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# Input signals to the plant circadian clock

**Andrew J. Millar***

*Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, UK*

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## Abstract

**Eukaryotes and some prokaryotes have adapted to the 24 h day/night cycle by evolving circadian clocks, which now control very many aspects of metabolism, physiology and behaviour. Circadian clocks in plants are entrained by light and temperature signals from the environment. The relative timing of internal and external events depends upon a complex interplay of interacting rhythmic controls and environmental signals, including changes in the period of the clock. Several of the phytochrome and cryptochrome photoreceptors responsible have been identified. This review concentrates on the resulting patterns of entrainment and on the multiple proposed mechanisms of light input to the circadian oscillator components.**

Key words: *Arabidopsis thaliana*, biological clock, Circadian rhythm, cryptochrome, light regulation, phytochrome.

## Introduction

Interfaces spawn wonders that are missing from the hinterlands, in evolution, in cuisine and in research. The circadian system is at an interface in the signalling network, between environmental response pathways and internal programmes. The chefs find 'fusion' at their interfaces, academics speak of 'interdisciplinarity' or they write of it to avoid the tangled syllables. Researchers in circadian rhythms are uncovering the signalling web that sets the endogenous clock to local time. The generic term for this process is 'entrainment' and it affects both recurring, daily adjustments (at what time should petals open?) and once-in-a-lifecycle decisions (when should an annual plant make the transition to flowering?). Signalling cross-talk in plants may be understood as a computation that combines internal and external input signals: the interface between the clock and the environment is a fascinating example.

Circadian clocks evolved as an adaptation to the planet's 24 h rotation and its attendant rhythms of light and temperature on the Earth's surface (Harmer *et al*., 2001; Young and Kay, 2001). Photo-autotrophic organisms must be exposed to sunlight for photosynthesis, so all plants are exposed to the day/night cycles, with the possible exceptions of buried, germinating seedlings and polar inhabitants. The circadian system allows organisms to anticipate these regular cycles, timing biological processes to a part of the cycle (a phase) that benefits from external light or warmth, or the absence of conflicting internal processes. This feat requires some detachment from the fluctuating present conditions. The 24 h biological rhythms that the circadian clocks control are not direct responses to any external factor and persist even under constant environmental conditions, with a period that often differs from 24 h. Being thus detached, the circadian system retains information from days past. Detachment cannot be complete or the rhythmic processes would never match the external opportunities, so entrainment of circadian clocks is crucial and is similar in all organisms. Molecular research on plant circadian rhythms is most advanced in *Arabidopsis thaliana*, though their physiology was largely defined in other species including *Ipomoea nil* (*Pharbitis nil*), *Kalanchoë* spp. and *Phaseolus* spp. (Bünning, 1935; Lumsden *et al*., 1995; Engelmann *et al*., 1998; Borland *et al*., 1999); recent studies have also developed molecular understanding in rice (Sugiyama *et al*., 2001).

## Plant circadian rhythms

Microarray experiments indicate that at least 6% of *Arabidopsis* genes are rhythmically expressed, with expression peaks at all phases throughout the day and night (Harmer *et al*., 2000; Schaffer *et al*., 2001). The circadian gene expression produces rhythms that pervade

* Fax: +44 024 7652 3701. E-mail: andrew.millar@warwick.ac.uk

 

plant physiology, some of which are obvious (such as the 'sleep movements' of legume leaves, noted since classical times), others less so. In several cases, genes that affect a common pathway or process are expressed at the same phase, suggesting that the phase might be important for the function of that process. Many genes encoding enzymes of phenylpropanoid biosynthesis had peak RNA levels before dawn, perhaps because it is advantageous to accumulate photoprotective flavonoids before the sun rises (Harmer *et al*., 2000). A large proportion of the rhythmically regulated genes also directly respond to environmental stress (Kreps *et al*., 2002). Rhythmic expression of these genes in anticipation of predictable environmental changes might thus prepare the plant to withstand a stress (or make best use of a resource), so circadian regulation would complement the plant's subsequent response to the stress. Recent experimental evidence can be interpreted to support this view (Green *et al*., 2002) and more is likely to follow from studies of *Arabidopsis* clock mutants and natural variants. Photoperiodism is a special case, in which a circadian rhythm is combined with light signalling. The photoperiod sensor allows plants to respond to the annual cycle of day length, by making flowers, tubers or frost-tolerant buds at appropriate seasons. The selective advantages of correct seasonality are very clear; recent reports have significantly enhanced understanding of this mechanism (a recent review is Hayama and Coupland, 2003). Correct entrainment is crucial for photoperiodism, indeed general physiology indicates that a key difference between light-dominant plants (most of which flower in long days) and dark-dominant plants (most of which flower in short days) is in the entrainment of their photoperiodic rhythm (Thomas and Vince-Prue, 1996).

## The molecular clock mechanism

The known clock mechanisms of mammals, insects, fungi, cyanobacteria, and plants include a gene circuit with negative feedback, involving 24 h rhythms in the levels of positively and negatively acting transcriptional regulators (Harmer *et al*., 2001; Young and Kay, 2001). These 'clock genes' or 'clock-associated genes' maintain the molecular oscillation that drives all other circadian rhythms. Any pathway that entrains the circadian clock must affect the expression of at least one of these clock components. The molecular components of the clock seem distinct to each taxon, so the shared architecture has arisen at least four times in evolution, providing a rich basis for comparative research. Their mechanisms of entrainment are quite different. Mammals and insects share several orthologous clock genes; light signals by activating the transcription of *Per* genes in mammals, but by inducing the degradation of a different protein, Timeless, in *Drosophila* (Young and Kay, 2001).

The candidate oscillator components in *Arabidopsis* are two small gene families founded by the DNA-binding proteins LHY and CCA1 (Schaffer *et al*., 1998; Wang and Tobin, 1998), and the pseudo-response regulator protein TOC1 (Matsushika *et al*., 2000; Strayer *et al*., 2000). The first reasonable model to link these components explicitly (Alabadi *et al*., 2001) can be summarized as follows (reviewed in Hayama and Coupland, 2003; Eriksson and Millar, 2003): *LHY* and *CCA1* are expressed rhythmically with a circadian peak around dawn and are also rapidly light-induced. The cognate proteins are produced within 2–3 h, they bind to and inhibit transcription from the *TOC1* promoter. As LHY and CCA1 protein levels fall towards the end of the day, *TOC1* RNA abundance rises and is maintained until the middle of the night. *TOC1* transcription is not acutely regulated by light. TOC1 protein is proposed to activate *LHY* and *CCA1* transcription, perhaps indirectly. This model captures a number of data sets but is incomplete; notably, *lhy;cca1* double mutants that lack both gene functions were shown to retain a short-period rhythm (Alabadi *et al*., 2002; Mizoguchi *et al*., 2002). Overexpression of *CCA1* or *LHY* abolishes all rhythms yet tested (Schaffer *et al*., 1998; Wang and Tobin, 1998), with the possible exception of rhythmic expression of the *EARLY-FLOWERING 3* (*ELF3*) gene (Hicks *et al*., 2001): the significance of the latter datum remains to be determined. The mechanisms of *LHY* and *CCA1* activation are unknown, but require at least three further genes that are expressed at around the same phase as *TOC1*; *ELF4*, for example, encodes a 111-residue protein without obvious sequence homologies (Doyle *et al*., 2002). Further genes, such as *TIME FOR COFEE* (*TIC*), regulate *LHY, CCA1* and *TOC1* expression and profoundly affect the circadian system, although they have yet to be cloned and sequenced (Hall *et al*., 2003). Among the genes described in this paragraph and their products are the molecular targets for entrainment.

## Environmental signals for entrainment

Nature provides a very complex set of signals over the day/night cycle, including variation in temperature, light quality and light quantity at varying rates of change. Thus a combination of signals in the day/night cycle resets the clock reliably, entraining it to match the environmental cycle. If the circadian clock were delayed relative to the environment, biological processes would occur later than normal, so entrainment must advance the phase of the clock. By contrast, a circadian clock that was advanced relative to the environment would trigger rhythmic processes too early, so entrainment must delay its phase. A phase delay in each successive cycle results in a change of period. This would be required, for example, to entrain the 21 h circadian clock of the fungus *Neurospora crassa* to a 24 h day/night cycle.

The assays used to study entrainment in the laboratory focus on single stimuli, for example, a brief pulse of light

in otherwise constant darkness. Such a transient signal affects the oscillator components transiently, resulting in a change of phase: the clock is advanced or delayed, sometimes by many hours. By contrast, exposure to constant light affects the oscillator components all the time, thus altering the circadian period compared to constant darkness. One of the most detailed and revealing experiments measures the phase-response curve (PRC): a test pulse of light or temperature is applied at all possible phases across the circadian cycle in otherwise constant darkness, showing how the clock responds to light at each point. In some cases, the details of entrainment can be predicted from the PRC alone. A substantial part of the classical literature of circadian biology investigates this area, showing why the PRC for light pulses has a characteristic shape in all organisms, for example (Johnson, 1999). Entrainment of the circadian clock is a particular case in the mathematical field of dynamical systems. Circadian entrainment is one of the areas of biology where mathematical predictions have been tested experimentally and confirmed (Winfree, 1987, gives a simple account with good graphics).

### *Temperature*

All circadian rhythms can be entrained by warm/cold cycles; *Arabidopsis* rhythms entrain well to temperature cycles in which day and night temperatures differ by 4 °C or less. These have been used experimentally to test whether the defects of circadian mutants were specific to light signalling: the phenotype of the *toc1-1* allele was not (Somers *et al*., 1998; but see Mas *et al*., 2003), whereas the phentoype of *elf3* and *phyB* mutants was (McWatters *et al*., 2000; Salome *et al*., 2002). A detailed phase response curve to temperature pulses was recently published (Michael *et al*., 2003). However, understanding of the entrainment mechanism is in its infancy and presents different problems with light entrainment: potential photoreceptors are rare but all biochemical reactions are temperature-sensitive so potential thermosensors might be common. Intriguing new evidence indicates that photoreceptor signalling can be temperature sensitive (Mazzella *et al*., 2000; Halliday *et al*., 2003), which raises the possibility that light-sensing and temperature-sensing are interdependent in plants.

### *Light*

Light signalling pathways from both phytochrome and cryptochrome photoreceptors regulate clock components to achieve entrainment in plants (reviewed in Fankhauser *et al*., 2002). The phytochromes (phy) respond to red and far-red light, whereas the cryptochromes (cry) absorb in the UV-A/blue wavelengths. *Arabidopsis* has five phytochrome genes, *PHYA–PHYE* (Nagy *et al*., 2002; Quail, 2002) and two cryptochromes, *CRY1* and *CRY2* (Lin, 2002). The cry's and phy's together account for almost all of de-etiolation: a *cry1;cry2;phyA;phyB* quadruple mutant develops almost as an etiolated seedling in white light, even though it retains the three minor phytochromes, phyC, phyD and phyE. Notwithstanding its striking morphology, the mutant exhibits entrained and free-running circadian rhythms of leaf movement. This suggests that free-running circadian rhythms do not require input from the major photoreceptors (Yanovsky *et al*., 2000).

*Arabidopsis* plants in constant light (i.e. the 'standard' laboratory conditions of white fluorescent light at *c*.100 μmol $m^{-2}$ $s^{-1}$) have a period close to 24 h, whereas the period can exceed 30 h after several days in darkness (Millar *et al*., 1995). Much recent work has used the period assay to define which photoreceptors affect the clock, under which lighting conditions: this work has been reviewed extensively elsewhere (Somers, 1999; Yanovsky and Kay, 2001; Devlin, 2002). The major photoreceptors involved in de-etiolation all signal to the seedling clock, shortening its period under red light (phyA and phyB, but also to a lesser extent D and E; phyC has not been tested in detail) and blue light (cry1 and cry2). There are two types of overlap between the phy and cry pathways. Firstly, phyA accumulates to such high levels under very low light conditions that its minor absorption of blue light causes significant period shortening. Secondly, and more intriguingly, cry1 is required for the wild-type response to low red light, although its absorption spectrum has no peak in the red: the current suggestion is that phyA signalling requires functional cry's in a non-photoreceptor role (Devlin *et al*., 2000). Further photoreceptor interactions have been described from molecular and/or genetic assays, though their impact on the clock is not yet clear.

Ten years after work started on *Arabidopsis* rhythms, the first phase-response curves were published (Covington *et al*., 2001; Devlin *et al*., 2001). Previous work on other plant species had shown that plant phase responses shared the common pattern from other organisms (reviewed in Engelmann and Johnsson, 1998). A light pulse causes little phase change during the middle of the subjective day but, in the evening, light delays the clock and light in the morning advances it, with an apparent transition from large delays to large advances in the middle of the night. PRCs are therefore extremely revealing and allow detailed predictions of entrainment patterns. However, many of the rhythms used in *Arabidopsis*, such as *CAB* gene expression, are related to photosynthesis and rapidly lose amplitude in darkness. This complicates the phase-response experiments, which normally involve giving a single light pulse in prolonged darkness: after a couple of days, there was no longer a rhythm to measure. The development of a marker that was stably rhythmic for many days in darkness, *CCR2* gene expression, allowed the measurement of PRCs for 1 h light pulses, administered

to dark-adapted plants (Covington *et al*., 2001). Red and blue light gave similar results, again suggesting that both phy and cry photoreceptors participate. Repeating these experiments in photoreceptor mutants and with different amplitudes of light pulse should now identify the particular contribution of each photoreceptor species, but eventually assays will be required that include the more complex crepuscular light conditions found in nature.

## Targets for light signalling?

Light input must affect a component of the oscillator if it is to reset the clock. Defining the targets of entrainment in the plant clock remains a challenge, although not for lack of candidates (Kim *et al*., 2003*a*). The first potential mechanism can be described as the 'PIF3 hypothesis' (reviewed in Nagy *et al*., 2002). In summary, phytochrome-interacting factor 3 (PIF3) is a DNA-binding protein of the basic helix-loop-helix class. PIF3 dimers bind directly to promoter fragments of *CCA1* and *LHY in vitro*, to a G-box sequence that is also present in many light-activated genes. The light-activated (Pfr) form of phyB can interact with the promoter-bound PIF3 (Martinez-Garcia *et al*., 2000). As mutants with altered *PIF3* function affect developmental light responses *in vivo*, this was clearly a potential mechanism of photoentrainment. The *CCA1* and *LHY* genes should then be critical for entrainment, and indeed *cca1;lhy* loss-of-function mutants have a very early phase, but they retain light-entrained circadian rhythms, showing these genes are not uniquely required. Other considerations also argue for further entrainment mechanisms: CCA1 and LHY proteins fall to very low levels by the end of a 12 h day in wild-type plants (Wang and Tobin, 1998; Kim *et al*., 2003*a*), yet wild-type plants remain sensitive to much longer photoperiods (see below). The model of *PIF3* as a co-activator of light responses has recently been complicated by results suggesting that *PIF3* antagonizes at least some light responses (Kim *et al*., 2003*c*).

A second possible pathway involves three proteins of the ZEITLUPE (ZTL) family (Somers *et al*., 2000), ZTL, Flavin-binding-Kelch-F-box (FKF) and LOV-Kelch protein 2 (LKP2). These contain a Period-ARNT-Sim (PAS)-related domain, similar to the domain that binds a flavin chromophore in the phototropin photoreceptors (Briggs and Christie, 2002). *ztl* mutant phenotypes are light-dependent, supporting a possible photoreceptor role. However, the ZTL protein has the potential to interact with both phyB and cry1, which might cause light-dependence more indirectly (Jarillo *et al*., 2001). The two other domains of ZTL, an F-box and seven kelch repeats, suggest an involvement in ubiquitin-mediated protein degradation (Kim *et al*., 2003*b*). The proteins that are targeted for degradation should soon be identified. If these include clock-related proteins and the photoreceptor function is also proven, one or more of the ZTL family are very likely to function in entrainment.

The four *Arabidopsis PSEUDO-RESPONSE REGULATOR* (previously called *APRR* but more correctly *PRR*, Eriksson *et al*., 2003) genes homologous to *TOC1* prompt other speculation. They are expressed rhythmically, in an intriguing sequence every 2–3 h from dawn to dusk, *PRR9-PRR7-PRR5-PRR3-TOC1* (Makino *et al*., 2002). Alterations in *TOC1* function have greater effects on oscillator function than manipulation of individual PRRs (Eriksson *et al*., 2003), but their joint function remains to be elucidated in double mutants. PRR9, PRR5, and TOC1 have light-dependent effects on the circadian period (Mas *et al*., 2003; Eriksson *et al*., 2003). Three PRRs have been linked to light signalling in other ways: *PRR9* expression is light-activated but inhibited by overexpression of *TOC1* (Makino *et al*., 2002), *PRR7* has been identified as a modifier of phytochrome signalling in hypocotyl elongation (Kaczorowski and Quail, 2003), strong *toc1* mutant alleles can alter light responses (Mas *et al*., 2003) and the TOC1 protein can also interact with PIF3 and related bHLH transcription factors (Makino *et al*., 2002). PRRs might thus function in part downstream of phytochromes, either modifying phy signalling through PIF3 or in a parallel input pathway. It is quite possible that the multiple input photoreceptors affect the plant circadian clock by several mechanisms.

## Rhythmic regulation of light input

The *PHY* and *CRY* photoreceptor genes are themselves targets of circadian regulation at the level of RNA abundance (Bognar *et al*., 1999; Hall *et al*., 2001; Toth *et al*., 2001), although any circadian regulation at the protein level is of much lower amplitude (Bognar *et al*., 1999; Sharrock *et al*., 2002). The phy and cry photoreceptors are post-translationally modified by phosphorylation and nuclear translocation (Nagy *et al*., 2002), so other types of rhythmic regulation remain possible. Furthermore, a circadian 'gating' pathway rhythmically inhibits the activity of the light input pathways around subjective dusk, making the clock less sensitive to light at this phase. The gating pathway depends upon *ELF3* (McWatters *et al*., 2000; Covington *et al*., 2001). Gating is essential for normal entrainment under long photoperiods and for continued rhythmicity in constant light, because the oscillator arrests about 10 h after dawn in *elf3* mutants if light is present (McWatters *et al*., 2000). The ELF3 protein can interact with phyB (Liu *et al*., 2001), potentially inhibiting its function in the subjective evening. Circadian clocks in other organisms also have rhythmically gated light signals (human sleep effectively limits light input, for

example), but understanding the full effects of gating is not trivial.

## The phase of entrainment

The joint input from all the input pathways entrains the plant's rhythms to a particular phase relative to the environmental day/night cycle, known as the phase of entrainment. The phase of entrainment does not alter the internal sequence of events, but determines how that sequence relates to the environmental cycle: does dawn fall before or after the peak of *CCA1* expression, for example, and does dusk arrive before or after *TOC1* expression? Circadian rhythms in nature are always entrained, so the circadian clock contributes to plant physiology mainly by regulating the phase of entrained rhythms: the period of the clock under constant conditions is rarely, if ever, observed outside the laboratory. Many parts of the circadian system combine to determine this phase, not only the light and temperature signalling pathways but also the circadian oscillator. Variation in any of these factors should alter the phase of entrainment and hence the adaptive value of the circadian system, so it is important to understand how phase is controlled. There is a rich literature on formal studies of entrainment that dissect the various contributions of different factors (Pittendrigh, 1981).

The alteration in photoperiod, which occurs naturally in the seasonal cycle and alters the phase of entrainment, is possibly the most physiologically relevant variation. *CAB* gene expression peaks at a phase about 40% of the way through the predicted light interval, independent of the length of the entrainment photoperiod, when phase is measured in constant darkness after entrainment to several cycles of a test photoperiod (Millar and Kay, 1996). This indicates that dawn and dusk do not 'drive' this rhythm, because its phase would then be a constant time interval from either dawn or dusk. Rather, at least two signals must participate in entrainment, from a selection comprising the sharp transitions at dawn and dusk and the intervals of continuous light and darkness. Both phy and cry photoreceptors are presumably involved in setting the phase under white light:dark cycles. A 2 h early phase of entrainment has recently been reported in *phyB* mutants, directly implicating phyB in entrainment (Hall *et al*., 2002; Salome *et al*., 2002). More extreme phase changes can be created experimentally, using non-24 h cycles (so-called T cycle experiments, used extensively in Roden *et al*., 2002). The *timing of cab expression 1* mutant (*toc1-1*) has a period of approximately 21 h, for example, so under 24 h entraining cycles it entrains at an earlier phase than wild-type plants with a period of ~24.5 h. Under 21 h environmental cycles, however, *toc1-1* plants have a normal phase of entrainment (Somers *et al*., 1998; Yanovsky and Kay, 2002).

## Applications and challenges

Studies in cyanobacteria show that clock mutants gain a competitive growth advantage under light:dark cycles that match their circadian period. If and only if it has a normal phase of entrainment, a clock mutant can outgrow the wild type (Ouyang *et al*., 1998). Retaining an optimal phase of entrainment very likely drives balancing natural selection on clock genes, which should be revealed in the extensive natural variation for circadian period in wild *Arabidopsis* accessions and among crop varieties (our unpublished results; Bünning, 1935; Swarup *et al*., 1999). Given this variation and the wide range of processes under circadian control, crop performance might be improved by matching circadian rhythms to local growing conditions.

More detailed understanding of entrainment will be required to predict the behaviour of a particular variety, for a particular rhythm of interest. Mathematical modelling will grow in importance, given the complexity of the interactions involved. The flexibility and diversity of the circadian system will also call for much more carefully-defined experiments. In one paradoxical development, entrainment has been shown to affect the period of subsequent circadian rhythms under constant conditions. This phenomenon, termed an after-effect of entrainment, has been classically described in mammals, but was recently reported in *Arabidopsis* (Roden *et al*., 2002; Michael *et al*., 2003). The use of different rhythms to test entrainment in different assays is raising a different problem. Very many (if not all) plant cells have a functional circadian system and input photoreceptors. The clocks controlling the expression of genes in different anatomical locations are functionally independent and their periods differ slightly, probably reflecting differences among the cell types involved (Sai and Johnson, 1999; Thain *et al*., 2000, 2002; Hall *et al*., 2002; Michael *et al*., 2003). This issue was highlighted all too clearly by experiments on *CAB* expression in wheat and tobacco seedlings in the first days after germination (Kolar *et al*., 1998, and references therein): two oscillators controlled a biphasic rhythm, but only one of the clocks was reset by a light pulse. As entrainment studies become more detailed and quantitative, it will be necessary to define which, and how many, circadian clocks are being studied.

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## Note added in proof

PRR3 and PRR7 are shown to modulate circadian period, at least in the light, by a recent article by Michael *et al*. (2003).

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