

A Suite of Photoreceptors Entrain the Plant Circadian Clock

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Abstract Circadian rhythms in plants are relatively robust, as they are maintained both in constant light of high fluence rates and in darkness. Plant circadian clocks exhibit the expected modes of photoentrainment, including period modulation by ambient light and phase resetting by brief light pulses. Several of the phytochrome and cryptochrome photoreceptors responsible have been studied in detail. 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 circadian rhythms, biological clocks, *Arabidopsis thaliana*, light regulation, phytochrome, cryptochrome

Plants are run by light, to a degree that is difficult for us to comprehend, animals that we are. It is worth a moment's consideration, because this context probably shapes the molecular machinery that is available for the plant circadian system to deal with light and certainly affects the intellectual and experimental tools available to researchers in this area. So, solar energy fuels the whole plant metabolic network, thanks to light captured by chlorophyll in the light-harvesting complexes of the chloroplast, and the metabolic network is far more complex than animal metabolism. Chlorophyll is the mass market of photochemistry: The light-harvesting complex proteins are among the most abundant on Earth (they are also known as chlorophyll *a/b*-binding proteins [CAB], after their primary function).

Away from the chloroplast, 3 families of photoreceptors cater to the regulatory signaling network, including the circadian clock. The number of photons they capture is tiny, compared to the bulk of chlorophyll. Although these photoreceptors can strongly influence metabolism, this influence is usu-

ally very indirect. The 3 families are the phytochromes, the cryptochromes, and the phototropins (a UV-B photoreceptor is also at large, but its identity is unknown). Together, these photoreceptors track the changing intensity and spectrum of light; they pick up its direction and in some cases even its plane of polarization (Kendrick and Kronenberg, 1994). Their niche market in light signaling can be more directly compared to vision, for they provide information to control plant behavior, information such as the proximity of neighboring plants or the optimal direction of travel, as well as the current availability of the all-important solar fuel. Behavior in animals often involves locomotion, which is an option most plants lose after the dispersal stages of pollen and seeds are past. Rather, plants behave by altering their baroque chemical repertoire and by modifying development. In the latter area also, plants have far more scope than many animals because both growth and organogenesis continue through most of their life: Travel means elongation. If current appendages are inappropriate for a changed environment, the devel-

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opment of new organs can be altered to suit it better. Small, dense "sun leaves" might give way to broader, thinner "shade leaves" to improve light capture in a newly shaded habitat, for example, or leaves might be dropped altogether in favor of frost-tolerant buds if the shortening photoperiod indicates the approach of winter.

De-etiolation is the best known of the light-regulated developmental transitions because it occurs reproducibly and rapidly in the lab (Kendrick and Kronenberg, 1994). This is the transformation of an "etiolated" seedling, germinating with no leaves, elongating rapidly in darkness toward the soil surface with its apex trailing upside-down for protection, into a young plant in the light, with leaves expanding from the righted apex and chloroplasts developing as fast as possible. The photoreceptors control a substantial fraction of the transcriptome in this age of plant (Quail, 2002), perhaps more than at any other time. Elongation of the hypocotyl (the seedling stem) over a few days provides a facile, quantitative, 1-dimensional record of light perceived: Roughly speaking, the more light, the less elongation. A "blind" plant literally stands out, spindly and tall above neighbors with normal light perception, so this method of detecting photoreceptor-deficient mutants is among the easiest of genetic screens. It also gives the fastest indication that a mutant plant isolated by another phenotype, such as aberrant circadian regulation, may be deficient in light signaling. The most common molecular assay tests the activation of highly expressed, light-regulated genes such as *CAB* following a brief light pulse. These and several related approaches have identified mutants in the genes that encode the photoreceptor apoproteins of the 3 families mentioned above.

THE PLANT PHOTORECEPTORS

The regulatory photoreceptors comprise the phytochromes (phy), which absorb red to far-red light most efficiently; the cryptochromes (cry); and the phototropins (phot), which absorb in the UV-A/blue wavelengths. phot1 and phot2 are light-activated, membrane-associated ser/thr protein kinases, which control stomatal opening, chloroplast movement, and phototropic growth in response to directional light rather than overall hypocotyl elongation (Briggs and Christie, 2002). Their activity can be modified by phy and cry function. Alone, the phototropins have no known

association with circadian input (unpublished work cited in Devlin and Kay, 2001), except the distant link that their 2 FMN chromophores are bound by PAS-related protein domains that are similar to those of the *Neurospora crassa* White Collar 1 protein (see Liu, 2003 [this issue]). Crys also bear 2 chromophores, in this case a flavin and a pterin, in a protein related to photolyase DNA repair enzymes (Lin, 2002). Plant crys are more closely related to photolyases than to animal crys. Neither retains photolyase activity, though their molecular mechanism is postulated to retain the ancestral electron transfer mechanism. Either cry holoprotein can be located in the nucleus; they differ in that cry2 is notably light labile, whereas cry1 is stable.

Arabidopsis has 5 phytochrome genes, *PHYA-PHYE* (reviewed in Nagy and Schafer, 2002; Quail, 2002). The photoreceptor holoproteins phyA-phyE share a linear tetrapyrrole chromophore, which is covalently bound to a protein domain with sequence similarity to the GAF domain, which may have been involved in sensing intracellular bilin levels, before this bilin receptor with its ligand bound was adopted as a photoreceptor (Montgomery and Lagarias, 2002). The CikA protein that is implicated in the resetting of cyanobacteria (Schmitz et al., 2000) shares several protein domains with higher-plant phys but may use a different signaling mechanism. Among the higher-plant phys, phyA alone is light labile but accumulates to a very high abundance in etiolated seedlings. phyA and phyB are the most abundant and important species in light-grown seedlings, such that a *phyA;phyB* double mutant hardly inhibits hypocotyl elongation under red light. Both phyA and phyB have recently been shown to move from the cytosol into the nucleus after light activation (reviewed in Kircher et al., 2002; Nagy and Schafer, 2002). The crys and phys 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 3 minor phytochromes: phyC, phyD, and phyE (Yanovsky et al., 2000). Notwithstanding its striking morphology, the mutant exhibits entrained and free-running circadian rhythms of leaf movement. This can be interpreted as evidence that free-running circadian rhythms do not require photoreceptor input, but the minor phys—and perhaps further photoreceptor classes—prevent a definitive conclusion at this stage.

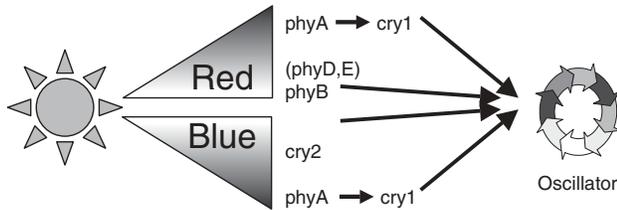


Figure 1. Light input to the *Arabidopsis* circadian clock. Several photoreceptors have been shown to alter the free-running period under high fluence rates of red or blue light (at center), so many or all of these will be active in daylight. cry2 functions under intermediate fluence rates of blue light. phyA is principally responsible for transducing low light signals, requiring cry1 presumably as a signaling component. Adapted from Devlin (2002).

INPUT TO THE PLANT CLOCK

Circadian rhythms in plants are affected by light:dark signals, with much overt similarity to the entrainment of other organisms. In constant conditions, the light environment alters the period of free-running rhythms, which provides a simpler and more robust assay for light input than the phase-response curve (PRC), though it does not directly test entrainment. *Arabidopsis* plants in constant light (the standard laboratory conditions are white fluorescent light of 50-100 micromoles/m²/sec), for example, 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 tested the period of gene expression rhythms via *LUC* reporter genes, to define which photoreceptors affect the clock under which lighting conditions (see Fig. 1). This work has been reviewed extensively elsewhere (Somers, 1999; Yanovsky and Kay, 2001; Devlin, 2002; Fankhauser and Staiger, 2002). Not surprisingly, 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) and blue light (cry1 and cry2). There are also 2 types of overlap between the phy and cry pathways. First, phyA accumulates to such high levels under very low light conditions that its minor absorption of blue light causes significant period shortening. Second, 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 signaling requires func-

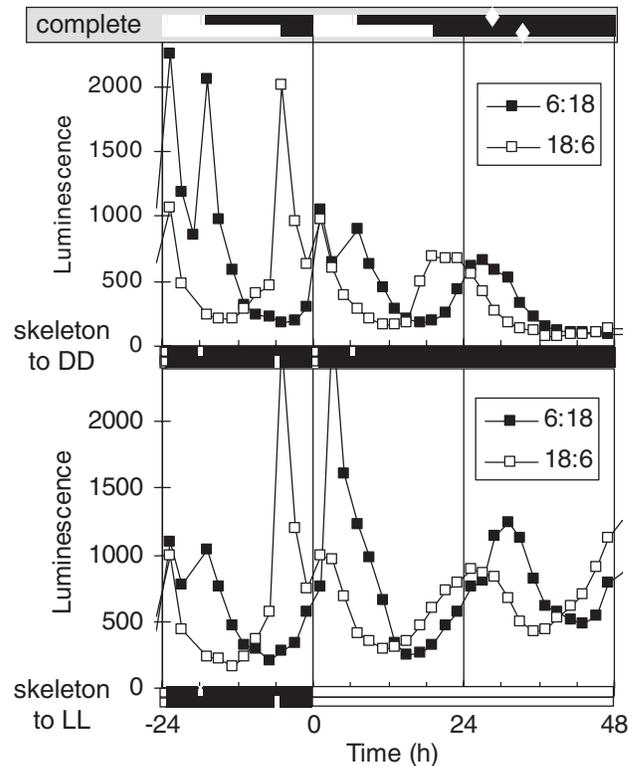


Figure 2. Entrainment of *CAB:LUC* rhythms by skeleton photoperiods in *Arabidopsis*. Upper bars show intervals of light and darkness under complete photoperiods of 6L:18D above 18L:6D ("complete"). The predicted phase of peak *CAB* expression after a transfer from each complete photoperiod to DD is marked by a diamond, showing that phase is earlier in 6L:18D than in 18L:6D (Millar and Kay, 1996). Bars below each graph show intervals of light and darkness in skeleton photoperiods of 30 min white light (e.g., skeleton 18:6 photoperiod is 0.5L:17.5D:0.5L:5.5D). Luminescence levels are shown for transgenic *CAB:LUC* plants on the last of 6 entraining cycles, followed by a transfer to constant darkness (upper) or constant light (lower). Each light pulse acutely activates *CAB* expression. After the transfer to constant conditions, the peak of *CAB* expression is close to the predicted phase after the 6:18 skeleton photoperiod. The clock performs a "phase jump" under 18:6 skeleton entrainment, interpreting it as 6:18, so the peak in the 18:6 samples occurs ca, 6 h earlier than the 6:18. Transfer to LL causes a phase delay in both samples, compared to DD (A. J. Millar and S. A. Kay, unpublished data).

tional crys in a nonphotoreceptor role (Somers, Devlin, et al., 1998; Devlin and Kay, 2000). Several other interactions have been described from molecular and/or genetic assays (some of which are shown in Fig. 2). The plant clock clearly exhibits behaviors similar to the parametric light effects and the "Aschoff rule" described for animals, and the simple, period assay has been useful in studying its mechanism (see below).

The period assay is operationally very similar to the ubiquitous hypocotyl length test; it was quite familiar to the many contemporary circadian researchers who came from previous projects in the phytochrome field, bringing a rich heritage of protocols and biological materials (especially mutants). They were also trained in the light pulse protocols, however, and 10 years after work started on *Arabidopsis* rhythms, the first PRCs were published (Covington et al., 2001; Devlin and Kay, 2001; an earlier preview appeared as Panda et al., 1998).

PHASE RESETTING

Brief light pulses were known to reset plant clocks, resulting in PRCs of type 1 or type 0, depending on the pulse amplitude. As predicted by Winfree, a singularity has been demonstrated in the circadian system that controls the rhythmic opening of *Kalanchoe blossfeldiana* flowers (reviewed in Engelmann and Johnsson, 1998). So plant clocks share nonparametric entrainment also. However, experimental difficulties in measuring phase shifts slowed progress in the molecular genetic model, *Arabidopsis*, so there remains much work to be done. Many of the rhythms we assay in *Arabidopsis*, such as *CAB* gene expression, are related to photosynthesis and have both light and circadian regulation. Owing to the absence of light activation, they lose amplitude in darkness after a few cycles, which has prevented the testing of a stable phase shift in a normal PRC protocol. The first PRC, then, used a 3-h bright light pulse to reset rhythms that were maintained by a constant background of dim red light (Devlin and Kay, 2001). The resulting type 0 PRC had little dead zone but was otherwise quite typical.

If a single light pulse produces strong resetting, then a sequence of pulses with an appropriate period should entrain the clock. Furthermore, the characteristic shape of circadian PRCs is such that a skeleton photoperiod consisting of a pulse at the time of dawn and another at dusk should result in a similar phase of entrainment to a complete photoperiod. The latter prediction holds for short photoperiods: The phase produced by the equivalent skeleton photoperiod is very similar. Pittendrigh showed that this predictability breaks down for long photoperiods (e.g., Pittendrigh, 1981) because the clock "interprets" a skeleton "18:6" photoperiod as a 6L:18D photoperiod and performs a "phase jump." *Arabidopsis* rhythms conform to this experimental regularity (Fig. 3).

CAB:LUC plants grown under 12L:12D cycles then entrained to 6L:18D or 18L:6D photoperiods revealed slightly different phases when tested in a subsequent interval of constant light or darkness (see below; Millar and Kay, 1996). 6L:18D leads to an earlier phase of entrainment (marked by diamonds in Fig. 3), and the plants entrained to a "6:18" skeleton photoperiod at the predicted phase. However, the plants entrained to the "18:6" skeleton photoperiod had an earlier phase, following a transfer to constant light (Fig. 3, lower panel). The data from plants transferred to constant darkness (Fig. 3, upper panel) showed that both groups of plants had entrained identically, relative to the shorter dark interval between the pulses. The phase difference between the samples directly reflects the fact that this shorter interval occurred 6 h earlier in the "18:6" compared to the "6:18" skeleton photoperiod. We interpret the result as follows: Both samples started growth with the same phase under 12L:12D cycles, and in one sample, the clock made a small phase advance to entrain under the "6:18" skeleton photoperiods. The plants under the "18:6" skeleton photoperiods found no stable phase of entrainment corresponding to the small phase delay that was predicted from plants in 18L:6D. Rather, they continued resetting until the 6-h "night" interval between the skeleton pulses occurred in the subjective day, which is the same, stable phase as in "6:18" skeleton photoperiods. Mathematics describes the situation precisely: The stable fixed point corresponding to the 18L:6D phase of entrainment disappeared at a saddle node bifurcation (B. Shulgin, A. J. Millar, and D. A. Rand, unpublished data). When the mechanism of resetting is better known, it will be possible to describe the same phenomenon in molecular terms.

The development of a marker that was stably rhythmic for many days in darkness, *CCR2* gene expression, recently allowed the measurement of PRCs for shorter light pulses given without background illumination (Covington et al., 2001). Hour-long red and blue light pulses give similar results, a type 0 PRC with a small dead zone, again suggesting that both phy and cry photoreceptors participate. These experiments must now be repeated in photoreceptor mutants and with different amplitudes of light pulse to identify the particular contribution of each photoreceptor species. It is hoped that a more complete data set will suggest a framework for understanding, or at least rationalizing, the multiplicity of input photoreceptors for the plant clock.

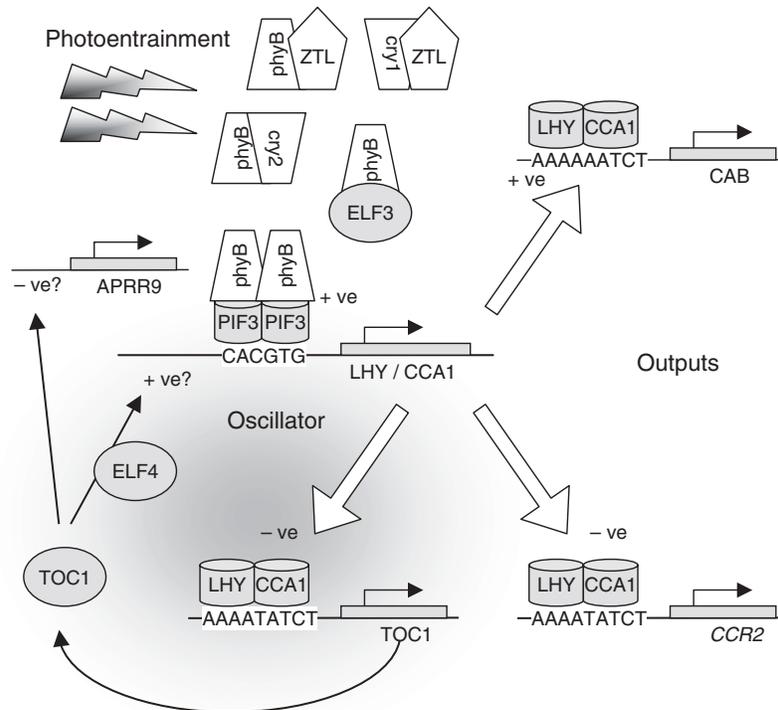


Figure 3. Components of the *Arabidopsis* circadian system. Some of the photoreceptors (cry1 and phyB) and photoreceptor-interacting proteins are shown at top left. ZTL may also function as a photoreceptor. phyB interacts with PIF3 bound to the G-box promoter sequence to activate (+ve) *LHY* and *CCA1* genes in response to light, providing a possible mechanism for photoentrainment. Interaction with ELF3 suppresses phyB function around subjective dusk. Genes that are known to be critical for ongoing rhythmicity are shown in the shaded area (*LHY/CCA1* and *TOC1*), with the feedback loop proposed by Alabadi and colleagues (2001). Interactions of *LHY* and/or *CCA1* protein demonstrated *in vitro* are shown in large arrows; interactions demonstrated genetically are shown in small arrows. *TOC1* and *ELF4* proteins are required for the activation of *CCA1* transcription, for example, but their mechanism of action is unclear. *TOC1* can also inhibit expression of its light-activated homologue, *APRR9*. Two rhythmic output genes are shown, *LHY* and/or *CCA1* activate *CAB* gene transcription (+ve) in the morning but inhibit transcription of *CCR2* (-ve).

PHASE OF ENTRAINMENT

The joint input from the array of photoreceptors entrains the plant's rhythms to a particular phase relative to the environmental day/night cycle, known as the phase of entrainment. Plants in most latitudes cannot avoid the day/night cycle, so free running is not a physiological state (with the possible exception of buried seeds, but we know little of their rhythms). The phase of entrainment is therefore the most important expression of circadian regulation in nature. It is predicted to depend not only on the zeitgebers and input pathways but also on the period of the oscillator (τ) or, more specifically, on the difference in period between the oscillator and the entraining cycle ($T-\tau$) (Pittendrigh, 1981). Variation in any one of these factors should alter the phase of entrainment; several have been tested in *Arabidopsis*.

Phase under Altered Photoperiod

The most physiologically relevant variation is the alteration in photoperiod, which occurs naturally in the seasonal cycle and alters the phase of entrainment. *CAB* gene expression peaks at a phase about 40% of the way through the predicted light interval, for example, when it is measured in constant darkness after entrainment to several cycles of a test photoperiod (Millar and Kay, 1996). This indicates that the steps at dawn and dusk do not predominate and drive this rhythm because its phase would then be a constant time interval from either dawn or dusk. Rather, at least 2 zeitgebers must participate in entrainment, from a selection comprising the sharp transitions at dawn and dusk and the intervals of continuous light and darkness. Prolonged constant conditions do alter period (see above), although it has not been demonstrated that the duration of a single night is sufficient to lengthen the period. The dark-light transition at

dawn has a major effect; for example, a single additional transition into constant light considerably reduces the phase change caused by preceding photoperiods (Millar and Kay, 1996) and skeleton photoperiods (Fig. 3). The light-dark transition at dusk has less effect: A single transition to darkness applied at various times hardly affected the phase of wild-type *Arabidopsis*, for example (McWatters et al., 2000). 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).

Phase under Altered T Cycles

Varying the zeitgeber period has been useful experimentally as a means of changing the phase of entrainment (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 period ~24.5 h). Under 21-h zeitgeber cycles, *toc1-1* plants have a normal phase of entrainment. This was taken to indicate that the period defect alone accounted for the altered phase and the mutation did not affect the input pathway (Somers, Webb, et al., 1998). This experiment was originally carried out using temperature cycles, but similar studies have recently used light:dark cycles with similar results (Yanovsky and Kay, 2002). In contrast, the early phase of the *phyB* mutant was rescued by temperature entrainment under 24-h temperature cycles, indicating that the mutation affected only the light input pathway (Salome et al., 2002).

THE PHYSIOLOGY OF PHOTOENTRAINMENT

Two issues arise particularly from seasonal variation in photoperiod. First, it may be favorable to alter the phases of rhythms relative to each other, for example, to maintain coincidence of one rhythm with dawn and of another with dusk. This is the issue addressed by the 2-oscillator model for nocturnal rodents, in which oscillators "e" and "m" control the start and end of the major activity bout at the beginning and end of the night, respectively (Meijer and Schwartz, 2003 [this issue]). The structure of the relevant circadian

output pathways may be critical in this respect, as an output pathway that includes a slave oscillator may cause more subtle phase changes than a simple delay mechanism. There is little information on relative phase changes so far in plants, but a wide range of gene expression markers is now available to address the issue. Two of the markers, *CCR2* and *PHYB*, are subject to negative feedback regulation that does not depend directly on the clock and suggests the potential for a slave oscillator (Heintzen et al., 1997; Hall et al., 2002).

Second, entrainment by different photoperiods is of particular interest in the context of seasonal rhythms that respond to photoperiod. Flowering is the best-known photoperiodic response in plants. Much physiological evidence shows that "dark-dominant" plants (a well-studied example is *Ipomoea nil*), most of which flower in short days, respond to a critical night length (Lumsden, 1998). This is measured using a circadian rhythm of floral response that is strongly reset by the light-dark transition, effectively restarting from lights-off. This rhythm is thus more like the driven clock in *Neurospora crassa* (see Liu, 2003 [this issue]) than the entrainment of *CAB* expression that was described above in *Arabidopsis*. *Arabidopsis*, in contrast, flowers preferentially in long days, its photoperiodic mechanism is light dominant, and key molecular components have been identified (Mouradov et al., 2002). Light-dominant species respond to day length rather than night length, and their photoperiodic response rhythm is more strongly reset by lights-on than lights-off, similar to the *Arabidopsis CAB* expression rhythm. The molecular mechanism underlying this striking difference in entrainment is not yet known, not least because molecular studies of short-day plants are in their infancy (and in the leading example, rice, the resetting behavior of its photoperiod response rhythm is not well studied). However, mutants of *Arabidopsis* that lack *EARLY-FLOWERING 3 (ELF3)* gene function show the dark-driven pattern of entrainment (McWatters et al., 2000), implicating this circadian gating gene in the mechanism (see below).

Operationally, then, light input pathways in plants allow both the familiar forms of resetting, Aschoff-style modulation of period, and abrupt resetting by light pulses or steps. At least 6 members of the phy and cry photoreceptor families contribute, with complex interactions and overlaps in the wild-type plant. What is their target in the oscillator mechanism, the equivalent of Tim protein degradation in *Drosophila*, or *frq*

transcription in *Neurospora*? Here we leave the open territory and enter a growing thicket of molecular interactions, with sun flecks of data, many of which are still quite disconnected.

THE MOLECULAR TARGETS

The molecular components of the plant oscillator are appropriately in the center of our thinking because one of these must respond to light input to effect resetting. A current difficulty is that many of the known clock-affecting genes are implicated to a greater or lesser extent in light signaling or light responses. Foremost among the candidate oscillator components are 2 small gene families founded by the DNA-binding proteins LHY and CCA1 and the pseudo-response regulator protein TOC1. The first reasonable model to explicitly link these components (Alabadi et al., 2001) is outlined in Figure 2. It can be summarized as follows: 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 to 3 h; they bind to and thus inhibit transcription from the TOC1 promoter. As LHY and CCA1 protein levels fall toward 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 indirectly. This model captures a number of data sets but was known to be incomplete; notably, *lhy;cca1* double mutants that lack both gene functions were subsequently shown to retain a weak, short-period rhythm under constant light (Alabadi et al., 2002; Mizoguchi et al., 2002). The mechanisms of CCA1 activation are unknown but require 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).

The best-supported mechanism for light entrainment can be described as the "PIF3 hypothesis" (reviewed in Martinez-Garcia et al., 2000; Nagy and Schafer, 2002). In summary, phytochrome-interacting factor 3 (PIF3) is a DNA-binding protein of the bHLH class, which also contains a PAS domain, though not in the same domain arrangement as the animal bHLH-PAS proteins of the animal *Clock* family. 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. As mutants with altered PIF3 function affect light responses in vivo, this is clearly one potential mechanism of photoentrainment. This mechanism integrates well with the model of the circadian oscillator mechanism outlined above; stronger experimental support for its importance in circadian resetting will be welcome.

A second possible pathway involves the ZEITLUPE (ZTL) protein, which contains a PAS-related domain that was shown to bind a flavin chromophore in the phototropin proteins (Somers et al., 2000). *ztl* mutant phenotypes are light dependent, supporting a possible photoreceptor role. However, both red and blue light were effective (Somers et al., 2000), and the ZTL protein also has the potential to interact with both phyB and cry1, which might cause an indirect light dependence of its function (Jarrillo et al., 2001). The 2 other domains of ZTL, an F-box and 7 kelch repeats, suggest an involvement in ubiquitin-mediated protein degradation. Although this suggests a similarity to the mechanism of light-mediated Tim protein degradation in *Drosophila* (see Ashmore and Sehgal, 2003 [this issue]), the ubiquitin pathway is such a basic element of cell function that no significant link can be assumed.

The 4 PSEUDO-RESPONSE REGULATOR (APRR) genes homologous to TOC1 prompt other speculation. They are expressed rhythmically, in an intriguing sequence every 2 to 3 h from dawn to dusk, APRR9-APRR7-APRR5-APRR3-TOC1 (e.g., Makino et al., 2002). Alterations in TOC1 function have greater effects on oscillator function than manipulation of other APRRs, but their joint function remains to be elucidated in double mutants. Three APRRs have been linked to light signaling: APRR9 expression is light activated but inhibited by overexpression of TOC1 (Makino et al., 2002), APRR7 has recently been reported as a modifier of phytochrome signaling in hypocotyl elongation (Kaczorowski and Quail, 2002), TOC1 protein interacts in vitro with PIF3 (Makino et al., 2002), and strong *toc1* mutant alleles can alter light responses (Mas et al., 2003). The domains of phytochrome proteins that are required for light signaling have sequence similarity to bacterial histidine kinases (Montgomery and Lagarias, 2002). In bacterial 2-component systems, histidine kinases signal via a "transmitter" domain to response regulator proteins; this system is conserved in some plant-signaling path-

ways (Lohrmann and Harter, 2002). APRRs are homologous to response regulators, although they lack a critical amino acid. A simple speculation is that the ancestral signaling pathway might have adopted a different biochemical mechanism but retained the same sequence of protein-protein interactions: APRRs might thus function in part downstream of phytochromes, either modifying phy signaling through PIF3 or in a parallel input pathway.

There remains considerable scope for surprise in unraveling the mechanism(s) of light input to the plant circadian clock. The early definition of the input photoreceptors suggests that the surprises will not be slow to arrive and has provided an array of experimental tools that greatly facilitate the process. One of the issues will be to integrate the early and partial biochemical insights into a framework that includes the more intricate aspects of physiology, such as the possibility that the input pathways also vary over time.

RHYTHMIC GATING OF LIGHT INPUT

The photoreceptor genes are themselves targets of circadian regulation, as the RNA abundance of all the *PHY* and *CRY* genes is rhythmic, though with varying amplitudes and peak phases (Bognar et al., 1999; Hall et al., 2001; Toth et al., 2001). The functional interpretation of this observation is complicated by the fact that although PHYB protein is rhythmically synthesized, its bulk level is not rhythmic, presumably due to the long half-life of the protein (Bognar et al., 1999). This result was recently confirmed for PHYE; PHYA and PHYC showed at most low-amplitude rhythms in constant light (Sharrock and Clack, 2002). The phy and cry photoreceptors are posttranslationally modified by phosphorylation and nuclear translocation (Kircher et al., 2002; Nagy and Schafer, 2002), however, so it remains possible that the daily amount of newly synthesized protein forms a rhythmic pool that is functionally distinct from preexisting protein.

A circadian gating pathway that depends on the *EARLY-FLOWERING 3 (ELF3)* gene strongly inhibits the activity of the light input pathways around subjective dusk (McWatters et al., 2000; Covington et al., 2001). This gating is essential for normal entrainment under long photoperiods and for continued rhythmicity in constant light because the oscillator arrests at about CT10 in *elf3* mutants if light is present (McWatters et al., 2000). The *ELF3* pathway therefore functions as a zeitnehmer, as proposed by Roenneberg

and Merrow (1998), and it is essential for the relative insensitivity of *Arabidopsis* rhythms to the light-dark transition (see above). In *elf3* mutants, *Arabidopsis* rhythms can be driven by the light-dark transition, similar to the photoperiod response rhythm of dark-dominant plants. The ELF3 protein interacts with phyB, and although the effect of this interaction is unknown, it strongly suggests that phyB protein can affect the clock in the subjective evening (Liu et al., 2001). It will now be very interesting to determine whether this late effect has the same mechanism as resetting at lights-on and whether other proteins cause a symmetrical, though less pronounced, gating of light input around subjective dawn.

FUTURE PROSPECTS

The plant circadian clock clearly uses many photoreceptors, which may require several input pathways to the oscillator. Much work remains to elucidate their mechanisms; however, mapping the molecular interactions in increasing detail does not automatically lead to understanding of such a complex system. One possible route toward such understanding would be to produce a computational model of the specific, molecular effects of the various photoreceptor pathways on oscillator components, then to test how well the model's behavior could account for the plant's entrainment. In other words, build up a model of the system from the biochemistry and cell biology of phototransduction, then use the model to reveal the contribution of particular biochemical entities to particular aspects of entrainment. The entrainment of the plant would ideally be tested in a simplified experimental protocol (such as far-red:dark cycles that would activate only one photoreceptor, phyA), to reduce the amount of "building" required. Accuracy in such a model gives real confidence, as no "fudges" were involved to fit the model to the plant's entrainment. This high standard is not attainable now, for lack of both molecular information and detailed entrainment studies, but this may change—photoreceptors are, after all, a classical subject for highly quantitative, biophysical studies. Modeling is useful now as an aid to thought and experimental design because the "fudges" required explicitly delimit our areas of ignorance.

A further prompt to simplify and standardize our experimental protocols comes from the diversity of clocks in plants. Very many (if not all) plant cells have

a functional circadian system and input photoreceptors. The clocks controlling gene expression in different anatomical locations are functionally independent and their periods differ slightly, probably reflecting differences among cell types, possibly including differences in light input pathways (Thain et al., 2000, 2002). 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): 2 oscillators controlled a biphasic rhythm, but only 1 of the oscillators was reset by light. As photoentrainment studies become more detailed and quantitative, we may need to consider which, and how many, circadian clocks we are studying.

REFERENCES

- Alabadi D, Oyama T, Yanovsky MJ, Harmon FG, Mas P, and Kay SA (2001) Reciprocal regulation between TOC1 and LHY/CCA1. *Science* 293:880-883.
- Alabadi D, Yanovsky MJ, Mas P, Harmer SL, and Kay SA (2002) Critical role for CCA1 and LHY in maintaining circadian rhythmicity in *Arabidopsis*. *Curr Biol* 12:757-761.
- Ashmore LJ and Sehgal A (2003) A fly's eye view of circadian entrainment. *J Biol Rhythms* 18:206-216.
- Bognar LK, Hall A, Adam E, Thain SC, Nagy F, and Millar AJ (1999) The circadian clock controls the expression pattern of the circadian input photoreceptor, phytochrome B. *Proc Natl Acad Sci U S A* 96:14652-14657.
- Briggs WR and Christie JM (2002) Phototropins 1 and 2: Versatile plant blue-light receptors. *Trends Plant Sci* 7:204-210.
- Covington MF, Panda S, Liu XL, Strayer CA, Wagner DR, and Kay SA (2001) ELF3 modulates resetting of the circadian clock in *Arabidopsis*. *Plant Cell* 13:1305-1315.
- Devlin PF (2002) Signs of the time: Environmental input to the circadian clock. *J Exp Bot* 53:1535-1550.
- Devlin PF and Kay SA (2000) Cryptochromes are required for phytochrome signaling to the circadian clock but not for rhythmicity. *Plant Cell* 12:2499-2509.
- Devlin PF and Kay SA (2001) Circadian photoperception. *Annu Rev Physiol* 63:677-694.
- Doyle MR, Davis SJ, Bastow RM, McWatters HG, Kozma-Bognar L, Nagy F, Millar AJ, and Amasino RM (2002) The ELF4 gene controls circadian rhythms and flowering time in *Arabidopsis thaliana*. *Nature* 419:74-77.
- Engelmann W and Johnsson A (1998) Rhythms in organ movement. In: *Biological Rhythms and Photoperiodism in Plants*, PJ Lumsden and AJ Millar, eds, BIOS Scientific, Oxford, UK.
- Fankhauser C, Staiger D (2002) Photoreceptors in *Arabidopsis thaliana*: Light perception, signal transduction and entrainment of the endogenous clock. *Planta* 216:1-16.
- Hall A, Kozma-Bognar L, Bastow RM, Nagy F, and Millar AJ (2002) Distinct regulation of *CAB* and *PHYB* gene expression by similar circadian clocks. *Plant J* 32:529-537.
- Hall A, Kozma-Bognar L, Toth R, Nagy F, and Millar AJ (2001) Conditional circadian regulation of *PHYTOCHROME A* gene expression. *Plant Physiol* 127:1808-1818.
- Heintzen C, Nater M, Apel K, and Staiger D (1997) AtGRP7, a nuclear RNA-binding protein as a component of a circadian-regulated negative feedback loop in *Arabidopsis thaliana*. *Proc Natl Acad Sci U S A* 94:8515-8520.
- Jarillo JA, Capel J, Tang RH, Yang HQ, Alonso JM, Ecker JR, and Cashmore AR (2001) An *Arabidopsis* circadian clock component interacts with both *CRY1* and *phyB*. *Nature* 410:487-490.
- Kaczorowski K and Quail PH (2002) 13th Symposium on *Arabidopsis* Research, Abstract 9-04, 28 June-2 July, Seville, Spain.
- Kendrick RE and Kronenberg GHM, eds (1994) *Photomorphogenesis in Plants*, Vol. 2, Kluwer Academic, Dordrecht, the Netherlands.
- Kircher S, Gil P, Kozma-Bognar L, Fejes E, Speth V, Husselstein-Muller T, Bauer D, Adam E, Schafer E, and Nagy F (2002) Nucleocytoplasmic partitioning of the plant photoreceptors phytochrome A, B, C, D, and E is regulated differentially by light and exhibits a diurnal rhythm. *Plant Cell* 14:1541-1555.
- Kolar C, Fejes E, Adam E, Schafer E, Kay S, and Nagy F (1998) Transcription of *Arabidopsis* and wheat *Cab* genes in single tobacco transgenic seedlings exhibits independent rhythms in a developmentally regulated fashion. *Plant J* 13:563-569.
- Lin C (2002) Blue light receptors and signal transduction. *Plant Cell* 14:S207-S225.
- Liu XL, Covington MF, Fankhauser C, Chory J, and Wagner DR (2001) ELF3 encodes a circadian clock-regulated nuclear protein that functions in an *Arabidopsis* *PHYB* signal transduction pathway. *Plant Cell* 13:1293-1304.
- Liu Y (2003) Molecular mechanisms of entrainment in the neurospora circadian clock. *J Biol Rhythms* 18: 195-205.
- Lohrmann J and Harter K (2002) Plant two-component signaling systems and the role of response regulators. *Plant Physiol* 128:363-369.
- Lumsden PJ (1998) Photoperiodic induction in short-day plants. In *Biological Rhythms and Photoperiodism in Plants*, PJ Lumsden and AJ Millar, eds, pp 167-182, BIOS Scientific, Oxford, UK.
- Makino S, Matsushika A, Kojima M, Yamashino T, and Mizuno T (2002) The *APRR1/TOC1* quintet implicated in circadian rhythms of *Arabidopsis thaliana*: I. Characterization with *APRR1*-overexpressing plants. *Plant Cell Physiol* 43:58-69.
- Martinez-Garcia JF, Huq E, and Quail PH (2000) Direct targeting of light signals to a promoter element-bound transcription factor. *Science* 288:859-863.
- Mas P, Alabadi D, Yanovsky MJ, Oyama T, and Kay SA (2003) Dual role of TOC1 in the control of circadian and photomorphogenic responses in *Arabidopsis*. *Plant Cell* 15:223-236.

- McWatters HG, Bastow RM, Hall A, and Millar AJ (2000) The *ELF3 zeitnehmer* regulates light signalling to the circadian clock. *Nature* 408:716-720.
- Meijer JH and Schwartz WJ (2003) In search of the pathways for light-induced pacemaker resetting in the suprachiasmatic nucleus. *J Biol Rhythms* 18:235-249.
- Millar AJ and Kay SA (1996) Integration of circadian and phototransduction pathways in the network controlling *CAB* gene transcription in *Arabidopsis*. *Proc Natl Acad Sci U S A* 93:15491-15496.
- Millar AJ, Straume M, Chory J, Chua N-H, and Kay SA (1995) The regulation of circadian period by phototransduction pathways in *Arabidopsis*. *Science* 267:1163-1166.
- Mizoguchi T, Wheatley K, Hanzawa Y, Wright L, Mizoguchi M, Song HR, Carre IA, and Coupland G (2002) *LHY* and *CCA1* are partially redundant genes required to maintain circadian rhythms in *Arabidopsis*. *Dev Cell* 2:629-641.
- Montgomery BL and Lagarias JC (2002) Phytochrome ancestry: Sensors of bilins and light. *Trends Plant Sci* 7:357-366.
- Mouradov A, Cremer F, and Coupland G (2002) Control of flowering time: Interacting pathways as a basis for diversity. *Plant Cell* 14:S111-S130.
- Nagy F and Schafer E (2002) Phytochromes control photomorphogenesis by differentially regulated, interacting signaling pathways in higher plants. *Annu Rev Plant Physiol Plant Mol Biol* 53:329-355.
- Panda S, Somers DE, and Kay SA (1998) 9th Symposium on *Arabidopsis* Research, Abstract 268, 24-28 June, Madison, WI.
- Pittendrigh CS (1981) Circadian systems: Entrainment. In *Handbook of Behavioral Neurobiology*, J Aschoff, ed, Vol 4, pp 95-124, Plenum, New York.
- Quail PH (2002) Phytochrome photosensory signalling networks. *Nat Rev Mol Cell Biol* 3:85-93.
- Roden LC, Song HR, Jackson S, Morris K, and Carre IA (2002) Floral responses to photoperiod are correlated with the timing of rhythmic expression relative to dawn and dusk in *Arabidopsis*. *Proc Natl Acad Sci U S A* 99:13313-13318.
- Roenneberg T and Mrosovsky M (1998) Molecular circadian oscillators: An alternative hypothesis. *J Biol Rhythms* 13:167-179.
- Salome PA, Michael TP, Kearns EV, Fett-Neto AG, Sharrock RA, and McClung CR (2002) The out of phase 1 mutant defines a role for *PHYB* in circadian phase control in *Arabidopsis*. *Plant Physiol* 129:1674-1685.
- Schmitz O, Katayama M, Williams SB, Kondo T, and Golden SS (2000) *CikA*, a bacteriophytochrome that resets the cyanobacterial circadian clock. *Science* 289:765-768.
- Sharrock RA and Clack T (2002) Patterns of expression and normalized levels of the five *Arabidopsis* phytochromes. *Plant Physiol* 130:442-456.
- Somers DE (1999) The physiology and molecular bases of the plant circadian clock. *Plant Physiol* 121:9-19.
- Somers DE, Devlin PF, and Kay SA (1998) Phytochromes and cryptochromes in the entrainment of the *Arabidopsis* circadian clock. *Science* 282:1488-1490.
- Somers DE, Schultz TF, Milnamow M, and Kay SA (2000) *ZEITLUPE* encodes a novel clock-associated PAS protein from *Arabidopsis*. *Cell* 101:319-329.
- Somers DE, Webb AAR, Pearson M, and Kay SA (1998) The short-period mutant, *toc1-1*, alters circadian clock regulation of multiple outputs throughout development in *Arabidopsis thaliana*. *Development* 125:485-494.
- Thain SC, Hall A, and Millar AJ (2000) Functional independence of circadian clocks that regulate plant gene expression. *Curr Biol* 10:951-956.
- Thain SC, Murtas G, Lynn JR, McGrath RB, and Millar AJ (2002) The circadian clock that controls gene expression in *Arabidopsis* is tissue specific. *Plant Physiol* 130:102-110.
- Toth R, Kevei E, Hall A, Millar AJ, Nagy F, and Kozma-Bognar L (2001) Circadian clock-regulated expression of phytochrome and cryptochrome genes in *Arabidopsis*. *Plant Physiol* 127:1607-1616.
- Yanovsky MJ and Kay SA (2001) Signaling networks in the plant circadian system. *Curr Opin Plant Biol* 4:429-435.
- Yanovsky MJ and Kay SA (2002) Molecular basis of seasonal time measurement in *Arabidopsis*. *Nature* 419:308-312.
- Yanovsky MJ, Mazzella MA, and Casal JJ (2000) A quadruple photoreceptor mutant still keeps track of time. *Curr Biol* 10:1013-1015.