All in good time: the *Arabidopsis* circadian clock

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Biological time-keeping mechanisms have fascinated researchers since the movement of leaves with a daily rhythm was first described >270 years ago. The circadian clock confers a ~24-hour rhythm on a range of processes including leaf movements and the expression of some genes. Molecular mechanisms and components underlying clock function have been described in recent years for several animal and prokaryotic organisms, and those of plants are beginning to be characterized. The emerging model of the *Arabidopsis* clock has mechanistic parallels with the clocks of other model organisms, which consist of positive and negative feedback loops, but the molecular components appear to be unique to plants.

Jet lag might be an annoying manifestation of our internal clocks, but an effective method for tracking time is important to enable organisms to adjust to changing environmental conditions. The most extensively studied biological systems for time keeping are circadian clocks (Box 1). They have been observed in both prokaryotes and eukaryotes, and they control a wide variety of biological processes. By definition, a circadian rhythm is one that persists (free-runs) with a period of ~24 h under constant conditions, in the absence of external timing cues[1]. The first circadian rhythm to be recorded in any organism was leaf movement[1]. Leaves of many plants, including *Arabidopsis*, have an open configuration during the day, producing a maximum surface for incident light, and fold to a more closed angle at night. When the plants are transferred to constant light, these leaf movements continue as if they were still being exposed to day–night cycles.

Another attribute of circadian rhythms is that environmental signals such as light–dark or temperature cycles can reset or 'entrain' the phase of the rhythm to match the environmental cycle. Although the clock can be entrained by changes in temperature, a third defining characteristic of a circadian rhythm is that, unlike other biological processes, its period hardly varies over a range of temperatures, a phenomenon known as temperature compensation[1].

The importance of a circadian clock for plants can be gauged by the fact that so many facets of plant development are under its control. Indeed, one of the advantages of studying circadian regulation in plants is the wide variety of processes that have been shown to have circadian rhythms (Fig. 1).

The classic conceptual model of the clock that gives rise to these circadian rhythms comprises three basic components – the input pathways, the oscillator and the output pathways (Fig. 2). The oscillator is the system's pacemaker and is responsible for generating the circadian rhythm. However, to be biologically meaningful, the phase of the rhythm must be synchronized with the outside world. Thus, the input pathways transduce time-keeping signals from the environment to the oscillator. These signals arise most commonly from diurnal light–dark transitions or changes in temperature, but other environmental cues such as imbibition of seeds can also set the clock[2]. Completing the circadian clock model are the output pathways, which provide a link between the oscillator and the various biological processes whose rhythms it controls. During the past few years, there has been a tremendous increase in our understanding of the molecular basis of the clock in a wide range of organisms including *Neurospora*, *Drosophila* and *Synechococcus*[3]. This article focuses primarily on the efforts to identify the components of the *Arabidopsis* circadian clock and to understand how they interact with each other.

What is the oscillator in *Arabidopsis*?

In spite of the large number of genes that have been implicated in clock function in *Arabidopsis*, defining components of the oscillator itself has been a challenge. Studies of other model eukaryotic organisms such as *Neurospora*, *Drosophila* and mice[3] have given rise to a common theme with respect to the molecular mechanisms of their circadian oscillators. At its core, the oscillator is a feedback loop consisting of positive and negative elements (Fig. 3). Proteins encoded by clock genes act as negative elements that repress their own expression by blocking transcriptional activators that act as

Box 1. Glossary of terms commonly used in chronobiology

Circadian
Literally, 'about a day' (24 hours).

Clock
Generally refers to the entire circadian system, although it is sometimes used to mean the oscillator.

Clock gene
A rhythmically expressed negative element of a transcription–translation feedback loop. This term is also used to refer to a gene that encodes any oscillator component.

Entrainment
The setting of the oscillator to match environmental cycles of light and dark, or of temperature.

Input pathways
The sequence of events via which information from the environment, such as changes in light and temperature, is transduced to the oscillator.

Oscillator
The cell-autonomous timekeeper responsible for generating self-sustained rhythmicity. Also called the pacemaker.

Output
The pathways linking the oscillator with the various biological processes it controls.

Pacemaker
See oscillator. This term is also used to describe a central oscillator that is coupled to and can entrain an array of peripheral oscillators; for example, the suprachiasmatic nucleus in the brain of mammals, which controls multiple peripheral clocks in cells of other tissues and organs.

Phase
The relationship of some point in a rhythm to a marker, such as another rhythm. For instance, the relationship of the peak expression of a gene to daybreak during a day–night cycle.

Rhythm
The regular oscillations of a process.

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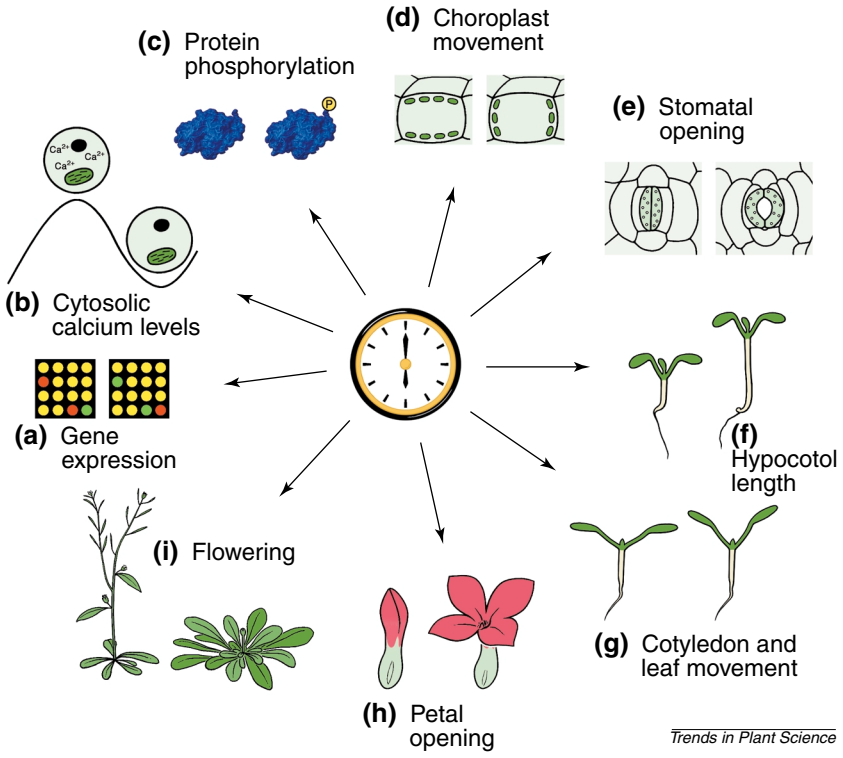

Fig. 1. Plant clocks control a plethora of biological processes[43]. (a) The expression of several genes shows circadian rhythms. Two examples are the genes encoding the light-harvesting chlorophyll-*a*/*b*-binding proteins (*Lhcb* or *CAB*) and nitrate reductase (*NIA2*). Many of these genes are associated with photosynthesis and its related biochemical and physiological activities. It is possible that the timing of expression of such genes (for instance, the predawn rise in *Lhcb*), indicates a role for the clock in the coordination of metabolism to maximize photosynthetic yields. The use of fluorescent differential display[44] and high density DNA arrays (depicted in the figure) to monitor global expression profiles should give us an indication of the range of genes showing circadian control. (b) Cytosolic concentrations of free calcium have been shown to oscillate with a circadian rhythm in *Arabidopsis*[45]. Considering the importance of calcium as both a secondary messenger and a cofactor for many enzymes, this might be a means by which the clock regulates a variety of cellular processes. (c) The clock regulates the phosphorylation of some proteins. The best-studied example is in *Kalanchoe fedtschenkoi*, which exhibits circadian activity of a kinase that phosphorylates phosphoenol pyruvate carboxylase[46]. At a higher level of organization, chloroplast movement[47] (d), stomatal opening[1] (e), hypocotyl elongation[48] (f) and cotyledon and leaf movements[1] (g) in *Arabidopsis* all show circadian rhythms. In *Kalanchoe*, petal opening shows a circadian rhythm (h). The clock is also vital for synchronizing developmental processes such as flowering time (i). Indeed, mutations in all the putative clock-associated genes cause altered photoperiodic control of flowering. The clock's role in the control of flowering has been extensively reviewed[49].

positive elements. The subsequent decrease in clock transcripts and proteins alleviates inhibition of the transcriptional activators, thereby reinitiating the oscillator cycle.

In addition to this autoregulatory negative feedback loop, there is another interconnecting feedback loop in which positive and negative elements assume opposite roles. An example of this concept can be seen in *Neurospora* (Fig. 3), in which the FRQ protein acts as a negative element to repress activation of *FRQ* transcription by WC-1 and WC-2 heterodimers (positive elements). FRQ also acts as a positive element that promotes synthesis of WC-1 from existing *WC-1* mRNA (Ref. 4). Such interconnecting loops were first discovered in *Drosophila*[5] and have also been found in mammalian systems[6]. It has been suggested that interlocking circadian feedback loops provide robustness to the oscillations and stability to the output[4].

Positive and negative oscillator elements have not been unequivocally identified in *Arabidopsis*. However, LHY and CCA1 (two closely related proteins that possess a Myb-related DNA-binding domain[7,8]) do fulfill some of the proposed criteria for establishing a candidate protein as an oscillator component[9]. The first criterion is that the component itself shows circadian oscillations. *LHY* and *CCA1* show robust circadian oscillations of both transcript and protein levels in plants kept in continuous light. They also fulfill a second criterion: that the component controls its own levels by feedback inhibition of its synthesis. In constant light, over-expression of either *CCA1* or *LHY* results in repression of their own and each other's endogenous expression[8,10].

The third criterion, that clamping (holding) the amount of the putative oscillator component at any particular level from null to high stops the clock and thus rhythmicity, is at least partially fulfilled by these genes. Over-expression of *CCA1* and *LHY* does indeed stop all overt rhythmicity that has been measured, including leaf movement, hypocotyl elongation and the expression of genes that peak with different phases[8] (E.M. Tobin and A. Millar, unpublished). However, in a *CCA1*-null line, robust rhythmicity of output gene expression is maintained, albeit with a shorter period than in wild-type plants[11]. This finding indicates that, although CCA1 is clearly an important element of clock function, LHY oscillations might be able to compensate for the loss of CCA1. Thus, LHY and CCA1 might have overlapping functions. The effect of clamping both LHY and CCA1 levels at null is, as yet, unknown.

A final criterion is that inducing transient changes in the levels of an oscillator component at a particular time in the clock cycle should quickly (within one cycle) cause a phase shift in the clock's oscillations and consequently of the overt rhythms. Work is currently under way to examine whether CCA1 and LHY fulfill this criterion and to establish them more firmly as bona fide clock components.

The first circadian mutant described in *Arabidopsis* was *toc1* (timing of *CAB/Lhcb* expression). This mutation has been shown to shorten the period of a variety of clock-controlled processes suggesting that TOC1 acts close to or as part of the oscillator[12]. The *TOC1* gene has recently been isolated and found to encode a nuclear protein with a region that is common to the CONSTANS-LIKE family of transcriptional activators[13]. It also contains a motif that is similar to the receiver domain of response regulators from two-component signal transduction systems. However, the conserved aspartate residue that normally undergoes phosphorylation in other characterized response regulators is substituted in TOC1. Consistent with this finding, TOC1 [also identified as APRR1 (Ref. 14)] and other pseudoreceivers are unable to

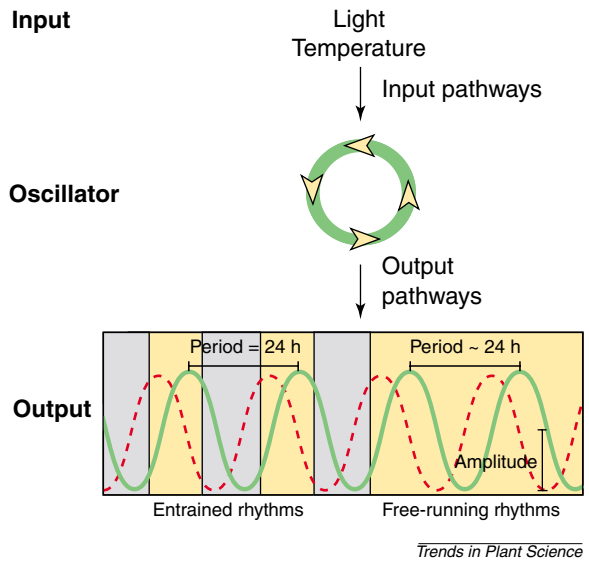

Fig. 2. Components of a circadian system. This simple model includes an input pathway (from light and/or temperature) to a circadian oscillator. The oscillator generates signals that are transduced via output pathways to produce overt circadian rhythms (output). The output is depicted as two idealized rhythms (red and green lines) with different phases. Yellow and grey boxes represent light and dark (diurnal) intervals, respectively. Under diurnal conditions, the period of the oscillator (the time between comparable points in the repeating cycle) matches the period of the entraining cycles. Under constant conditions, the clock oscillates with an endogenous period close to 24 h. Amplitude is half the distance between the peak and trough.

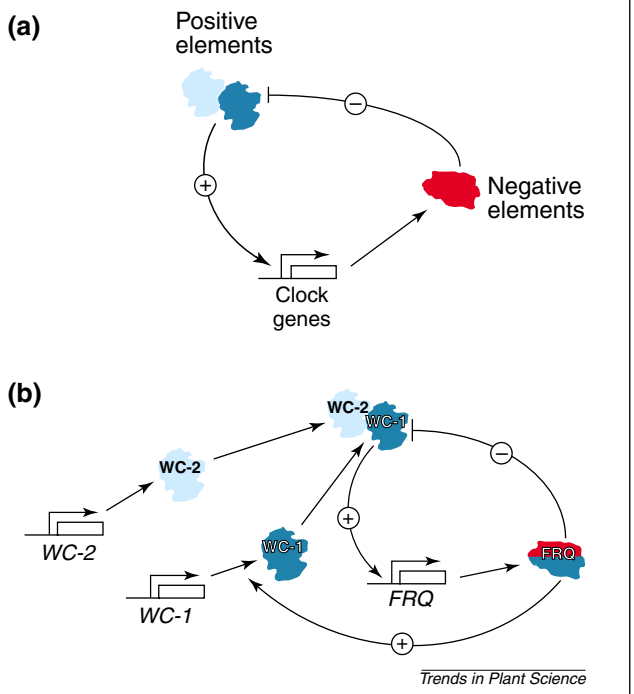

Fig. 3. Molecular models of eukaryotic circadian oscillators. (a) General scheme showing the core feedback loop central to eukaryotic circadian oscillators. Paired positive elements (CLOCK and CYC in *Drosophila*, WC-1 and WC-2 in *Neurospora*, CLOCK and BMAL in mice) form heterodimers that act as transcriptional activators to induce clock gene expression. The protein products (negative elements) of the clock genes (PER and TIM in *Drosophila*, FRQ in *Neurospora*, mCRYs and mPERs in mice) in turn block the action of the positive elements, thus indirectly repressing their own expression. (b) An emerging theme in circadian research is the existence of interconnecting feedback loops. A model of the *Neurospora* circadian system (*modified, with permission, from Ref. 4*) illustrates this. The WC-1–WC-2 heterodimer activates expression of the clock gene *FRQ*. The FRQ protein has two roles. In one loop, it acts as a negative regulator of the WC-1–WC-2 heterodimer. In a second loop, FRQ acts as a positive regulator of WC-1 protein synthesis from existing *WC-1* mRNA. Positive elements are depicted in blue and negative elements in red. Because FRQ acts as both a positive and negative element, it is depicted in red and blue.

undergo phosphorylation *in vitro*[14], and the function of these putative receiver domains remains unclear. *TOC1* transcript levels oscillate robustly in plants kept in continuous light[13]. Moreover, *TOC1* mRNA cycles with a shortened period in the *toc1-1* mutant, indicating that the *TOC1* product contributes to its own circadian expression. Thus, TOC1 is another candidate component of the oscillator.

There are also genes in *Arabidopsis* that fulfill some of the criteria for being oscillator components but that are clearly not part of the pacemaker. The RNA and protein levels of a putative RNA-binding protein, CCR2 (AtGRP7), show circadian oscillations, and over-expression of *CCR2* represses its own expression and that of the closely related *CCR1* (*AtGRP8*) gene[15,16]. However, *CCR2* over-expression has no effect on other clock-regulated genes such as *Lhcb* and *CAT3*. In addition, the period of *CCR2* RNA oscillations is shortened in the *TOC1*- and *CCA1*-null mutants[11,15]. Taken together, these results suggest that CCR2 acts as a 'slave' (or sub) oscillator rather than as part of the pacemaker itself.

As well as identifying putative oscillator components, recent work has started to show how these components might be regulated. In organisms as varied as *Synechococcus*, *Drosophila*, *Neurospora* and hamsters, phosphorylation of oscillator proteins is important for clock function. In *Synechococcus*, autophosphorylation of the clock protein kaiC is necessary for circadian rhythms[17]. Homologs of casein kinase I are implicated in the clocks of *Drosophila*[18] and hamster[19]. In *Drosophila*, the *DBT* gene is responsible for phosphorylating PER and is thought to affect its stability[18].

In plants, too, phosphorylation appears to play a regulatory role in the clock mechanism. CKB3, a regulatory subunit of casein kinase II (CK2) was recently identified as a CCA1-interacting protein[20]. CCA1 and LHY can be phosphorylated *in vitro* by both recombinant CK2 and by a CK2-like activity in *Arabidopsis* extracts. CK2 was shown to have a role in the regulation of circadian rhythms by over-expressing CKB3 in *Arabidopsis*[21]. Constitutive expression of *CKB3* shortens the period of expression of *CCA1*, *LHY* and several clock-controlled genes, although it is not known whether this effect of *CKB3* over-expression is a direct consequence of altered phosphorylation levels of CCA1 and/or LHY. However, given our knowledge of the role of phosphorylation in other circadian systems, it is reasonable to surmise that changes in the phosphorylation states of CCA1 and LHY might affect their activity, stability and/or cellular location.

In addition to identifying elements of the oscillator and studying their regulation, recent work has started to address the architecture of the circadian system in plants. It is likely that each cell in the plant has at least one oscillator[1]. Using reporter genes to monitor gene expression in transgenic *Arabidopsis* plants, it has been shown that these oscillators act autonomously and

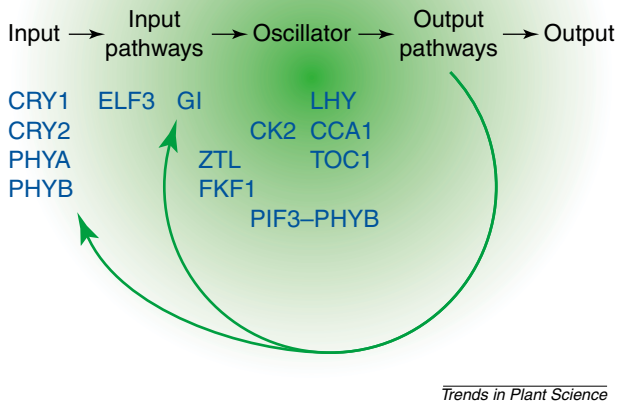

Fig. 4. A model of the putative roles of proteins involved in the *Arabidopsis* circadian system. Shading indicates that, at our current state of knowledge, the borders between what forms the oscillator and the input and output pathways are 'fuzzy'. Proteins have been placed within the shaded area according to how close they might be to the oscillator. The darker the shading (towards the center), the closer to the oscillator. Thus, CCA1, LHY and TOC1, which might be part of the oscillator itself[7,8,13] are located at the center of the shaded area. Similarly, ZTL and FKF1, although assigned to the input pathways, are also placed near the oscillator because they might interact with phosphorylated oscillator proteins, targeting them for degradation[39,40]. Oscillator proteins might be phosphorylated by CK2 (Refs 20,21). The PIF3–PHYB complex might also interact directly with the *CCA1* promoter[32] and is therefore placed close to the oscillator. The green arrows indicate that components such as PHYB and GI, assigned as inputs or as part of input pathways, respectively, are themselves affected by the clock[10,35,37]. Many of the putative clock components show circadian rhythmicity of transcript levels but the available evidence suggests that *ZTL* transcripts do not cycle[40].

independently of any centralized pacemaker[22]. This contrasts with the mammalian system, for example, in which centralized pacemakers located in the suprachiasmatic nucleus of the brain can entrain multiple clocks in peripheral parts of the animal[23].

How is the clock entrained?

In their natural habitats, plants experience wide variations in both light quality and light quantity at different times of the day and at different locations. Light is the most important factor in the entrainment of plant clocks. In addition to the role of light–dark cycles in setting the clock, light quantity also affects the period of the clock, with the clock running more slowly (i.e. with a longer period) under lower light intensities[12]. Members of both the phytochrome (PHY) and cryptochrome (CRY) families of plant photoreceptors have been shown to act as part of the input pathways to the circadian clock[24]. Considering the importance of light in circadian regulation, it is not surprising that multiple photoreceptors function in clock control. Interestingly, although most of the proteins involved in plant circadian rhythms appear not to be conserved in the circadian systems of non-plant organisms, cryptochromes have been identified as being essential for clock function in both *Drosophila*[25,26] and mice[6].

Studies in *Arabidopsis* using single mutants of the two cryptochromes and two of the five phytochromes show that PHYA mediates signals from low-fluence blue and red light to the clock, PHYB perceives high-fluence red light, and CRY1 perceives high-fluence blue light. At present, it seems that CRY2 has a more minor role in mediating input signals to the circadian oscillator[27]. However, it is striking that in white light, a *phyA phyB cry1 cry2* quadruple mutant retains robust circadian rhythmicity of leaf movement[28]. There appears to be no change in period and the phase of the clock can still be reset. This same mutant is severely impaired in light-regulated developmental processes such as de-etiolation. These results suggest that either the other phytochromes (PHYC, PHYD, PHYE) or an as-yet-unidentified photoreceptor(s) can function in the input pathways to the *Arabidopsis* clock. The results also indicate that cryptochromes are not components of the oscillator, in contrast to *Drosophila*[25,26] and mice[6], in which CRY proteins are essential for oscillator function. In *Drosophila*, CRY proteins also function as photoreceptors for resetting the circadian clock[29].

Circadian systems are also adapted to cope with environmental temperature changes. On the one hand, the period of the clock remains remarkably stable under a wide range of ambient temperatures[12]. In *Neurospora,* such temperature compensation might be regulated by changing the ratios of two forms of the FRQ protein[30]. No such system has so far been uncovered in *Arabidopsis* and the mechanism(s) by which temperature compensation is achieved in plants is not understood. On the other hand, abrupt temperature changes can entrain the clock[1]. However, the mechanisms by which this occurs are unknown, and no temperature sensor equivalents of photoreceptors have been isolated.

By contrast, progress has been made in uncovering a possible mechanism for the transduction of signals from photoreceptors to the clock. It has been shown that the expression of *CCA1* is induced by red light acting on phytochrome[8]. In its red-light-activated form, PHYB can be translocated to the nucleus, suggesting that it might directly affect gene regulation[31]. However, PHYB itself has not been shown to bind DNA. With the recent cloning of the gene encoding the phytochrome-interacting factor PIF3, a piece of the puzzle has fallen into place[32]. PIF3 is a basic helix–loop–helix putative transcription factor that, *in vitro*, can bind simultaneously to the red-light-activated form of PHYB and to a conserved motif that is present in the promoters of *CCA1* and *LHY*. Furthermore, in plants with reduced levels of PIF3, there is a decrease in the red-light-induction of *CCA1* and *LHY* expression. This provides a possible molecular explanation for the long-established interaction between phytochrome and the circadian system.

Genetic screens have uncovered additional proteins in *Arabidopsis* that are involved in the transduction pathways. For example, mutations in *EARLY FLOWERING 3* (*ELF3*) result in arrhythmia in light but not in the dark[33,34], suggesting that ELF3 is also a component of an input pathway from light perception. There is a large overlap in the phenotypes of *elf3* and *phyB* mutants: both have long hypocotyls and petioles, both flower early, and both are defective in their responses to red light. However, double mutants of *elf3* and *phyB* show an additive phenotype, suggesting that ELF3 and PHYB act in distinct pathways[34].

The complexity that is likely to be encountered in plant circadian systems as research progresses is illustrated by work on the *GIGANTEA* (*GI*) gene[10,35,36]. Transcript levels of this nuclear protein[36] show circadian oscillations, with peak expression in

the evening[10]. Moreover, in plants over-expressing either *CCA1* or *LHY*, oscillations of *GI* mRNA are disrupted[10]. Thus, *GI* expression is under control of the clock. Consistent with this idea, *GI* mRNA rhythms are also perturbed in *elf3* mutants, suggesting that GI acts downstream of ELF3 (Ref. 10). Complicating this scenario, however, is the finding that *gi* mutants exhibit an altered period and reduced amplitude of *CCA1* and *LHY* expression, which would suggest that GI is functioning in the input pathway(s) to the clock[10,35]. Intriguingly, GI is not the only putative input component that oscillates. *PHYB* transcript levels also show circadian rhythmicity[37]. It is tempting to speculate that we are seeing the outlines of interlocking loops in the *Arabidopsis* circadian system rather than a unidirectional input–oscillator–output pathway. However, in order for PHYB and/or GI to form part of an outer output-feeding-to-input loop, it would not only be necessary for their transcript levels to oscillate but also for their protein levels, activity or cellular location to show circadian rhythmicity[38]. No such data is available for GI, but weak rhythmicity in total PHYB protein has been reported, and this might be physiologically significant[37].

Two recent papers reported the cloning of *Arabidopsis* genes that encode novel proteins involved in modulating the circadian clock[39,40]. Both proteins, ZTL and FKF1, contain a subclass of the PAS domain known as the LOV (light, oxygen, voltage) domain. The PAS domain mediates protein–protein interactions and is highly conserved among clock components in other model organisms. PAS-like LOV domains have been found in several blue-light photoreceptors [NPH1 (*Arabidopsis*), PHY3 (*Adiantum*) and WC-1 (*Neurospora*)], leading to speculation that ZTL and FKF1 might have a role in controlling input from light signals to the clock. In support of this hypothesis, the period of clock-controlled gene oscillations in the *ztl* mutant is more dependent on fluence rate than in wild-type plants. The fact that ZTL and FKF1 might be involved in light perception could be particularly significant given that the *phyA phyB cry1 cry2* quadruple photoreceptor mutant retains a functioning circadian system[28].

The other two domains common to FKF1 and ZTL are an F-box and six repeated kelch motifs. F-boxes are found in a wide range of proteins that recruit target proteins to ubiquitination complexes[39,40]. Kelch motifs can mediate protein–protein interactions. Thus, FKF1 and ZTL were suggested to be involved in recruiting clock-associated proteins for ubiquitination and proteolytic degradation. Substrate recognition by F-box proteins is strictly phosphorylation dependent, and it is possible that clock protein targets of FKF1 and ZTL include phosphorylated CCA1 and/or LHY.

Perspectives

The field of plant molecular chronobiology has come of age. There has been a tremendous increase in our knowledge of *Arabidopsis* circadian systems over the past few years, and some key clock-associated components have been identified (Fig. 4). Although it is likely that important clock components remain to be found, the broad outlines of the workings of the *Arabidopsis* clock are coming into focus. The general oscillator mechanism observed across phyla, consisting of an autoregulatory transcription–translation negative-feedback loop, seems to hold true in *Arabidopsis*, if indeed TOC1, CCA1 and LHY are part of the oscillator. The involvement of CK2 in the *Arabidopsis* clock is consistent with the role of phosphorylation in other clock systems. Moreover, the *Arabidopsis* circadian system appears to be more complex than a simple linear input–oscillator–output (Fig. 2), as is the case in other model organisms (Fig. 3).

Where the *Arabidopsis* circadian system is clearly different is that, apart from the CRY proteins, no homologs of the *Arabidopsis* proteins involved in the circadian clock have so far been found to exist or to have a role in the circadian systems of other organisms. In other words, the molecular components that form the clock machinery might be unique to higher plants. In this context, it is important to determine whether other plant species have homologs for each of the *Arabidopsis* clock components and, if so, whether they have similar functions. In insects, there is a high degree of homology between *PER* in *Drosophila* and in the silk moth (*Antheraea pernyi*), yet the regulation of *PER* in the brains of these two organisms is dramatically different[41]. This suggests that the same molecular component can have diverse modes of regulation in different species.

In *Arabidopsis*, it will be important to determine whether TOC1, CCA1 and LHY fulfill all the criteria for bona fide oscillator components. None of the mutations in the genes encoding these proteins results in arrhythmic expression of clock-controlled genes, suggesting that they might be functionally redundant or that there might be as-yet-unidentified oscillator genes. Interestingly, the *Arabidopsis* genome contains several genes encoding proteins with a single copy of the Myb repeat sequence and that have homology to CCA1 and LHY (Ref. 27). These genes might also play a role in the clock. Mutant screens are being carried out in many laboratories with the goal of identifying additional clock components in *Arabidopsis*. One such screen used naturally occurring genetic variation between *Arabidopsis* strains and accessions to identify additional loci that affect circadian period[42]. It is likely that we will see more of this type of genetic analysis in the future.

The availability of technologies to analyze global gene expression should also become a powerful tool in clock research. It should facilitate the identification of new genes affected by the clock and should also help in characterizing the contextual function of an identified clock protein (i.e. how it interacts with other proteins within the dynamic network of proteins forming the circadian system). Indeed, the question of how the genes involved in the *Arabidopsis* clock are regulated is only starting to be addressed. Clearly, we still have a way to go before the plant clock is as well understood as clocks in other model organisms but we are catching up. Time will tell what new discoveries await us.

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