feedback Loops Model

With all these posttranscriptional modifications, is rhythmic transcription really necessary for clock function? This question has gained urgency with the discovery that although a feedback loop regulates rhythmic transcription of the cyanobacterial clock genes (*kaiA*, *kaiB*, and *kaiC*), purified Kai proteins drive temperature-compensated circadian rhythms in the phosphorylation of KaiC in vitro (86).

Some data suggest that transcriptional feedback loops may not be essential for clock function in plants. In a classic study, researchers found that circadian rhythms in the giant green alga *Acetabularia* persist for several days after removal of its nucleus (130). In higher plants, there are cases of mutations causing changes in period without obvious changes in the expression levels of clock genes. For example, *toc1-1* and *toc1-2* display similar short-period phenotypes (67, 126); however, only *toc1-2* causes the reduction in *CCA1* and *LHY* mRNA levels predicted by the transcriptional feedback loops model (1). Similarly, mutations in the clock-associated genes *FIONA1* (*FIO1*), *LIGHT INSENSITIVE PERIOD 1* (*LIP1*), and *XCT* affect free-running period without noticeably affecting expression levels of *CCA1*, *LHY*, and *TOC1* in constant conditions (49, 52, 67).

Conversely, there are a number of cases where large changes in clock gene expression levels do not cause significant changes in free-running period. For example, rhythmic gene expression with an approximately wild-type period (albeit with low amplitude) occurs in plants constitutively overexpressing *CCA1* (38). Similarly, overexpression of two clock-regulated transcription factors, *MYB3R2* and *bHLH69*, strongly reduces *LHY* and *TOC1* expression but has no effect on free-running period (36). A similar incongruity between clock gene expression levels and period phenotype is seen in plants mutant for *SENSITIVE TO FREEZING 6* (*SFR6*) (58). Data inconsistent with the transcriptional feedback loop models have also been reported in other eukaryotes (62).

How can these data be reconciled with the substantial amount of data indicating transcriptional feedback loops are central to clock function? Recent findings from cyanobacteria may point the way. Although purified Kai proteins exhibit circadian rhythms of KaiC phosphorylation in vitro (86), normal clock function in cyanobacteria likely relies on both rhythmic protein phosphorylation and gene transcription (56). It may well be that dual biochemical and transcriptional cycles are also required for robust circadian rhythms in higher plants.

Pieces Still to Be Fit Into the Puzzle

Other clock-associated genes must also be fit into the circadian network. *LUX ARRHYTHMO* (*LUX*) is an evening-phased gene encoding a Myb-like transcription factor essential for rhythmicity that may act near TOC1 in the oscillator (42, 93). Mutations in *PRR5* cause a short-period phenotype, but its mode of action is currently unknown (20, 78, 145). SPINDLY (SPY), a protein with O-linked β-N-acetylglucosamine transferase activity, interacts with GI and affects clock pace (137). Clock pace is also regulated by TEJ, a poly(ADP-ribose) glycohydrolase (97), and FIO1, a putative S-adenosyl-L-methionine–dependent methyltransferase (52), via unknown mechanisms. Another locus, *TIME FOR COFFEE* (*TIC*), is important for maintenance of circadian period and amplitude (14). *sfr6* plants have reduced tolerance to freezing and show altered response of free-running period in response to sucrose (58). Finally, overexpression of several Myb-like and bHLH factors disrupts clock function (36, 60, 150); it will be interesting to determine whether loss-of-function mutants also show circadian defects. Further genetic and biochemical studies are required to place these genes within the clock network.

Conservation of Clock Genes

Despite the similarities in circadian physiology and transcriptional feedback loops in diverse organisms, genes with proposed primary roles in the clock are not conserved across higher taxa. However, cryptochromes mediate blue light input into the clocks of both plants and insects, while CK2 phosphorylates clock proteins in animals, plants, and *Neurospora* (see Reference 131 for a review). Notably, these proteins play important roles in diverse signaling pathways, suggesting that their involvement in circadian clocks in diverse species may represent convergent evolution.

What about conservation of clock genes in photosynthetic organisms? Homologs of the molecular components of the circadian network described above can be found in both monocot and dicot plant species, including crassulacean acid metabolism (CAM) plants, chestnut, *Pharbitis*, poplar, rice, and *Lemna* (7, 41, 76, 84, 103, 118). Studies in the green alga *Chlamydomonas reinhardtii* have revealed that Myb-like transcription factors and a component of the SCF E3 ubiquitin ligase complex are important for clock function, suggesting some similarities to the *Arabidopsis* clock (71). However, obvious homologs of *TOC1*, *GI*, and *ZTL* are not found in this alga, and many *Chlamydomonas* genes implicated in clock function are not known to play a role in the clock in higher plants (71, 138). Further investigation of the molecular makeup of the circadian system in algae and nonvascular plants will provide interesting insights into the evolution of the plant clock.

THE CIRCADIAN SIGNALING NETWORK

As noted above, clock genes cannot be neatly classified as input, central clock, and output components. Instead, they tend to have multiple functions within the circadian system. Many genes thought of as clock components also play roles in light signaling. For example, TOC1 has biochemically separable roles in light signaling and control of clock pace (67, 69). ZTL, in addition to regulating TOC1 levels in a light-dependent manner, is important for clock function in constant darkness (120). ZTL also plays separable roles in red light–dependent signaling and the central clock (50). Similarly, GI, LIP1, and XCT appear to have discrete functions in light regulation of photomorphogenesis and clock function (49, 67, 92). PRR5 and PRR7 have also been suggested to act in phytochrome signaling pathways (46, 48).

Clock genes can also directly regulate clock output pathways. GI plays biochemically separable roles in clock function and the regulation of flowering time (68, 82). This finding may indicate that its interactions with ZTL and FKF1, the F-box proteins influencing these two processes, rely upon different amino acid residues (54, 116). CCA1 and LHY likely directly

control expression of many clock output genes (see below). Thus it is quite common for clock genes to have multiple functions within the plant circadian system (**Figure 3***b*). Similar multifunctionality can be seen for clock components in other organisms (148).

HOW MANY CLOCKS?

One way to assess the number of clocks in an organism is to compare the rhythmicity of multiple circadian outputs; different free-running periods imply these rhythms are controlled by different oscillators. Leaf movement rhythms have a longer free-running period than rhythms in stomatal conductance, photosynthesis, or expression of the *CAB2* gene (80, 122). Follow-up studies revealed that rhythmic changes in cytosolic free calcium levels and *CAB2* expression also have different periods, as do rhythmic expression of the *CAB2* and *CHALCONE SYNTHASE* (*CHS*) genes (110, 134). A comparison of clock regulation of the *CAB2* and *CATALASE3* (*CAT3*) promoters also suggests they are controlled by oscillators with different properties (77). These data imply that plants are composed of oscillators with different biochemical properties. However, because these rhythmic outputs are primarily generated by different cell types, it may be that clock composition varies between cells rather than distinct clock mechanisms existing within a single cell.

A recent study provides support for the idea that the biochemical nature of the circadian system differs between cell types. In *prr3* plants, genes with widespread expression patterns have a modest short-period phenotype, whereas a stronger phenotype is seen for genes preferentially expressed in the vasculature (98). *PRR3* is expressed most strongly in the vasculature, supporting the idea that it acts primarily in this tissue and raising the possibility that other clock genes have cell-specific functions. In addition, some allele-specific period phenotypes have been reported. *toc1-1* has differential effects on rhythms in free cytosolic calcium concentration and gene expression, whereas *toc1-2*

causes similar period-shortening of these outputs (142). Similarly, the *gi-2* mutation has differential effects on leaf movement and gene expression rhythms, whereas the *gi-1* allele has a similar effect on both (99). Despite these findings, clock composition is likely broadly similar in different cell types. In most circadian mutants, multiple clock outputs are affected in a similar manner (97, 134). These data suggest the clocks driving rhythmicity in diverse cell types are fundamentally similar, sharing many components but exhibiting some biochemical differences.

The presence of multiple clocks within a single organism leads to the question of how these oscillators are coordinated with each other. This coordination may occur via entrainment by external cues. The presence of photoreceptors in many plant cell types and the light-piping properties of leaf and vascular tissues may allow light signals to coordinately entrain most of the plant, even in woody species (128). Similarly, temperature entrainment could coordinate clocks in diverse plant tissues. The lack of strong coupling between transcriptional rhythms in a single organ (26, 133) suggests that entrainment by environmental cues, rather than by an endogenous signal, is important for coordinate regulation of rhythms throughout an individual plant.

LIGHT, TEMPERATURE, AND THE CLOCK

Environmental cues set the phase of the clock to the appropriate time of day. Are such cues necessary to start the clock as well? Recent studies have demonstrated circadian rhythms in transcription that can be detected within two days after imbibition, around the time the emerging radicle breaks the seed coat (114). These rhythms can be entrained by imbibition, release from stratification, or a light pulse (77, 114, 151). Circadian rhythms can even be observed in etiolated seedlings that have never been exposed to a temperature step or light treatment, indicating that the circadian clock in plants is truly endogenous (114).

Nonetheless, changes in light and temperature can have strong effects on the circadian system; light effects in particular have been intensively studied. Light input to the clock occurs via multiple types of photoreceptors. As described above, ZTL is a photoreceptor that controls TOC1 stability in a blue light–regulated manner. The phytochrome and cryptochrome photoreceptors also control red and blue light signaling to the clock (13, 119). The signaling pathways downstream of these photoreceptors are less clear. EARLY FLOWERING 3 (ELF3) and EARLY FLOWERING 4 (ELF4), two unrelated proteins of unknown function, both negatively regulate light input to the clock and help maintain robust rhythms in constant conditions via unknown mechanisms (10, 17, 73, 74). A third pioneer protein, SENSITIVITY TO RED LIGHT REDUCED 1 (SRR1), is a positive regulator of signaling in response to red and white light and plays an unspecified role in the setting of clock pace (124). A recently identified gene, *LIP1*, has distinct roles in light signaling to the clock and photomorphogenesis. *LIP1* encodes a plant-specific GTPase localized to both the cytoplasm and the nucleus (49). Finally, XCT is a ubiquitously expressed protein of unknown function that acts both in light input and clock function in constant conditions (67).

Although the light signaling pathways responsible for clock resetting are still unclear, a number of targets have been identified. Expression of *CCA1*, *LHY*, *PRR9*, and *GI*, all genes thought to act within the clock transcriptional feedback loops (**Figure 4**), is induced by light (53, 65, 132, 140). Light also promotes degradation of *CCA1* mRNA and increases the translation rate of *LHY* mRNA (53, 144). Finally, as noted above, the stability of many clock proteins is light regulated. These data suggest light resetting of the clock occurs via modulation of multiple clock genes at multiple regulatory levels.

Less well studied is the mechanism by which temperature affects the plant clock. Transcription of *CCA1*, *LHY*, *TOC1*, and *GI* is temperature sensitive (31, 96, 112), although the underlying mechanisms are unknown. However, PRR7 and PRR9 are clearly important for responding to temperature signals. *prr7 prr9* mutants do not entrain to temperature cycles and do not respond to temperature pulses (112), suggesting a role in response to temperature signaling. Temperature regulation of gene expression may also play an important role in temperature compensation. The maintenance of similar free-running periods at different temperatures may be achieved by an antagonistic balance of differential expression between morning- and evening-phased genes (31).

DAILY AND SEASONAL RHYTHMS

Many, perhaps most, aspects of plant growth and development are influenced by the clock. These include processes that occur with daily rhythms, such as photosynthesis, stem growth, and scent emission (143). Seasonal processes, such as the transition from vegetative to reproductive growth and the onset of dormancy, are also regulated by the circadian clock (72). In addition, many signaling pathways are modulated by the clock so that plant sensitivity to stimuli varies across the circadian cycle, a process known as gating. How is clock modulation of these processes achieved?

Clock Regulation of Gene Expression

A large fraction of the plant transcriptome is regulated by the circadian clock, which likely plays an important role in clock regulation of plant physiology. A recent microarray study suggests that approximately one-third of expressed genes are clock controlled in *Arabidopsis* (9); this estimate correlates well with results from an enhancer trap screen suggesting that 36% of *Arabidopsis* promoters are circadian regulated (75). The fraction of clock-regulated genes may be even higher because these studies were performed using whole seedlings. Tissue-specific circadian regulation is prevalent in animals (125); an examination of rhythmic gene expression in isolated plant tissues or cell types

is likely to reveal many more clock-regulated genes.

How is clock regulation of these hundreds of genes, with peak phases of expression occurring at all times of the subjective day and night, achieved? Several promoter motifs associated with phase-specific expression have been identified. The evening element, or EE, is found in the promoters of approximately one-quarter of dusk-phased genes and confers evening-phased expression on a reporter (9, 18, 37, 38, 76). The EE is present in the promoters of evening-phased clock genes such as *TOC1*, *GI*, and *LUX* and is thought to play a role in the transcriptional loops of the central clock. The dawn-phased CCA1 and LHY proteins bind to these EE promoter sequences, leading to repression of evening-expressed genes (1, 38, 42, 100). A small family of clock-regulated *CCA1*-like genes can bind the EE in vitro (30, 60, 150); however, their relative contributions to clock regulation of evening-phased genes remain unclear.

A few other regulatory motifs have been implicated in phase-specific expression. The morning element, or ME, is overrepresented in the promoters of morning-phased genes and confers dawn-phased rhythms on a reporter gene (9, 38, 76), whereas the protein box (PBX) is prevalent in the promoters of night-phased genes and confers midnight-phased rhythms on a luciferase reporter (76). The GATA and G-boxes have been implicated in the regulation of afternoon-phased and morning-phased gene expression, respectively (9, 18, 43, 76), although these predictions need experimental validation. The identification of the transcription factors that bind to these circadian promoter motifs will shed light on the mechanisms underlying clock function and how the circadian system influences plant physiology.

Interactions with Other Signaling Networks

Perhaps not surprisingly considering the large fraction of the genome under clock control, the circadian system influences and is influenced by many signaling and metabolic pathways. The interactions between light and clock signaling pathways are particularly intimate, because not only is plant sensitivity to light gated by the clock, but also many clock genes play roles in light signaling (described above). This phenomenon is seen in multiple species and may be due to light signaling genes having been co-opted by the circadian system during evolution (39). Temperature-sensing pathways are modulated by the clock as well. Both clock resetting in response to changes in temperature and plant susceptibility to extreme heat or cold vary in a circadian manner (77, 106, 107). Rhythmic cold resistance is likely due to circadian gating of cold-induced transcription factors that confer freezing tolerance (23). More complex interactions between pathways have also been reported. For example, the ability of low temperature to induce cold acclimation is regulated by light quality in a clock-dependent manner (24). Thus there is cross talk between the clock, cold, and light signaling networks.

The clock modulates many hormone pathways as well. The abundance of ethylene, brassinosteroids, gibberellins, and auxin, all hormones implicated in stem elongation, is modified by the clock (3, 6, 47, 135). Plant responsiveness to endogenous and exogenous auxin is also under circadian control (8). Moreover, analysis of genes regulated by both the clock and various hormone signaling pathways suggests that the clock influences the abscisic acid, cytokinin, methyl jasmonate, and salicylic acid signaling pathways, with important implications for plant development and responses to biotic and abiotic stresses (9). Data suggest that cytokinin signaling feeds back to regulate the clock itself (35, 113), another example of a clock output also acting as an input.

Recent studies are exploring the relationships between light, hormone, and clock signaling pathways in the control of plant growth. *REVEILLE1* (*RVE1*), a *CCA1* homolog and clock output gene, has been implicated in the auxin-mediated control of hypocotyl elongation (R. Rawat, J. Schwartz I. Sairanen, Y. Cheng, C.R. Andersson, Y. Zhao, K. Ljung,

& S.L. Harmer, manuscript submitted). The rapid growth of plant hypocotyls in response to simulated shading by neighbors is gated by the clock via induction of a transcription factor in a time-of-day sensitive manner (115). Clock and light signaling also cooperate in the regulation of plant growth by controlling the transcription and protein degradation, respectively, of two basic helix-loop-helix transcription factors (91). The combined action of these pathways results in different phasing of peak stem growth depending on day length (91).

Complex relationships between the clock and other signaling pathways are being revealed in other studies as well. Light, temperature, and sugar availability all alter the pattern of expression of clock-regulated genes (5, 58, 76). A clock component, CCA1, regulates expression of key genes involved in nitrogen assimilation; in turn, a pulse of organic nitrogen can modify the phase of *CCA1* expression (33). Similarly, the abundance of the cytosolic signaling molecules Ca^{2+} and cyclic adenosine diphosphate ribose is clock regulated, and perturbation of these cycles alters circadian parameters (15). Thus metabolites and second messengers can feed back to modify clock function, another example of circadian regulation of physiological processes that in turn affect clock function. The circadian system can therefore be considered an integral part of a large signaling network that optimizes plant responses to the environment.

SUMMARY POINTS

1. The circadian clock provides plants with a growth advantage, likely due to correct phasing of clock outputs to the most suitable time of day.

2. The plant clock is cell autonomous; plant clock genes act in self-sustaining transcriptional and posttranscriptional feedback loops. Clock genes are conserved within angiosperms but not across higher taxa.

3. The circadian system is best described as a network, with extensive feedback regulation between the oscillator and clock input and output pathways.

4. Light and clock signaling pathways are closely linked. Almost all known clock components are either transcriptionally or posttranscriptionally light regulated. In addition, many clock genes also act in light signaling pathways.

5. The circadian system acts as a signal integrator, interacting with many other signaling networks to restrict plant responses to environmental stimuli to the most appropriate time of day. These signaling pathways in turn can feed back to affect clock function.

FUTURE ISSUES

1. The current model of the plant clock needs refinement. The predicted but currently unidentified clock components must be found and characterized, and the interlocked transcriptional feedback loop model needs to be modified to accommodate discrepant data.

2. Many genes implicated in central clock function encode pioneer proteins with no known biochemical functions. An understanding of their molecular functions will tremendously improve our understanding of the circadian system.

3. Data suggest the molecular make-up of the clock varies between different organs and tissues. The field will need to move beyond the whole-plant level to define the molecular composition of the clock and related signaling pathways in single cell types.

4. Finally, we need to further investigate interactions between the circadian clock and other signaling and developmental networks to better understand how the clock modifies environmental responses. These studies will help us understand how the circadian clock provides an adaptive advantage.

DISCLOSURE STATEMENT

The author is not aware of any biases that might be perceived as affecting the objectivity of this review.

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NOTE ADDED IN PROOF

A recent paper (1) shows that when plants are grown so that the shoots but not roots are exposed to light, only a subset of the central clock-associated genes are rhythmically expressed in roots. This suggests that clock composition depends both upon environment and organ type.

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Contents

Errata

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