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Biological clocks in *Arabidopsis thaliana*

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This review is dedicated to the memory of Colin Pittendrigh, whose outstanding contribution to research on circadian rhythms includes the term 'circadian'.

CONTENTS

SUMMARY

Biological rhythms are ubiquitous in eukaryotes, and the best understood of these occur with a period of approximately a day – circadian rhythms. Such rhythms persist even when the organism is placed under constant conditions, with a period that is close, but not exactly equal, to 24 h, and are driven by an endogenous timer – one of the many 'biological clocks'. In plants, research into circadian rhythms has been driven forward by genetic experiments using *Arabidopsis*. Higher plant genomes include a particularly large number of genes involved in

Abbreviations: bHLH, transcription factor with basic helix-loop-helix DNA-binding domain; CAB, chlorophyll *a*/*b*-binding protein; *CAT*, catalase-encoding gene; *CCA* ,circadian clock-associated gene; *CCG*, clock-controlled gene; *CCR*, cold- and circadian-regulated gene; *CCRE*, circadian clock-regulated promoter element; CGF, *CAB* GATA factor (*CAB2* DNA-binding complex); *cry*, *cryptochrome*; CUF, *CAB* upstream factor; *elf3*, *early flowering* 3; *LHY*, *late-elongated hypocotyl*; *luc*, firefly luciferase-encoding gene; PAS, *per*-ARNT-*sim*, PER protein's PAS domain; *PHY*, phytochrome-encoding gene; PRR, photoperiodic response rhythm; *tim*, *timeless*; *toc*, *timing of CAB*; *wc*, *white collar*.

metabolism, and circadian rhythms may well provide the necessary coordination for the control of these – for example, around the diurnal rhythm of photosynthesis – to suit changing developmental or environmental conditions. The endogenous timer must be flexible enough to support these requirements. Current research supports this notion most strongly for the input pathway, in which multiple photoreceptors have been shown to mediate light input to the clock. Both input and output components are now related to putative circadian oscillator mechanisms by sequence homology or by experimental observation. It appears that the pathways linking some domains of the basic clock model may be very short indeed, or the mechanisms of these domains may overlap. Components of the first plant circadian output pathway to be identified unequivocally will help to determine exactly how many output pathways control the various phases of overt rhythms in plants.

Key words: circadian rhythm, transcriptional control, photoreceptor, photoperiodism.

I. THE CIRCADIAN SYSTEM

Biological rhythms pervade plant physiology, covering an astonishing range of timescales, from rapid oscillations in ion fluxes to the seven-year cycles of flowering in some bamboos (Sweeney, 1987). This review covers only a small sample of rhythms in *Arabidopsis thaliana* – the system of choice for the majority of molecular genetic studies in higher plants, with its extensively characterized small genome and large number of available mutants. *Arabidopsis* exhibits biological rhythms with the frustrating moderation of a genetic model species: their effects are detectable, but lack the drama of those of more specialized plants in which rhythms were so obvious to the early investigators. *Arabidopsis* does have a full complement of rhythms: the corkscrew pattern of inflorescence and hypocotyl growth (circumnutation), for example, causes the apex of the growing organ to flex through a full circle in as little as 20 min (Schuster & Engelmann, 1997); the longest-period rhythm reported in *Arabidopsis* is a seasonal pattern of seed germination (Baskin & Baskin, 1983) – this particular rhythm is probably driven by the environmental conditions, because these change so much with the seasons in temperate latitudes. Here, rhythms that match the daily and yearly cycles in the environment are considered; they are not driven by the rotation or the orbit of the Earth, but rather by an endogenous timer – one of the many 'biological clocks'.

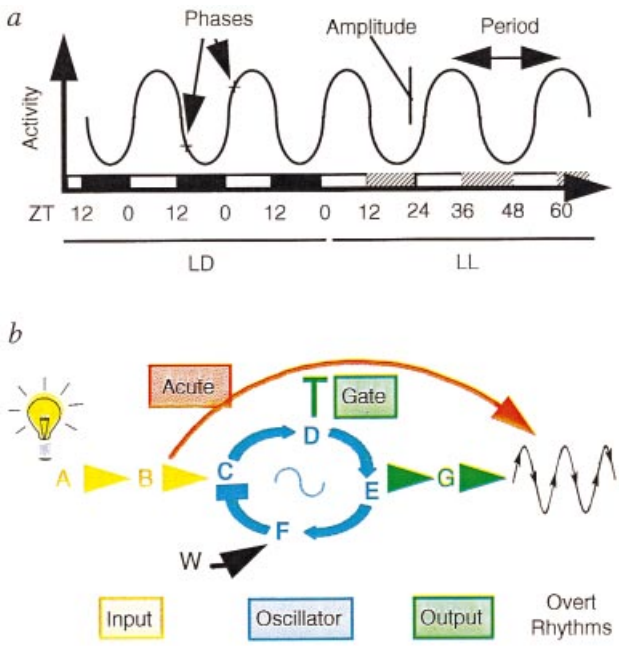

Figure 1. Terminology and components of the circadian system. (a) An overt rhythm ('Activity') is shown during entrainment to light–dark cycles (LD) and after transfer to constant light (LL) (filled boxes, darkness; open boxes, light; hatched boxes, predicted dark intervals during constant light). Phase points are marked (+). Time is marked in zeitgeber time (ZT, hours after the last lights-on); the free-running period is longer than 24 h. (b) The simplest model of the circadian system includes an input pathway (yellow) from light (bulb), a circadian oscillator (blue), and an output pathway (green) to the overt rhythm (wave). The oscillator is shown as a feedback loop, with negative feedback of component F upon component C. Component W is required for oscillator function, although it is not part of the oscillator loop. A distinct pathway that mediates an acute response to light is shown (red; the acute response in the output marker is not shown); a circadian gating pathway rhythmically inhibits the acute response (green). More extensive glossaries can be found in Sweeney (1987), Edmunds (1988) and Lumsden & Millar (1998).

Biological clocks that cycle over a whole year (circannual rhythms) have been implicated in other species, but have not been extensively studied (Gwinner, 1986). The rhythms that are best understood are those with a period of approximately a day (circadian rhythms), which represent an adaptation to the Earth's rotation and its associated rhythms of light and temperature (Edmunds, 1988). A common terminology describes all biological rhythms (Fig. 1a), but circadian rhythms are distinguished by three, shared characteristics:

- They persist even when the organism is placed under constant conditions, with a period that is close, but not exactly equal, to 24 h. 'Diurnal' ('driven') rhythms, in contrast, are direct responses to a rhythmic environmental signal, and do not persist in constant conditions.
- Their period is temperature compensated (it varies little at a range of uniform temperatures).
- Their phase can be reset by light, temperature and other environmental signals (Johnson *et al.*, 1998b). Phase resetting in light–dark cycles

matches the period of the endogenous rhythm to the environmental cycle, a situation known as 'stable entrainment'.

These fundamental properties are shared very broadly, as circadian rhythms are ubiquitous in eukaryotes (Dunlap, 1996; Young, 1998) and have also been demonstrated in cyanobacteria (Johnson *et al.*, 1996). A recent boom in research activity has been widely reviewed, in dedicated journal issues (Loros, 1995; Carré, 1996) and elsewhere (Dunlap & Loros, 1998). Recent reviews of plant rhythms concentrate on molecular and genetic data (Beator & Kloppstech, 1996; Kreps & Kay, 1997; Millar & Kay, 1997; Millar, 1998b) but broader coverage is found in introductory texts (Salisbury & Ross, 1992; Mohr & Schopfer, 1995), a classic monograph (Sweeney, 1987) and a more recent book (Lumsden & Millar, 1998).

1. *Components of the circadian system*

The biological mechanism responsible for circadian rhythms is known as the circadian system or circadian clock: a basic model is shown in Fig. 1b. The three functional domains of the system are identified as the oscillator, which generates a period of approximately 24 h, the input signalling pathways that carry light signals to reset the oscillator (change its phase), and the output pathways, which carry timing signals from the oscillator to clock-regulated processes in the cell. This has been a very useful, heuristic model, but it is increasingly clear that the biological mechanisms might blur the conceptual distinctions between the domains. The overt rhythms are often the only accessible manifestation of the circadian system, and are (until oscillator components are identified: section IV) the experimental basis for much rhythm research. There is an understandable emphasis on rhythms that can be monitored automatically, because several days of data are required to determine phase and period accurately. It is only possible to be confident that the overt rhythm reflects the stable phase and period of the oscillator. The output pathway and other, independent regulators can alter the waveform of the overt rhythm (including the amplitude and skewness of the peaks). The instantaneous phase of the overt rhythm also need not reflect the oscillator, as several 'transient' cycles can be required before the overt rhythm fully reflects a phase shift of the oscillator (Johnson *et al.*, 1998a).

II. OVERT CIRCADIAN RHYTHMS

The circadian rhythms in *Arabidopsis* reflect their diversity in other plants; a large proportion of these rhythms are related to photosynthesis (Sweeney, 1987; Wilkins, 1992). For example, the cotyledons and leaves of young plants move up and down rhythmically under constant light (Fig. 2; Engelmann *et al.*, 1992) such that the leaves are horizontal at midday. The change in leaf angle is probably caused by asymmetric elongation of the petioles, as *Arabidopsis* lacks the pulvini characteristic of the Fabaceae: the changes in membrane properties involved in pulvinar movements are better understood (Coté, 1995; Engelmann & Johnsson, 1998). *Arabidopsis* stomata open during the 'subjective' day and a rhythm in stomatal aperture persists under constant light (Somers *et al.*, 1998; Webb, 1998), as it does in many other species (Hennessey & Field, 1992). Other morphological rhythms include the rhythmic elongation of the hypocotyl in young seedlings (Dowson-Day & Millar, 1999) and of the inflorescence stem in mature plants (Degli Agosti *et al.*, 1997).

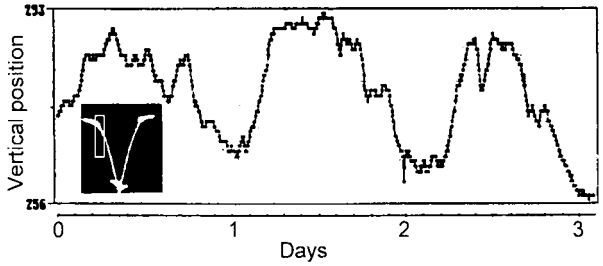

Figure 2. Circadian rhythm of leaf movement in *Arabidopsis*. The position of a primary leaf was monitored in a video imaging system, in constant dim light; the inset shows a processed video image of a young plant, taken from one side. The data presented are the mean vertical position of white pixels within the image analysis field (white box in inset), in images taken every 20 min. Time 0 = time of transfer from the greenhouse. *Reproduced, with permission, from Engelmann* et al. (*1992*).

Circadian rhythms are so widely conserved that they are assumed to have adaptive value, but their exact contribution (apart from photoperiodic timing: section III) remains largely a matter for speculation. Several plant species, including tomato, grow very poorly in the absence of environmental time cues, or in light–dark cycles that differ markedly from 24 h, indicating that some type of timing in the circadian range is required for normal growth (e.g. Highkin & Hanson, 1954). Circadian rhythms could contribute by coordinating physiological processes with the external day–night cycle: several rhythms, including that of expression of the chlorophyll *a*/*b*-binding protein gene *CAB* (see Abbreviations), might prepare the plant for photosynthesis in anticipation of dawn, to take maximum advantage of sunlight. This is external coordination. In addition, circadian timing can maintain a particular temporal sequence of internal events. Other metabolic processes might be restricted to the night phase, not to avoid light, but to prevent interference with, for example, photosynthesis: this is internal co-ordination. The rhythms can be modified as part of an adaptive

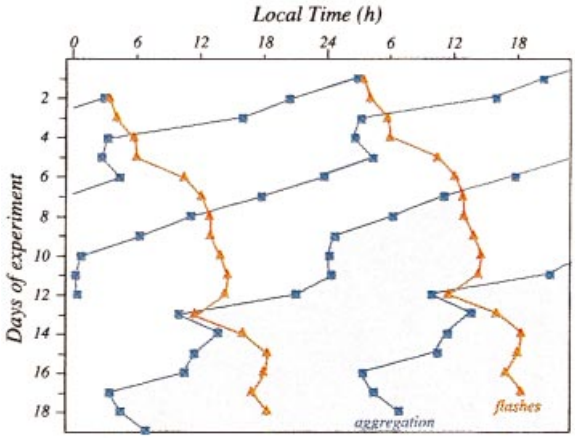

Figure 3. The unicellular *Gonyaulax polyedra* contains two circadian oscillators. The peak phases of the circadian rhythms in aggregation (blue squares, period approx. 21 h) and bioluminescence (red triangles, period approx. 24.6 h) were measured in the same culture, under dim red light. The different periods indicate that different oscillators control the two rhythms. *Modified*, *with permission*, *from Hastings* (*1994*).

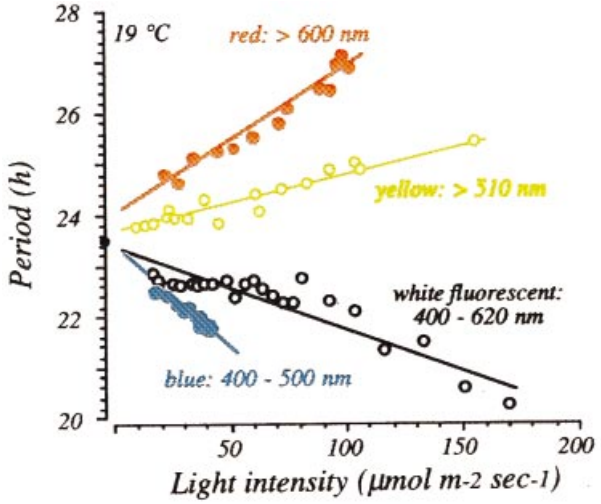

Figure 4. Two photoreceptors control the period of the *Gonyaulax polyedra* bioluminescence rhythm. The relationship between period and fluence rate is shown for four light qualities. The opposite gradients in red and blue light indicate that different photoreceptors mediate red- and blue-light input to the clock. *Modified*, *with permission*, *from Roenneberg & Hastings* (*1988*).

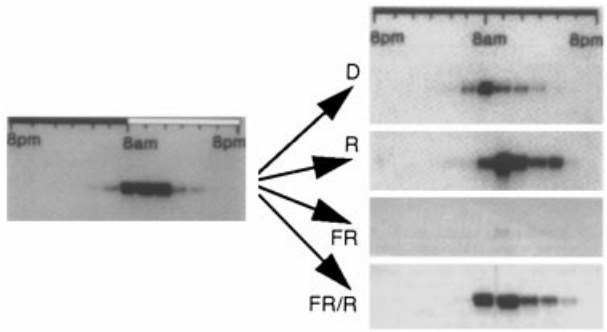

Figure 5. Control of *Cab-1* mRNA abundance by phytochrome and the circadian clock. Wheat plants were grown for 7 d in light–dark cycles. Plants were harvested for RNA extraction during a further light–dark cycle (left panel) or after transfer to constant darkness (D). The rest of the plants were given red (R), far-red (FR) or far-red followed by red (FR/R) light treatments immediately before transfer to darkness. RNA gel blots were hybridized with a *Cab-1*-specific probe. Far-red light reduces the levels such that only a trace of RNA is detectable after subjective dawn, but this reduction is red-reversible, as expected of a low-fluence phytochrome response. *Modified*, *with permission*, *from Nagy* et al. (*1988*).

strategy: the phase of some events is predictably reversed in CAM plants, which fix carbon at night (Wilkins, 1992). Recent evidence indicates that a circadian system with a period close to the environmental cycle confers a competitive advantage in cyanobacteria, by both external and internal coordination (Johnson *et al.*, 1998b; Yan *et al.*, 1998).

1. *Circadian rhythms in* Arabidopsis

(a) *Gene expression and luciferase markers*. The recent concentration on the circadian regulation of gene expression (Fejes & Nagy, 1998) is due in part to three advantages of clock-controlled genes (*CCG*s). First, *CCG*s provide molecular entry points to the last steps of the circadian output pathways, through the molecular analysis of circadian clock-regulated promoter elements (*CCRE*s) and their associated proteins. There is an established suspicion that very few signal transduction steps separate the *CCG*s from the oscillator mechanism (Loros, 1995): as the oscillator appears to depend on transcriptional regulation (section IV), the same mechanism might regulate *CCG*s. Second, *CCG* promoters containing a *CCRE* can be used to create novel rhythmic markers, in combination with an appropriate reporter gene. Non-invasive, bioluminescent reporter genes – such as that encoding the firefly luciferase (*luc*) – have been critical in identifying the *Arabidopsis CCRE*s and rhythm mutants (Millar *et al.*, 1992; White *et al.*, 1996). The dinoflagellate *Gonyaulax* has a natural rhythm of bioluminescence (Figs 3, 4), but luciferase transgenes have also been used to monitor circadian rhythms in cyanobacteria (Kondo *et al.*, 1993), *Drosophila* (Plautz *et al.*, 1997) and mouse (Geusz *et al.*, 1997). Appropriate *CCG* promoters can be selected to mark specific circadian phases or tissues that would otherwise lack visible, rhythmic markers. Finally, the functions controlled by *CCG*s suggest which physiological or metabolic processes are subject to circadian regulation (Fejes & Nagy, 1998). This knowledge might then direct more restricted, physiological studies to new rhythms with potential adaptive significance. The expression of catalase (CAT) genes at the end of the day is one rhythm that awaits such a follow-up investigation.

(b) *Rhythmic expression of* CAB *genes*. Diurnal and circadian rhythms at the mRNA level were first

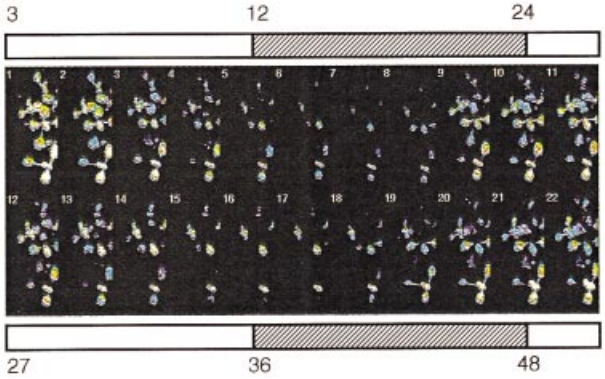

Figure 6. The bioluminescence of transgenic *cab2 : : luc* plants recapitulates the circadian regulation of *CAB* transcription. Four, 9-day old *cab2 : : luc* plants were treated with the luciferase substrate, luciferin, and imaged for 2 d after transfer from a 12-h light–12-h dark cycle to constant light. Each plant has luminescent cotyledons and one pair of leaves. Eleven images were captured per day (left to right), at the times shown (hours since the last lights-on). The hatched box shows the predicted dark interval of the preceding light–dark cycle. Blue represents low luminescence on this pseudo-colour scale; red and white represent high luminescence. *S. C. Thain & A. J. Millar, unpublished.*

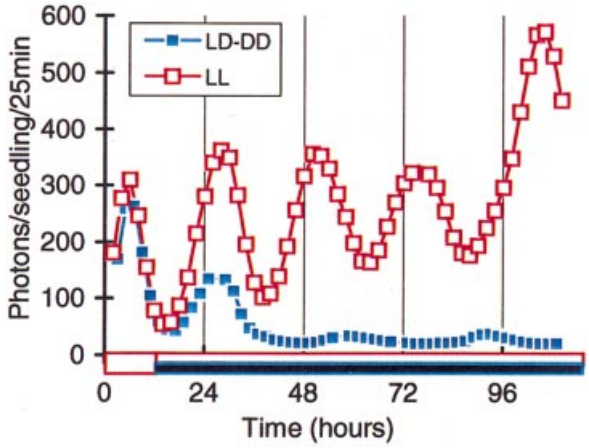

Figure 7. *cab2 : : luc* bioluminescence in constant light and constant darkness. *cab2 : : luc* seedlings were grown for 5 d under 12-h light–12-h dark cycles, and imaged (as in Fig. 6) in constant light (open, red symbols) or under one light–dark cycle followed by constant darkness (filled, blue symbols). Bioluminescence was quantified by image-processing software. The rhythm in darkness shows a long period and rapid damping. Time axis: open box, light interval; filled box, dark interval. *A. Hall* et al., *unpublished.*

reported for the *CAB* genes of the photosynthetic light-harvesting complexes (Kloppstech, 1985). The CAB (or light-harvesting complex (LHC)) proteins are among the major proteins associated with the thylakoid membranes, and are encoded by a nuclear, multigene family. The abundance of *CAB*-family mRNA is minimal at night in plants grown in a 12-h light–12-h dark cycle (Fig. 5). The abundance of mRNA starts to rise 2–4 h before dawn, peaking before the middle of the light period and beginning to decrease well before lights-off. The oscillations persist under constant light and constant darkness (Figs 5–7). The anticipation of light–dark and dark–light transitions, and the persistence of the rhythm under constant conditions, strongly indicate that *CAB* gene expression is controlled by a circadian rhythm. The bioluminescence of young *Arabidopsis* plants carrying a *cab2 : : luc* fusion transgene recapitulates the rhythmic regulation (Figs 6, 7). The green luminescence is so weak that it does not activate plant photoreceptors (Millar *et al.*, 1992; Anderson *et al.*, 1997), and can be detected only by ultra-low light video imaging (Figs 6, 7; Millar *et al.*, 1992) or by luminometry (Carré & Kay, 1995).

The levels of *CAB* expression are reduced in prolonged darkness because of the decrease in phytochrome activation, so the rhythm is 'damped' to a low amplitude. A pulse of red light before the dark period abrogates damping without greatly affecting the timing of expression, whereas far-red light converts phytochrome to the inactive, P_r form, and hastens the damping; the effect is reversible by red light (Fig. 5; Nagy *et al.*, 1988). The rate of damping varies considerably among species, and is already marked after one dark cycle in *Arabidopsis* (Fig. 7). Constant darkness also lengthens the period (section V). Robust circadian rhythms in *CAB* expression are the rule in almost all species under constant light (Oberschmidt *et al.*, 1995): *CAB* genes are now an important model for studies of plant *CCG*s (Fejes & Nagy, 1998). *CAB* rhythms have also been observed in dark-grown plants, but it is unclear how closely their regulation is related to the rhythm in constant light. Genetic (Hicks *et al.*, 1996) and photobiological (Kolar *et al.*, 1995, 1998) experiments show that the two rhythms can be uncoupled.

(c) *Clock-controlled genes for multiple phases.* Circadian regulation has been discovered for many plant genes, often serendipitously. Light-regulated genes and genes that encode proteins associated with photosynthesis are frequently circadian regulated (Fejes & Nagy, 1998). There are species-specific differences, such as in the expression of genes encoding the small subunit of Rubisco (*rbcS*). This is not rhythmic in all species and, where rhythmicity is observed, the circadian regulation can be transcriptional or post-transcriptional (Pilgrim & McClung, 1993). In addition, molecular screens aimed at identifying genes associated with photoperiodic floral induction have recovered a number of *CCG*s in *Pharbitis nil* (Zheng *et al.*, 1993), *Sinapis alba* (Heintzen *et al.*, 1994a,b) and *Lolium temulentum* (Perilleux *et al.*, 1996). When fully sequenced, these RNAs will suggest the scope of circadian regulation in plant genomes.

Three classes of *CCG*s include genes expressed in the evening. Rhythmically expressed, glycine-rich proteins with homology to RNA-binding domains

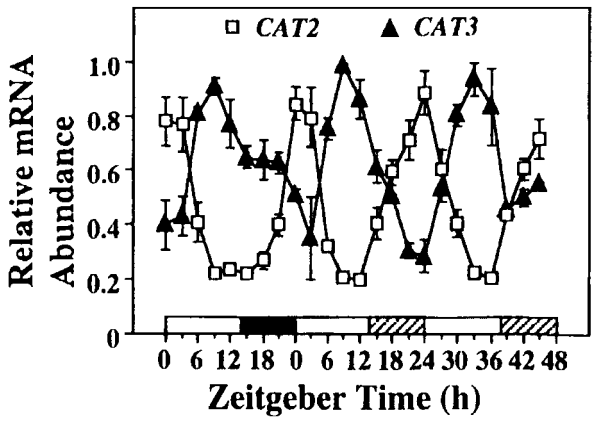

Figure 8. Opposite phases of *CAT2* and *CAT3* mRNA abundance rhythms in *Arabidopsis*. Plants were grown in soil under 14-h light–10-h dark cycles and harvested during one light–dark cycle and after transfer to constant light. Probes specific for *CAT2* (open symbols) and *CAT3* (filled symbols) were hybridized to RNA slot blots and hybridization intensity was quantified, relative to a constitutive control transcript. Time axis: open box, light interval; filled box, dark interval; hatched box, predicted dark interval in constant light. Data are means ± SEM; $n = 3$. *Reproduced, with permission, from Zhong & McClung (1996).*

are maximally expressed just before lights-off in *Sinapis* (*Sagrp* genes; Heintzen *et al.*, 1994b) and *Arabidopsis* (*ATGRP* or *CCR* (cold- and circadian-regulated) genes; Carpenter *et al.*, 1994; Kreps & Simon, 1997). The rhythm in *CCR*2 is likely to be modulated by an autoregulatory loop within the circadian output pathway: the overexpression of *CCR2* causes arhythmia in the abundance of endogenous *CCR2* RNA, but does not affect other circadian rhythms (Heintzen *et al.*, 1997). The RNA-binding motifs suggest that this autoregulation might be post-transcriptional (Pilgrim & McClung, 1993; Mittag *et al.*, 1994). The second class of cycling transcripts encodes germin-like proteins, which are also expressed in the evening (Heintzen *et al.*, 1994a; Ono *et al.*, 1996). These cell wall-associated proteins might be involved in remodelling wall structure, a likely correlate of the many circadian rhythms in plant growth (Engelmann & Johnsson, 1998). *CAT* genes form a small, multigene family in maize, tobacco and *Arabidopsis*: in each case, one member of the family is circadian regulated with a peak early in the night (*CAT3* in maize and *Arabidopsis*; Fig. 8) (Boldt & Scandalios, 1995; Zhong & McClung, 1996). Other *CAT* genes are constitutively expressed (Acevedo *et al.*, 1991) or are rhythmic with a peak in the day (Fig. 8; Zhong & McClung, 1996). The function of such gene-specific rhythms is unclear, especially where the rhythmic and non-rhythmic RNAs encode identical proteins and are expressed in similar tissues (Millar & Kay, 1991).

Comparison with other genomes suggests that the growing number of plant *CCG*s is not unusual. About 10% of various RNA samples have revealed rhythmic regulation – in the filamentous fungus *Neurospora crassa* (Bell-Pedersen *et al.*, 1996b), the dinoflagellate *Gonyaulax polyedra* (Milos *et al.*, 1990) and the fruit fly *Drosophila melanogaster* (Van Gelder *et al.*, 1995). The functions of the cognate gene products are often unknown. New types of technology will now permit truly genome-wide screens for *CCG*s, particularly in *Arabidopsis* (Ramsay, 1998).

2. *Output mechanisms*

As many processes are rhythmic in plants, so output signal transduction pathways must carry the timing signals from the circadian oscillator to the immediate effectors of those processes. At large in the cell, the timing signals join many other types of information in the signalling network, often converging on the same targets. This is particularly true of light signalling (phototransduction): dual control by the circadian clock and by light allows the plant both to anticipate the regular light–dark cycle and to respond rapidly to the changing light environment. Current understanding of plant photoreceptors and light-regulated gene expression offers a real advantage for studies linking circadian rhythms with photo-transduction. Similar studies of circadian output pathways should also be possible in any system with well-characterized signal transduction pathways, such as those that control the membrane properties of guard cells and pulvinar cells. These underlie the rhythms of stomatal aperture and leaf movement, respectively (Coté, 1995). Long-term assays – over several days – are being developed in protoplasts (Kim *et al.*, 1993; Mayer & Fischer, 1994), and might soon provide an informative comparison to the molecular rhythms.

(a) *The acute response to light*. Rhythmic processes in many organisms respond rapidly and directly to light, independently of the circadian clock (Fig. 1b). Adult *Drosophila*, for example, tend to move after light–dark transitions in a 'startle' response that is superimposed on the circadian rhythm of locomotion (e.g. Konopka *et al.*, 1994). The expression of *Neurospora ccg1* and *ccg2* is induced by light even in null mutants of the clock gene *frequency*, indicating that the acute response is not dependent on the circadian oscillator (e.g. Arpaia *et al.*, 1995). Acute responses to light are well documented for rhythmic markers in higher plants, including stomatal aperture (Assmann, 1993), leaf movement (Kim *et al.*, 1993) and the expression of *CAB* genes (Terzaghi & Cashmore, 1995). *CAB* genes are principally regulated by the phytochrome family of red and far-red photoreceptors, whereas stomatal opening is promoted mainly by blue light.

The expression of *CAB* genes in dark-grown plants clearly separates the acute activation of *CAB* by a pulse of red light from the subsequent circadian

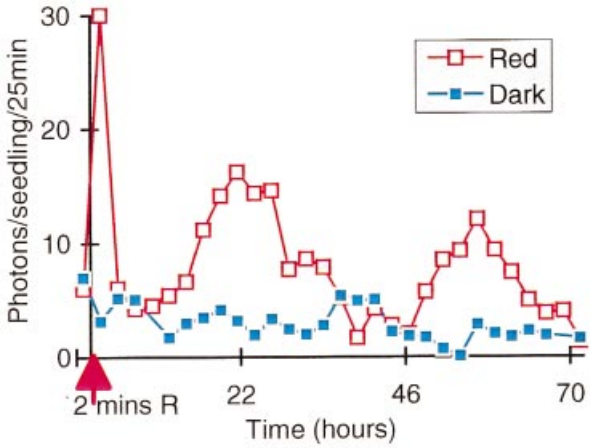

Figure 9. Phytochrome causes an acute activation of *CAB* in etiolated seedlings. The bioluminescence of dark-grown *cab2::luc* seedlings was imaged in darkness with (red; open, red symbols) or without (dark; filled, blue symbols) a 2-min pulse of red light at time 0. The acute response peaks at 1.5 h after the pulse and is followed by a long-period, circadian rhythm. *Modified, with permission, from Millar & Kay (1996).*

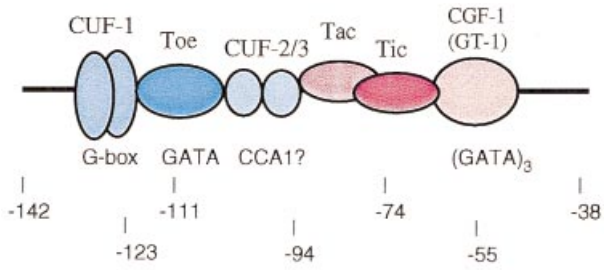

Figure 10. DNA-binding complexes at the *CAB2* promoter. The complexes are named above the line; sconserved sequence motifs and base numbers are indicated below the line, including a putative *CCA1*-binding sequence. CUF-1 is probably related to the leucine-zipper, G box-binding proteins, and is shown as a dimer. Abbreviations: CUF, *CAB* upstream factor; CGF, CAB GATA factor; $(GATA)_3$, a triple-GATA motif. *Modified, with permission, from Carré & Kay (1995).*

regulation (Fig. 9). Phytochromes PhyA and PhyB are the principal species required for the acute response in *Arabidopsis* – the *phyA–phyB* double mutant greatly diminishes the acute response (Reed *et al.*, 1994; Anderson *et al.*, 1997). *Cis*-element analysis has identified a conserved, triple GATA motif in the *CAB2* promoter (Fig. 10) as a necessary DNA sequence element for the acute response in dark-grown seedlings (Anderson & Kay, 1995; Anderson *et al.*, 1997). A mutation in the GATA sites is therefore predicted to mask the effect of the *phyA–phyB* double mutant, but this awaits confirmation.

Comparative sequence analysis indicates that the GATA motif is evolutionarily related to the binding site of the trihelix protein GT-1, which binds to many light-regulated promoters (Terzaghi & Cashmore, 1995; Arguello-Astorga & Herrera-Estrella, 1996). The *CAB2* DNA-binding complex, *CAB* GATA factor (CGF) is antigenically related to a cloned GT-1 protein (Teakle & Kay, 1995). Together with a non-light-regulated factor, GT-1 is sufficient to confer acute responsiveness to light (Puente *et al.*, 1996) and to phytochrome microinjection (Wu *et al.*, 1996), via a G protein- and Ca^{2+}-dependent pathway (Barnes *et al.*, 1997; Mustilli & Bowler, 1997). This component of *CAB* regulation is comparatively well understood.

(b) *Dissection of the* CAB *circadian clock-regulated element*. It took the field seven years to 'reduce' the size of the smallest known CCRE from 357 bp (Nagy *et al.*, 1988), to 36 bp (Carré & Kay, 1995). Luciferase reporter fusions have relieved the painstaking toil of RNA timecourses, because the *in vivo* activity of promoter fragments can now be tested in the luminescence imaging assay. These fusions have underlined a more fundamental and interesting problem, which is the difficulty in separating plant *CCRE*s from light-regulated sequence elements. The smallest promoter fragments that support circadian rhythmicity of reporter genes in transgenic plants will also confer responsiveness to light (Fejes *et al.*, 1990; Anderson & Kay, 1995). Gel-shift assays show that a number of DNA-binding complexes can form on the relevant 36 bp in the *Arabidopsis CAB*2 promoter, from −111 to −74 (Fig. 10; Carré & Kay, 1995). This does not include the adjacent CGF binding site (Fig. 10), and promoters that carry mutations in the CGF site retain circadian regulation, albeit at lower expression amplitude (Anderson & Kay, 1995; Anderson *et al.*, 1997). *CCRE*-mediated *CAB* expression is not uniquely dependent on PhyA and PhyB, so any phototransduction activity that is required might be provided by phytochromes C, D or E. An intriguing question is whether the circadian regulator(s) include repressor functions, as is expected for *CAB* and observed in some mammalian contexts (Molina *et al.*, 1993), or whether it will activate transcription, as in *Neurospora ccg2* (Bell-Pedersen *et al.*, 1996a) and other mammalian promoters (Fonjallaz *et al.*, 1996; Darlington *et al.*, 1998; Gekakis *et al.*, 1998).

The identity and DNA sequence specificity of the *CCRE*-binding proteins has been unclear, but a reverse genetic approach might now be converging with the classical promoter analysis. A novel *myb* relative, *CCA1* (circadian clock-associated gene), has been cloned for its involvement in light regulation, but it binds to a motif in the *CCRE* that is widely conserved among *CAB* genes (Wang *et al.*, 1997). It should soon become clear whether the cloned protein corresponds to the CUF-2 or CUF-3 *CCRE*-binding complexes of Carré & Kay (1995). The abundance of the CCA1 protein is regulated by a circadian rhythm, with a peak shortly after dawn (Wang & Tobin, 1998), consistent with a role in the circadian regulation of *CAB*. CCA1 expression is

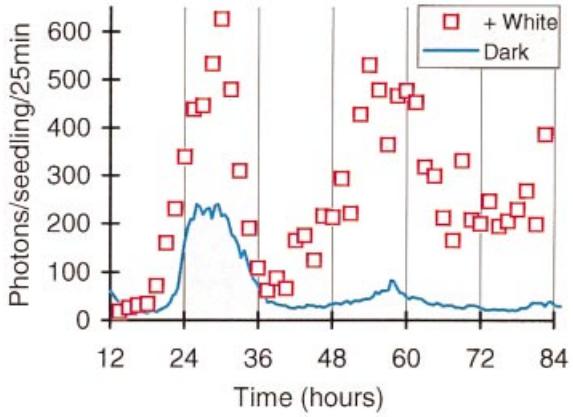

Figure 11. Circadian gating of acute *CAB* gene activation by light. *cab2 : : luc* seedlings were grown under light–dark cycles and transferred to a period of extended darkness at the normal time of lights-off. Controls were assayed in constant darkness (Dark, blue line). Replicate samples were exposed to 30 min of white light; the peak level of acute activation is shown, at each time of light treatment (+White; open red symbols). Maximal responsiveness to light occurs at the phases of maximal expression in the dark controls, and there is very little response in the first two subjective nights. *Modified, with permission, from Millar & Kay (1996).*

also acutely activated by phytochrome, however, and antisense reduction of CCA1 abundance reduces the acute response of *CAB* (Wang *et al.*, 1997). Some of the promoter elements that mediate circadian regulation might therefore be involved in the acute response as well, and these include binding sites for the transcriptional activator CCA1. Likewise, light-regulated transcription factors may participate in the circadian oscillator mechanism (see section IV), which gives a fresh significance to the study of *CCRE*s and their cognate DNA-binding proteins.

3. *Circadian gating of signalling pathways*

Acute responses to light are not dependent on the circadian clock, as the *Neurospora ccg*s indicate. They are often modulated by the clock, however, leading to a rhythm in the responsiveness to light. For comparison, a similar circadian rhythm in the responsiveness to light is widely accepted to be the basis for photoperiodic time measurement in plants. The acute light responsiveness of both stomatal opening and *CAB* gene expression is greatest during the subjective day (Gorton *et al.*, 1993; Millar & Kay, 1996). White light treatments of *cab2 : : luc* seedlings in dark adaptation result in maximal induction of *CAB* transcription at the phases when *CAB* expression is highest in the dark control plants (Fig. 11). Most significantly, there was little or no response to light during the first night, which is equivalent to the dark period of a light–dark cycle. A rhythmic signal from the circadian clock therefore interacts with the phototransduction pathway to create a circadian 'gate': the acute response is observed only at circadian phases when the gate is open.

(a) *Gating mechanisms*. The simplest model suggests that the circadian gate acts negatively, antagonizing the activation of *CAB* during the night (Fig. 1b). A previous 'gating' hypothesis (Kay & Millar, 1993) suggested that the circadian rhythm of *CAB* expression in constant light could be caused entirely by the rhythmic antagonism of a positively acting phototransduction pathway. As implied by the model, the acute response is present in light-grown plants, and does appear to be continuously active under constant light (Millar & Kay, 1996). Its contribution to *CAB* expression levels should then be phase-dependent, being greater during the subjective day, when the circadian gate is open. The phase-dependent activation will specifically increase the expression levels at the peak of the rhythm, causing an increase in rhythmic amplitude rather than in the overall expression level. Removing the acute response, by mutation of the CGF binding site or phytochromes A and B, does indeed reduce rhythmic amplitude in constant light (Anderson & Kay, 1995; Anderson *et al.*, 1997). The acute response therefore contributes to amplitude, but two lines of evidence indicate that circadian gating of the acute response is not the only rhythmic regulator of *CAB*. First, a clear rhythm of *CAB* expression remains in the CGF or *phyA–phyB* mutants. Second, the detailed waveform of *CAB* expression in light–dark cycles (Millar & Kay, 1996) indicates that the 'basal' rhythm in darkness has a different trough phase from the circadian gating rhythm. The 'basal' rhythm is presumably mediated by the 36 bp *CCRE*, not by CGF; it may depend on phytochromes C, D or E, but is predicted not to require ongoing exposure to light.

The gating mechanism could theoretically affect any level of phototransduction from the photoreceptor to the *CAB* promoter. The circadian gate is not specific to *CAB*, so the mechanism cannot be restricted to the *CAB* promoter: rhythms in light responsiveness have also been described for hypocotyl unhooking and elongation in response to red light (Horwitz & Epel, 1978; Wildermann *et al.*, 1978). The abundance of the photoreceptor proteins themselves appeared an unlikely target. The dynamics of the phytochrome proteins were thought to be faster (PHYA is turned over within minutes of light exposure) or slower (PHYB is stable for more than a day) than a circadian rhythm (Vierstra, 1994).The transcriptional activity of phytochrome promoter fragments in *luc* fusions was arhythmic in dark-grown tissue (Kolar *et al.*, 1998), and though the relative abundance of the P_r and P_{fr} forms of phytochrome changed over time, these changes did not match the rhythmic pattern of *CAB* transcription (Anderson *et al.*, 1997). This indicated that the

abundance of active phytochrome was not sufficient to account for the *CAB* expression patterns, and that the phytochrome signalling pathway must also change over time, at least in this tissue (Anderson *et al*., 1997). The PhyB promoter, surprisingly, drives circadian rhythms of *luc* activity with high amplitude in light-grown tissue (A. J. Millar *et al*., unpublished). Circadian rhythms in the photochemical properties of phytochrome have also been reported (Horwitz & Epel, 1978; King *et al*., 1982), again suggesting that the photoreceptor and/or an interacting molecule might be subject to circadian control. A second messenger for the phototransduction pathway could also be controlled by the circadian clock, as suggested by the rhythm in cytosolic calcium concentration (Johnson *et al*., 1995), and interactions among downstream effectors (Fig. 10) are likely. Intermediates of the phototransduction pathway must now be tested for circadian gating during the acute response, in order to determine where circadian regulation intersects with light regulation.

(b) *Two mechanisms of circadian regulation*. The circadian clock might therefore have two different types of output in the cell: a direct regulation, creating a 'basal' circadian rhythm, and an indirect effect, modulating the response to a non-rhythmic signal (Fig. 1b). It will be extremely interesting to determine whether circadian gating extends to further pathways in addition to phototransduction. One might speculate that circadian gating would allow a wide range of responses to be 'fine-tuned' appropriately for different times of day, even if the response was primarily to a non-rhythmic signal. Where that signal was present continuously, circadian changes in responsiveness might lead to a circadian rhythm in all the targets of the signalling pathway. Specific components of circadian output pathways must now be identified, in order to clarify the distinction between gated and basal rhythms.

III. PHOTOPERIODISM

Photoperiodic responses are triggered by a duration of light or darkness that is longer or shorter than a 'critical' duration. Two broad types of photoperiodic response can be distinguished in plants, both of which include a circadian rhythm in the responsiveness to light (Thomas & Vince-Prue, 1995). This complex area has been well reviewed (Thomas & Vince-Prue, 1996; Thomas, 1998). Here, the current models that are relevant to circadian timing are summarized, because recent genetic screens have recovered several mutants that affect both circadian rhythms and photoperiodism.

1. *Photoperiodic response rhythms*

(a) *Measuring the duration of darkness*. Most short-day plants are 'dark dominant', meaning that their photoperiodic timing system measures a critical duration of darkness (night length: Lumsden, 1998). A circadian rhythm in the responsiveness to light can be measured experimentally in these species, using light pulses to 'break' a long night (King *et al*., 1982; Thomas, 1991). This photoperiodic response rhythm (PRR) is reset at lights-off, and free-runs in darkness. The phase of maximal responsiveness to light occurs first at 7–10 h after lights-off: if this phase passes in darkness, the plant perceives a long night. In nature, a long night corresponds to a short day, and short-day plants are induced to flower under these conditions. If light is present at the sensitive phase, the plants perceive a short night and do not flower. This is an example of the 'external coincidence' model, in which an external signal (light) must coincide with the light-sensitive phase of the PRR.

Light has two effects in this model (Lumsden & Furuya, 1986). First, light (in a night break) can prevent the induction of flowering: phototransduction presumably causes an acute response that inhibits the mechanism of floral induction. The response is not uniform at all phases, because this acute-response pathway is subject to circadian gating. The gating introduces a timing component, determines the critical night length and leads to the PRR that is observed experimentally (Thomas, 1991). Second, a prior light interval is required to give the lights-off signal that resets the circadian oscillator. Dark-dominant plants are extremely sensitive to red-light night breaks. The light requirement for a lights-off signal, by contrast, has less wavelength specificity.

(b) *Measuring the duration of light*. 'Light-dominant' species are mostly long-day plants, including *Arabidopsis* (Fig. 12; Carré, 1998). Their differences from dark-dominant plants suggest that the critical timing occurs during the light period, so that light-dominant plants measure a critical day length. First, they tend to be much less sensitive to night breaks: several hours of illumination during prolonged darkness are usually required to cause a long-day response. Second, specific wavelengths of light are required during the photoperiod for maximal floral induction (far-red light is often required). Third, the effectiveness of far-red light for floral induction varies with a circadian rhythm that free-runs under constant light. In light-dominant plants, therefore, the most dramatic PRR can be the inductive effect of far-red illumination during a prolonged light period (Fig. 13; Deitzer, 1984), as opposed to the inhibitory effect of red light in prolonged darkness that is characteristic of dark-dominant plants.

The control of flowering in *Arabidopsis* is complex, because vernalization and a 'constitutive' pathway also contribute, and photoperiodism is not the major regulator in all ecotypes. The involvement of photoperiodism can be clearly demonstrated under

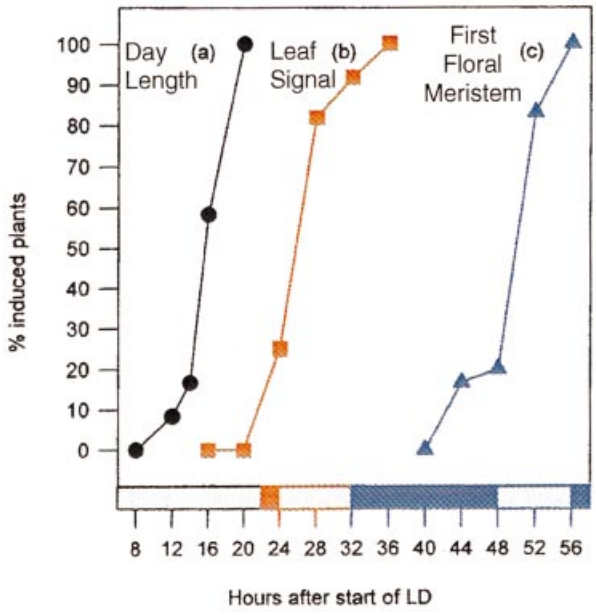

Figure 12. The photoperiodic control of flowering in *Arabidopsis*. Plants (ecotype Columbia) were grown under 8-h light–16-h dark cycles for two months, and given a single longer day, then returned to short days. Separate experiments measured the percentage of plants induced (a) to flower by a single photoperiod of various durations (filled circles), (b) to flower after the removal of all the leaves that had been exposed to one 22-h light–2-h dark cycle (red squares), and (c) to initiate floral meristems at various times after one 22-h light–2-h dark cycle and return to 16-h ligth–8-h dark (blue triangles). The results indicate that: (a) 50% induction occurs after one cycle of approx. 8-h light–16-h dark; (b) the floral stimulus has left the leaves by about 28 h after dawn on the long day, and (c) that the apex has responded to the signal by about 50 h. Flowering was measured by visual examination of the apex 2.5 weeks after the long day in (a) and (b), or by microscopic examination of the apical sections in (c). *Modified, with permission, from Corbesier* et al. (*1996*).

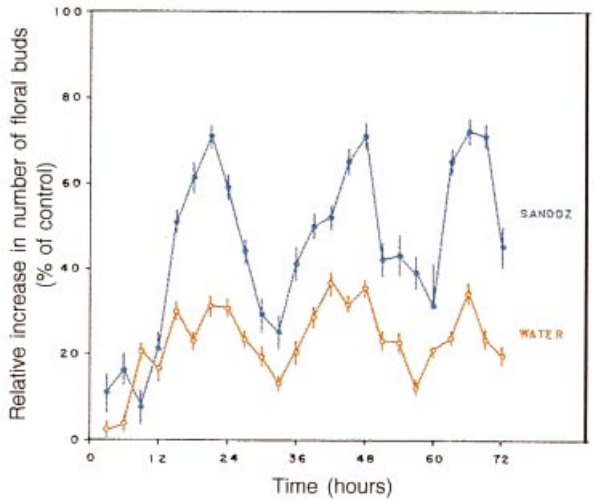

Figure 13. The photoperiodic response rhythm in *Arabidopsis*. Plants were grown under white fluorescent light on agar alone (WATER; red, open circles) or on agar containing the herbicide Norflurazon (SANDOZ; blue, filled circles), which results in photobleaching. The plants were treated with 6 h of supplementary far-red light, at different times during 72 h of constant white light. The flowering response of the plants was measured as the number of buds formed many days later, relative to controls without far-red light supplement (0%) and with 72 h far-red light supplement (100%). The supplement induced flowering most effectively during the subjective night, and the destruction of chloroplasts by photobleaching affected the amplitude but not the phase of the rhythm. Time 0 = lights-on; data are the mean ± SEM, n = 50 for each point, plotted in the middle of the 6 h far-red pulse. *Modified, with permission, from Deitzer* (*1984*).

appropriate conditions, however (Fig. 12): the day length required to induce 50% flowering was about 16 h for this ecotype, indicating that induction occurs at this phase. Day-length perception depends on the leaves, as in other species. Floral evocation at the apex becomes independent of the leaves about 26 h after the start of an inductive, long day (only 10 h after the inductive phase), indicating that the inducing signal has been transmitted by this time (Fig.12). The first floral meristem is distinguishable about 50 h after the start of the long day (about 34 h after the inductive phase). Phytochrome is very likely to entrain the relevant circadian oscillator (section V). It might be particularly challenging to distinguish between the contribution of a particular phytochrome to entrainment and its effect on the acute promotion or inhibition of flowering. The high sensitivity of the acute, night-break response allowed these two effects to be distinguished by their fluence requirement in *P. nil*, a dark-dominant species (Lumsden & Furuya, 1986). A combination of *Arabidopsis* mutants may be required to provide such clarity: fortunately, a number of photoperiodism mutants have been identified.

2. *Photoperiodism mutants in* Arabidopsis

The ultimate targets of phytochrome phototransduction should soon be identified among the *Arabidopsis* genes that control meristem identity (Hempel *et al.*, 1997; Coupland, 1998). In order to identify the intermediate steps between the photoreceptors and their targets, genetic screens have been performed for photoperiod-insensitive (aphotoperiodic) mutants, which flower at the same time under long days and short days (Carré, 1998; Coupland, 1998). Such an aphotoperiodic mutation might theoretically affect any non-redundant step in the acute response pathway between the photoreceptor and its targets, or it might disrupt the circadian gating mechanism. Only a subset of aphotoperiodic mutants are expected to carry a primary defect in the circadian system, therefore, but every mutant that is arhythmic in the relevant circadian system should be aphotoperiodic. If the PRR measures the duration of light, the simplest model suggests that mutants with an earlier or later phase in the PRR should shorten or lengthen the

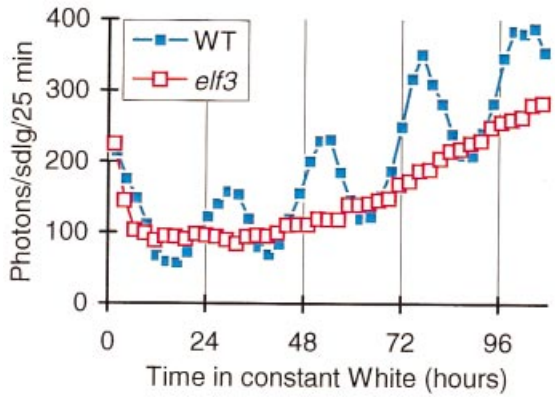

Figure 14. The *elf3* mutant is arhythmic under constant light. *cab2::luc* activity was imaged in wild-type (WT; filled, blue squares) and *elf3* (*elf3*; open, red squares) seedlings, essentially as in Figure 6. *K. Hicks* et al., *unpublished*.

critical day length, respectively. This model can be tested either by measuring the photoperiodic response of circadian rhythm mutants, such as *toc1–1* (section IV), or by assaying the circadian rhythms of mutants identified by altered photoperiodism.

(a) *Aphotoperiodic mutants*. Phytochrome mutants have severe effects on light-regulated traits, such as hypocotyl elongation, and can alter flowering time. No single *phy* mutation is aphotoperiodic, however, indicating that multiple phytochromes contribute to photoperiodism (King & Bagnall, 1996; Whitelam & Devlin, 1997). The first truly aphotoperiodic mutants (Rédei, 1962; Koornneef & Peeters, 1997) are late-flowering, but have no dramatic effects on light-regulated traits, and at least one (*constans*) does not affect other circadian rhythms (Somers & Kay, 1998). These genes might encode components specific to the photoperiodic response mechanism. Aphotoperiodic mutants in a second class affect both photomorphogenetic phenotypes and circadian rhythms. The recessive, *early flowering* 3 (*elf3*) (Zagotta *et al.*, 1996) and the dominant *LATE-ELONGATED HYPOCOTYL* (*LHY*) overexpressor (Schaffer *et al.*, 1998) were identified by their aphotoperiodic flowering. They are early- and late-flowering, respectively, compared to the wild type under long days. A transgenic line overexpressing *CCA1* (CCA1-OX: section II) has a similar phenotype to the *LHY* overexpressor (Wang & Tobin, 1998). All three mutations have a striking effect on the circadian system – *CAB* expression and leaf movement were shown to be arhythmic when plants were grown under light–dark cycles and transferred to constant light (Fig. 14) (Hicks *et al.*, 1996; Schaffer *et al.*, 1998; Wang & Tobin, 1998). *CAB* expression was also arhythmic in dark-grown *elf3* plants after a red-light pulse (Anderson *et al.*, 1997), though the acute response to light was still present. In agreement with predictions, then, three of the aphotoperiodic mutants also abolished overt rhythms that are not obviously related to flowering, suggesting that they might be mutants in the circadian system.

(b) *Phenotypic effects of* elf3, LHY *and* CCA1. An uncertainty remains, because the aberrant circadian system might be either the primary defect that causes aphotoperiodism or the secondary effect of another alteration – this unknown alteration might cause aphotoperiodism by a clock-independent mechanism. If an altered circadian system is the primary defect in these mutants, three predictions follow: first, all of the mutant phenotypes should be recognizably caused by aberrant circadian timing; second, the gene products should have an identifiable function in the circadian system; and third, restoring the relevant circadian rhythmicity to the mutants by any means should be sufficient to restore photoperiodism. The first prediction assumes that the gene has a single function, which may be an oversimplification, but it cannot be rejected yet. The second prediction is discussed further here (section IV) and the third cannot yet be tested.

The elf3, CCA1-OX and LHY-OX mutant phenotypes are all clock related (arhythmic *CAB* expression and leaf movement, and aphotoperiodism), in agreement with the first prediction. The three mutants also have defects in hypocotyl elongation and both *elf3* and plants expressing an antisense *CCA1* gene alter the acute response of *CAB* to light (Wang *et al.*, 1997). These traits are typical of phototransduction mutants and are not expected in circadian mutants, suggesting that the primary defect may be near the start of a phototransduction pathway. The mutation's effects on hypocotyl elongation and the acute response could be independent of effects on photoperiodism and the circadian system (section V). Recent data on the scope of circadian regulation provides an alternative explanation, because the circadian clock affects both the acute response and hypocotyl elongation. The acute response of *CAB* to light has an increased amplitude and slightly faster kinetics in dark-grown *elf3* plants (Anderson *et al.*, 1997). The circadian gating pathway modulates the amplitude and kinetics of the acute response (section II), so a clock defect could affect the acute response via the gating phenomenon. More direct investigation of circadian gating in the mutant lines is currently in progress. The *elf3*, *CCA1-OX* and *LHY-OX* lines also have elongated hypocotyls when grown under light–dark cycles (Zagotta *et al.*, 1996; Schaffer *et al.*, 1998; Wang & Tobin, 1998). The long-hypocotyl phenotype is usually associated with photoreceptor mutants and is notably absent in the short-period mutant, *toc1–1* (section IV). Hypocotyl elongation in all three lines is much closer to the wild type under long photoperiods or constant illumination, which is a phenotype previously observed only in the *phyA* mutant (Johnson *et al.*, 1994), but the elf3 hypocotyl

phenotype lacks the wavelength-specificity typical of a photoreceptor mutant (Zagotta *et al*., 1996). Rather surprisingly, the circadian clock has recently been shown to regulate hypocotyl elongation as well (Schuster & Engelmann, 1997; Dowson-Day & Millar, 1999). The three arhythmic mutants lack this circadian rhythm, suggesting that the absence of rhythmicity causes the long hypocotyl phenotype (Dowson-Day & Millar, 1999). It is therefore possible that a primary defect in the circadian system of the *elf3*, *CCA1-OX* and *LHY-OX* lines causes all of the observed phenotypes, and that a detailed examination of these mutants will reveal further processes that are normally regulated by the circadian system.

(c) *Aphotoperiodism without arhythmia*? Full-blown arhythmia under all circumstances should not be required for an aphotoperiodic phenotype, so long as whatever circadian output remains is insufficient to induce the photoperiodic system. The problem for these studies is that the available rhythmic markers might not be representative of the unidentified circadian output for the PRR. The flowering and hypocotyl phenotypes in *elf3* are most severe in short photoperiods, for example, but the waveform of *CAB* expression is closest to the wild type under these conditions, because of the acute response at dawn (Hicks *et al*., 1996). However, the *elf3* mutant does not lack all circadian rhythms. Both dark-grown *elf3* plants and light-grown, but dark-adapted, plants retain circadian rhythms of *CAB* expression. Six alleles of *elf3* have indistinguishable phenotypes, suggesting that they are null alleles, and that this rhythmicity is not caused by a partial loss of function. Even *elf3* plants in light–dark cycles exhibit a near-normal increase of *CAB* expression in anticipation of dawn, before losing the rhythm in the light period (Hicks *et al*., 1996). All of the arhythmic phenotypes in *elf3* are conditional upon illumination of the plants, supporting the notion that the relevant timing for photoperiodism occurs during the day, when circadian timing is disrupted. The primary defect in *elf3* might therefore disrupt an interaction between light and the circadian system (Hicks *et al*., 1996), either in the circadian input pathway or by another mechanism (Millar, 1998b): if so, *ELF3* expression need not be rhythmically regulated. Both *CCA1* and *LHY* are rhythmically expressed: their possible function in the circadian system is discussed in section IV.

Bünning's external coincidence model requires only one circadian timing system and one, gated phototransduction pathway, which combine to create the PRR. Several photoreceptors are known to be involved in photoperiodism, because molecular and genetic tools are available to assay the functions of specific photoreceptor species. The data discussed suggest that a single circadian mechanism controls very many rhythms throughout the plant. New, genetic and molecular tools will soon reveal whether the timing system for photoperiodism is indeed simpler than its photoreceptors.

IV. OSCILLATOR THEORY AND PRACTICE

The circadian oscillator mechanism is being unravelled in several model systems, in parallel. Given the tremendous diversity in the overt rhythms and the input photoreceptors (Johnson, 1995; Foster, 1998), only the mechanism and functional organization of the circadian oscillator retain a 'lingering hope' of ubiquity (Pittendrigh, 1960). Limited regions of DNA sequence homology now link candidate oscillator components across great evolutionary distances, suggesting that a conserved mechanism might exist.

Five widely accepted criteria have been developed from an input–oscillator–output model of the circadian system (Fig. 1b) in order to identify components of the oscillator mechanism (Block *et al*., 1993; Kay & Millar, 1995; Dunlap, 1996). Not only is an oscillator component expected to oscillate with the same period as the overt rhythm, but this oscillation must also be necessary for overt rhythmicity. Pegging an oscillator component to any constant activity level (in or out of the normal range) should therefore cause arhythmia. The activity of the component should be rapidly affected (in less than one cycle) by any environmental signal that shifts the stable phase of the rhythm. A transient change in the activity of the component should be sufficient to cause a predictable phase shift, in the absence of environmental signals. A sustained change in the mean level of its oscillation might also alter the free-running period, and this is certainly an empirical regularity among the candidates tested so far. The pharmacological, molecular and genetic approaches guided by these criteria have identified several components that are likely to be central to oscillator function in one or more species.

1. *Transcription and translation of the clock mechanism*

Many pharmacological agents have been tested for effects on circadian rhythms, and the abolition of rhythmicity by translation inhibitors is perhaps the data set that resonates most with current research (Edmunds, 1988). Inhibitor-induced arhythmia was not merely caused by an inhibition of the rhythmic output, while the motion of the oscillator continued undetected: reversible inhibitors demonstrated that the circadian oscillator stopped, and only restarted when the inhibition was relieved. Transcription inhibitors gave similar results.

The giant alga *Acetabularia mediterranea* has been important for such studies among the photosynthetic organisms (Vanden Driessche *et al*., 1997). The *A.*

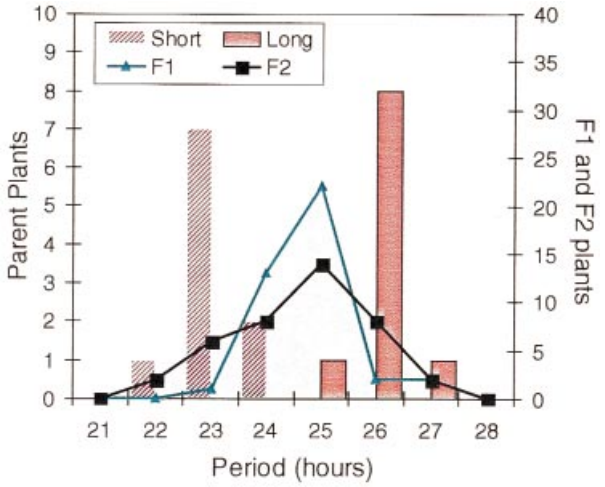

Figure 15. Bünning's period variants in *Phaseolus*. True-breeding lines were selected for long (Long; purple hatched columns) and short (Short; red columns) periods of leaf movement. Ten plants of each line were assayed (left scale). 40 plants were tested in the F1 generation (F1; triangles) and F2 (F2; squares) generations of a cross between the lines (right scale). The period distribution in the F2 geneation is not significantly different from a 1:2:1 combination of the parental and F1 distributions. *Redrawn from Bünning* (*1935*).

mediterranea cell is so large that the nucleus can easily be removed, or transplanted between cells. The nucleus is not required for free-running rhythms of photosynthesis, which persist in anucleate cell fragments; when it is present, however, the nucleus determines the phase of the rhythm (Edmunds, 1988). These experiments suggest that ongoing nuclear transcription is involved in the circadian system, but is not required for the free-running oscillator. Rhythmic translation is essential, because translation inhibitor treatments rapidly cause arhythmia. Inhibitor experiments have been perfected in studies of the marine snail, *Bulla gouldiana* (Whitmore & Block, 1996). Both transcription and translation are required for the oscillator in the *B. gouldiana* eye, during overlapping phases within the subjective day (Khalsa *et al.*, 1996). The implication is that a 'clock gene' or genes are expressed at specific phases, and that the synthesis of the cognate protein and RNA species determines the phase of the oscillator. In other words, these molecular events are central components in the biochemical mechanism of circadian timing.

Oscillator models suggest that the oscillator components will participate in a negative feedback loop, such that the accumulation of one component is self-limiting, directly or indirectly (Dunlap, 1996; Lakin-Thomas, 1998). The accumulation of a clock protein might therefore reduce the expression of the cognate gene, and falling levels of the protein should subsequently allow a fresh cycle of expression. The loop must incorporate sufficient delays to create a 24-h cycle, and must be temperature compensated: neither is a typical feature of gene expression. A positive input is also required for a self-sustaining oscillator like the circadian clock in order to avoid damping out under the negative regulation alone, but this input need not be rhythmic. Candidate molecules now exist for each of these functions.

2. *The genetics of circadian oscillators*

(a) *The first clock mutants in bean*. Genetics has been, and remains central to, the identification of the oscillator RNAs and proteins. Bünning (Bünning, 1935) first recognized an opportunity in the variable period of his runner beans (*Phaseolus multiflorus* (formerly *Phaseolus coccineus*)). He selected true-breeding lines with 23-h and 26-h periods of leaf movement, clearly revealing a genetic component in this circadian rhythm (Fig. 15), and crossed the lines to determine how many genes were involved. The F2 generation gave a distribution of period length that matched the 1:2:1 segregation expected from two co-dominant alleles at a single locus (Fig. 15). Bünning was disappointed by the result, however, because his methods (the 40 F2 plants were 'a large sample') lacked the resolution to distinguish a 1:2:1 pattern from the more complex segregation of a multigenic trait. This paper should not have been a disincentive to further studies (Bünning suggested using a different organism), but in fact the field moved away from genetics for thirty years.

(b) *The* Drosophila *and* Neurospora *models*. The filamentous fungus *Neurospora* and the fruit fly *Drosophila* are not only classic genetic models, but automated assays are also available for their circadian rhythms of sporulation and locomotor activity, respectively. 'Brute-force' screens in *Drosophila* and *Neurospora* reawakened interest in clock genetics, because their mutant phenotypes were intuitively attractive and fulfilled the criteria for an oscillator component so well. A null allele of an oscillator gene was expected to be arhythmic, but the same would be true for a gene required in the output pathway. The null alleles of the *per* and *frq* genes caused arhythmia in *Drosophila* and *Neurospora*, respectively, but other alleles at the same loci altered period length (reviewed in Dunlap, 1996; Young, 1998). This distinguished *per* and *frq* from output pathway components, and has now become the paradigm for identifying clock genes in genetic screens. The rhythm-altering mutants at other loci (18 in *Neurospora* and 7 in *Drosophila*) have received less attention, because their alleles do not have such a range of phenotypes (Dunlap, 1996; Lakin-Thomas, 1998). The semi-dominance of the period alleles at some loci is another empirical regularity across all species, although the genes in question may not all encode oscillator components. The clock-mutant screens might not be saturated, so a few more genes

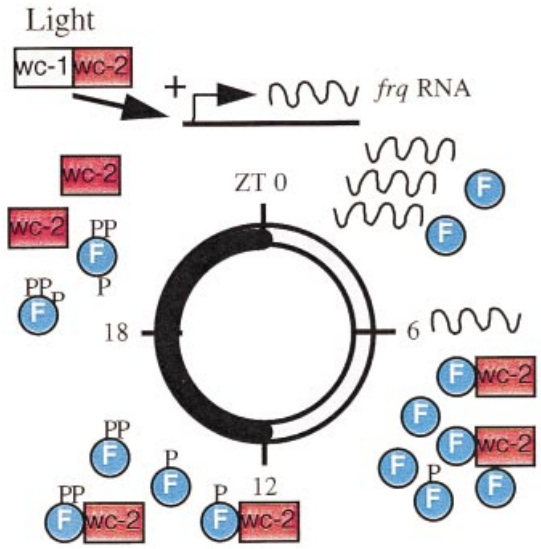

Figure 16. The *Neurospora frq*/FRQ model. wc-1 and wc-2 products activate *frq* transcription, so *frq* RNA accumulates in the early subjective day. FRQ protein (blue circles) levels peak late in the subjective day, causing repression of *frq* transcription (shown here as binding of FRQ to WC-2, although the mechanism is unknown). FRQ is then phosphorylated (P) and degraded, allowing another cycle of *frq* transcription. wc-1 and wc-2 products also mediate the activation of *frq* by light. The inner circle shows a 12-h light–12-h dark cycle as a phase reference.

in the *per* and *frq* class might be recovered: *timeless* (*tim*), *double-time* (*dbt*), *cycle* (*cyc*) and *jerk* (*jrk*) in *Drosophila* are recent examples.

The characterization of *per*, *frq* and *tim* has proceeded rapidly (Rosato *et al*., 1997), although not always smoothly. *frq* transcription has fulfilled all of the classical criteria for an oscillator component in *Neurospora* (Fig. 16): it is rhythmic, with a peak close to dawn, and altering the mean level of transcription affects the period of the rhythm (Merrow *et al*., 1997). Rhythmic transcription is necessary and autoregulated, because constant *frq* transcription at any level from a heterologous promoter (a promoter from a different species) does not support rhythmicity, and overexpression of FRQ represses transcription from the *frq* promoter (Aronson *et al*., 1994). *frq* transcription is induced by phase-shifting light pulses, and experimentally arranged steps in the rate of *frq* transcription set the phase of the rhythm in a predictable manner (Aronson *et al*., 1994; Crosthwaite *et al*., 1995). The negative feedback of FRQ protein on *frq* transcription, and the recovery of *frq* transcription after FRQ is removed, together incorporate sufficient time delays to account for the circadian period (Merrow *et al*., 1997). It is formally possible that few or no other components are required in order to specify phase uniquely (Merrow *et al*., 1997). For example, unknown mechanisms must also operate to create the delays but these may be permissive (Fig. 1b). Two translational initiation sites produce long and short forms of the FRQ protein in a ratio controlled by the ambient temperature. The two protein species differ in their temperature responses, providing a mechanism for temperature compensation as well (Liu Y *et al*., 1997).

The *Drosophila* model of a *per*- and *tim*-dependent oscillator is broadly similar (Rosato *et al*., 1997; Young, 1998). The rhythmic expression patterns of *per* and *tim* peak during the night, in contrast to *frq*, and are also slightly different from each other (So & Rosbash, 1997). The accumulation of their protein products is delayed by the instability of *per* protein, for which the *dbt* protein kinase is required (Price *et al*., 1998). TIM and PER then interact via the PER protein's PAS domain (*per*-ARNT-*sim*; Pellequer *et al*., 1998): this interaction is important for the transport of PER to the nucleus (Rutila *et al*., 1996; Saez & Young, 1996). Neither PER nor TIM has been shown to bind DNA directly. Rather, they inhibit transcription from the *per* and *tim* promoters by interacting with the products of the *cyc* and *jrk* genes, which are PAS-containing transcriptional activators (Allada *et al*., 1998; Darlington *et al*., 1998; Rutila *et al*., 1998). The primary mechanism of light input is the destruction of the TIM protein (Huter-Ensor *et al*., 1996; Lee *et al*., 1996; Myers *et al*., 1996). The rhythm of *per* transcription was thought to be an essential part of the oscillator, but the discovery of a circadian-regulated, post-transcriptional element in *per* RNA has recently called this part of the model into question: *per* constructs that are transcribed uniformly still support circadian rhythms (So & Rosbash, 1997; Stanewsky *et al*., 1997; Cheng & Hardin, 1998). Studies of *per* homologues in the silkmoth and house fly independently suggested a similar conclusion: so long as *per* RNA is present, other levels of regulation might be sufficient to maintain circadian rhythms in these insects (Sauman & Reppert, 1996; C. P. Kyriacou, pers. comm.). Temperature compensation depends on the PAS domain (Huang *et al*., 1995) and on a variable region of Thr-Gly repeats (Sawyer *et al*., 1997; Peixoto *et al*., 1998).

FRQ shares little sequence similarity with either PER or TIM, except in the Thr-Gly repeat region, so there has not yet been a molecular reconciliation of the oscillator models in the fly and the fungus. The *Neurospora* system has the advantage that most or all cells of the fungus are similarly rhythmic, whereas there are significant differences among *Drosophila* cell types. For example, a small number of neurones in the brain are principally responsible for driving normal locomotor activity rhythms (Ewer *et al*., 1992), but *per* is expressed very widely, and not always rhythmically, throughout the fly. Genetic studies of whole organisms are therefore concentrated on the circadian system of the critical neurones, whereas biochemical assays pool many cell types, sometimes obscuring the regulation of *per*

(Hall, 1995). Transgenic *per*::*luc* flies now provide a non-invasive marker for *per* transcription, a process that is at the least very closely associated with the oscillator (Plautz *et al.*, 1997). The bioluminescence rhythms give high spatial resolution in optically accessible cells, but not in the deeply buried clock neurones. A simpler model of the insect clock might therefore be required, for example in cell cultures (Saez & Young, 1996; Darlington *et al.*, 1998).

(c) *New model species for rhythm research.* Genetic model systems to study the circadian oscillator have developed much more recently in mouse, in *Arabidopsis* and in cyanobacteria. Circadian rhythms were only recently discovered in cyanobacteria (reviewed in Johnson *et al.*, 1996). The stunning progress in this system (Kondo *et al.*, 1994; Liu *et al.*, 1995) is driven by one of the most convenient rhythm assays (a *psbA–luciferase* reporter) and an outstanding command of molecular biology, including the complete genome sequence, albeit of a different species. Components of the cyanobacterial oscillator should soon be characterized, but this system is not just a genetic 'fast track'. The adaptive value of circadian rhythms has also been directly confirmed by competition studies between clock mutant strains with different periods. Either a long-period or a short-period strain can have a competitive advantage, if the light–dark cycle matches its free-running period (Johnson *et al.*, 1998b; Yan *et al.*, 1998).

The mouse system was partly developed by a 'forward' genetic screen, which identified a short-period mutant, *Circadian locomotor output cycles kaputt* (*Clock*), in a screen of over 700 animals (Vitaterna *et al.*, 1994). Saturation mutagenesis for rhythm mutants is impractical in mouse, so Bünning's experiment in *Phaseolus* was revisited: the allelic differences between two strains of mice have revealed several quantitative trait loci that affect the period of circadian rhythms (Hofstetter *et al.*, 1995; Mayeda *et al.*, 1996). A 'reverse' genetic approach has recently been initiated by the identification of molecular homologues of *per*, among the bHLH-PAS family of transcription factors. The genes concerned carry a basic helix-loop-helix DNA-binding domain and the PAS domain implicated in protein–protein interaction. The most interesting of the family members are *mPer1* and *mPer2*, which are both rhythmically regulated in the relevant part of the brain and *mPer1* at least is also light-induced (Shearman *et al.*, 1997; Shigeyoshi *et al.*, 1997). The molecular clone of the *Clock* gene revealed that it too was a bHLH-PAS protein, with a lower degree of homology to insect *per* (King & Bagnall, 1996). The CLOCK protein was recently shown to activate *mPer1* transcription as a heterodimer with another bHLH-PAS protein, BMAL1 (Gekakis *et al.*, 1998). In a convincing moment of experimental convergence, BMAL1 and CLOCK were simultaneously shown to be most closely related to *Drosophila cyc* and *jrk*, respectively, which activate *per* transcription in *Drosophila* (Allada *et al.*, 1998; Darlington *et al.*, 1998; Rutila *et al.*, 1998). There is a temporary hiatus, however, while the function of the mPer genes is tested *in vivo*. Homologous recombination techniques should soon produce an allelic series of mutations in each gene, which could replicate the long-period, short-period and arhythmic phenotypes characteristic of *per* mutants in the fly.

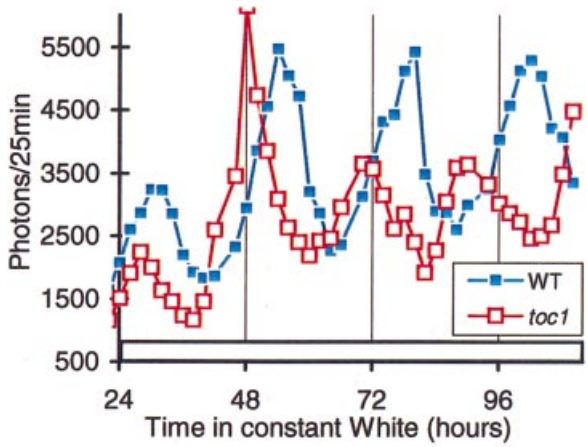

Figure 17. *toc1–1 Arabidopsis* seedlings have a short period. *cab2*::*luc* activity was imaged in wild-type (WT; filled, blue symbols) and *toc1–1* (*toc1–1*; open, red symbols) seedlings, as in Figure 6. *J. Fielding & A. J. Millar, unpublished.*

3. *Rhythm mutants and candidate genes in photosynthetic organisms*

(a) Chlamydomonas. The second photosynthetic organism on the clock mutant scene was the unicellular green alga, *Chlamydomonas reinhardtii*. Four period genes were identified from a combination of mutagenesis and testing various strains, but each is represented only by a single allele (reviewed in Somers & Kay, 1998). Rhythm research in *C. reinhardtii* might be a natural extension of the cyanobacterial studies: the system has much to offer, including well-characterized entrainment patterns (Johnson & Kondo, 1992).

(b) Arabidopsis. *Arabidopsis* had no documented circadian rhythms until rhythmic gene expression and leaf movements were reported (Millar & Kay, 1991; Engelmann *et al.*, 1992; reviewed in McClung & Kay, 1994). The *cab2*::*luc* transgenic marker allowed a preliminary genetic screen to identify more than twenty lines with aberrant rhythms, including both long- and short-period mutants (Millar *et al.*, 1995a; Millar & Kay, 1997). A short-period mutation (Fig. 17) in the *timing of CAB* gene (*toc1–1*) has been best characterized, but the bank of mutants includes other *toc1* alleles and mutants in at least two other genes (Somers & Kay, 1998; Somers *et al.*, 1998). The *toc1* mutant is not *CAB*-specific – it also

shortens the period of rhythms in leaf movement (Millar *et al.*, 1995), *CCR2* expression (Kreps & Simon, 1997) and stomatal opening (Somers *et al.*, 1998) – but the plants appear normal in both light-regulated traits and gross morphology. The circadian input pathway is probably unaffected, because the fluence response curve for period is very similar in *toc1–1* and in the wild type (Somers *et al.*, 1998). The photoperiodic induction of flowering is altered in *toc1–1* (Somers *et al.*, 1998), though this was masked in the mutant's original genetic background. The correlation of circadian rhythm defects with altered photoperiodic responses is therefore maintained in this clock mutant, as well as in the mutants originally identified for flowering phenotypes (section III).

The *toc* screen was insensitive to arhythmic mutants: *elf3* and the *LHY* and *CCA1* overexpressors are the only such lines reported in *Arabidopsis* (section II). Moreover, the sequence similarity between *CCA1* and *LHY* suggests that they may be functionally redundant, which would hinder their identification as loss-of-function alleles. Most interestingly, both the *LHY* and *CCA1* genes are rhythmically expressed in wild-type plants, with peaks of expression shortly after subjective dawn (Schaffer *et al.*, 1998; Wang & Tobin, 1998). *CCA1* expression correlates broadly with *CAB* expression, but slightly precedes *CAB*, just as it does in the acute response to light (Wang *et al.*, 1997). The expression of the endogenous *CCA1* and *LHY* genes is repressed in the overexpression lines, suggesting that the rhythm may be produced by negative autoregulation (Schaffer *et al.*, 1998; Wang & Tobin, 1998). This is expected of an oscillator component but is not unique to such genes: it is also observed for *CCR2*, for example (section II; Heintzen *et al.*, 1997). *LHY* and *CCA1* are unlikely to function in the terminal steps of the circadian output pathway, because their overexpression affects a number of different rhythmic phenotypes, in contrast to *CCR2* (section III). The CCA1 protein also accumulates rhythmically, but lacks any substantial delay with respect to the RNA rhythm (Wang & Tobin, 1998): a delay must be incorporated at some point, in order to draw out an autoregulatory loop over a circadian period. CCA1 is known to be light induced, again as expected of an oscillator component. It remains to be determined whether experimental manipulation of *CCA1* and *LHY* (and, later, *ELF3*) activity can alter circadian phase or period in predictable ways, and whether their rhythmicity is absolutely required for overt rhythms in the organism.

Molecular data on the identity and expression of *toc1* and *elf3* are now eagerly awaited. The *toc1* mutant has so far conformed with expectations conditioned by the *per* and *frq* model, whereas *elf3* suggests that the interactions between light signalling and circadian rhythms are more complex than the simplest circadian models allow. A concerted effort of biochemistry and molecular genetics is building a new framework, or at least extending the old one: the principles of the new model may also transcend species boundaries, and light-regulated transcription factors seem to play a central role.

4. *How many clocks?*

The organization of the broader circadian system is the focus of increasing interest. Most species probably carry multiple oscillators, perhaps one in every cell (Millar, 1998a): pulvinar protoplasts, for example, can maintain circadian rhythms (Kim *et al.*, 1993; Mayer & Fischer, 1994). The oscillators might include more than one mechanism, for even the unicellular *Gonyaulax* can exhibit two circadian periods, indicative of two oscillators (Roenneberg & Morse, 1993; Fig. 3). Many lines of evidence indicate that the coupling or independence of the oscillators is crucial for normal circadian rhythms (Pittendrigh, 1993; Roenneberg & Mittag, 1996; Liu C *et al.*, 1997; Millar, 1998a). The functional organization of these cellular clocks has barely been addressed in plants. The rhythms of different organs can become desynchronized, but the coherence of rhythms within an organ suggests that clocks are locally coupled, as they are in other species (Block *et al.*, 1993; Liu C *et al.*, 1997).

V. PHOTOTRANSDUCTION PATHWAYS

The input pathway entrains the circadian oscillator, in the simplest model, and is most often discussed in terms of two ubiquitous phenomena (Johnson *et al.*, 1998b). First, the input pathway mediates phase shifts of the oscillator in response to light pulses or steps in light intensity, and second, the intensity and quality of ambient light can alter the period of the oscillator, again via the input pathway. Natural photoperiods include both types of signal and others besides, which together lead to stable entrainment. The balance of phase shifts caused by the various signals causes the period of an entrained oscillator to match the period of the day–night cycle (Millar & Kay, 1997; Millar, 1998b). Temperature and other environmental factors can also contribute to entrainment (Sweeney, 1987), however, and light often affects the amplitude of circadian rhythms as well as phase and period.

1. *Photoreceptors for circadian input pathways*

(a) *Damping and the control of amplitude.* Many circadian rhythms in higher plants persist for much longer in continuous light than in darkness, in contrast, for example, to the conidiation rhythm of *Neurospora* and the locomotor activity rhythm of *Drosophila*. These processes become arhythmic in

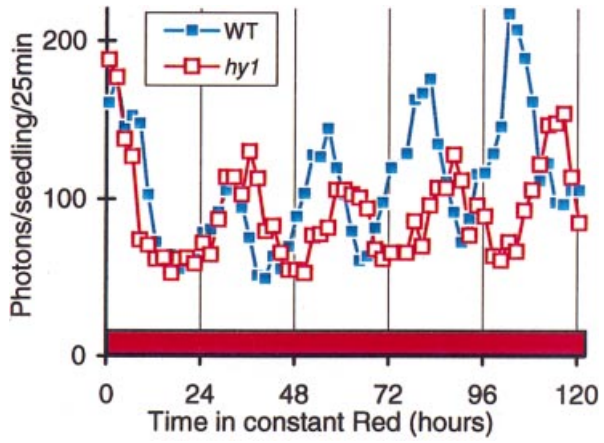

Figure 18. Phytochrome deficiency lengthens circadian period in *Arabidopsis*. *cab2::luc* activity was imaged in wild-type (WT; filled, blue symbols) and *hy1–100* (*hy1*; open, red symbols) seedlings, as in Figure 6 but under constant red light. The partial phytochrome deficiency of *hy1–100* leads to a period 1.5–2 h longer than the wild type. Data from representative seedlings are shown. *A. J. Millar* et al., *unpublished*.

continuous light, but for different reasons: rhythmic conidiation was thought to be masked in the fungus, because light directly induces conidiation (Sargent & Briggs, 1967), whereas increasing light fluence rates lengthen the fly's period until the rhythm becomes undetectable (Konopka *et al*., 1989). The rhythm of *CAB* expression in dark-adapted *Arabidopsis* shows both a drop in expression level that eventually masks the rhythm, and an increase in period from 24.5 h in the light to up to 30 h in darkness (Fig. 7). The activation of *CAB* expression by phytochrome is well characterized (section II), and *CAB* expression levels decrease as this activation is progressively lost in darkness (Millar & Kay, 1997).

The rhythmic expression of *CAT3*, in contrast, damps to a high level in darkness. Mutations in PhyA and in the blue-light receptor, cryptochrome 1 (*cry1*) prevent damping (Zhong *et al*., 1997), indicating, surprisingly, that the PhyA and Cry1 photoreceptors are required for the damping of this rhythm in wild-type plants. Their phototransduction pathways seem most likely to mask rhythmic regulation (similar to the *Neurospora* example), perhaps by acting at the *CAT3* promoter. An effect on the oscillator is also possible. The period of *CAT3* expression remains close to 24 h for at least two days in darkness, when damping is prevented, in contrast to the *CAB* rhythm. This might well be another example of internal desynchronization, indicating that the oscillator that controls *CAT3* might differ from that controlling *CAB* (Millar, 1998a).

(b) *The control of period*. At least two photoreceptors provide circadian input in most photosynthetic organisms, possibly in order to buffer their circadian systems against changes in light conditions (Johnson *et al*., 1998b). This was graphically demonstrated in *Gonyaulax*: increasing fluence rates of red light lengthened the period of the bioluminescence rhythm, whereas blue light shortened the period (Fig. 4; Roenneberg & Hastings, 1988). Chlorophyll and a blue light receptor are thought to mediate these responses, which together maintain a more stable period at a range of white light intensity (Roenneberg & Mittag, 1996). A similar balance was revealed in the higher plant, *Coleus*: continuous red light shortened the period of the leaf movement rhythm, whereas blue light lengthened the period (Halaban, 1969).

Wild-type *Arabidopsis* plants have similar periods of *CAB* expression (24.5–25 h) under red, blue and white light, compared to the 30-h period of white light-grown plants transferred to darkness (Millar *et al*., 1995b; Somers *et al*., 1998). Partial phytochrome deficiency in the *hy1* mutant lengthens the period of *CAB* expression under red light, as expected of a partially 'blind' mutant (Fig. 18). The *hy1* mutant slightly shortened the period under blue light, however, and the opposite effects of phytochrome depletion in red and blue light strongly implicate a non-phytochrome, blue photoreceptor. As a result, *hy1* has a wild-type period under white light, so it would not be recovered as a period mutant in these conditions (Millar *et al*., 1995b). The phytochrome family probably provides circadian input in all higher plants, and work is in progress to identify the specific phytochrome species responsible; the identity of the blue photoreceptor remains unknown (Somers & Kay, 1998).

Developmental regulation of phototransduction pathways has been reported in several contexts (e.g. Frohnmeyer *et al*., 1992), and developmental factors might also affect circadian input. The low-level expression of *cab2::luc* in dark-grown *Arabidopsis* has a 24-h period (Hicks *et al*., 1996), for example, in contrast to the 30-h period of plants that have undergone photomorphogenesis before transfer to darkness. A single red-light exposure is sufficient to trigger both photomorphogenesis and arhythmia in *elf3* (Anderson *et al*., 1997).

These dramatic effects on circadian period suggest that some of the mutants identified by their altered periods probably affect light input, not the circadian oscillator. The light-conditional arhythmia in *elf3* has been taken to suggest that such a mutated function may alter period to the point of arhythmia (as in *Drosophila* in constant light; Fig. 19). Null alleles of the photoreceptors *phyB* and *cry1* are even semi-dominant (Koornneef *et al*., 1980), like the period alleles of the canonical clock genes. The phenotype of an input pathway mutant is likely to be conditional upon the lighting conditions, as in *hy1* (Millar *et al*., 1995b). The *toc1–1* mutation, by contrast, shortens the period equally over the entire fluence range, suggesting that *TOC1* does not affect the light-input pathway (Somers *et al*., 1998).

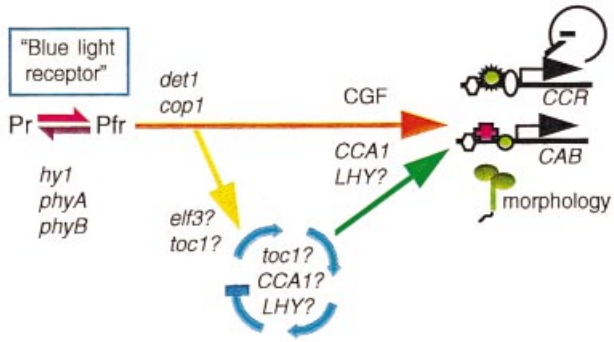

Figure 19. Genes and mutations associated with the *Arabidopsis* circadian system. The components of the circadian system are shown in the same colours as in Figure 1b. A few targets of circadian regulation are shown (morphology and the expression of *CAB* and *CCR* genes), with the additional autoregulatory loop of *CCR* expression. Unidentified, blue light receptor(s) and phytochromes provide input to the circadian clock. PhyA, PhyB and CGF are specifically required for the acute response of *CAB* to light. The *det1* and *cop1* genes affect both the circadian period and the level of *CAB* expression. *elf3* affects an interaction of light and the circadian system, but assignment to the input pathway is speculative. The *toc1* mutation and the overexpression of *CCA1* and *LHY* probably disrupt the function of the oscillator, although it remains possible that *toc1* has an input function, and that *CCA1* and *LHY* have output functions. *CCA1* is also known to affect the acute response of *CAB*; the highly homologous *LHY* may also do so.

2. *An essential accessory to the oscillator*

Formal oscillator theory indicates that a self-sustaining oscillator requires negative feedback and a delay (Section IV). A positive factor is also necessary to sustain the oscillation, which would damp out under the negative regulation alone; the positive input need not be a part of the negative feedback loop (Fig. 1b). The *Neurospora white collar* (*wc*) mutants suggest that the input pathway provides both the necessary positive input to the *frq*/FRQ cycle and the light signals for entrainment. *wc1* and *wc2* are the only two genes recovered in screens for 'blind' *Neurospora* mutants, making these likely components of the input pathway (Fig. 16). Sporulation is arhythmic in the *wc* mutants in light and darkness; *frq* RNA is almost undetectable. The *wc* products are therefore required for *frq* activation (Crosthwaite *et al.*, 1997). *frq* activation appears to be the only function lacking in *wc1*, but transient expression of *frq* ('priming' the system) does not initiate a rhythm in the *wc-2* mutant, indicating that another function in addition to *frq* is required for rhythmicity. The *wc-2* product, or a *wc2*-dependent function, may therefore be the activator that sustains the oscillator, and is antagonized daily by FRQ negative feedback (Crosthwaite *et al.*, 1997), just as *jrk* and *cyc* are antagonized by *per* in *Drosophila*. The *wc* genes encode zinc-finger DNA-binding proteins (Ballario *et al.*, 1996; Linden & Macino, 1997), with PAS domains. *wc-1* has further homology to the LOV domain, which is a putative flavin-binding motif found in proteins that sense light, oxygen or voltage, including the *Arabidopsis* light-response gene *NPH1* (Huala *et al.*, 1997). *wc-1* might therefore be close to the photoreceptor for circadian input in *Neurospora* (Crosthwaite *et al.*, 1997).

NPH1 does not appear to contain a PAS domain and its function in circadian input is only now being tested (Somers & Kay, 1998). However, the phytochromes do contain PAS homology, (Lagarias *et al.*, 1995), PhyB is translocated to the nucleus in a light-dependent fashion (Sakamoto & Nagatani, 1996) and phytochromes are known to provide circadian input. Even if PAS is a molecular red herring, which occurs too widely to be a reliable indicator of specific function, the *wc* results indicate that components of the input pathway might have a very direct effect on the nuclear oscillator mechanism. This functional organization might well be conserved in plants (and other species). The wild-type fungus maintains robust, free-running rhythms in darkness, for which the *wc* functions are required. Plant rhythms often persist poorly under these conditions, suggesting that the activator function equivalent to the *wc*'s might be light-dependent. This function could be aberrant in the *elf3* mutant, leading to its conditional arhythmia. The involvement of light-regulated transcription factors *CCA1* and *LHY* in circadian oscillator mechanisms links these pathways very closely: the difficulties in dissecting the light-activated elements of plant promoters from clock-regulated elements (section II) might not be coincidental. Given the complexity of both red and blue photoreceptors in plants, it is possible that a few photoreceptor species are specialized for circadian input and are necessary for the circadian system to function, whereas any input function of the other photoreceptors is secondary and dispensable. Similar specialization is strongly indicated in rodents, where the opsin-like receptor that entrains the circadian clock is known to be located in the retina, but has yet to be unequivocally identified (Foster, 1998).

VI. CONCLUSIONS

Higher plant genomes include a high percentage of genes involved in metabolism in comparison with other organisms (Bevan *et al.*, 1998). Circadian rhythms can provide the necessary external and internal coordination to orchestrate metabolic processes, for example around the diurnal rhythm of photosynthesis. The relative phases of some rhythms might need to be adjusted to suit changing developmental or environmental conditions (Roenneberg & Mittag, 1996). The plant circadian system must be flexible enough to support these diverse timing requirements.

Our current understanding confirms this suggestion most strongly for the input pathway, because

multiple photoreceptors clearly mediate light input to the clock. The circadian input has still to be located within the biochemical events of the phototransduction pathways (Barnes *et al*., 1997), and the signalling circuits around the oscillator must be identified. Input to the clock can be finely modulated, in some cases by a circadian rhythm (Millar, 1998b). This feedback of output upon input creates an 'outer loop', which may be present in the plant circadian system. Components of the first plant circadian output pathway will soon be identified unequivocally. These should help to determine how many output pathways control the various phases of overt rhythms in plants, and may distinguish direct circadian output from circadian gating pathways. Both input and output components are now related to putative circadian oscillator mechanisms by sequence homology or by experimental observation (Fig. 19). The pathways linking some domains of the basic clock model (Fig. 1b) may be very short indeed, or the mechanisms of these domains may overlap.

It seemed unlikely that the circadian system would have so few components that its rhythmic output could not be regulated flexibly. If this turns out to be the case, however, multiple copies of the circadian system might provide the versatility that was lacking in a single one. If one plant oscillator mechanism is derived from the cyanobacterial ancestor of the chloroplast, higher plant clocks may also have other mechanisms.

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