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What makes the Arabidopsis clock tick on time? A review on entrainment

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ABSTRACT

Entrainment, the synchronization of a circadian clock with the external environment, is a crucial step in daily life. Although many signals contribute to entrainment, light and temperature are typically the strongest resetting cues. Much progress has been made concerning light resetting in the model plant *Arabidopsis thaliana*. Multiple photoreceptors (phytochromes, cryptochromes, LOV-domain proteins) are involved in light perception. The clock genes *CCA1*, *LHY* and *TOC1* are all probable targets of light signalling, although the details of these pathways are not completely established. Temperature can entrain the clock, but little is known about the mechanism underlying this resetting; no obvious clock gene candidate for temperature resetting has been identified. Although circadian research has emphasized oscillations in free-running conditions, in the real world the circadian clock is entrained. During entrainment, short or long period mutants exhibit a 24-h period, but a mutant phenotype is often manifested as an altered phase relationship with the entraining cycle; short and long period mutants show leading and lagging phases, respectively, and this may be detrimental under some conditions. Arrhythmic CCA1-overexpressing plants display increased lethality under very short photoperiods, consistent with the circadian clock being of adaptive significance to life on a rotating world.

Key-words: circadian rhythm; clock; cryptochrome; entrainment; light signalling; phytochrome; temperature sensing.

INTRODUCTION

A rhythm under the control of a circadian oscillator must obey three fundamental rules. First, this rhythm must persist in the absence of environmental time cues. In most cases, the persistence of the rhythm is assayed at constant temperature under continuous light or darkness. Under these conditions, the clock oscillates with its free-running period. This may seem curious, given that clocks evolved in a world where constant conditions do not exist and their presumed role is to temporally synchronize the organism with the environmental cycle and, for example, to allow the anticipation of dawn and dusk. One possible explanation calls upon the way the system has been built. All circadian oscillators described to date are based on a feedback loop, which will create an oscillation in continuous conditions, even though they are only exposed to such conditions in the artificial laboratory situation. Our ability to release plants, flies or flying squirrels into a setting freed of environmental time cues is also crucial to our understanding of the workings of the clock, as often it is only in these free-running conditions that the consequence of a mutation can be readily seen and interpreted.

The second rule a rhythm must follow to be considered circadian is that it must maintain a fairly constant period length over a range of physiologically relevant temperatures. This temperature compensation of period length assures a relative insensitivity to strong changes in temperature. However, this does not mean that the rhythm will not be affected by temperature cycles from the outside environment. And this leads us to the third and, for this review, most important aspect of a circadian rhythm: entrainment. The deviation of the free-running period from 24 h means that an endogenous clock would rapidly fall out of phase with local time. However, external time cues serve to synchronize an organism's endogenous clock with its environment. Although feeding cues can reset the clock of some animals, the most obvious entraining signals are those provided by the alternation of day and night: light-dark cycles and temperature cycles. Setting or resetting a circadian clock refers to the ability of the environmental signals to effect a stable change of the phase of the organism's internal clock. As will become clear in this review, resetting of a clock is achieved through the modification of the mRNA and/or protein and/or activity levels encoding one or more of the clock components.

Plants have an intricate system of photoreceptors and signalling pathways dedicated to the perception of light quality and quantity coming from the environment. Light induces the expression of some of the constituents of the Arabidopsis circadian oscillator and, thus, causes a stable phase shift that synchronizes the endogenous clock with the world it grows in (Fankhauser & Staiger 2002). Temperature also serves as a resetting signal, although the sensing mechanism is unknown. After presenting our current knowledge of light and temperature input to the clock, we will attempt to bring some key points in light of the rotating world in which plants normally thrive. For example, what

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really happens to a plant with a short or a long period under the strict 24-h entrainment caused by day-night cycles? Does it matter that plants have a fully functional wild-type clock?

THE ARABIDOPSIS CIRCADIAN CLOCK: A PRIMER

All entraining stimuli eventually alter the expression of some clock component, causing the necessary phase shift in the clock to synchronize the organism to the (new) external cycle. A quick primer on the Arabidopsis circadian system will therefore define a set of clock components that are potential targets of entraining stimuli. A simple model of the Arabidopsis circadian system is illustrated in Fig. 1. For more details on the workings of the Arabidopsis clock, several recent reviews provide fuller discussion (McClung, Salomé & Michael 2002; Eriksson & Millar 2003).

Let us first consider two transcription factors, CCA1 (for Circadian Clock Associated protein 1, Wang & Tobin 1998) and LHY (for Late Elongated Hypocotyl, Schaffer *et al*. 1998). These single-Myb domain proteins oscillate in a circadian fashion and show peak transcription, mRNA and protein abundance in the early morning, around dawn (Fig. 2c). CCA1 then becomes phosphorylated by Casein Kinase II (CK2, Sugano *et al*. 1998). This phosphorylation is a prerequisite for DNA binding. LHY does not appear to be phosphorylated *in vivo*, but can be used as a substrate by CK2 *in vitro* (Sugano *et al*. 1998). Both proteins target their own promoters, as well as each other's, and block further expression. In addition, they repress the expression of the third component in the system, the Pseudo-Response Regulator TIMING OF CAB EXPRESSION 1 (TOC1, also known as APRR1 or, more simply, PRR1, Matsushika *et al*. 2000; Strayer *et al*. 2000) directly through *TOC1* promoter binding. *TOC1* oscillates with peaks in transcription, mRNA and protein abundance near dusk, approximately 180° out of phase with CCA1 and LHY (Fig. 2c). Upon turnover of CCA1 and LHY protein, the repression of *TOC1* expression is alleviated, resulting in the accumulation of *TOC1* message and protein (Más *et al*. 2003). TOC1 acts as a positive regulator of *CCA1* and *LHY* expression, which closes the loop and starts the next cycle (Alabadí *et al*. 2001). It is not known whether this positive regulation of *CCA1* and *LHY* by TOC1 is direct or indirect. This reciprocal interaction between the three proteins has been postulated to comprise the core of the oscillator in Arabidopsis.

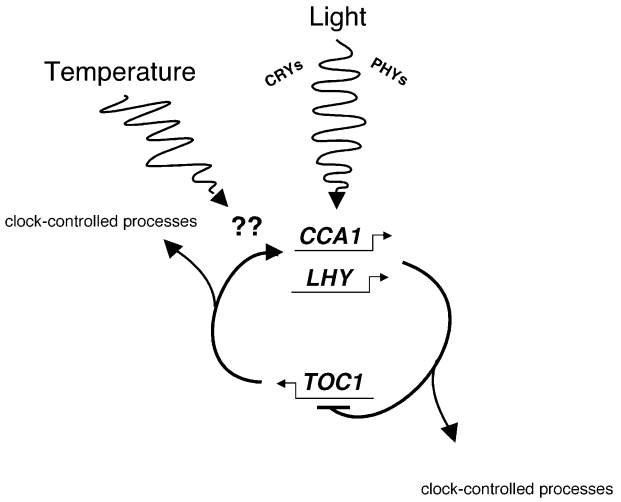

Figure 1. A simple model of the circadian oscillator in Arabidopsis. The oscillator is composed of the positive element TOC1, inducing the expression of the negative elements CCA1 and LHY. The negative elements are repressors of the positive element. The core oscillator is therefore based on an interconnected feedback loop. Light and temperature input allow the oscillator to synchronize its phase to match the outside environment. The oscillator regulates the clock-controlled processes/output rhythms.

Partial redundancy among clock components is evident for *CCA1* and *LHY*. Although loss of function alleles of *CCA1* or *LHY* confer a short period, *cca1 lhy* double loss of function plants are arrhythmic (Mizoguchi *et al*. 2002). Similarly, *TOC1* loss of function does not lead to arrhythmicity in all conditions, suggesting that TOC1 function is redundantly specified in at least some conditions. *TOC1* is the founding member of the *PSEUDO-RESPONSE REGULATOR* (*PRR*) family (Matsushika *et al*. 2000). Loss of function alleles of *PRR3*, *PRR5*, *PRR7* and *PRR9* all lead to alterations of circadian period or phase, demonstrating that the *TOC1*-related genes participate in some way in the clock (Eriksson *et al*. 2003; Ito *et al*. 2003; Michael *et al*. 2003b; Yamamoto *et al*. 2003).

LIGHT INPUT TO THE CLOCK: PHOTORECEPTORS GALORE

Photoreceptors perceive light quality and quantity. The white light under which we commonly grow our plants can be broken down into three active wavelengths or light qualities: far-red, red and blue light. Red and far-red light is perceived via the phytochromes (Wang & Deng 2002) and blue light is perceived by the cryptochromes, phototropins, and other receptors (Lin & Shalitin 2003). Three classes of photoreceptors have been shown to affect the period and/or phase of rhythms: the phytochromes, cryptochromes, and a novel class of blue-light photoreceptors containing a LOV domain.

The phytochromes and cryptochromes

The phytochrome gene family in Arabidopsis consists of five genes, *PHYA-PHYE* (Clack, Mathews & Sharrock 1994). The use of luciferase as a non-invasive reporter gene for clock-regulated transcription has greatly facilitated the study of the individual contribution of each PHY on the expression of *LHCB*, which is the best-studied circadian output rhythm in plants (Millar *et al*. 1992). In plants and day-active organisms, the free-running period of a rhythm is inversely correlated with the light intensity (Aschoff 1960). Thus, a mutant defective in a photoreceptor involved

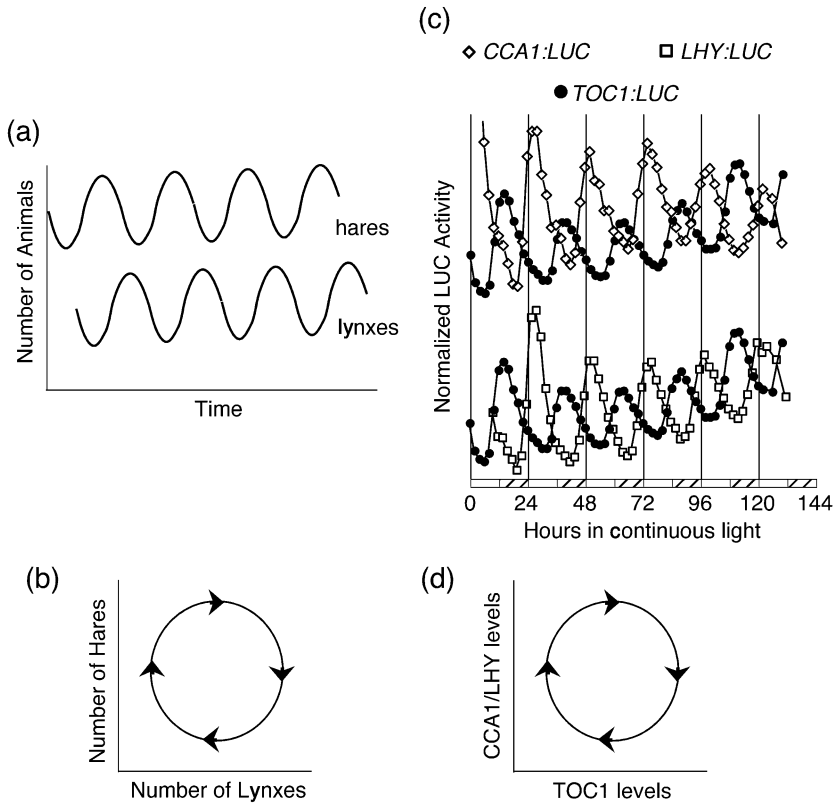

Figure 2. A limit-cycle approach to the Arabidopsis circadian system. (a) Oscillations in hares and lynxes. Because of their fast breeding time, the hare population quickly rises. This ample food supply allows the lynx population to increase as well. However, as the predatory pressure increases, the hare population plateaus and declines, with a subsequent crash in the lynx population, whose food supply is quickly disappearing. Once most lynxes have died from starvation, hares can again multiply because of the release of the predatory pressure.(b) A two-dimensional limit-cycle interpretation of the hare/lynx system.(c) The circadian clock in Arabidopsis relies on the cyclic expression of the genes encoding two transcription factors CCA1 and LHY, which peak in the morning, and the gene encoding the Pseudo-Response Regulator TOC1, which peaks in the evening. The rhythmic pattern of the three genes is consistent with the current model (Alabadí *et al.* 2001, 2002): as *CCA1* and *LHY* expression rises, *TOC1* expression becomes repressed, reaching its lowest point (trough) when *CCA1* and *LHY* are at their peak. Then, as the single Myb-domain proteins are degraded, *TOC1* expression is de-repressed. As *TOC1* reaches its peak, *CCA1* and *LHY* expression is induced to initiate the next cycle.(d) A two-dimensional limit-cycle interpretation of the Arabidopsis circadian system. By convention, ZT0 is designated as the top of the circle, when CCA1 and LHY levels are highest.

in light input to the clock would perceive less light than is present and would, as a consequence, display a longer period. The analysis of single and multiple *phy* mutants in red, far-red or blue lights showed that the loss of one or more photoreceptors indeed caused a lengthening in period. PHYA normally functions under low-fluence red and blue light, whereas PHYB is important for higher fluences of red light (Somers, Devlin & Kay 1998a). The free-running period of *phyA phyB phyD* and *phyA phyB phyE* triple mutants is even longer than that of the *phyA phyB* double mutant, but still responds to increasing light intensities, suggesting modest roles for PHYC, PHYD and PHYE (Devlin & Kay 2000).

The classical blue light photoreceptors, the cryptochromes, are part of a three gene-family in Arabidopsis (Brudler *et al.* 2003; Lin & Shalitin 2003). The most recent member, CRY3 or CRY-DASH, localizes to chloroplasts and mitochondria and is therefore unlikely to participate in light input to the nuclear clock (Kleine, Lockhart & Batschauer 2003). *cry1* mutants have a slightly longer period under both low and high fluence rates of blue light (Devlin & Kay 2000). CRY2 also has a role in clock-regulated *LHCB* expression, as the period of the *cry1 cry2* double mutant is longer than that of the *cry1* single mutant under all fluence rates of blue light (Devlin & Kay 2000). A more detailed analysis of the response of *cry2* mutants in red and blue lights found a weak period shortening in low fluence rates of red and blue lights (Más *et al.* 2000). Similarly, CRY1 plays a role in red light signalling, and loss of *cry1* results in a period lengthening of *LHCB* expression under low-fluence red light (Devlin & Kay 2000). Importantly, *cry1cry2* double mutants are still rhythmic, albeit with a long period, indicating that they are not integral components of the Arabidopsis circadian oscillator. This contrasts with the mammalian clock (van der Horst *et al.* 1999), where a mouse *Cry1Cry2* double knockout is arrhythmic in continuous conditions.

In nature, plants seldom grow under red or blue lights. In white light, photoreceptor mutations generally cause a weaker or distinct effect on the circadian clock compared

to their effects in red or blue light. For example, *phyB* mutants display a normal period length under white light for all rhythms tested, even though they have a long period in high-fluence red light (Hall *et al*. 2002; Salomé *et al*. 2002). Physical interaction between photoreceptors has been established for PHYA/CRY1 and PHYB/CRY2 (Ahmad *et al*. 1998; Más *et al*. 2000) and these interactions appear to be of importance for the control of *LHCB* transcription in white light. For instance, CRY1 acts downstream of PHYA in the regulation of *LHCB* period length (Devlin & Kay 2000). Loss of CRY1 function alone causes a lengthening of period length over the low to intermediate fluence range, but not under high fluence white light (Devlin & Kay 2000), which is in sharp contrast with the long period phenotype displayed by *cry1* mutants under all fluence rates of blue light. Loss of CRY2, on the other hand, creates a period lengthening in white light but not in blue light (Más *et al*. 2003). Now that the respective contributions of the PHYs and CRYs in red or blue lights have been established, it would be very informative to start characterizing their response to mixed red or blue lights. That is, what happens to the period of *LHCB* transcription under combined red and blue light, or blue and far-red lights? This exercise could help in understanding how a plant controls its period length under white light.

Loss of all phytochrome activity in the chromophore biosynthesis mutants *hy1*, *hy2* and *hy6* does not affect period length significantly (P.A.S. and C.R.M., unpublished; Millar *et al*. 1995). Even the loss of the four major Arabidopsis photoreceptors PHYA, PHYB, CRY1 and CRY2 does not affect the period of leaf movement (Yanovsky, Mazzella & Casal 2000). These results suggest that CRY1 and CRY2 (in the case of the strong *hy6* mutant) or PHYC, PHYD and PHYE (in the case of the *phyA phyB cry1 cry2* quadruple mutant) are sufficient to correctly pace the clock in white light. PHYB and CRY1 also play roles in white light in the regulation of circadian phase for a number of rhythms. *phyB* mutants display a leading phase in cotyledon movement and in *PHYB* and *LHCB* transcription, whereas *cry1* mutants have a lagging phase for the same rhythms (P.A.S. and C.R.M., unpublished; Hall *et al*. 2002; Salomé *et al*. 2002). The phase phenotype is due to a defect in light signalling, as it is evident only after entrainment to light-dark cycles but not to temperature cycles (P.A.S. and C.R.M., unpublished; Hall *et al*. 2002; Salomé *et al*. 2002).

DOWNSTREAM OF THE PHYTOCHROMES AND CRYPTOCHROMES?

Plant photoreceptors are not limited in their function to the control of circadian period and phase, but also affect plant morphogenesis in the light. One of the best characterized growth responses to light is the inhibition of hypocotyl length elongation (Neff & Chory 1998). The length of the hypocotyl is inversely proportional to the amount of light perceived, and is longer in loss of function photoreceptor mutants than in wild type. Mutations in PHYB or in intermediates in the PHYB signalling cascade have longer hypocotyls in red light, and to a lesser extent in white light. Most of these mutants have not been assessed for circadian behaviour. Two PHYB signalling cascade components, SENSITIVITY TO RED LIGHT REDUCED 1 (SRR1) and PRR7, have been described as having effects on the clock (Kaczorowski & Quail 2003; Staiger *et al*. 2003). Both *SRR1* and *PRR7* were identified based on the long hypocotyl phenotype of mutants carrying loss of function alleles in red light. SRR1 is a protein of unknown function with no known functional domains, but with putative orthologues in yeast, flies, mouse and humans. SRR1 can be found both in the cytosol and the nucleus, whereas PRR7 is exclusively nuclear. Both proteins are necessary for full PHYB function, as both mutants display a long hypocotyl in red light, although not to the same extent as strong *phyB* mutants. In addition, they show the same leading circadian phase in gene expression seen in *phyB* mutants in white light (Hall *et al*. 2002; Salomé *et al*. 2002; Kaczorowski & Quail 2003; Staiger *et al*. 2003). SRR1 is also important in the control of period length in white light, as the *srr1* mutation shortens the period of the clock. Neither protein interacts directly with PHYB, nor do they form the nuclear foci seen for PHYB after illumination with red light (Chen, Schwab & Chory 2003; Kaczorowski & Quail 2003; Staiger *et al*. 2003), indicating that other components must lie between the photoreceptor and SRR1 and PRR7. The circadian phenotypes of these mutants remain to be determined under red or blue lights.

PHYTOCHROME INTERACTING FACTOR 3 (PIF3) was the first published phytochrome-interacting protein that interacts in the yeast two-hybrid system with the C-terminal part of PHYB (Ni, Tepperman & Quail 1998). PIF3 is in the large (162-member) bHLH family of transcription factors (Toledo-Ortiz, Huq & Quail 2003), and binds to the G-box found in the promoters of light-induced genes (Martínez-García, Huq & Quail 2000). PIF3 is always nuclear and binds DNA even in the absence of PHYB (Ni, Tepperman & Quail 1999). Upon illumination with red light, PHYB translocates to the nucleus (Nagy & Schäfer 2000), where it complexes with PIF3 already bound to G-boxes. This PHYB-PIF3 complex induces the expression of light-responsive genes like *CCA1* and *LHY*, whose promoters contain each a G-box (Martínez-García *et al*. 2000). The extent of induction of *CCA1* and *LHY* expression following transfer to red light was decreased in plants expressing an antisense construct for PIF3 (line A22), therefore decreasing the levels of active PIF3. These findings suggest a very short signalling cascade between the photoreceptor and the effector genes. Additionally, these findings suggest a mechanism of light resetting of the clock. Light activates PHYB, which translocates to the nucleus, binds to PIF3 already bound to the *CCA1* and *LHY* promoters and induces their transcription. However, several publications have recently cast some doubt as to the precise role of PIF3 in light input to the clock. A T-DNA insertion allele, *pif3-1*, has a short hypocotyl in red light, in striking contrast with the very long hypocotyl seen in line A22 under the same conditions (Ni *et al*. 1998; Kim *et al*. 2003a). A possible reconciliation of

these data would be that line A22 affects multiple bHLH members and not just *PIF3*, and that the down-regulation of a second bHLH protein is responsible for the hypocotyl phenotype in line A22. Consistent with this interpretation, line A22 is also free of circadian phenotypes in white light, as seen by mRNA levels of the clock genes *CCA1*, *LHY* and *TOC1* (Oda *et al*. 2004). Using cotyledon movement as a non-invasive assay, no circadian phenotypes could be seen in single loss of function alleles of *PIF3* or *PIF4* (P.A.S. and C.R.M., unpublished), under either white or red light. Loss of function alleles in another member of the *PIF3* family, *PIL1*, also lack circadian defects in white light (Yamashino *et al*. 2003). Importantly, each of these genotypes (line A22, *pif3-1*, *pif4/srl2* and *pil1-1*) is entrained by light-dark cycles and takes on the proper phase when released in continuous light (P.A.S. and C.R.M., unpublished; Yamashino *et al*. 2003; Oda *et al*. 2004), indicating that light input to the clock is not compromised. Taken together, these results suggest that PIF3 is not necessary for transduction of the light signal to the clock. Redundancy among the *PIF3* family members is possible, and it will require the analysis of multiple loss of function plants to address this experimentally.

THE ZTL/ADO/LKP FAMILY OF LOV DOMAIN, F-BOX-CONTAINING PROTEINS

Earlier we noted that the *phyA phyB cry1 cry2* quadruple mutant retains rhythmicity and is entrainable to light-dark cycles. It is possible that PHYC, PHYD and PHYE provide light input to the clocks in the quadruple mutant, but it is also possible that photoreceptors other than the PHYs and CRYs can contribute. The phototropins (PHOT1 and PHOT2) detect blue light but do not seem to play a role in light input to the Arabidopsis clock (Devlin & Kay 2001). There are also UV-sensitive photoreceptors, but these are still poorly defined and there has been no evidence indicating that plant clocks respond to UV illumination.

Are there other good candidates to serve as circadian photoreceptors? Two genes come to mind: *ZEITLUPE*/*LOV-KELCH PROTEIN 1*/*ADAGIO 1* (*ZTL/LKP1/ADO1*), and *LKP2/ADO2* (Kiyosue & Wada 2000; Somers *et al*. 2000; Jarillo *et al*. 2001; Schultz *et al*. 2001). These genes are members of a three-gene family of proteins containing F-boxes and LOV and Kelch domains. The LOV domain is a flavin-binding light-sensitive domain, the Kelch domain is a protein–protein interaction motif, and the F-box implicates these proteins in ubiquitin-mediated proteasomal degradation. The family's third member, *FLAVIN-BINDING KELCH REPEAT F-BOX 1*, *FKF1*(*LKP3/ADO3*), is now considered an output component important for regulation of flowering time, but does not affect the clock (Nelson *et al*. 2000; Imaizumi *et al*. 2003).

The secondary structure of the family members points to a possible role in light perception. All three proteins are composed of a LOV domain, also found in the blue-light clock photoreceptors WC-1 and WC-2 from *Neurospora*, as well as in the Arabidopsis blue light photoreceptors PHOT1 and PHOT2 (Crosthwaite, Dunlap & Loros 1997; Froehlich *et al*. 2002; Corchnoy *et al*. 2003). In the case of WC-1 and the PHOTs, the LOV domain was shown to undergo light-induced photochemistry and conformational change *in vitro*. Similar results were obtained with the LOV domain of FKF1 (Cheng *et al*. 2003; Imaizumi *et al*. 2003). Based on the high sequence similarity between the LOV domains of ZTL, LKP2 and FKF1, it is likely that all three LOV domains can undergo such a conformational change *in vitro*, although this has yet to be shown in the context of the full-length protein. In addition, it remains to be determined if FKF1, ZTL and LKP2 function as photoreceptors *in vivo*.

Loss of function alleles of *ZTL* produce rhythms with a long period in cotyledon movement, *CCR2* mRNA levels and *LHCB* transcription in the light (Somers *et al*. 2000). More recently, *ztl* alleles were also shown to lengthen the period of *CCR2* in the dark (Somers, Kim & Geng 2004). Overexpression of ZTL causes arrhythmic *LHCB* expression, a long hypocotyl in red light and delayed flowering (Somers *et al*. 2004). Similar phenotypes are seen in plants overexpressing the related gene *LKP2* (Schultz *et al*. 2001), suggesting that LKP2, like ZTL, may lie close to the clock. Loss of function alleles of *LKP2* do not affect rhythmicity (Jarillo *et al*. 2002), indicating possible redundancy with *ZTL*. This redundancy is further supported by the fact that loss of *ZTL* affects the clock, but does not lead to arrhythmicity. Analysis of a *ztl lkp2* double mutant will be informative.

WHEN LIGHT INFLUENCES THE CLOCK

Entrainment of the clock by light–dark cycles requires that one or more clock component must be responsive to light. For example, transcription of the Neurospora clock gene *FREQUENCY* is induced by light, whereas the Drosophila clock protein TIMELESS (TIM) is degraded via the proteasome in response to light (Crosthwaite, Loros & Dunlap 1995; Hunter-Ensor, Ousley & Sehgal 1996; Zeng *et al*. 1996).

In Arabidopsis, light can potentially affect the clock components on three separate levels. First, *CCA1* expression is induced by red light (Wang *et al*. 1997). The transcription of *CCA1* and *LHY* is rapidly induced in response to far-red light, and this induction is greatly reduced in *phyA* mutants (Tepperman *et al*. 2001). Other genes found to be induced by far-red light include the transcription factor *HY5* and the *TOC1*-related gene *TOC1-L*, also known as *PRR9* (Matsushika *et al*. 2000). *PRR9* is induced in response to red and far-red light, and both PHYA and PHYB contribute to this induction (Ito *et al*. 2003). The transduction of the light signal from phytochromes to *CCA1*, *LHY* and *PRR9* (whose promoter contains 2 G-boxes) may involve several members of the *PIF3* family, but recall that single loss of function alleles in three of the *PIF3* family members had no circadian defects.

A second level of action of light on the clock may lie at the translational level. Overexpression of LHY leads to arrhythmicity, concurrent with high constitutive levels of

transgene-derived *LHY* and repression of the endogenous *LHY* gene (Schaffer *et al*. 1998). It was therefore surprising to discover that LHY protein levels in the LHY overexpressor were higher in the light than in the dark and therefore displayed a diurnal rhythm (Kim *et al*. 2003b). This rhythm in LHY accumulation is not likely to be circadian, as it does not persist in continuous light and does not show the dawn anticipation seen in wild-type plants. Rather, it is likely that the light–dark cycle drives the oscillation in LHY protein abundance. Nevertheless, these results demonstrate that light need not act on transcription to cause a change in the levels of a clock component, and provides another avenue by which light can reset the clock.

A third very recent example of light affecting a clock component is the ZTL-mediated degradation of TOC1. TOC1 and ZTL interact in the yeast two-hybrid system. It is interesting that the CCT domain of TOC1 has a very high PEST score (+16.67; http://www.at.embnet.org/embnet/tools/bio/PESTfind/) and that the mutation in *toc1-1* lies very close to the beginning of this putative PEST sequence (Strayer *et al*. 2000). A second PEST sequence, with a weaker score of +5.01, can be found at the end of the pseudo-receiver domain. Because the *toc1-1* allele is semi-dominant (as opposed to the strong, and putative null allele, *toc1-2*, which is recessive), at least some TOC1 protein must remain in *toc1-1* plants. It will be interesting to assay TOC1 protein levels in *toc1-1* in order to test the effect of a mutation so close to a potential PEST sequence on protein accumulation. TOC1 and ZTL show rhythms in their abundance, with peaks after subjective dusk (Kim, Geng & Somers 2003c; Más *et al*. 2003). TOC1 protein levels are elevated and only weakly oscillating in a *ztl* mutant, consistent with a role for ZTL in the degradation of TOC1. This degradation is induced in the dark, and somehow repressed in the light. Of the three functional domains in the ZTL protein, the LOV domain is sufficient to mediate the ZTL/TOC1 interaction (Más *et al*. 2003). However, the kelch repeats are also critical, as mutations in this domain (found in the *ztl-1* allele) abrogates the interaction (Somers *et al*. 2000; Más *et al*. 2003). The interaction between ZTL and TOC1 via the LOV domain may therefore be modulated by the light status. One possibility is that, in the light, the LOV domain of ZTL is converted to its photo-activated form and presents a conformation that is unable to bind to TOC1. Upon transfer into the dark, the photo-activated LOV domain slowly reverts to its inactivated form, while newly synthesized photo-inactive ZTL accumulates. Closer examination of the effects of blue light on the physical interaction between TOC1 and the LOV domain of ZTL could be very informative. Direct light modulation of protein–protein interactions has been established for the interaction between PHYB and PIF3 (Martínez-García *et al*. 2000). This repression of TOC1 degradation may provide a third level of control for light into the clock. At dusk, TOC1 degradation rates increase, and a pulse of light after dusk would slow this degradation, resulting in a phase delay. Clearly, this third mode of action is by no means sufficient to explain all light resetting of the clock, as *ztl* mutant alleles show a decreased peak in the levels of *CCA1* and *LHY* mRNA at dawn, although TOC1 levels are higher (Somers *et al*. 2004).

Conditional arrhythmicity: ELF3 and the limit cycle model

So far mutants that show complete disruption of circadian behaviour under all conditions have not been found in Arabidopsis or other plants, which contrasts with the isolation of arrhythmic mutants in Neurospora, *Synechococcus elongatus*, Golden hamster and flies. Null alleles of the clock genes *FREQUENCY*, the *Kai* cluster, *tau*, *PERIOD* and *TIMELESS*, are sufficient to prevent the generation of an oscillation and confer arrhythmicity (Reddy *et al*. 1984; Loros & Feldman 1986; Ralph & Menaker 1988; Myers *et al*. 1995; Ishiura *et al*. 1998). In contrast, this does not hold true for the Arabidopsis *CCA1*, *LHY* and *TOC1* genes, and has been explained for *CCA1* and *LHY* by the partial redundancy between the two genes (Alabadí *et al*. 2002; Mizoguchi *et al*. 2002).

To date, loss of function mutations in the gene *EARLY FLOWERING 3* (*ELF3*, Hicks *et al*. 1996) confer arrhythmicity for all rhythms tested in the light. However, the phenotype of *elf3* is conditional, because *elf3* mutants are rhythmic in the dark (Hicks *et al*. 1996; Covington *et al*. 2001). Overexpressing ELF3 does not lead to a strong circadian phenotype, and only leads to a modest period lengthening, most evident in blue light (Covington *et al*. 2001). The period lengthening observed in these plants suggested that ELF3 acts as a clock-gated negative regulator of light input to the clock (Liu *et al*. 2001). The absence of ELF3 would thus cause light input into the clock to be always on, resulting in arrhythmicity. This model is consistent with the amplitude of the acute response of *LHCB* transcription of etiolated seedlings given a pulse of red light. The acute induction of *LHCB* is stronger in the *elf3-1* mutant and weaker in the ELF3-overexpressing seedlings than in wild type (Covington *et al*. 2001).

We would like to reconsider these results and assign to ELF3 a more defined role, seen from the view of a limit cycle model. Limit cycles are mathematical models used to describe a two-dimensional system in a simplified form (Lakin-Thomas 1995). A classic biological example is the interaction between the populations of lynxes and hares (Krebs *et al*. 2001). As hares reproduce, the number of lynxes rises because the food supply allows them to proliferate. As the lynx population rises, the hare population crashes because of the strong predatory pressure. What then follows is a crash in the lynx population that is unable to sustain itself on the diminished hare population. In turn, the predatory pressure on the hares is alleviated and their numbers start to increase again. The variation in the populations may be drawn in a one-dimensional model in which hare and lynx numbers oscillate out of phase with one another (Fig. 2a). For ecological accuracy, it is necessary to acknowledge that the hare population also is influenced by food supply, so Lotka and Volterra were only partly correct

in their model of endless hare and lynx cycles based purely on predator–prey dynamics (Krebs *et al*. 2001). Nonetheless, the dynamics of the lynx and hare populations describe sine waves very similar to those of clock-regulated gene transcription (Fig. 2c). The same idea can be conveyed on a two-dimensional scale, where the number of hares is plotted as a function of the number of lynxes (Fig. 2b); this describes a limit-cycle. One can make a similar limit-cycle graph for the Arabidopsis clock, with CCA1/LHY levels plotted as a function of TOC1 levels (Fig. 2d). Such a model incorporates many of the aspects of the feedback loop that have been described between the three genes (Alabadí *et al*. 2001, 2002).

One interesting aspect of a limit cycle is the existence of a point at the centre of the circle, called the singularity. When the system is perturbed by light or temperature pulses, the relative levels of the state variables (hares and lynxes, or CCA1/LHY and TOC1, in the examples) are driven off the circle. In some cases the state of the oscillator ends in the centre of the circle, resulting in arrhythmicity. For light pulses, the singularity is reached when a pulse of just the right intensity is given around ZT14-20. The existence of the singularity has been empirically tested in plants. In *Kalanchoë*, 2 min of red light applied at subjective midnight abolished the rhythm in petal movement (Englemann, Karlsson & Johnsson 1973). In *Chlamydomonas*, a light treatment of 6 h starting at approximately ZT14 has distinct consequences for the phototaxis rhythm, depending on the fluence rate used (Johnson & Kondo 1992). At fluence rates lower than 85 μmol m^{-2} s^{-1}, the phase of the rhythm is delayed, and the amplitude is decreased. At fluence rates higher than 85 μmol m^{-2} s^{-1}, the amplitude is also decreased but the phase of the rhythm is now advanced. At the fluence of 85 μmol m^{-2} s^{-1}, the rhythm is lost, with an amplitude so low that no phase value can be correctly measured. The interpretation was that the circadian clock had been driven very close to the centre of the limit-cycle, resulting in arrhythmicity (Johnson & Kondo 1992).

So how does this apply to ELF3 and light input to the clock? ELF3 is thought to be a negative regulator of light input. However, *elf3* plants have very low levels of the light-induced genes *CCA1* and *LHY*, and high levels of the *CCA1*- and *LHY*-repressed gene *TOC1* (Schaffer *et al*. 1998; Alabadí *et al*. 2001). One would imagine that if ELF3 were simply a negative regulator of light input, then both *CCA1* and *LHY* should be expressed at high levels in *elf3* mutants, resulting in low levels of *TOC1*. It would instead appear that *elf3* mutants are stuck at a time when *CCA1* and *LHY* are low, and *TOC1* is high. The time during the day when such a situation is reached is around ZT14 (Fig. 2c), suspiciously close to the time in *Kalanchoë* and *Chlamydomonas* when the singularity can be reached. Perhaps, then, ELF3 is a gate-keeper, preventing light pulses from driving plants to the singularity.

Phase Response Curves (PRCs) in wild-type and the *elf3-1* mutant are consistent with a role for ELF3 as a gate-keeper to protect plants from the singularity. To generate a phase response curve, plants are entrained to light-dark cycles for a few days to establish the phase of the clock and then transferred into continuous conditions. After 0–3 d in continuous conditions, subsets of plants are treated every few hours with a stimulus and then returned to continuous conditions. The stimulus may be a light pulse of red or blue light, a dark pulse, a low- or a high-temperature pulse.

Using the *CCR2:LUC* transgene, blue- and red-pulse PRCs were generated for wild-type, *elf3-1* and *ELF3* overexpressing plants (Covington *et al*. 2001). At most time-points, all three genotypes respond to the light pulses and show similar phase-shifts. Around ZT16-18, when wild-type switches from phase delays to phase advances (another indication that the singularity is close), *elf3–1* plants become arrhythmic in response to the light pulses (Covington *et al*. 2001). This result is consistent with release from entrainment experiments, which showed that the oscillator in *elf3* mutants stops at dusk (McWatters *et al*. 2000). If ELF3 were a negative regulator of light input, then *elf3-1* plants should show strong resetting (a type-0 PRC), as any light-pulse should reset the clock to the same point in the cycle. Reciprocally, ELF3 overexpressing plants should show only weak resetting (a type-1 PRC). In reality, the PRCs of *elf3–1* and wild-type plants only differ dramatically around ZT16-18, at a time when ELF3 protein accumulates in wild-type (Liu *et al*. 2001). It is possible that the absence of ELF3 at that one time allows the circadian system to be driven too close to the singularity, resulting in arrhythmicity. Thus, we propose that, in wild type, ELF3 ensures that the light signal around ZT16-18 is attenuated, thereby preventing the system from reaching the singularity.

The interpretation of the role of ELF3 in the context of a limit cycle agrees with the conditional arrhythmicity seen under varying photoperiods. Although *elf3* mutant plants are arrhythmic (as measured with *LHCB:LUC*) when released in continuous light, they are rhythmic in continuous dark and in entraining cycles (Hicks *et al*. 1996). The quality of the rhythm is strongly dependent on the photoperiod. In short days, the rhythm in *elf3-1* is close to wild type, with clear anticipation of dawn and dusk (Hicks *et al*. 1996). However, as photoperiod increases, the waveform in *elf3-1* becomes distorted, with strong acute induction in response to dawn followed by progressive accumulation until dusk, when there is a rapid reduction in *LHCB* transcription.

Approaching arrhythmicity: TIC, ELF4 and GIGANTEA

A second gene, *TIME FOR COFFEE* (*TIC*), has partially overlapping function with *ELF3*, as well as *ELF3*-independent roles (Hall *et al*. 2003). A mutation in *TIC* causes a decreased amplitude and shorter period for a number of rhythms, including *LHCB* and *CCR2* transcription and cotyledon movement. The *elf3 tic* double mutant is completely arrhythmic for *LHCB* transcription in the light and in the dark, indicating functional overlap in the dark. Using release from entrainment experiments, it was established that the clock in *tic* mutants arrests in the subjective morn-

ing, whereas the clock in *elf3* mutants arrests in the subjective night (McWatters *et al*. 2000; Hall *et al*. 2003). We have suggested above that the role of ELF3 is to keep the clock from the singularity when it reaches subjective night; quite possibly TIC has a similar gate-keeping role to prevent a stimulus during the subjective day from driving the clock to the singularity. This would predict that TIC expression would be maximal earlier during the light part of the cycle; the testing of this prediction will have to await the cloning of *TIC*.

A T-DNA insertion in *ELF4* causes phenotypes similar to those displayed by *elf3* mutants, raising the exciting possibility that ELF4 and ELF3 act in close proximity in a pathway controlling clock function (Doyle *et al*. 2002). ELF4 expression is under the control of the clock, and shows a peak abundance similar to that of ELF3, around ZT12. *elf4* mutants display rhythms with a much weaker amplitude than wild-type in the first few days following release from entrainment, before turning arrhythmic (Doyle *et al*. 2002). In addition, *elf4* mutants show a much wider range of period lengths while still rhythmic, suggesting that ELF4 normally functions in period determination. The phenotypes observed for *elf4* mutants are not as extreme as those seen in *elf3*, but other *ELF4*-related genes may partially compensate for the loss of ELF4.

Loss of function mutations in *GIGANTEA* (*GI*) greatly decrease the amplitude of cotyledon movement and mRNA levels of *CCA1*and *LHY* (Fowler *et al*. 1999; Park *et al*. 1999; Tseng *et al*. 2004). In plants carrying the putative null *gi-11* allele, rhythms are maintained upon release from entrainment for a few days before reaching arrhythmicity (Fowler *et al*. 1999). In addition, loss of function *gi* alleles cause late flowering (Fowler *et al*. 1999; Park *et al*. 1999). The place of GI within the clock has been debated: *GI* is clock-regulated and is therefore a clock output (Park *et al*. 1999). The effect on the period of *LHCB* transcription of the *gi-1* and *gi-2* alleles is strongly dependent on the fluence rate, indicating that GI may be part of a light input pathway (Park *et al*. 1999). Finally, GI is required for high-amplitude expression of *CCA1* and *LHY* (Fowler *et al*. 1999; Park *et al*. 1999; Mizoguchi *et al*. 2002), suggesting a role in the oscillator itself. Although the effect of mutations in *GI* on *TOC1* expression is not known, it is possible that GI and TOC1 act together to induce *CCA1* and *LHY*. GI protein is nuclear-localized but does not concentrate in any foci or subnuclear compartment (Huq, Tepperman & Quail 2000). GI protein abundance is under control of the clock and peaks in the evening at the same time as TOC1 (Putterill, Milich & David 2002). Because GI shows no homology to transcriptional activators and has no obvious DNA-binding domain, it may recruit other proteins to potentiate the TOC1-dependent induction of *CCA1* and *LHY*. Protein modifications like the O-linked *N*-acetylglucosamine (GlcNAc) decoration catalysed by SPINDLY (SPY) may play a role. SPY and GI physically interact in the two-hybrid assay, and a genetic interaction is evident between the two genes, as the reduction of *O*-GlcNAcylation in *spy* mutants enhances the small amplitude phenotype conferred by the *gi-2* mutation in cotyledon movement (Tseng *et al*. 2004). Loss of *SPY* function also lengthens the period, while SPY overexpression shortens the period length accompanied by a stronger amplitude in cotyledon movement (Tseng *et al*. 2004). A testable prediction for a potential role of SPY as modifier of a clock protein would be that a *spy* mutation should enhance the phenotype of a partial loss of function *TOC1* allele like *toc1-1*.

Phase response curves for light pulses: what do they tell us?

In the previous section, we used the Phase Response Curve (PRC) to probe the state of the clock in *elf3* mutants (Covington *et al*. 2001). A few Arabidopsis PRCs have been published, but far more from many species have been assembled into the PRC Atlas (Johnson 1990). The shape of the PRC reflects the levels of the state variables of the circadian system. PRCs can usually be broken down into three parts: phase advances, dead zone and phase delays. Here we consider the PRC elicited by a light pulse, and make use of the known effects of light in the two model organisms, Neurospora and Drosophila, to speculate about the Arabidopsis clock.

First let us consider the light-PRC from Neurospora (Crosthwaite *et al*. 1995). Light, via the activation of the White Collar complex of proteins formed by WC-1 and WC-2 (Crosthwaite *et al*. 1995; Froehlich *et al*. 2002), rapidly and directly induces the transcription of the clock gene *FREQUENCY*; resulting in increased accumulation of *FRQ* mRNA and protein. One can match the three portions of the PRC with the relative levels of FRQ mRNA (Fig. 4a). If applied when *FRQ* is rising, the light pulse will bring its levels to peak value, in effect advancing the clock by a few hours, to the time when *FRQ* is normally at peak value (Fig. 3a). Because FRQ accumulates after dawn, a light pulse in the late night induces FRQ early, resulting in a phase advance. A second part of the PRC is referred to as the 'dead zone', when pulses have only weak effects on the phase. For Neurospora, this time corresponds to the peak in *FRQ* message. The third and final part of the PRC consists of phase delays. After dusk, when the expression of *FRQ* is declining, a light pulse leads to FRQ accumulation, retarding its normal decline.

A second type of resetting is seen in Drosophila, a night-active organism. *tim* transcription and mRNA abundance are not directly affected, but TIM protein is rapidly degraded in response to light (Fig. 3b, Hunter-Ensor *et al.* 1996). If one plots the PRC against TIM protein levels (Fig. 4b), it becomes evident that the dead zone of the PRC corresponds to the trough in TIM protein. In effect, light cannot drive the levels of TIM any lower, and therefore cannot shift the clock. The biggest phase shifts are observed when TIM protein is at its peak; light will bring TIM levels down and reset the clock to the middle of the day. Phase advances align well with the declining TIM levels, while phase delays align with rising TIM levels. The logic behind light resetting in Drosophila is the inverse of light resetting

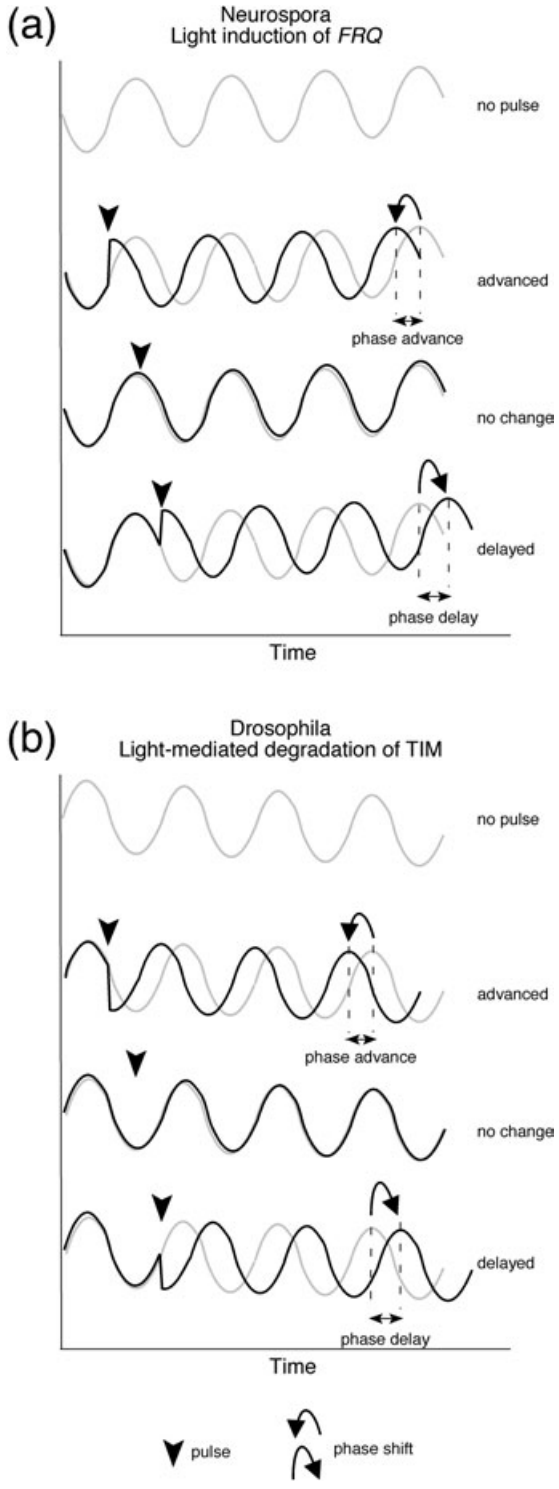

Figure 3. Generation of a phase response curve (PRC) in response to light pulses in Neurospora and Drosophila. (a) Generation of a light PRC in Neurospora. Light rapidly induces the transcription of the clock gene *FRQ*. A phase advance will be obtained if the light pulse is administered when *FRQ* levels are rising. A phase delay will be seen if light is given while *FRQ* levels are declining. No phase shift will occur if *FRQ* levels are already at their peak.(b) Generation of a light PRC in Drosophila. Light causes the rapid degradation of TIM protein. A phase delay will be seen when light is given while TIM protein levels are rising. A phase advance will be obtained when light is administered while TIM protein levels are declining. No phase shift will be seen if TIM levels are already at their lowest.

in Neurospora: light induces the accumulation of *FRQ* message, whereas light induces the degradation of TIM protein.

If one then plots the PRCs in response to red light and blue light against the normal oscillatory behaviour of the Arabidopsis clock genes *CCA1*, *LHY* and *TOC1*, a clear picture emerges, and is illustrated in Fig. 4c–e. The dead zone for the red pulse PRC matches the peak accumulation of *CCA1* and *LHY*, which are both red light inducible. The dead zone for the blue PRC appears to occur a little later than in the red light PRC, and could indicate that *CCA1* and *LHY* are not the primary targets of blue light resetting. The phase advance part of the PRCs aligns well with the rise of *CCA1* and *LHY* message; the phase delay part of the PRCs also line up very well with the declining levels of the two transcription factors. Therefore, the light PRC of Arabidopsis may, at least in part, be explained by modulation of the levels of *CCA1* and *LHY*, consistent with the mechanism for light resetting of the Neurospora clock (Crosthwaite *et al.* 1995). This hypothesis could be tested with a PRC to discrete pulses of CCA1 or LHY transcription driven from an inducible promoter applied at different times of day. Is induction of CCA1 or LHY sufficient to produce the predicted phase shifts?

TEMPERATURE INPUT TO THE CLOCK: THE GREAT UNKNOWN

Most research in plants and other organisms has focused on the impact of light and light resetting of the clock. The effects of temperature steps and temperature entrainment have been extensively studied in *Kalanchoë* (*Bryophyllum*), where the rate of CO_2 assimilation is under circadian control and is sensitive to temperature (Rensing & Ruoff 2002). Temperature steps as small as 0.5 °C can entrain the *Kalanchoë* clock, showing the exquisite sensitivity of the system. In Arabidopsis, cotyledon movement can be entrained with 4 °C temperature steps (McClung *et al.* 2002). The circadian oscillation in the transcription of *LHCB* and *CAT3* is similarly responsive to temperature (Michael & McClung 2002). However, the mechanism of action of the temperature steps on the clock is currently unknown. Temperature entrainment of the circadian clock probably occurs through a mechanism distinct from cold acclimation, as plant responses to cold require exposure to lower temperatures (Thomashow 1999) than necessary to entrain the clock.

Because the circadian system is entrained by temperatures cycles, one or more clock components should respond to the temperature steps. In Arabidopsis, transcription of the clock genes *CCA1*, *LHY* and *TOC1* is entrained by thermocycles, and takes on the same phase as during light-

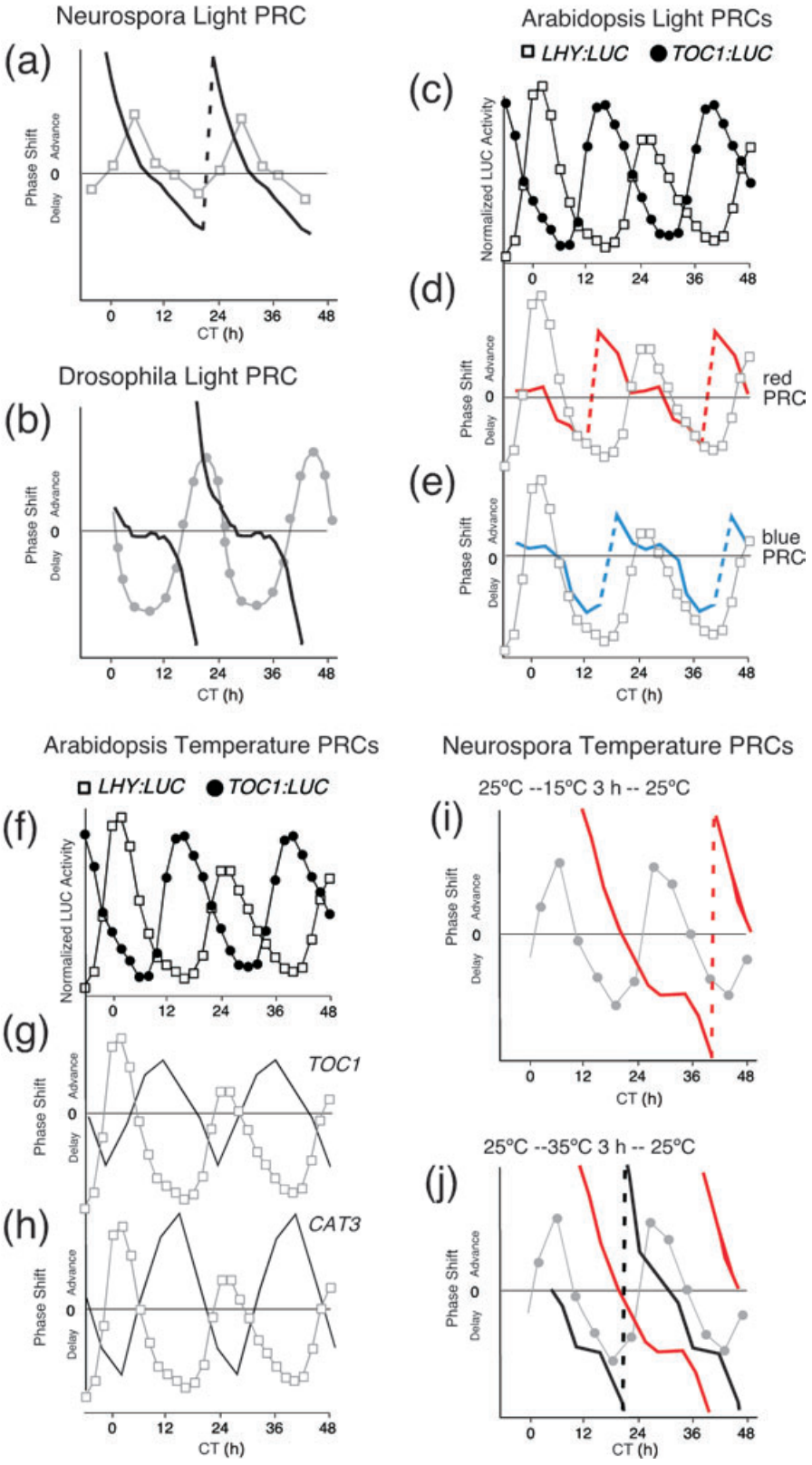
Neurospora Light PRC
(a)
Phase Shift
Advance
0
Delay
0 12 24 36 48
CT (h)
Drosophila Light PRC
(b)
Phase Shift
Advance
0
Delay
0 12 24 36 48
CT (h)
Arabidopsis Light PRCs
LHY:LUC
TOC1:LUC
(c)
Normalized LUC Activity
0 12 24 36 48
(d)
Phase Shift
Advance
0
Delay
red PRC
(e)
Phase Shift
Advance
0
Delay
blue PRC
0 12 24 36 48
CT (h)
Arabidopsis Temperature PRCs
LHY:LUC
TOC1:LUC
(f)
Normalized LUC Activity
0 12 24 36 48
(g)
Phase Shift
Advance
0
Delay
TOC1
(h)
Phase Shift
Advance
0
Delay
CAT3
0 12 24 36 48
CT (h)
Neurospora Temperature PRCs
25°C --15°C 3 h -- 25°C
(i)
Phase Shift
Advance
0
Delay
0 12 24 36 48
CT (h)
25°C --35°C 3 h -- 25°C
(j)
Phase Shift
Advance
0
Delay
0 12 24 36 48
CT (h)

Figure 4. Light, but not temperature phase response curves (PRCs) may be explained by the expression pattern of CCA1 and LHY. (a) Light resetting of the Neurospora clock is achieved through light induction of *FRQ* transcription. *FRQ* mRNA levels at different times of day are indicated by the grey curve with open squares. The light-pulse PRC is shown as a black line. Note that the strongest phase changes occur when FRQ levels are at the trough, while the weakest resetting is seen when FRQ message reaches its peak. Redrawn from (Crosthwaite *et al*. 1995).(b) Light resetting of the Drosophila clock is achieved through degradation of TIM protein. TIM protein levels at different times of day are indicated by the grey line and filled circles. The light-PRC is shown as the black line. Note that the strongest resetting is observed when TIM protein is at its peak. Redrawn from the PRC Atlas (Johnson 1990).(c) Expression of *LHY* and *TOC1* during 2 d in continuous light. Note the out-of phase expression of the two genes, seen here with the luciferase gene fusions *LHY:LUC* (open squares) and *TOC1:LUC* (filled circles). *LHY:LUC* peaks near dawn, while *TOC1:LUC* peaks 12 h later.(d) A red-light pulse PRC of *CCR2:LUC*, redrawn from (Covington *et al*. 2001). The PRC has been double-plotted. The dead zone of the PRC, when changes in phase are smallest, occurs at the time when *LHY* is nearing its peak accumulation. The phase advances are seen during the ascent in *LHY:LUC* signal, while the phase delays are seen when *LHY:LUC* signal decreases. The red-light pulse PRC may therefore be explained in terms of the effects of red light on the expression of *LHY*, and presumably *CCA1*. The same correspondence between the light PRC and *FRQ* expression is seen in Neurospora. *FRQ* expression is rapidly induced by light pulses (Crosthwaite *et al*. 1995).(e) A blue-light PRC of *CCR2:LUC*, redrawn from (Covington *et al*. 2001). The PRC has been double-plotted. The dead zone of the PRC also aligns well with the peak in *LHY*. The phase advances and delays also follow the expression of *LHY*, although the shifts are weaker than in red light.(f) Same as (c), re-plotted here for reference.(g) Temperature-pulse (cold pulse) PRC of *TOC1:LUC*, redrawn from (Michael *et al*. 2003a). Pulses consisted of 4 h at 12 °C, then the plants were returned to 22 °C. Small advances and delays can be seen, but no clear dead-zone can be found. The strongest phase shifts do not match the peak or trough of LHY or TOC1 expression.(h) Temperature-pulse step down PRC of *CAT3:LUC*, redrawn from (Michael *et al*. 2003a). The phase shifts are slightly stronger than for *TOC1:LUC*, but again no clear dead zone can be found. The biggest phase advances align with the peak in *TOC1* transcription.(i) and (j) Temperature PRCs from *Neurospora* redrawn from (Johnson 1990). The filled grey circles represent FRQ protein levels and are adapted from (Froehlich, Loros & Dunlap 2003). (i) A cold-pulse PRC. *Neurospora* race tubes were transferred from 25 to 15 °C for 3 h before being returned to 25 °C. The time of least phase change corresponds to the trough in FRQ protein.(j) A hot-pulse PRC. *Neurospora* race tubes were transferred from 25 to 35 °C for 3 h before being returned to 25 °C. The time of least phase change corresponds to the peak in FRQ protein.

dark cycles, but does not show acute induction or repression at the temperature steps (P.A.S. and C.R.M., unpublished). This would suggest that temperature might entrain the clock at the post-transcriptional level. Perhaps by looking at a PRC for temperature pulses, one might be able to predict when the temperature responsive clock component peaks. PRCs to 4-h cold pulses (plants growing at 22 °C subjected to 12 °C for 4 h, before returning to 22 °C) show the strongest changes in the phase of *TOC1:LUC* and *CAT3:LUC* transcription at ~ZT16 (Fig. 4f–h, Michael, Salomé & McClung 2003a). The strongest advances in phase are seen in the evening, while the strongest delays are seen in the morning; but overall the changes in phase are fairly modest and constitute a type-1 PRC, which shows weak resetting. This is reminiscent of PRCs executed in Neurospora with long (6 h) cold (5 °C lower than the growth conditions) temperature pulses (PRC #C/Nc-6; Johnson, 1990). Shorter pulses of stronger amplitude (10 °C lower than ambient) cause bigger changes in the phase of the conidiation rhythm (PRC #C/Nc-6; Johnson, 1990), and tend towards a type-0 PRC, showing strong resetting of the rhythm. Cleaner and more informative PRCs may therefore be generated with shorter pulses of more extreme temperature.

The Neurospora clock can be reset by light and temperature. In contrast to light pulses that affect the transcription of *FRQ*, temperature steps do not change the levels of *FRQ* message. Instead, FRQ protein responds to temperature and accumulates to higher levels at higher temperatures (Liu *et al*. 1998). The highest FRQ protein levels at lower temperatures are lower than the trough levels of FRQ at higher temperatures. This leads to the following model: after a transition to the higher temperature FRQ levels are lower than necessary to ensure negative feedback, causing the clock to reset to the morning to allow more FRQ to be made. Effectively this resets the clock to dawn. During a step down, any level of FRQ from the high temperature will be higher than the peak accumulation at the lower temperature. The cell therefore has enough FRQ to initiate negative feedback, and the clock is reset to the evening. If one were then to superimpose a temperature PRC from Neurospora and the FRQ protein levels, one would see that the peak in FRQ protein is close to the point of least resetting in response to a high temperature pulse (Fig. 4i). The point of least resetting in the case of a low temperature pulse is close to the trough in FRQ protein (Fig. 4j). Thus the PRCs correlate with FRQ protein levels. During low temperature pulses, FRQ protein levels decrease. Upon return to the original temperature, the levels of FRQ protein will be read as the trough of the FRQ protein oscillation. Therefore, a trough before low temperature pulses will remain a trough after returning to the initial temperature. For high temperature pulses, FRQ protein will rise at the higher temperature. Upon returning to the initial temperature, the levels of FRQ protein will be interpreted as peak value in the cycle. Therefore, a peak before the temperature step will remain a peak after returning to the initial temperature. Does this help us identify the gene(s) in Arabidopsis that respond to temperature? Not yet, because the low temperature tPRCs show two points each day when changes in phase are minimal, around ZT6 and again around ZT22. The expression of the clock genes *CCA1*, *LHY* and *TOC1* do not easily align with the tPRC replotted in Fig. 4f–h. This could indicate that none of the known clock genes is primarily responsible for temperature resetting.

From other systems, a few temperature sensors have been identified. They are based upon a conformational

change induced by the change in temperature. The sensor molecule can be the 5′ UTR of a gene to regulate its translation (Johansson *et al*. 2002), or a protein whose unfolding prevents the formation of the active dimer necessary to induce gene expression (Hurme *et al*. 1997). These examples lack the expected sensitivity for a circadian temperature sensor, as changes as low as 0.5 °C can entrain the rhythm of CO_2 assimilation in *Kalanchoë*. Two other examples come from cyanobacteria. In *Synechocystis* sp. PCC 6803, a histidine kinase and its associated response-regulator were identified as playing a role in cold sensing during a screen for mutants unresponsive to temperature steps (Suzuki *et al*. 2000). A number of genes are induced in response to cold temperatures (22 °C for *Synechocystis*). The response regulator, *rer1*, possesses a DNA-binding domain in its N-terminus and may therefore directly induce transcription of the cold-induced genes. Changes in transcription are again only observed after high amplitude changes in temperature, and may not easily explain how the circadian clock can be so precisely set by even small variations in temperature. One mutant from *Synechococcus elongatus, cikA,* is the only known clock mutant to be insensitive to temperature pulses. Disruption of *cikA* by transposon mutagenesis affects the phase, period and/or amplitude of transcriptional rhythms, demonstrating an important role for cikA in the proper establishment of a rhythm (Schmitz *et al*. 2000). In addition, *cikA* mutant strains fail to reset in response to temperature pulses, indicating that cikA is critical in sensing or responding to the environmental inputs of light and temperature cycles (Schmitz *et al*. 2000; Ditty, Williams & Golden 2003). One of the functional domains of the cikA protein shows homology to the receiver domain of response regulators. cikA lacks the aspartic acid that is normally phosphorylated by the cognate histidine kinase of classical two-component cascade (Schmitz *et al*. 2000; Hwang, Chen & Sheen 2002), making cikA strikingly similar in this regard to the Arabidopsis PRRs. The mode of signal transduction through cikA and the PRRs remains ambiguous. Nonetheless, the *cikA* mutant does not respond to temperature; by analogy this could suggest that one or more of the Arabidopsis PRRs could act in temperature sensing. Because *toc1-1* and *toc1-2* show normal temperature sensing (P.A.S. and C.R.M., unpublished, Somers *et al*. 1998b), the other *prr* mutants may by themselves or in combination show decreased sensitivity to temperature pulses.

Using cotyledon movement to test mutants for temperature entrainment, it was found that most clock mutants known to date (*cca1-1*, *lhy-20*, *ztl-4*, *toc1-2*, all *prr* single mutants, multiple *gi* alleles) are entrained by temperature cycles (P.A.S. and C.R.M., unpublished). The *cca1 lhy* double mutant (Mizoguchi *et al*. 2002) has not been directly tested for the ability to entrain to temperature. Plants lacking ELF3, or overexpressing CCA1, LHY or ZTL, are arrhythmic during and after temperature entrainment (P.A.S. and C.R.M., unpublished), making it difficult to assess whether there is a defect in response to temperature. Thus, we lack a genetic handle on the response to temperature pulses.

ENTRAINMENT AND LIVING ON A ROTATING WORLD

And now comes the interesting part: how do a few oscillators, nine potential circadian photoreceptors and an unknown temperature sensor help in entraining the clock to the light-dark and temperature cycles that plants and most organisms experience? We will go through the life cycle of a plant, and see when and where entrainment can occur.

All plants start as a seed. Buried in the ground, the germinating seed will probably not see light for a few days, until it breaks the ground surface. The circadian clock of an imbibed seed is nevertheless running, and all cells within the seed are synchronized (Zhong *et al*. 1998). However, no oscillations are observed for the mRNA levels of *CAT2* or *CAT3*, or for the transcription rate of *LHCB* in etiolated seedlings (Millar & Kay 1996; Zhong *et al*. 1998). In the case of *CAT3*, the high stability of the mRNA in the dark probably masks the oscillations (Michael & McClung 2002). *CAT2* and *LHCB* are light-induced, and their expression is low in dark-grown seedlings. Experiments conducted in tobacco using the Arabidopsis *LHCB* promoter indicate that the clock which regulates *LHCB* transcription is not sensitive to light for the first 36 h after imbibition (Kolar *et al*. 1998). In effect, light pulses given at any time within 36 h of imbibition fail to reset the phase of *LHCB*, and only pulses delivered later than 36 h after imbibition will cause a shift in the phase of *LHCB* (Kolar *et al*. 1998). One question that remains unanswered is when oscillations in *LHCB* transcription are detected in Arabidopsis. Kolar *et al*. (1998) demonstrate that tobacco seedlings show rhythmic expression of *LHCB* even in the dark, quite a different situation from Arabidopsis. If this applies to Arabidopsis seedlings, then the phase of *LHCB* expression, and possibly other photosynthesis-associated genes, will not initially be synchronized with the environment. There may be a window of time (36 h in tobacco) during which the seedlings are refractory to entrainment by light-dark cycles. Perhaps it may not be necessary for a 1- to 2-day-old-seedling to be synchronized with the light-dark cycles to maximize its photosynthetic abilities, when its cotyledons are just starting to turn green. Interestingly, not all genes fail to be synchronized by the environment at the early seedling stage. For instance, the phase of transcription of *CAT3* appears to be set by temperature rather than light (Michael *et al*. 2003a). The phase of *CAT3* transcription tracks the release from stratification and not the time of imbibition, in contrast to *LHCB* and *CAT2* (Michael *et al*. 2003a). Release from stratification typically changes the light status (from dark to light) and temperature (from 4 to 22 °C), but the temperature step associated with the release is likely responsible for the setting of the clock controlling *CAT3* expression (P.A.S. and C.R.M., unpublished). This would suggest that the phase of genes not directly involved

in photosynthesis can be set by the temperature cycles that the young seedling will face. In the extreme situation when light–dark cycles are not provided, the relative temporal coordination (phase angle) between *LHCB* and *CAT3* expression is quite different from that seen in plants properly entrained (Michael *et al*. 2003a). In real life however, the young seedling will eventually become sensitive to light and will be subjected to light-dark cycles. The photoentrainment will then reset the phase of *LHCB* and coregulated genes, but would only have a modest effect on the phase of genes like *CAT3*, which are already in phase with the thermocycles.

We have previously noted how mutations in photoreceptors cause a period lengthening in free-running conditions, but constant conditions are not normal growth conditions for most plants outside of the subpolar regions. What becomes of a period mutant during entrainment? Analysis of the *toc1-1* mutant showed that a short period mutant displays a normal period during entraining cycles, but the phase of the rhythm is altered; a short period mutant like *toc1-1* will exhibit a leading phase (Somers *et al*. 1998b). A long period mutant will, on the other hand, exhibit a lagging phase, with an otherwise normal entrained period length. From this it ensues that photoreceptor mutants that confer a long period in white light would be predicted to display a lagging phase in real life. But even in the absence of a functional clock, plants display rhythms during entraining conditions. For instance, *elf3* and *gi* mutants are entrained by light–dark cycles or temperature cycles (Hicks *et al*. 1996; McWatters *et al*. 2000; Tseng *et al*. 2004). Plants lacking both CCA1 and LHY still exhibit daily rhythms: the expression pattern of *CCA1* and *LHY* in light–dark cycles in wild-type and the *cca1 lhy* double mutant are identical, with both genes peaking at dawn (Mizoguchi *et al*. 2002). Only the phases of evening genes like *TOC1* and *GI* are affected in the double mutant, and peak much earlier than in wild type, possibly because of the lack of repression from CCA1 and LHY.

Two other genotypes that are arrhythmic in free-run show oscillations in light–dark cycles. In plants with high constitutive levels of *LHY*, both *LHCB* and *CCR2* show diurnal rhythms. The phase of *LHCB* seems to be dictated by dawn, while the phase of *CCR2* follows dusk regardless of the length of the entraining cycle (Kim *et al*. 2003b). Overexpression of the related gene *CCA1* similarly causes arrhythmicity in free run, but oscillations can be driven by a light-dark cycle (Green *et al*. 2002). The phase of the morning gene *CAT2* is relatively unaffected by the high levels of CCA1, but an alteration of waveform and phase becomes more obvious for genes normally peaking at later times during the day. For instance, the expression of *LHCB* is delayed relative to wild type. The expression pattern for *CCR2* is more difficult to interpret, as it seems to also be induced in the dark in the CCA1 overexpressing background (Green *et al*. 2002). These results show that even in the absence of a running functional clock, the environment can drive rhythms. However, the same plants show an interesting photoperiod-dependent survival phenotype. Under long days and continuous light, plants overexpressing CCA1 or LHY fare very well, even better than wild-type based on seed production and plant size (Green *et al*. 2002). In very short days, however, these genotypes die early. A similar early death phenotype was observed in the *elf3* mutant (Green *et al*. 2002), indicating that a stopped clock may impair the plant's ability to survive under short day conditions, possibly due to a lack of adaptation to the new photoperiod. This early death phenotype might be observable under ecologically relevant photoperiods, and might be of evolutionary significance. One mutant that remains to be tested for its survival under very short photoperiod is *gi*. Plants carrying loss of function *gi* alleles are robust and in fact look very much like plants overexpressing *CCA1* or *LHY*. The *gi* mutants were, after all, identified as supervital mutants by Redei more than 40 years ago (Rédei 1962). One prediction is that *gi* mutants will die early under very short photoperiods.

In other systems another type of experiment was used to test for the advantage conferred by a functional circadian system. In *Synechococcus elongatus*, competition assays between strains of different period lengths showed that the strains whose internal period best matches the external entraining conditions outcompetes the other strain (Ouyang *et al*. 1998). It will be interesting to run similar experiments comparing the fitness of Arabidopsis accessions and mutants under laboratory conditions of different photoperiods and temperatures, as well as under field conditions (see, for example Weinig *et al*. 2002). For example, is the total day length a factor, so that long period mutants will fare better under days that are more than 24 h long?

If the reason behind maintaining a running circadian clock in an organism is to adapt and partition the expression of genes when they are needed, why not just rely on diurnal rhythms that would be driven by the succession of the light and dark? Using the model of an hourglass, the timing of genes relative to dawn and dusk could be modulated effectively. Gene expression could be modulated by the levels of a clock molecule, and sets of genes could respond only above or below some threshold of this molecule, thereby creating multiple phases. However, this would not readily allow adaptation to changing photoperiods. For instance, the phase of *LHCB* transcription is dependent on the photoperiod, and always coincides with the middle of the light period (Millar & Kay 1996). In the real world the photoperiod and thermoperiod change every day by only a few minutes. Photoreceptors and thermoreceptors allow the clock to adjust accordingly. When photoreceptors are missing, the plant will not fully respond to the new conditions; photoreceptor mutants should show weaker phase shifts than wild type. Even though this has not been tested systematically, one study indicates that this indeed occurs in *phy* and *cry* mutants (Yanovsky *et al*. 2001). Using leaf movement to monitor the clock, 2-week-old-plants entrained to light-dark cycles were given a 5-h pulse of light at the end of the last dark period, and released in continuous white light. For wild type seedlings, a pulse of red, far-red or blue light advances the phase of the leaf movement

rhythm. In *phy* and *cry* single and double mutants, this phase shift is decreased after a pulse of red or blue light, and is abolished in *phyA cry1 cry2* triple mutants in response to blue light, and in *phyA* mutants in response to far-red light. These results constitute the beginning of a light PRC for leaf movement, and demonstrate that mutations in photoreceptors do in fact diminish the ability of the plant to respond to changes in the perceived light. Knowing that *LUC* fusions to the promoters of the clock components *CCA1*, *LHY* and *TOC1* oscillate and recapitulate the expression patterns seen in Northern blots, the next step will be to complete the light PRCs in the *phy* and *cry* mutants for the clock genes themselves and, hence, directly probe the state of the oscillator. Photoperiod can change more than the phase of the rhythm during entrainment. It was shown recently that the protein levels of the two light-labile photoreceptors, PHYA and CRY2, are strongly influenced by the photoperiod. In long days and continuous light, PHYA and CRY2 levels are very low, reflecting their light-induced degradation (Quail 1997; Shalitin *et al*. 2002; Mockler *et al*. 2003). Under shorter photoperiods, both proteins accumulate to higher levels during the night, and participate in light perception in the early morning before being degraded. It is not clear how the photoperiod-dependent degradation of single photoreceptors influences the clock, but it could modulate the extent of resetting contributed by each individual photoreceptor. Light signalling would be relatively enhanced under short photoperiod because of the higher levels of PHYA and CRY2. If so, the pace of the clock should be hastened, which would be seen as a leading phase in short versus long photoperiods (the entraining conditions would ensure a constant period of 24 h). This mechanism may partially explain how some clock-regulated genes display distinct phases under different photoperiods. For example, the phase of *LHCB* is modulated so that the peak matches the middle of the light period (Millar & Kay 1996). This means that the peak occurs earlier (relative to dawn) under shorter photoperiods than under longer ones. The same modulation of phase was shown for rhythms in cytosolic calcium (Love, Dodd & Webb 2004). Oscillations in cytosolic calcium may play a signalling role, but they are unlikely to contribute to the photoperiod sensitivity of *LHCB* expression, as rhythmic *LHCB* transcription persists in the absence of cytosolic calcium rhythms (Sai & Johnson 1999). Other genes, like *CAT3*, display the same phase under all photoperiods (Michael *et al*. 2003a). Interestingly, *LHCB* is light induced whereas *CAT3* is not (Zhong *et al*. 1998). The effect of photoperiod on the expression of *CCA1*, *LHY* and *TOC1* is not known. Modulation of phase by photoperiod may act directly through the clock genes, in which case photoperiod-insensitive genes must be controlled by a separate oscillator. Alternatively, the phases of the clock genes may not be affected by photoperiod, but rather the new phase of the clock-controlled *LHCB* gene is achieved through a modulation of the output pathways.

Another possible explanation of the differential response to photoperiod calls upon the degradation of TOC1 and its repression by light (Más *et al*. 2003). The light induction of *CCA1* and *LHY* probably serves as a dawn signal. However, a dusk signal was also shown to be important, because the time of the peak mRNA abundance for *LHCB* in wheat seedlings was determined by the light to dark transition the previous day, and not by the following dark to light transition (Lam & Chua 1989). The levels of TOC1 protein may vary with the photoperiod, because the amount of time permissive for TOC1 degradation will change with the length of the dark period. In addition, the onset of TOC1 degradation will occur later in long days relative to short days. The exact timing of the initiation of TOC1 degradation may constitute the dusk signal. If this were true, then one would therefore expect that a strong allele of *TOC1,* such as *toc1-2,* would display the same phase for *LHCB* transcription in any photoperiod.

A dark-stimulated calcium burst might also play a role as a dusk signal. Upon transfer into the dark, stromal calcium transiently increases, while cytosolic calcium shows a slight dip, followed by a transient increase (Sai & Johnson 2002). Although the overall magnitude of the changes in cytosolic calcium is small, local gradients of calcium may exhibit bigger variations that might regulate protein activity. If the dusk-stimulated changes in calcium levels are responsible for photoperiodic sensing, the following prediction can be made. When calli are grown on medium lacking sucrose, the cytosolic calcium rhythm is lost (Sai & Johnson 1999). If cytosolic calcium rhythms transduce photoperiodic information to the clock, then the phase of *LHCB* transcription in these calli should be the same under all photoperiods.

The role of the light and temperature signalling pathways is to synchronize the circadian oscillator of the plant with the environment. Because plants are always subject to entraining cycles, their clocks will always display an exact 24-h period. However, modulation of the endogenous period of the clock can affect the phase of the rhythm in entraining conditions, as stated above. One might expect to see some evidence of selective pressure on the regulation of period or phase. We still now do not fully understand how distinct genes can be expressed to different times of day, although transcriptional regulation clearly contributes (Harmer *et al*. 2000; Michael & McClung 2002). A change in period would affect the phase of the genes under clock control selectively and gradually. A period change (shortening or lengthening) will affect the phase of the evening genes more than the morning genes, because of the strong resetting associated with the dawn signal. With a shorter period, the evening genes will be expressed earlier during the day; for a period lengthening, expression of the evening genes will be delayed. An advance in the phase of later genes could be advantageous for shorter photoperiods, which would occur in the winter or at latitudes closer to the equator. A delayed phase would become useful for longer photoperiods, either in the summer days or at latitudes closer to the poles. A significant correlation was indeed observed among 150 Arabidopsis accessions between period length and latitude,

which itself is correlated with day-length (Michael *et al*. 2003b). At higher latitudes and, hence, longer day-lengths, period length increased. This would be predicted to delay the phases of clock-regulated genes progressively over the duration of the day. This would have the effect of stretching the pattern of gene expression to better match the lengthened day. At latitudes closer to the equator, period length tended to be shorter, and would again modulate the phase of the clock-regulated genes to better match the environment. In essence, latitude and photoperiod have very similar effects on the clock.

IN CONCLUSION

Even though most research in circadian rhythms is accomplished in free-running conditions, organisms actually live in an entraining environment. Although it is very important to study the effect of light and temperature in continuous conditions, one should keep in mind that the ultimate goal is to bring it all back to our rotating world. After a decade of research emphasizing the mechanisms underlying the generation of the oscillation, fuller consideration of the consequences of the perturbation of the circadian system when in entraining conditions is past due. After all, the timing mechanism called a circadian rhythm is always set to a 24-h period in real life, where the real challenge exists.

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