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Review

Tansley review

The physiology of circadian rhythms in plants

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Contents

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Summary

Circadian rhythms regulate many aspects of plant physiology including leaf, organ and stomatal movements, growth and signalling. The genetic identity of some of the components of the core circadian oscillator has recently become known. Similarly, the photoperception and phototransduction pathways that entrain the oscillator to the day and night cycle are being determined. Less clear are the pathways by which the circadian oscillator regulates cellular physiology. Circadian oscillations in cytosolic free calcium might act to transduce the temporal outputs of the circadian oscillator. This hypothesis requires rigorous testing using novel noninvasive technologies. Plants might gain advantage from the circadian clock by being able to predict changes in the environment and coordinate physiological processes, presumably increasing survival and hence, reproductive fitness. Technical advances coupled with cell-specific measurement techniques will allow the advantages of the circadian regulation of physiology to be quantified.

Abbreviations

casein kinase 2α (CK2α); CRYPTOCHROME (CRY); PHYTOCHROME (Phy); ZEITLUPE (ZTL); LATE ELONGATED HYPOCOTYL (LHY); CIRCADIAN CLOCK

ASSOCIATED (CCA1); EARLY FLOWERING 3 (ELF3); EARLY FLOWERING 4 (ELF4); TIMING OF CAB EXPRESSION1 (TOC1); CCT (CO, COL and TOC1 binding domain); PHYTOCHROME-INTERACTING FACTOR3 (PIF3); nitrate reductase (NR); LIGHT HARVESTING COMPLEX B (LHCB); cytosolic free calcium concentration ($[Ca^{2+}]_i$); chloroplastic free Ca^{2+} ($[Ca^{2+}]_{chl}$); light (L); dark (D); continuous light (LL); continuous dark (DD), CAULIFLOWER MOSIAC VIRUS 35S (CaMV 35 s); the small subunit of ribulose-1,5 bisphosphate (*rbcS*); ethyleneglycol *bis* α-amionethyl *N, N, N′, N′*, tetraacetic acid (EGTA); inositol 1,4,5 trisphosphate ($Ins1,4,5P_3$); LUCIFERASE (LUC); *out of phase* (*oop1*); Cyclic ADP ribose (cADPR); abscisic acid (ABA); suprachiasmatic nucleus (SCN); *Samanea* pulvini inward rectifying channel (SPICK); *Arabidopsis* potassium transporter 2 (AKT2); *Samanea* pulvini outward rectifying channel (SPORK); stelar potassium outward rectifier (SKOR); *Samanea saman* Aquaporin 2 (SsAQP2); phototropin (PHOT); phosphatidylinositol 3 phosphate (PtdIns3P); phosphatidylinositol 4 phosphate (PtdIns4P); water use efficiency (WUE); multi electrode ion flux estimation (MIFE™); indole acetic acid (IAA); phospho*enol*pyruvate carboxylase (PEPC); ribulose-1,5-*bis*phosphate carboxylase/oxygenase (Rubisco).

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I. Introduction

Most eukaryotes and some prokaryotes have a circadian clock (Table 1). Circadian clocks have a period of *c.* 24 h and are entrained to the daily temperature and light/dark cycles of the rotating earth (Table 1). Circadian rhythms are most easily observed by placing an organism in constant conditions (usually constant light) after entrainment of the rhythm to temperature or light/dark cycles and then detecting those phenomena that are rhythmic with a period of about 24 h (Table 1). Circadian rhythms have been observed in physiological, developmental, biochemical and transcriptional activities.

The widespread occurrence of circadian behaviour suggests it is of considerable benefit to the organism. Presumably, the circadian clock allows an organism to anticipate rhythmic changes in the environment and this provides adaptive advantage. However, the mechanisms by which circadian behaviour is regulated, the processes controlled by circadian behaviour and the adaptive advantages gained by circadian control are poorly understood and are starting only now to be elucidated.

Table 1 Glossary and Definition of Terms (Lumsden & Millar, 1998)

Glossary	Definition
Acute response	A rapid, often transient response, usually to an environmental stimulus.
Amplitude	The amount of change in a circadian activity. Often calculated as the difference between the peak and the trough levels; more strictly, the maximal deviation from the mean.
Circadian clock (or biological clock)	The set of components that underlies all the properties of a circadian rhythm. A widespread model divides this into three parts: input, oscillator and output.
Circadian rhythms	Rhythms with a period of about 24 h are termed circadian (from c., about, and dies, a day).
Diurnal cycle	A biological rhythm driven entirely by environmental cues that does not persist in constant conditions. Sometimes used to describe rhythms seen in day/night cycles.
Entrainment	The coupling of one rhythm to the same period as another cycle, for example, the resetting of a circadian rhythm from its free-running period to exactly the 24-h period of the day/night cycle.
Gating	The restriction of a processes to a particular circadian phase. Often used to describe the rhythmic modification of acute or other responses.
Period	The time for one complete cycle of a rhythm. The reciprocal of frequency.
Phase	A particular reference point in the cycle, e.g. the day phase, the portion of the cycle occurring during the day. A phase shift refers to a movement of this reference point relative to another cycle.
Input	A signal that can reset or entrain a rhythm. Usually light or temperature. Sometimes called also, zeitgeber ('time-giver').
Oscillator	Or sometimes core oscillator, the biochemical and molecular components that generate a self-sustaining rhythm.
Output pathways	The intracellular signal transduction pathways communicating temporal information from the oscillator to the rest of the cell.
Ultradian	A rhythm with a period substantially less than 24 h.

In this article, I review the current understanding of the circadian regulation of the physiology of plants. Physiological process known to be regulated by the circadian clock can be placed in the following major categories: growth (hypocotyl elongation and leaf movements in *Arabidopsis*); organ movement (petal movements, leaflet movements in legumes); stomatal movements; transport processes involved in Crassulacean acid metabolism; transport processes in the root; intracellular signalling and the regulation of membrane properties. Detailed discussion of the circadian regulation of these physiological processes and source references can be found in Lumsden & Millar (1998) and Sweeney (1987). I have focused on the cell physiology of the circadian regulation of growth, organ movements and intracellular signalling. I have concentrated on these aspects of circadian physiology because there are likely to be shared mechanisms. The comparative physiology of the circadian regulation of growth, organ movements and signalling will identify common themes and unique aspects that will shed light on circadian physiology in general. Additionaly, the circadian regulation of gas exchange in CAM plants is also discussed.

The circadian clock of plants has a much wider role than just the regulation of physiology. A number of excellent reviews cover aspects of the circadian regulation of plant metabolism and biochemistry and the role of the clock in the photoperiodic control of the transition from vegetative growth to flowering (Lumsden & Millar, 1998; Millar, 1999; Harmer *et al.*, 2001; Hayama & Coupland, 2003).

II. The circadian clock

Studies in various organisms have lead to a consensus model of the circadian clock (Harmer *et al.*, 2001). The model describes an input pathway through which photoperception occurs and light signals are transduced to entrain a circadian oscillator to environmental cues; the circadian oscillator that generates rhythmicity and lastly, there are output pathways by which the temporal signals generated by the oscillator regulate cellular behaviour (Fig. 1). The basic mechanisms of clock function in plants and animals have common features in their organisation and functionality. However, the clock genes are not shared between these kingdoms. Only the cryptochromes (blue light photoreceptors involved in entrainment) and casein kinase 2α (CK2α) have been shown to be shared components of the clock between kingdoms (Sugano *et al.*,

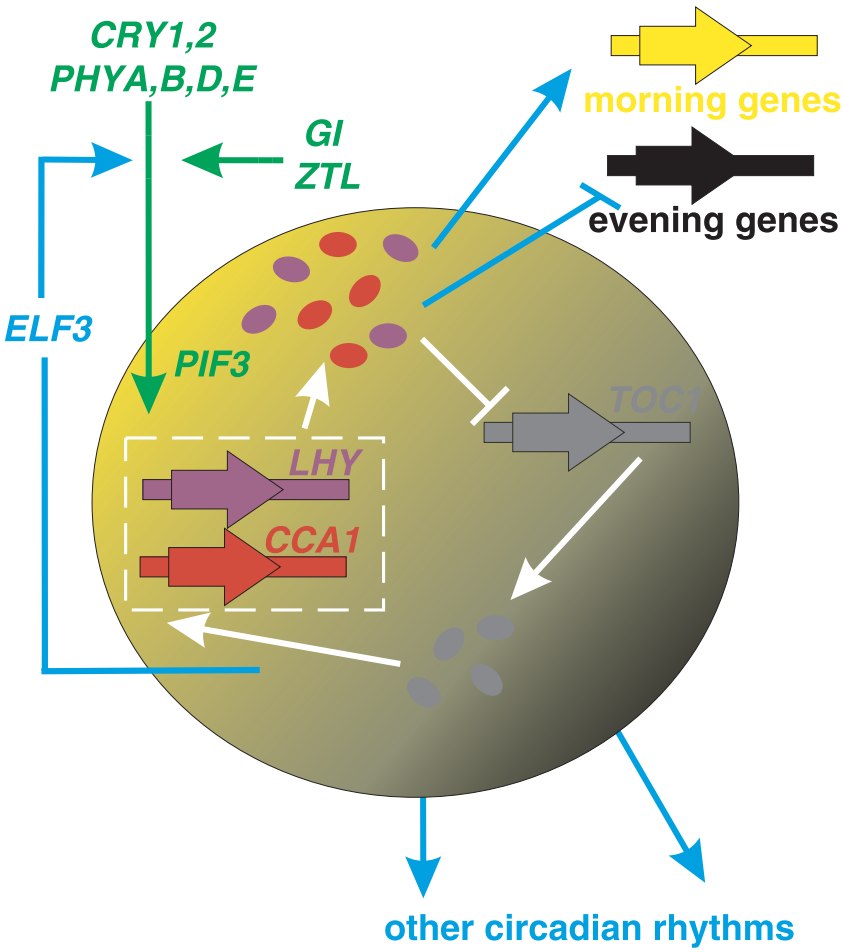

Fig. 1 A model of the circadian clock in Arabidopsis based on a negative-regulating transcriptional feedback loop. Light activates *LHY* (purple) and *CCA1* (red) expression at dawn. The accumulation of LHY/CCA1 proteins down-regulate *TOC1* (grey) expression. During the day *LHY/CCA1* expression reduces, allowing *TOC1* transcript levels to rise and reach a maximum at the end of the day, when *LHY/CCA1* are at their lowest. TOC1 probably promotes *LHY/CCA1* expression, allowing *LHY/CCA1* expression to reach a peak at dawn beginning the cycle again. Further details of the model are described in the text. Arrows indicate positive regulation. Bars indicate negative regulation. The model proposes a circadian clock consisting of input pathways (green), the core oscillator (contained within the circle, white arrows and bars) and output pathways (blue). The day is indicated by yellow and night by black. This figure is redrawn from the model proposed by Alabadi *et al*. (2001) and reviewed in Hayama & Coupland (2003).

1998; Lin *et al.*, 2002). This review will focus solely on plant circadian clocks. An excellent comparison of circadian clock structure, genes and function between kingdoms can be found in Harmer *et al.* (2001).

1. The plant circadian clock

(a) The input pathway In *Arabidopsis*, the circadian clock is entrained by the cryptochromes and phytochromes (Somers *et al.*, 1998a). Blue light entrains via CRYPTOCHROME1 (CRY1), CRY2 and PHYTOCHROME A (PhyA), whilst red light entrains via PhyA, PhyB, PhyD and PhyE (Millar, 1999). The ZEITLUPE (ZTL) protein interacts with both PhyB and CRY1 and the *ztl* mutation lengthens circadian periods in a light dependent manner suggesting that ZTL acts in the light input pathway (Somers *et al.*, 2000; Jarillo *et al.*, 2001). The mechanisms by which photoreceptor activation may regulate the circadian clock are reviewed and discussed further in Millar (1999); Harmer *et al.* (2001); Fankhauser & Staiger (2002) and Hayama & Coupland (2003).

The relationship between photoperception and entrainment of the clock is complicated by feedbacks from the oscillator that regulate photoperception. *PhyB* expression is rhythmic and is regulated by a circadian oscillator composed of *LATE ELONGATED HYPOCOTYL* (*LHY*) and *CIRCADIAN CLOCK ASSOCIATED* (*CCA1*) and other genes (Hall *et al.*, 2002) (see section II.1.(b) for a discussion of oscillator structure and function). It is likely that the circadian regulation of photoreceptor abundance fine-tunes circadian rhythms and light responses (Hall *et al.*, 2002). Another gene that modulates photoperception and transduction is *EARLY FLOWERING 3* (*ELF3*). *elf3* mutants of *Arabidopsis* are arrhythmic in continuous light but not in darkness. This is consistent with an effect of *elf3* on light input (Hicks *et al.*, 1996; Hicks *et al.*, 2001). *ELF3* transcript and protein levels are circadian-regulated and both ELF3 and PhyB can interact suggesting a mechanism by which ELF3 modulates light inputs to the clock (Liu *et al.*, 2001). ELF3 is thought to antagonize phototransduction in the early to the middle of the night acting as parting of the 'gating' mechanism essential for the maintenance of self-sustained oscillations in LL (McWatters *et al.*, 2000). Constitutive expression of *LHY* results in arrhythmia of most circadian responses in *Arabidopsis*, presumably by 'stopping' the clock. However, oscillations of *ELF3* transcriptional regulation were detectable in an *LHY* overexpressing background, providing supporting evidence for *ELF3* acting 'upstream' from the oscillator in a light input pathway (Hicks *et al.*, 2001). The rhythmicity of *ELF3* transcription in the *LHY* over expressing background raises questions concerning the mechanism by which the circadian regulation of *ELF3* transcription occurs.

(b) The core oscillator Genetic components thought to act in the circadian oscillator have been identified via screening for mutants with altered circadian behaviour (Fig. 1). In common with molecular central oscillators identified in other organisms, the plant circadian oscillator consists of transcriptional loops of negative feedback inhibition (Harmer *et al.*, 2001). In *Arabidopsis*, three candidate components of an oscillator have been identified (Fig. 1). These are *CCA1*, *LHY* and *TIMING OF CAB EXPRESSION1* (*TOC1*). CCA1 and LHY are closely related proteins with a MYB-like DNA binding motif (Hayama & Coupland, 2003). The similarities between LHY and CCA1 suggest that they might have partially redundant functions and this has been demonstrated genetically (Mizoguchi *et al.*, 2002). TOC1 is a pseudo response regulator belonging to a novel family. A plant-specific motif has been identified in TOC1. This CCT (CO, COL and TOC1) motif is thought to be involved in protein interactions and nuclear localization (Hayama & Coupland, 2003). The experimental evidence that CCA1, LHY and TOC1 are components of the circadian oscillator has recently been reviewed, to avoid repetition the reader is referred to Hayama & Coupland (2003).

A model for the plant circadian oscillator based on a transcriptional feedback between *LHY*, *CCA1* and *TOC1* has been proposed (Alabadi *et al.*, 2001). This model is based on the observations that LHY and CCA1 repress *TOC1* expression and TOC1 appears to promote *LHY/CCA1* expression (Alabadi *et al.*, 2001). Furthermore, the peaks of *LHY/CCA1* expression and *TOC1* expression are at opposite phases of the cycle. It is proposed that the cycle starts when light activates *LHY/CCA1* expression at dawn (Fig. 1). The accumulation of LHY/CCA1 represses *TOC1* expression which in turn results in reduced activation of *LHY/CCA1*. As a result, during the progression of the cycle there is a reduction in *LHY/CCA1* expression allowing *TOC1* transcript levels to rise and reach a maximum at the end of the day, when *LHY/CCA1* are at their lowest. TOC1 is predicted to enhance *LHY/CCA1* expression allowing *LHY/CCA1* expression to reach a peak at dawn beginning the cycle again (Alabadi *et al.*, 2001).

TOC1 probably activates *LHY/CCA1* expression by interaction with PHYTOCHROME-INTERACTING FACTOR3 (PIF3), a basic helix-loop-helix protein that binds to elements in the *LHY* and *CCA1* promoters and also interacts with phytochromes (Hayama & Coupland, 2003). *EARLY FLOWERING 4* (*ELF4*) may also activate *CCA1* expression, either as part of the central oscillator or as part of another mechanism that confers persistence to the oscillator. Lesions in *ELF4* result in low levels of *CCA1* expression and rhythms in LL with variable periods that quickly become arrhythmic (Doyle *et al.*, 2002).

It is proposed that LHY/CCA1 abundance also affect the expression of genes that are not part of the central oscillator (Alabadi *et al.*, 2001). LHY/CCA1 probably activate genes with a 'morning' phase (Table 1) and simultaneously repress 'evening' phase genes (Alabadi *et al.*, 2001). Translational feedback loops dampen if a delay is not imposed in the loop, therefore post-translational modifications might introduce delays to this loop. *CK2α* has been demonstrated to phosphorylate CCA1 in Arabidopsis suggesting a possible role for

CK2α in regulating the central oscillator, possibly by introducing a delay (Sugano *et al.*, 1998). In addition to post-translational modification of proteins, post-translational control of protein abundance is likely to play an important role in oscillator function. For example, phase-specific protein degradation mediated by the proteasome is responsible for circadian alterations in ZTL abundance (Kim *et al.*, 2003).

Important support for this model comes from the effects of *toc1* mutations and *LHY* and *CCA1* over expression on widespread and functionally different circadian responses in *Arabidopsis*, including flowering time, *CHLOROPHYLL A/B BINDING PROTEIN* (*CAB*) promoter activity, hypocotyl elongation, cotyledon movements, the expression of *LHY/CCA1* and *TOC1* themselves and stomatal opening and closing (reviewed in Millar (1999)). However, it is clear that the model is incomplete, because over expression of *TOC1* does not abolish circadian oscillations as predicted by the model (Makino *et al.*, 2002) and plants almost completely lacking *LHY* and *CCA1* function retain some rhythmicity (Alabadi *et al.*, 2002). This does not mean the model is incorrect, but it does suggest additional levels of complexity (Carré & Kim, 2002).

The presence of a core oscillator is still hypothetical. An alternative model is that there is no central clock and the circadian rhythms arise as a result of self-sustained feedback loops in the processes that in the *LHY/CCA1/TOC1* model are considered 'outputs' of the clock (Table 1). In this alternative model, the circadian behaviour itself produces the necessary feedback signals to generate oscillatory behaviour with a 24-h period. The repression of nitrate reductase (NR) expression by the catalytic activity of the NR protein has been proposed to be an example of self-regulatory feedback loop generating circadian behaviour that could occur in the absence of a central oscillator (Lillo *et al.*, 2001).

The self-sustained feedback loop model is an attractive model of circadian function but does not explain why genetic alterations to the *LHY/CCA1/TOC1* oscillator alter all output pathways so far tested (Millar, 1999). However, the *LHY/CCA1/TOC1* model of an oscillator and the self-sustained feedback loops model are not mutually exclusive because multiple oscillators might be present in plants. Evidence for the presence of multiple oscillators comes from experiments in which different physiological rhythms are decoupled. Rhythms of stomatal conductance and assimilation can become decoupled under certain conditions in *Phaseolus vulgaris* L. (Hennessey & Field, 1992). Similarly, the circadian periods of *PHYB* and *CAB* expression are different in *Arabidopsis* suggesting regulation by distinct but functionally similar circadian clocks (Hall *et al.*, 2002). These multiple oscillators are autonomous because circadian oscillations of *PhyB*, *CAB* and chalcone synthase expression in *Arabidopsis* can be entrained independently at the organ and suborgan level (Thain *et al.*, 2002). Furthermore, it appears that each plant cell contains at least one oscillator because circadian rhythms are detectable in stomatal movements and leaf pulvinus protoplasts even when these tissues are isolated from the rest of the plant (Gorton *et al.*, 1989; Mayer *et al.*, 1997).

Circumstantial evidence based on the presence of autonomous individual cell rhythms and the pleiotropic effects of genetic manipulation of the *LHY/CCA1/TOC1* oscillator suggest the presence of an *LHY/CCA1/TOC1* oscillator in every cell in the plant. This particular model is assumed for the description of the circadian regulation of physiology and signalling in this review. However, this assumption does not exclude the possibility of coupling between oscillators at the cellular/tissue level, the presence of other intracellular oscillators and the presence of unidentified, additional components of the *LHY/CCA1/TOC1* oscillator.

(c) The output pathways The output pathways act 'downstream' from the core oscillator to regulate many processes including transcription, physiology, biochemistry and development. However, the nature of the circadian output pathways and the mechanisms by which the circadian oscillator regulates cellular processes are largely unknown. At least some genes are probably regulated directly by components of the oscillator. For example, CCA1 activates *CAB* expression and may activate other genes that cycle in the same phase as *CAB* (Alabadi *et al.*, 2001). Similarly, so called 'evening elements' have been identified in the promoters of some *Arabidopsis* genes whose transcripts oscillate with a peak in the evening (Harmer *et al.*, 2000; Alabadi *et al.*, 2001). It is through this evening element that CCA1/LHY are predicted to repress *TOC1* expression.

However, the circadian control of plant physiology might involve processes in addition to the direct activation/repression of transcription by the transcriptional regulators that form the core oscillator. Processes as diverse as enzyme activity and ion transport are regulated by the circadian clock and there is evidence for both translational and post-translational control of the circadian regulation of these activities (Lillo *et al.*, 2001; Moshelion & Moran, 2001; Hartwell *et al.*, 2002; Moshelion *et al.*, 2002b).

The post-translational modifications of protein activity by circadian signals suggest the presence of intracellular signalling pathways that couple the circadian oscillator to the regulation of cellular physiology. There are data to suggest that Ca^{2+}-based signalling pathways may form part of at least one of the output pathways that transduce circadian signals. The whole plant cytosolic free calcium concentration ($[Ca^{2+}]_i$) oscillates with a circadian period (Fig. 2). Johnson and coworkers have used the bioluminescent Ca^{2+} reporter, aequorin, to measure $[Ca^{2+}]_i$ for many days in seedlings of tobacco (*Nicotiana plumbaginifolia*) and *Arabidopsis*. Seedlings were transformed with the cDNA for apoaequorin. Reconstitution to functional aequorin with the luminophore coelentrazine allowed luminescent monitoring of $[Ca^{2+}]_i$ from whole seedlings (Johnson, 2001). $[Ca^{2+}]_i$ oscillated with a circadian period in continuous white or red light but in continuous dark the $[Ca^{2+}]_i$ oscillations were damped and often were not detectable.

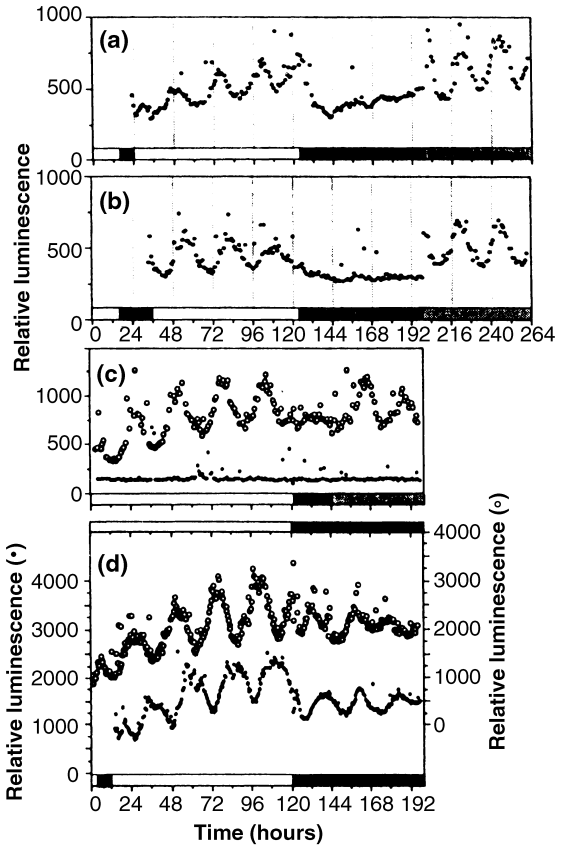

Fig. 2 Circadian oscillations of whole plant $[Ca^{2+}]_i$ in tobacco seedlings. The relative luminescence of transgenic seedlings containing aequorin was measured under white light (white boxes), dark (filled boxes) or red light (grey boxes). High relative luminescence indicates increased whole plant $[Ca^{2+}]_i$. (a) Circadian $[Ca^{2+}]_i$ oscillations in seedlings entrained in 16 h: 8 h LD. (b) As A except the final dark period of entrainment was extended by 12 h to shift the phase of the rhythm. (c) Aequorin transformed seedlings (open circles) and nontransformed seedlings (filled circles). (d) Circadian oscillations of seedling entrained to reversed 16 h: 8 h LD. The upper bar is light regime for the open circle trace and the lower bar is for the closed circle trace. Reprinted with permission from Johnson *et al.*, *Science* **269** 1863–1865. Copyright 1995 American Association for the Advancement of Science.

Circadian behaviour in plants is often more robust in continuous light (LL) and therefore it is perhaps not surprising that the oscillations of $[Ca^{2+}]_i$ were damped in the dark.

Following an entrainment of cycles of 16 h light (L) and 8 h dark (D) (LD 16 : 8), the circadian oscillations of whole plant $[Ca^{2+}]_i$ in both *Arabidopsis and* tobacco peaked at around 600 nM shortly after subjective dawn (Johnson *et al.*, 1995). However, the phase of the oscillations might vary in different cell types. When different promoters were used to drive apoaequorin expression in tissues, the phase of the circadian $[Ca^{2+}]_i$ rhythm was altered (Wood *et al.*, 2001). Because of the low levels of light emitted by aequorin it proved difficult to determine which cells were emitting the light signal, but clearly the use of different promoters resulted in different expression patterns and altered circadian phasing. These data highlight the autonomous nature of cellular oscillators (see section II.1.(b)).

The cytosolic and organelle free Ca^{2+} concentrations are regulated independently in plants (Pauly *et al.*, 2000; Sanders *et al.*, 2002). This is also true for the circadian regulation of Ca^{2+}. Chloroplastic free Ca^{2+} ($[Ca^{2+}]_{chl}$), measured by CaMV 35 s promoter driven expression of the apoaequorin transgene with the coding sequence of the transit peptide of the small subunit of ribulose-1,5 bisphosphate carboxylase (*rbcS*), does not exhibit rhythms in LL (Johnson *et al.*, 1995). Transition from LL to DD results in a rapid increase in $[Ca^{2+}]_{chl}$ that peaks at 5–10 μM. This transient rise in $[Ca^{2+}]_{chl}$ is followed by damped oscillations that have a circadian period (Johnson *et al.*, 1995; Sai & Johnson, 2002). The stromal Ca^{2+} transient occurs on a daily basis in LD and the size of the transient following the light to dark transition is increased with lengthening time in light (Sai & Johnson, 2002). The mechanism by which the LD transition-induced stromal Ca^{2+} transient occurs is unknown but this transient is unlikely to be involved in circadian behaviour (Sai & Johnson, 2002). Conversely, circadian rhythms in nuclear free Ca^{2+} concentration were not detectable in either DD or LL in plants in which the aequorin was predominantly localised to the nucleus using a nucleoplasm coding region (Wood *et al.*, 2001). It is surprising that circadian oscillations in $[Ca^{2+}]_i$ occur in LL, whilst circadian oscillations in $[Ca^{2+}]_{chl}$ have so far been detected only in DD. The mechanism and purpose of this differential regulation of Ca^{2+} stores by circadian signals in different light environments are unclear.

III. The regulation of cellular physiology by circadian oscillations in cytosolic free Ca^{2+}

Circadian oscillations of $[Ca^{2+}]_i$ (section II.1.(c)) are a striking and intriguing phenomenon but little is known about their role in plant physiology. Circadian oscillations of $[Ca^{2+}]_i$ are not the only rhythmic alterations of $[Ca^{2+}]_i$ in plant cells. Shorter-term oscillations with a period of about 1–20 min are induced in plant cells in response to extracellular stimuli (McAinsh *et al.*, 1995; Staxén *et al.*, 1999; Allen *et al.*, 2000; Kiegle *et al.*, 2000; Allen *et al.*, 2001; Ng *et al.*, 2001; Klusener *et al.*, 2002). Information concerning the generation, maintenance and roles of short-term stimulus-induced oscillations of $[Ca^{2+}]_i$ might be useful in understanding the biology of circadian oscillations of $[Ca^{2+}]_i$. Equally, circadian behaviour may provide insights for understanding complex phenomena such as 'bursting' of $[Ca^{2+}]_i$ in single cells (Schuster *et al.*, 2002).

The following key questions remain to be answered before the function of circadian regulation of $[Ca^{2+}]_i$ is known: what is the relative position of the circadian oscillations of $[Ca^{2+}]_i$ in the circadian signalling network?; what physiological responses are regulated by circadian oscillations of $[Ca^{2+}]_i$?; in which cells do circadian $[Ca^{2+}]_i$ oscillations occur?; what is the source

of Ca^{2+} for the circadian oscillations?; and is information encoded in circadian oscillations of $[Ca^{2+}]_i$?

1. The relative position of the circadian oscillations of $[Ca^{2+}]_i$ in the circadian signalling network

A central step in understanding the role of circadian oscillations in $[Ca^{2+}]_i$ will be positioning the oscillations with respect to the other components of the circadian clock. Circadian oscillations of $[Ca^{2+}]_i$ could plausibly form part of the input pathway by which light regulates the circadian oscillator, the Ca^{2+} oscillations could be a component of the central oscillator or the oscillations could be a part of the intracellular signalling network by which the circadian oscillator regulates cellular behaviour. Alternatively, the oscillations could act at more than one point in the pathway (Fig. 3).

Mutant analysis will be important in positioning the circadian oscillations of $[Ca^{2+}]_i$ in the circadian signalling pathway. A first step will be to monitor circadian behaviour of Ca^{2+} in light signalling mutants (e.g. *phyA*, *phyB*, *phyD*, *phyE*, *cry1* and *cry2*), 'clock-stopped' plants (e.g. *CCA1* and *LHY* over expressers), period mutants (*toc1*, *lhy-12*, *cca1–1* and *ztl*) and phase mutants (e.g. *out of phase* (*oop1*) (Salome *et al.*, 2002)).

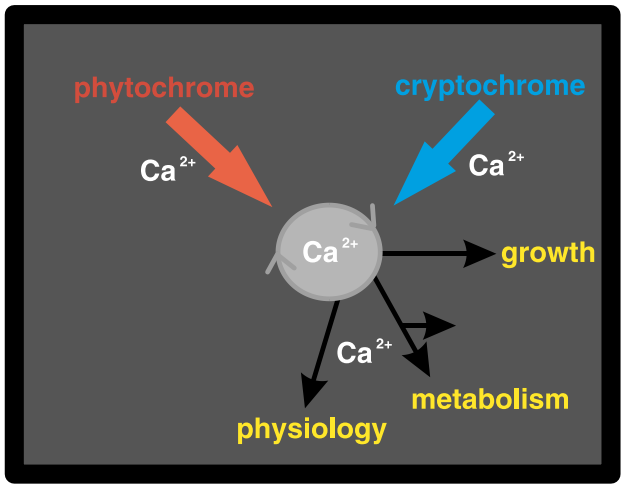

Fig. 3 Possible relative positions for alterations in $[Ca^{2+}]_i$ in the circadian signalling network. Ca^{2+} may act in a single location or multiple points in the circadian signalling network. Not all the suggested positions for Ca^{2+} in the circadian signalling have been demonstrated experimentally. Increases in $[Ca^{2+}]_i$ transduce light signals and might act in the input pathways by which phytochrome (red) and cryptochrome (blue) entrain the oscillator. Blue light signal transduction following phototropin activation is Ca^{2+}-dependent but it is thought that phototropin activation does not entrain the oscillator. Circadian oscillations of $[Ca^{2+}]_i$ might be a component of the core oscillator (light grey). Lastly, circadian oscillations in $[Ca^{2+}]_i$ might act in intracellular signalling pathways that transduce the temporal signals generated by the clock (black). It is likely that some circadian output pathways are Ca^{2+}-independent whilst others might by Ca^{2+}-dependent. See the text for a discussion of the position of Ca^{2+} in the circadian signalling network.

Positioning genetic lesions with respect to each other and also with respect to the circadian oscillations of $[Ca^{2+}]_i$ will represent a major advance in defining the circadian signalling network.

The relative position of the mutant allele in the circadian signalling pathway will often reflect the site of action of the protein encoded by the wild-type gene, but this may not always be the case (Webb & Baker, 2002). First, gain-of-function mutations might implicate the gene in the circadian signalling pathway when the wild type gene product plays no role. Second, the presence of the mutation might result in compensatory alterations in gene expression or recruitment and reorganisation of the circadian signalling pathway that mask the function of the wild type gene. Third, pleiotropic or complex effects of the mutation might make interpretation of the phenotype difficult. This has already proved to be the case with some circadian genes, for example positioning *elf3* in the circadian network is proving less than straight forward (Hayama & Coupland, 2003). Therefore, the relative position of circadian genes and oscillations of $[Ca^{2+}]_i$ in the circadian signalling network might require confirmation by demonstrating activity of the wild type protein at the predicted position in the circadian signalling network (Webb & Baker, 2002).

There is evidence that suggests Ca^{2+} acts in the input pathway linking light perception to the circadian oscillator. Red light elevates $[Ca^{2+}]_i$ in wheat protoplast (Shacklock *et al.*, 1992), suggesting that transient elevations in $[Ca^{2+}]_i$ act in the phytochrome signal transduction pathway. Pulses of red light, the Ca^{2+} ionophore A23187 or 10 mol m^{-3} $CaCl_2$ (all treatments that would be expected to elevate $[Ca^{2+}]_i$) change the circadian phase of leaf movement in *Robinia pseudoacacia* (Gomez & Simon, 1995). The phase is advanced if the Ca^{2+} agonists are applied during the subjective day and delayed if the agonists are applied during subjective night (Gomez & Simon, 1995) These studies are suggestive of a role of $[Ca^{2+}]_i$ in setting the phase of the circadian cycles of leaflet movements but it is not clear whether this is related to the phenomenon of circadian oscillations of $[Ca^{2+}]_i$. Blue light elevates also $[Ca^{2+}]_i$ via phototropin mediated signalling (PHOT1, formally known as NPH1 and PHOT2, formally known as NPL1) though phototropins are not thought to entrain the oscillator (Stoelzle *et al.*, 2003).

The phytochrome-mediated elevations in $[Ca^{2+}]_i$ and circadian oscillations of $[Ca^{2+}]_i$ may be independent phenomena with Ca^{2+} acting in at least two different points in the circadian signalling network (Fig. 3). Alternatively, the circadian modulation of *PHYB* expression (Hall *et al.*, 2002) could result in rhythmic sensitivity to red light and presumably rhythmic alterations in the Ca^{2+} signal downstream. This model places circadian oscillations of $[Ca^{2+}]_i$ in the phytochrome signalling pathway with outputs of the core oscillator 'gating' or modifying the sensitivity of the pathway to light. Alternatively, the oscillator might regulate $[Ca^{2+}]_i$ via a direct

mechanism that is independent of circadian oscillations of *PHYB* expression. Mechanistically these two models for the circadian regulation of $[Ca^{2+}]_i$ are very different but functionally the result is the same; oscillations of $[Ca^{2+}]_i$ are a driven by outputs of the core oscillator.

The assumption in this review is that the oscillations of Ca^{2+} act predominantly as an output of the clock that regulate cellular physiology. This is an appealing working hypothesis, particularly in cells such as stomatal guard cells and leaf pulvini in which Ca^{2+}-regulated ion channel activity underlies the circadian behaviour (see sections IV.2 and IV.1) (Snaith & Mansfield, 1986; Moshelion & Moran, 2001; Schroeder *et al.*, 2001).

If alterations in $[Ca^{2+}]_i$ act as an output of the clock then it is probable that Ca^{2+} acts as a second messenger to transduce and amplify temporal signals generated by the oscillator. An alternative hypothesis is that the circadian oscillations in $[Ca^{2+}]_i$ are the result of an homeostatic mechanism. Therefore, the hypothesis that Ca^{2+} acts as a second messenger in a circadian signalling pathway requires rigorous testing. For a molecule to be a second messenger it has to meet several criteria. The criteria and suggested experiments designed to test whether Ca^{2+} acts as a second messenger in a circadian signalling pathway are outlined below:

i. The molecule must be present in low cytosolic concentrations. $[Ca^{2+}]_i$ in plants is typically maintained at very low 'resting' values of about 150 nm;

ii. The cytosolic concentration must be elevated by the stimulus. This criterion requires modification for circadian signalling because the stimulus is an oscillator. Artificial manipulation of clock components should elevate $[Ca^{2+}]_i$ in a manner consistent with the phase of the clock component;

iii. Removal of the stimulus should return the cytosolic concentration of the molecule to 'resting' levels. In circadian experiments, this criterion can be interpreted that $[Ca^{2+}]_i$ oscillations will be absent in 'clock-stopped' phenotypes;

iv. The signalling pathway should not proceed in the presence of the stimulus if the cellular concentration of the second messenger is prevented from increasing. In terms of circadian signalling, experimental manipulations that prevent circadian oscillations of $[Ca^{2+}]_i$ should prevent Ca^{2+}-dependent circadian behaviour;

v. Elevations in the cytosolic concentration of the second messenger should be sufficient to induce the final response in the absence of the first messenger. Experimentally induced oscillations in $[Ca^{2+}]_i$ with a period of 24 h should induce periodic rhythms in physiological outputs in the absence of light and temperature entrainment. This represents an extremely daunting experiment.

2. The physiological responses regulated by circadian oscillations of $[Ca^{2+}]_i$

Candidates for regulation by the circadian oscillations of $[Ca^{2+}]_i$ are those physiological processes that are known to be regulated by both the circadian clock and alterations in $[Ca^{2+}]_i$. Two prime candidates for a role for circadian regulation by $[Ca^{2+}]_i$ are leaf and stomatal movements (see sections IV.2 and IV.1).

The Ca^{2+}-sensitivty of the ion channel activities that underlie circadian stomatal movements strongly support a role for circadian oscillations of $[Ca^{2+}]_i$ in the circadian behaviour of stomata (Snaith & Mansfield, 1986; Webb, 1998; Schroeder *et al.*, 2001). It is not known whether guard cell $[Ca^{2+}]_i$ oscillates with a circadian period but the detected light emitted by the aequorin used to measure circadian oscillations of $[Ca^{2+}]_i$ comes from the epidermis and mesophyll cells near the epidermis (Wood *et al.*, 2001).

There is evidence for a role for Ca^{2+} in the circadian regulation of the movements of the leaves of legumes. Several legumes exhibit movements of their leaves, including *Samanea*, *Phaseolus* and *R. psoudoacacacia* (this is discussed further in section IV.1). $LaCl_3$ (a putative Ca^{2+} channel blocker) and ethyleneglycol *bis* (β-amionethyl *N*, *N*, *N′*, *N′*, tetraacetic acid (EGTA, a divalent cation chelator with high selectivity for Ca^{2+}) inhibit circadian rhythms of leaf movement in R. *pseudoacacia* and *Albizzia lophantha* implicating Ca^{2+} in the maintenance of this behaviour (Moysset *et al.*, 1994; Gomez & Simon, 1995). Alterations in $[Ca^{2+}]_i$ also probably act in the transduction pathways by which light entrains the circadian leaf movements and also induces the acute response of leaf movement (Moysset *et al.*, 1994; Gomez & Simon, 1995; Mayer *et al.*, 1997). Furthermore, inositol 1,4,5 trisphosphate ($Ins1,4,5P_3$, a Ca^{2+}-mobilizing hydrophilic diffusible messenger) has been suggested to be involved in the shrinking of *Phaseolus pulvinus* extensor protoplasts (Mayer *et al.*, 1997). These data have been obtained using the effects of inhibitors known to interfere with Ca^{2+} signalling and inositol metabolism in animals and plants. Lack of specificity and uncertain sites of action can complicate inhibitor studies. This is particularly a problem in investigations of Ca^{2+} influx across plasma membranes in plants, whilst La^{3+} and verapamil (inhibitors of L-type Ca^{2+} channels in mammals) do act as blockers of Ca^{2+} channels, they also block K^+ channels in plants (Terry *et al.*, 1992). Therefore, the effects of La^{3+} and verapamil on the circadian and acute responses of pulvini could be due also to block of the K^+ channels that catalyse the flux of K^+ that underlie the swelling and contraction of pulvini cells.

Clear evidence for a role for $[Ca^{2+}]_i$ in the circadian behaviour of pulvini requires measurement of $[Ca^{2+}]_i$ in pulvini cells and experiments designed to test the criteria outlined in section III.1. However, the weight of evidence suggests two roles for alterations in $[Ca^{2+}]_i$ in the circadian movements of legume leaves. Firstly, Ca^{2+} acts as a second messenger in the pathway by which phytochrome resets the clock resulting in phase shifts of leaflet movements (Gomez & Simon, 1995; Mayer *et al.*, 1997). Secondly, changes in $[Ca^{2+}]_i$ appear to be required for maintenance of circadian leaf movements (Moysset *et al.*, 1994). It is tempting to speculate that the requirement for Ca^{2+} to

maintain circadian leaf movements is because of a requirement for circadian oscillations of $[Ca^{2+}]_i$ in the pulvini.

Sai & Johnson (1999) have provided evidence that circadian oscillations of $[Ca^{2+}]_i$ are not involved in the circadian regulation of one of the best characterised circadian responses, the circadian regulation of the *LIGHT HARVESTING COMPLEX B* (*LHCB*) promoter. Plants that have been transformed with both *CaMV35S::apoaequorin* to report circadian oscillations of $[Ca^{2+}]_i$ and also *LHCB::LUCIFERASE* (*LUC*) to report circadian regulation of the *LHCB* promoter exhibit asynchrous rhythms in these circadian phenomenon. Furthermore, in calli generated from these plants, rhythms of LUC activity were detected but no rhythms of aequorin luminescence could be measured (Sai & Johnson, 1999). These data suggest that there is no functional link between the oscillations of $[Ca^{2+}]_i$ and the oscillations of *LHCB* promoter activity.

3. The location of circadian $[Ca^{2+}]_i$ oscillations

Identifying the cells in which the circadian oscillations of $[Ca^{2+}]_i$ occur is critical to understanding their function. The bioluminescent signal emitted by aequorin penetrates only a few cell layers, making imaging the location of the luminescent signal within an organ particularly difficult (Wood *et al.*, 2001). In my laboratory, we have imaged light emission from all organs of *Arabidopsis* seedlings transformed with *CAMV35S::APOAEQUORIN*. Our data demonstrate circadian oscillations of $[Ca^{2+}]_i$ occur in the cotyledons and leaves but we are unable to determine which cells in these organs contribute to the circadian Ca^{2+} signal (J. Love *et al.*, unpublished). Recently, Kiegle *et al.* (2000) described the use of GAL4 transactivation in enhancer-trap lines to target apoaequorin to specific cells (Fig. 4). By targeting apoaequorin to different cell types it might

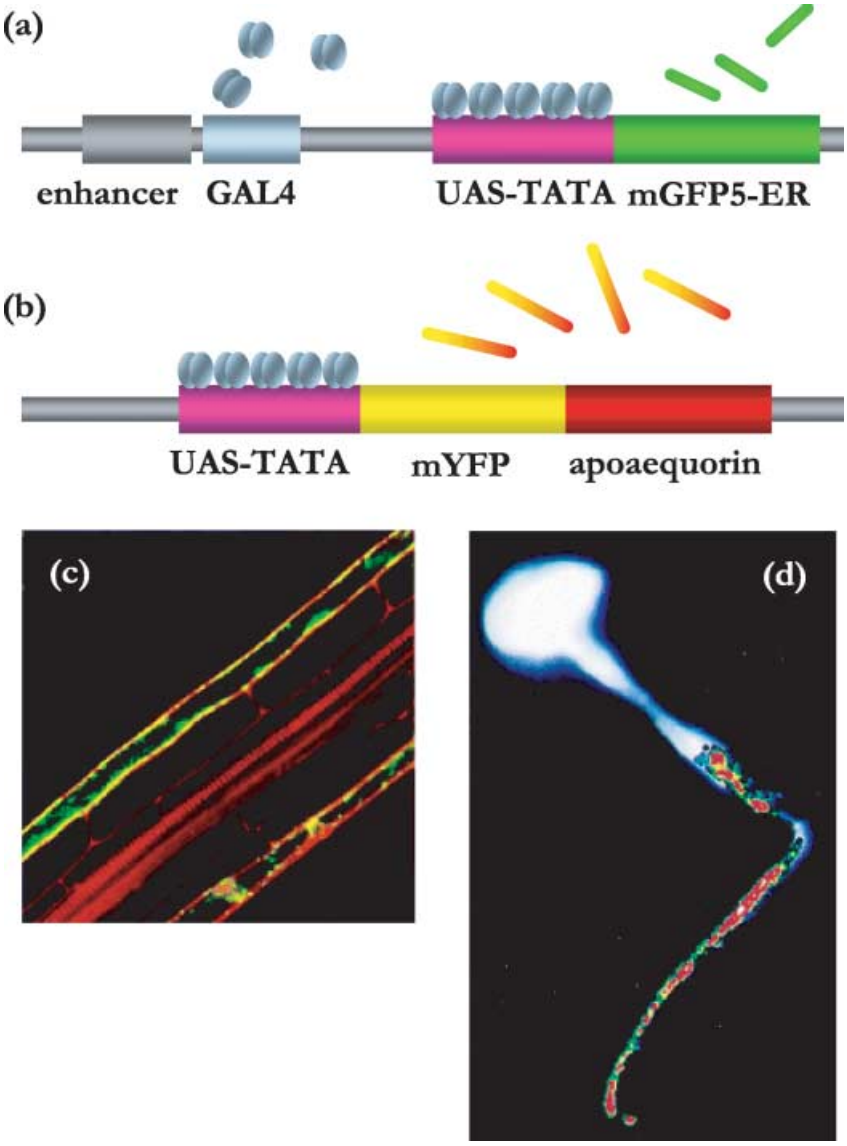

Fig. 4 GAL4 transactivation of apoaequorin allows the measurement of cell-specific Ca^{2+} signals. (a) An enhancer trap construct containing a GAL4-VP16 domain that was randomly inserted into the plant genome. When the GAL4 coding region locates near an endogenous enhancer element GAL4 is expressed. GAL4 expression is visualized using a linked mGFP5-ER driven by the GAL4-upstream activation sequence (UAS). (b) Transforming lines with specific cell types expressing GAL4 allows UAS-driven YFP-aequorin fusion gene expression only in GAL4-mGFP5-ER-expressing cells. (c) Laser scanning confocal microscopic images of aequorin targeted to the root epidermis. In the pseudo colour image the cell walls are stained red with propidium iodide. GFP is green and aequorin-YFP is yellow. (d) Ambient light images of seedlings overlaid with counts of total photons emitted by aequorin during a gradual cooling stress in a root epidermal aequorin expresser. The Ca^{2+} signal from aequorin is pseudo colour coded with blue representing 'resting' [$Ca^{2+}]_i$ and red representing high $[Ca^{2+}]_i$. Reproduced and redrawn with modifications from (Kiegle *et al*., 2000).

be possible to determine in which cells $[Ca^{2+}]_i$ oscillates. The luminescent signal from aequorin is low, even in plants in which the Ca^{2+}-reporter is expressed in all tissues, therefore measuring the circadian Ca^{2+} signal from a sub set of cells will be technically demanding.

4. The source of Ca^{2+} for the circadian oscillations

Johnson *et al.* (1995) estimated that over the circadian period, $[Ca^{2+}]_i$ oscillates between 150 nM at the trough and about 600 nM at the peak. The estimation of absolute changes in $[Ca^{2+}]_i$ were performed by determining the proportion of the total aequorin consumed at any point in time, which is related to the $[Ca^{2+}]_i$ by an empirically derived formula (Fricker *et al.*, 2002). However, the estimations of the magnitude of the circadian oscillations of $[Ca^{2+}]_i$ were based on the assumption that all tissues contribute equally to the circadian luminescence signal and this assumption is most likely incorrect because different tissues show different phases of circadian oscillations of $[Ca^{2+}]_i$ (Johnson *et al.*, 1995; Sai & Johnson, 1999; Wood *et al.*, 2001). Nevertheless, the estimates of the magnitude of the oscillations are the best that can be achieved given current technical limitations. These estimates are similar to those that have been made for estimating the size of increases in $[Ca^{2+}]_i$ induced by extracellular stimuli in plant cells (Webb *et al.*, 1996b). This suggests that similar mechanisms may generate both circadian oscillations of $[Ca^{2+}]_i$ and the shorter-term oscillations that occur in response to extracellular stimuli.

There are three main routes for Ca^{2+} entry into the cytosol (Sanders *et al.*, 2002). Ca^{2+} can enter from extracellular stores across the plasma membrane, from the vacuole across the tonoplast and also from ER stores. A number of different mechanisms have been identified for regulating Ca^{2+} entry from intra- and extracellular Ca^{2+} stores (Fig. 5). Some of these have been demonstrated to be involved in the generation and maintenance of oscillations of $[Ca^{2+}]_i$ induced by extracellular signals. Cyclic ADP ribose (cADPR) and Ins(1,4,5)P3 are thought to mobilise Ca^{2+} from internal stores during oscillations in guard cell $[Ca^{2+}]_i$ activated by abscisic acid (ABA) (Leckie *et al.*, 1998; Staxén *et al.*, 1999; Klusener *et al.*, 2002). An oscillating influx of Ca^{2+} across the plasma membrane in guard cells can contribute to oscillations of $[Ca^{2+}]_i$ in guard

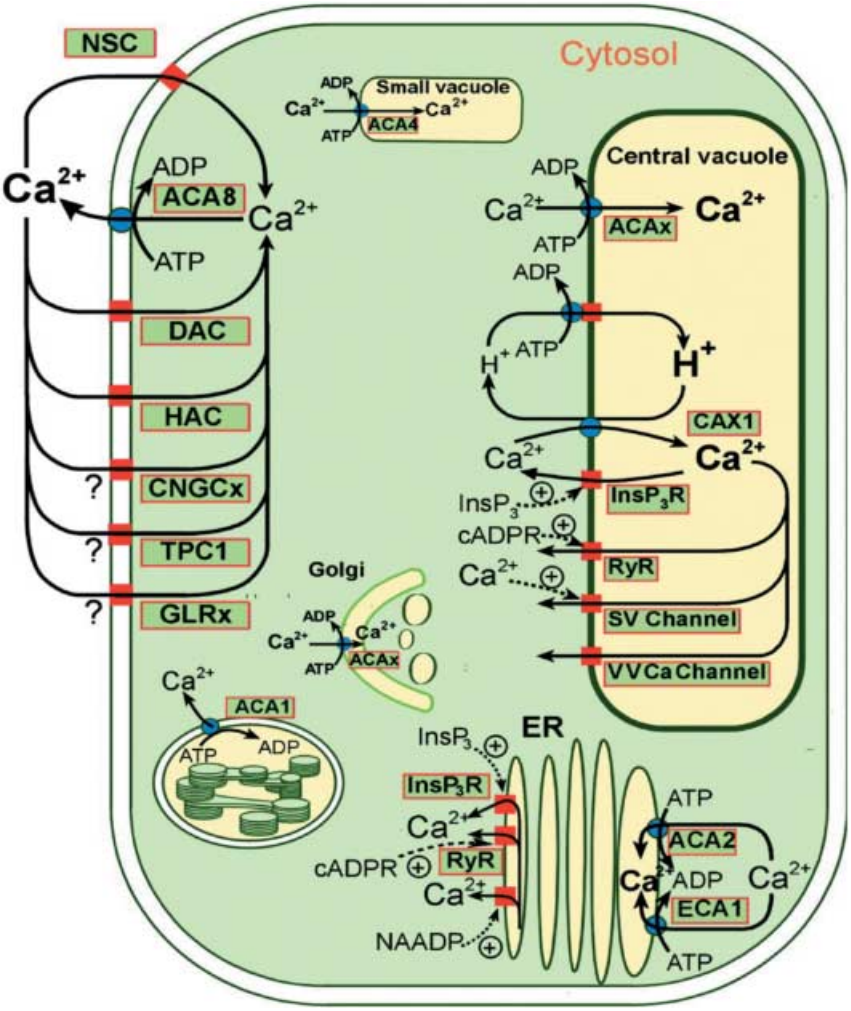

Fig. 5 Identified and predicted Ca^{2+} transport pathways in *Arabidopsis*. The diagram is a model proposed by (Sanders *et al*., 2002) to synthesise current electrophysiological and molecular characterisation of Ca^{2+} influx and efflux pathways in *Arabidopsis*. Blue circles represent energized transport systems. Red squares represent Ca^{2+}-permeable channels. Abbreviations, ACA (Arabidopsis Calcium ATPases). ECA (ER-type calcium ATPase). CAX1 (calcium exchanger1). NSC (nonselective cation channels), DAC (depolarization activated channels). HAC (hyperpolarization activated channels). TPC1 (two-pore channel1). CNGC (cyclic nucleotide gated channels). GLR (glutamate receptor) $InsP_3R$ (putative Ins(1,4,5)P3 receptor). RyR (putative ryanodine receptor activated by cADPR). NAADP (nicotinic acid adenine dinucleotide phosphate). SV channel (slowly activating vacuolar channel). VVCa channel (vacuolar voltage-gated Ca^{2+} channel). Reproduced from (Sanders *et al*., 2002). Copyrighted by the American Society of Plant Biologists. Reprinted with permission.

cells in response to elevations in extracellular Ca^{2+} (McAinsh *et al.*, 1995) and ABA (Klusener *et al.*, 2002). It is plausible that any of the mechanisms known to drive oscillations in plant cell $[Ca^{2+}]_i$ contribute to the circadian oscillations of $[Ca^{2+}]_i$. Alternatively, some of the other mechanisms that elevate $[Ca^{2+}]_i$ in plant cells that have not been assigned a role in oscillatory behaviour could contribute to the circadian regulation of $[Ca^{2+}]_i$. Some mechanistic differences must exist between the circadian $[Ca^{2+}]_i$ oscillations and oscillations in $[Ca^{2+}]_i$ caused by extracellular stimuli because the period of the oscillations are so different. Circadian $[Ca^{2+}]_i$ oscillations have, by definition, a period of about 24 h. Whilst those induced by extracellular stimuli have periods of between about 1 and 60 min (McAinsh *et al.*, 1995; Ehrhardt *et al.*, 1996; Hetherington *et al.*, 1998; Leckie *et al.*, 1998; Allen *et al.*, 1999; Staxén *et al.*, 1999; Allen *et al.*, 2000; Kiegle *et al.*, 2000; Allen *et al.*, 2001; Ng *et al.*, 2001; Klusener *et al.*, 2002).

How might a core oscillator generate rhythms of $[Ca^{2+}]_i$ with a period of 24 h? It is unlikely that core oscillator components interact directly with Ca^{2+} channels in the tonoplast, ER membrane or plasma membrane to mobilize Ca^{2+}. This is assumed because the core oscillator components are in the nucleus and based on the predicted protein functions, are unlikely to participate directly in the regulation of signalling pathways. More likely, the core oscillator components might regulate the expression of genes whose products function to regulate, either directly or indirectly, Ca^{2+} channel activity. Ca^{2+} channel activity in plants is typically regulated by membrane potential (e.g. HAC, DAC and SV), ligand binding (e.g. $Ins(1,4,5P)_3$, cADPR) or other post-translational modifications (glycosylation, de/phosphorylation, lipid binding, G-protein regulation, etc.) (Fig. 5). Clock genes probably regulate the abundance of proteins that lead to alterations in membrane potential, signalling intermediate abundance or proteins that bring about post-translational modification of ion channels. Recently, cADPR has been shown to participate in the circadian generation of oscillations of $[Ca^{2+}]_i$ in neurons in the hypothalamic suprachiasmatic nucleus (SCN) of the rat brain (Ikeda *et al.*, 2003). The SCN is the primary circadian clock in mammals. The rat SCN contains about 8000 neurons that fire with a circadian period. The participation of ryanodine receptors (cADPR-gated Ca^{2+} channels) in circadian $[Ca^{2+}]_i$ oscillations demonstrates that mechanisms similar to those that generate ultradian oscillations of $[Ca^{2+}]_i$ in plants can generate circadian oscillations of $[Ca^{2+}]_i$. It is not known if cADPR plays a similar role in plant cells.

5. Information encoding in circadian oscillations of $[Ca^{2+}]_i$

It has been proposed that information is encoded in oscillations of $[Ca^{2+}]_i$ in plant cells (McAinsh *et al.*, 1995; McAinsh & Hetherington, 1998). Information might be encoded in the period (sometimes this is described using the inverse measure, frequency; Table 1), amplitude (Table 1), phase and/or shape of the oscillation (Fig. 6) (Schuster *et al.*,

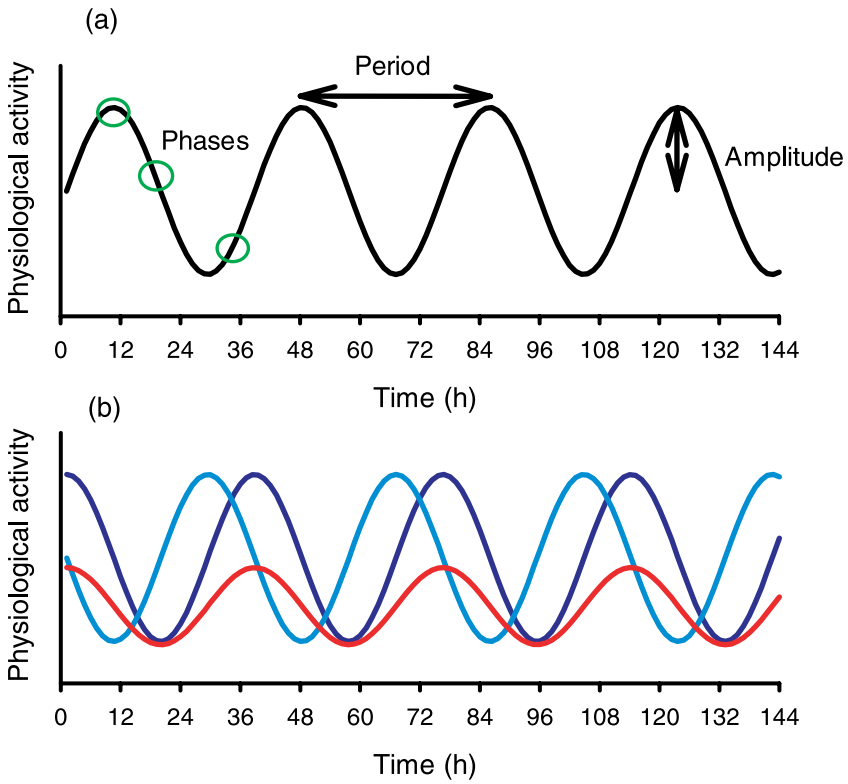

Fig. 6 Possible mechanisms for encoding information in circadian oscillations. (a) A theoretical rhythm in physiological activity showing the phase, period and amplitude. The amplitude measurement shown here is the maximal deviation from the mean. Often in biology the amplitude is calculated as the difference between the peak and the trough levels, or the size of the maximal increase above a 'resting' value. (b) Theoretical alternative fixed period (24 h) rhythms that could encode information. The blue rhythms are phase shifted and the red rhythm has a reduced amplitude.

2002). In stomatal guard cells information concerning stimulus strength is encoded in the period and amplitude of the oscillations and the period of oscillations of $[Ca^{2+}]_i$ regulates the final response of guard cells (McAinsh *et al.*, 1995; Staxén *et al.*, 1999; Allen *et al.*, 2001). Guard cell $[Ca^{2+}]_i$ oscillations may also encode complex information about stimulus type and strength of more than one stimulus simultaneously (Hetherington *et al.*, 1998).

Clearly, the period of the circadian $[Ca^{2+}]_i$ oscillations has the potential to transduce the period signal and provide periodic regulation of cellular events. Thus, essential timing information is likely to be encoded in the 24-h period of circadian $[Ca^{2+}]_i$ oscillations. Recent data from my laboratory suggest more information than just circadian period is encoded in the circadian oscillations of $[Ca^{2+}]_i$. Information concerning day length and light quality is encoded in the amplitude and phase of circadian Ca^{2+} oscillations in Arabidopsis (J. Love *et al.*, unpublished). Day length and light quality contribute to the photoperiodic control of flowering in day length sensitive species. The developmental transition from vegetative growth to reproductive growth is partially controlled by the circadian clock and one possibility is that the circadian regulated alterations in $[Ca^{2+}]_i$ act to in the transduce photoperiodic information to the cellular apparatus regulating cellular development. It appears, that just like short-term ultradian oscillations of $[Ca^{2+}]_i$, there is the potential to encode complex information in circadian oscillations of $[Ca^{2+}]_i$.

Elevations in $[Ca^{2+}]_i$ are distributed unevenly across the cell. It has been proposed that 'spatial encoding' may contribute to generating a stimulus-specific Ca^{2+}-signature (McAinsh & Hetherington, 1998). Furthermore, coupling of cells via 'calcium-waves' can also be important in oscillatory behaviour in mammalian cells (Schuster *et al.*, 2002). Advances in imaging technology will be required before the spatial variations in $[Ca^{2+}]_i$ during circadian oscillations can be visualized and their role be determined.

If information is encoded, there must also be mechanisms for decoding oscillations. In plants these mechanisms are obscure but models exist based on the post-translational modification of proteins (e.g. ion channels or enzymes) by counter-acting and differentially sensitive regulatory proteins (e.g. phosphatase and kinases) (McAinsh & Hetherington, 1998; Schuster *et al.*, 2002).

Ca^{2+} oscillations have been suggested to serve many functions including a role in Ca^{2+} homeostasis and a mechanism for encoding information (McAinsh & Hetherington, 1998). The circadian Ca^{2+} dynamics reported by aequorin do not suggest a homeostatic mechanism because $[Ca^{2+}]_i$ appears to remain elevated for several hours. However, it is not known whether the circadian oscillations of $[Ca^{2+}]_i$ reported by constitutive expression of aequorin truly represent the dynamics of $[Ca^{2+}]_i$ at a single cell level. At least two possibilities exist. Firstly, single cell $[Ca^{2+}]_i$ oscillates with a 24-h period as predicted by whole plant aequorin. Secondly, the whole plant aequorin signal reports the integrated collective signal of more complex $[Ca^{2+}]_i$ changes occurring in single cells. For example, the peak of the whole plant circadian oscillation of $[Ca^{2+}]_i$ might represent globally elevated $[Ca^{2+}]_i$, alternatively it might represent a relatively high frequency of short-term oscillations in $[Ca^{2+}]_i$ in a population of cells. It is possible that both short-term and long-term (circadian) oscillations of $[Ca^{2+}]_i$ are superimposed in a single cell to generate 'calcium-signatures' that describe both the physiological status of the plant and provides an internal measure of time. Complex alterations of oscillations by multiple signals has been reported in stomatal guard cells and it has been suggested that this is a mechanism for integrating information (Hetherington *et al.*, 1998).

IV. The circadian regulation of physiology

1. Circadian regulation of leaf and organ movements

Leaves and other organs of plants are able to move in response to environmental and other stimuli, including circadian signals. Leaf and organ movements are brought about in two main ways. Firstly, specialised organs called pulvini control the movements of leaves and leaflets in the families Fabacae, Maranthacae and Oxaldacae and also the movements of the flowers of Kalanchoë (Engelmann & Johnsson, 1998). Secondly, spatially and temporally separated growth responses can lead to circadian regulation of the movements of leaves of other species, including *Arabidopsis*, with alternating growth on the upper and lower lamina causing raising and lowering of the leaves (Engelmann & Johnsson, 1998). Similarly, hypocotyl growth in species such as *Arabidopsis* is under circadian control (Engelmann *et al.*, 1992).

Circadian entrainment of legume leaf movements occur via red light perception using phytochromes and blue light signalling (Gomez & Simon, 1995). Turgor changes in the cortical motor cells of small organs at the base of the leaves (pulvini) are responsible for the movements. The turgor changes are brought about by fluxes of K^+ and anions in a manner analogous to the movements of guard cells (Moshelion & Moran, 2001). The motor cells are parenchymatous cells located between the epidermis and a central core of supporting tissues in the pulvinus. Co-ordinated action of the motor cells results in leaflet movement. Swelling of the cells in the lower segment and shrinking of the cells in the upper segment results in upward movement of the leaflet. The extensor cells in the lower half of the pulvinus swell in response to light resulting in upward movement of the leaf. The upward movement of the leaf requires also the light-induced shrinking of the flexor cells in the upper part of the pulvinus. Downward leaf movements require the inverse responses of the cells (Mayer *et al.*, 1997).

Cell swelling (and therefore lengthening) is brought about by an increase in turgor as a result of increased water uptake

as a consequence of accumulation of osmotica, notably K^+ and Cl^-, in the vacuole. Increased turgor causes cell lengthening because the arrangement of cellulose microfibrils in the cell wall allows changes in cell length but not diameter. As the cells take up water, the cell lengthens and when water is lost and turgor decreases the cell shortens (Engelmann & Johnsson, 1998). The most likely sequence of events is that swelling is caused by hyperpolarisation of the plasma membrane as a result of the activity of an ATP-dependent plasma membrane pump driving H^+ efflux. This helps to provide a driving force for K^+ influx and results in opening of hyperpolarisation-activated K^+ channels (Engelmann & Johnsson, 1998; Moshelion *et al.*, 2002b). Cl^- uptake probably occurs via a proton symporter and provides chemical balance for K^+.

Candidate genes have been identified for the K^+ channels that control K^+ uptake and efflux in pulvinar cells. Samanea pulvini inward rectifying channel1 (SPICK1) and SPICK2 are *Arabidopsis* potassium transporter 2 (AKT2)-like channels that are likely to be hyperpolarisation-activated K^+-influx channels, whilst *Samanea* pulvini outward rectifying channel 1 (SPORK1) is a stelar potassium outward rectifier (SKOR)-like channel that might represent a depolarisation-activated channel and be responsible for K^+ efflux (Moshelion *et al.*, 2002b).

If expression of *SPICK1*, *SPICK2* and *SPORK1* represents a rate limiting step in K^+ fluxes it might be expected that K-channel transcripts would be diurnally and circadian-regulated with different patterns of regulation for hyperpolarisation- and depolarisation-activated channels and also different patterns of expression in extensor and flexor cells. Therefore, transcripts representing hyperpolarisation-activated channels would be associated with periods of swelling, mornings for extensor cells and afternoons for flexor cells. The converse would be true for transcripts representing depolarisation activation K-channels, with abundance expected to peak in the afternoon in extensor cells and in the morning in flexor cells, when shrinking occurs (Moshelion *et al.*, 2002b). A detailed study demonstrated diurnal and circadian regulation of *SPICK1*, *SPICK2* and *SPORK1* (Moshelion *et al.*, 2002b). In some cases it was possible to correlate the peaks in transcript abundance with predicted channel activity. For example, transcripts of *SPICK1* peaked in extensor cells at midnight in DD or DL; this could correlate with increased hyperpolarisation-activated K^+ channel activity in the morning. In other cases the relationship between transcript abundance and predicted role was less clear; the requirement for depolarisation-activated K^+ flux is predicted to be out of phase in extensor and flexor cells but the cycles of *SPORK1* transcript abundance in DD or DL were in phase, peaking at dawn in both cell types (Moshelion *et al.*, 2002b). The role of transcriptional, translational and post-translational regulation of K^+ channel abundance and activity in circadian control of pulvini will become clearer when probes that detect channel protein abundance become available.

The two populations of cortical cells in the pulvinus are physiologically similar but light results in K^+ uptake in the extensor cells and K^+ loss from the flexor cells. Circadian signals co-ordinately regulate the action of these physiologically similar, but opposite acting cells, to bring about circadian oscillations of leaf movement. The presence of two physiologically similar but differentially regulated cell populations, provides an opportunity for the use of comparative studies to dissect the mechanisms of the circadian control of physiology. Assuming the circadian physiology of the extensor and flexor cells is regulated by autonomous intracellular oscillators, how do these oscillators bring about opposite physiological responses at the same phase of the circadian cycle? At least four models describing how the circadian clock oppositely regulate the physiology of extensor and flexor cells exist (Fig. 7). Photoreception and transduction pathways might behave differently in extensor and flexor cells resulting in opposite phased entrainment of the core oscillator and consequently the output pathways and physiological response (Fig. 7(a), (b)). Alternatively, the elements of the core oscillator may respond differently to the light input pathway resulting in opposite phased cycling of the core oscillator in the flexor and extensor cells with downstream effects on the output pathways and physiology (Fig. 7(a), (c)). Two other models are proposed in which the core oscillator is in phase in extensor and flexor cells (Fig. 7(a), (d), (e)). In the first of these, an output pathway responds differently to the oscillator phase in extensor and flexor cells, resulting in opposite phased physiology (Fig. 7(a), (d)). The last model describes the possibility that the input pathway, the core oscillator and the output pathways are identical in flexor and extensor cells but the proteins catalysing the turgor changes respond differently to the output signalling pathways (Fig. 7(a), (e)). For example, K^+ channels in each cell type may show different sensitivities to $[Ca^{2+}]_i$ (Moshelion & Moran, 2001).

To determine which of these models is correct requires determining whether the circadian oscillator components are expressed in, or out of phase, in the two cell types. Out of phase expression of oscillator components in flexor and extensor cells would suggest that either there are differences in the light transduction pathways regulating circadian entrainment or that the oscillators in the two cell types respond differently to the light transduction pathway (Fig. 7(b), (c)). In phase expression of the circadian oscillator elements in the two cell types would suggest that the differences are downstream form the oscillator. Preferably, the abundance of oscillator components should be measured at the protein as well as the transcript level. Post transcriptional control of gene expression may be important in circadian behaviour and therefore analysis of protein abundance may be required to assign the full function of a clock gene.

Alterations in $[Ca^{2+}]_i$ have been implicated in both the maintenance of circadian behaviour of the pulvini of *Robinia pseudoacacia* and *Albizzia lophantha* (Moysset *et al.*, 1994; Gomez & Simon, 1995) and also the light-induced swelling of extensor protoplasts and dark-induced shrinking of *Phaseolus pulvinus* cells (Mayer *et al.*, 1997) (see also section III.2). However, the precise role of Ca^{2+} in the circadian control of

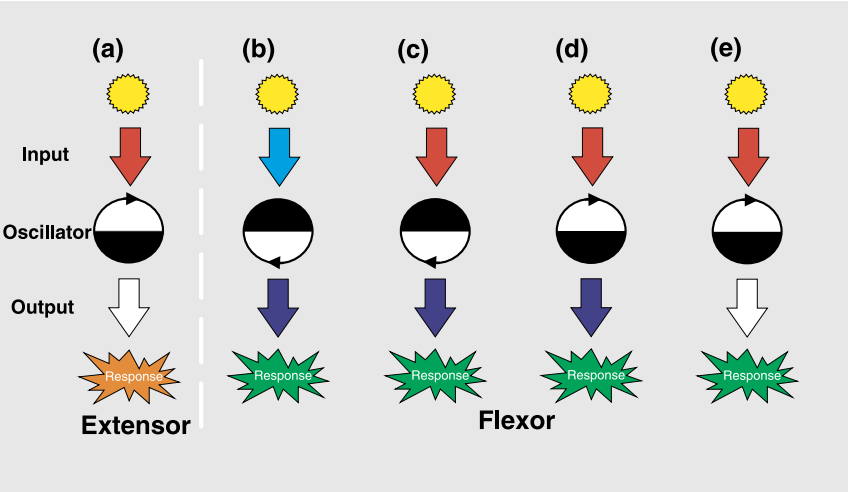

Fig. 7 Alternative conceptual models for the circadian regulation of physiology in the extensor (a) and flexor cells (b–e) of the pulvini of legumes. The pathway in the model has been conceptualised as a light-regulated input pathway (red or light blue arrows), a core oscillator (black and white circles), output signalling pathways (white or dark blue arrows) and the proteins that catalyse the ion fluxes that bring about the physiological response (orange or green flash boxes). Each flexor cell model (b–e) is described in relation to the extensor cell model (a). Same coloured arrows represent pathways that act similarly in the extensor and flexor cells in each model. The relative phase of the core oscillator in each model is represented by the black and white semicircles. (a) Circadian regulation of extensor cell physiology. Light entrains a core oscillator (possibly containing orhthologues of *LHY/CCA1/TOC1*) via an input pathway (red arrow). The core oscillator (black and white circle) regulates output signalling pathways (white arrow) that bring about the extensor cell response (orange flash). (b) Photoreception and transduction pathways (red and light blue arrows) behave differently in extensor and flexor cells resulting in opposite phased entrainment of the core oscillator and consequently oppositely phased output pathways (white and dark blue arrows) and physiological responses (orange and green flashes). In this model, the pathway transducing light signals to the core oscillator respond differently to light signals in the extensor and flexor cells. Thus, the opposite-phased physiology of extensor and flexor cells is brought about by altered phototransduction. (c) The phototransduction pathways in extensor and flexor cells are the same (red arrows) but the core oscillators are entrained to opposite phases in the two cell types. Elements of the core oscillator may respond differently to the light input pathway resulting in opposite phased cycling of the core oscillator in the flexor and extensor cells with downstream effects on the output pathways (white and dark blue arrows) and physiology (orange and green flashes). (d) The output pathways respond differently to the oscillator phase in extensor and flexor cells (blue and white arrows) resulting in opposite phased physiology (orange and green flashes). This model describes a scenario in which light transduction and entrainment of the oscillator are identical in flexor and extensor cells (red arrows). This results in the core oscillators in the extensor and flexor cells being in phase. However, the outputs from the core oscillator differ in the two cell types (white and dark blue arrows) resulting in opposite phase of the physiological response (orange and green flashes). In this model the peak of, for example, *TOC1* (or orthologue) expression would be in phase in extensor and flexor cells but signalling outputs would be at opposite phases. Under this circumstance, circadian oscillations of $[Ca^{2+}]_i$, possibly acting in a circadian output pathway might be out of phase in the two cell types. (e) The input pathways (red arrows), the core oscillator and the output pathways (white arrows) are in phase in extensor and flexor cells but the proteins that bring about the physiological response respond oppositely to the circadian pathway leading to the opposite-phased physiology (orange and green flashes). In this model the core oscillators in extensor and flexor cells would be entrained to the same phase by the input pathways. Similarly, the output pathways would generate identical outputs in phase in extensor and flexor cells but the proteins catalysing the ion fluxes would respond differently in the two cell types. For example, K^+ channels in the two cell types have different sensitivities to $[Ca^{2+}]_i$ (see section VI.1).

leaf movements of legumes is speculation. Intriguingly, depolarisation-activated K^+ channels in extensor and flexor cells are not activated directly by elevations in $[Ca^{2+}]_i$ (Moshelion & Moran, 2001). Depolarisation-activated K^+ efflux is an essential step in pulvini coritical cell shrinkage and therefore it is perhaps surprising that this is a Ca^{2+}-independent event. The equivalent channels in the plasma membrane of guard cells are also Ca^{2+}-independent but ABA-induced stomatal closure is Ca^{2+} dependent, presumably because of the Ca^{2+}-dependent steps required to bring about the membrane depolarisation that in turn activates the K^+ efflux channels (Webb *et al.*, 2001).

Associated with the large fluxes of ions are twice-daily massive movements of water across the tonoplast and plasma membrane of extensor and flexor cells. The fluxes of water occur during movement of the pulvinus in the morning and early evening (Moshelion *et al.*, 2002a). Recently, *Samanea saman* Aquaporin 2 (*SsAQP2*) has been identified as a circadian- and light-regulated aquaporin responsible for carrying some of this water flux in extensor and flexor cells (Moshelion *et al.*, 2002a). The evidence that *SsAQP2* contributes to the circadian regulation of pulvini movement is convincing: Heterologous expression of *SsAQP2* in oocytes resulted in high permeability to water; both $HgCl_2$ and phloretin inhibited extensor protoplast swelling and also reduced SsAQP2 permeability to water; *SsAQP2* transcripts were detected in those parts of the leaf associated with movement; transcript

abundance of *SsAQP2* is circadian regulated. Circadian regulation of aquaporin gene expression may be a common feature of the circadian regulation of plant physiology because aquaporins are abundant in the *Arabidopsis* genome and an *Arabidopsis* tonoplast aquaporin transcript also is regulated by the circadian clock (Harmer *et al.*, 2001).

In nonlegumes such as *Arabidopsis*, circadian cotyledon movements occur as a result of differential growth of the leaf lamina on each surface (Engelmann *et al.*, 1992; Engelmann & Johnsson, 1998; Dowson-Day & Millar, 1999). The movements of leaf cotyledons in *Arabidopsis* is closely related to another circadian growth response, the elongation of the hypocotyl (Dowson-Day & Millar, 1999; Jouve *et al.*, 1999). The circadian regulated growth responses of *Arabidopsis* are controlled by a *LHY/CCA1/TOC1* oscillator because circadian regulated growth responses are impaired by *CCA1* or *LHY* over expression and the *hy-1*, *toc1–1* and *elf3–1* mutations (Dowson-Day & Millar, 1999). The mechanisms underlying the circadian regulation of growth are unclear but may well involve the circadian-regulation of hormone production and transport (Jouve *et al.*, 1999).

2. Circadian regulation of stomatal physiology

Stomata are pores on the aerial surfaces of most plants and allow gas exchange between the plant and the atmosphere (Fig. 8). CO_2 is taken up through the pore for photosynthesis and water is lost via transpiration. The size of the stomatal pore is controlled by movements of the guard cells in response to environmental stimuli, including light, CO_2 concentration, temperature, wind speed and humidity. Additionally, stomatal aperture is regulated by the physiology of the plant, such as the degree of water deficit or the concentration of plant hormones including ABA, indoleacetic acid (IAA) and cytokinins (Mansfield *et al.*, 1990; Assmann, 1993; Schroeder *et al.*, 2001).

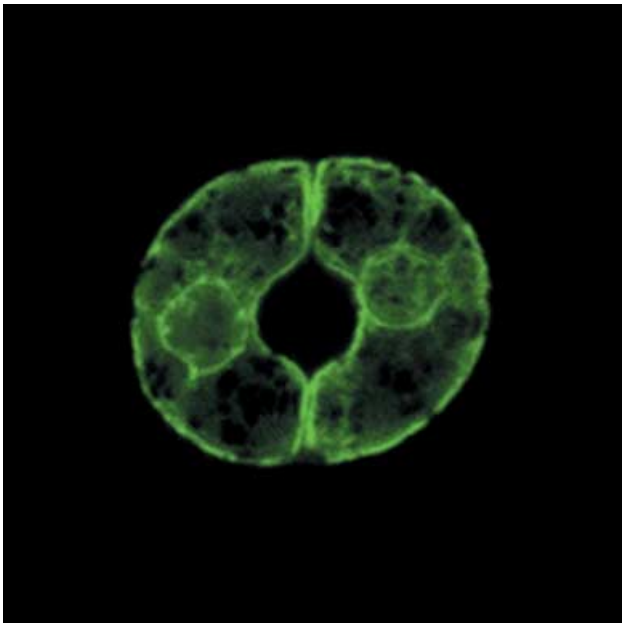

Fig. 8 A stoma of *Arabidopsis thaliana*. The image shows green fluorescent protein localised in the ER of the guard cells. The green fluorescence was visualised using confocal scanning laser microscopy. Note that the pore between the two guard cells is open because of high turgor in the cells. Opening of the pore is bought about by circadian and other signals.

The circadian clock influences the movements of stomata under constant conditions and also the sensitivity of guard cells to extracellular signals (Snaith & Mansfield, 1985; Snaith & Mansfield, 1986; Gorton *et al.*, 1989; Gorton *et al.*, 1993). Circadian control results in the stomata being open during the day and closed at night in well-watered C_3 and C_4 plants. In common with other circadian-regulated processes stomatal movements in LD cycles anticipate light-dark transitions (Somers *et al.*, 1998b) (Fig. 9). The predictive outputs of the circadian clock result in the stomata beginning to close in the middle of the photoperiod (Fig. 9). In Crassulacean acid metabolism plants, the circadian rhythm of stomatal movements is in a phase opposite to that of the rhythms of C_3 and C_4 plants (Thimann *et al.*, 1992; Wyka & Lüttge, 2003; section IV.4).

Circadian stomatal movements persist in LL (Martin & Meidner, 1971; Hennessey & Field, 1992) or DD (Martin & Meidner, 1972; Heath, 1984). The phase of the circadian rhythms can be shifted by altering the photoperiod of the entrainment cycle (Heath, 1984; Holmes & Klein, 1986) or by interrupting LL with a several hours of darkness, and DD with a period of light (Martin & Meidner, 1971; Martin & Meidner, 1972). The period of the rhythm in LL is unaffected by changes in temperature over the physiological range because it is temperature compensated (Gorton *et al.*, 1989; Hennessey & Field, 1992).

There is probably a separate circadian oscillator located within the guard cell (Gorton *et al.*, 1989). The most compelling evidence for the presence of at least one autonomous oscillator in each guard cell is the persistence of circadian stomatal movements in detached epidermis in which the guard cells are physiologically isolated from other leaf tissue and also each other (Gorton *et al.*, 1989). This oscillator is probably of the *LHY/CCA1/TOC1* structure because *toc1–1* shortens the circadian period of stomatal rhythms in LL by *c.* 3 h compared to the wild-type (Somers *et al.*, 1998b). Whilst *CCA1* over expression results in arrhythmia in both stomatal conductance and assimilation in LL (A.N. Dodd & A.A.R. Webb, unpublished). There also might be feedback among the stomatal oscillators and those oscillators that regulate other circadian behaviour in plants (Hennessey & Field, 1992; Wilkins, 1992).

(a) Circadian control of stomatal sensitivity to extracellular signals The response of the stomata to extracellular signals depends on the phase of the circadian cycle at which the signal is applied. The circadian clock can thus modulate (or 'gate')

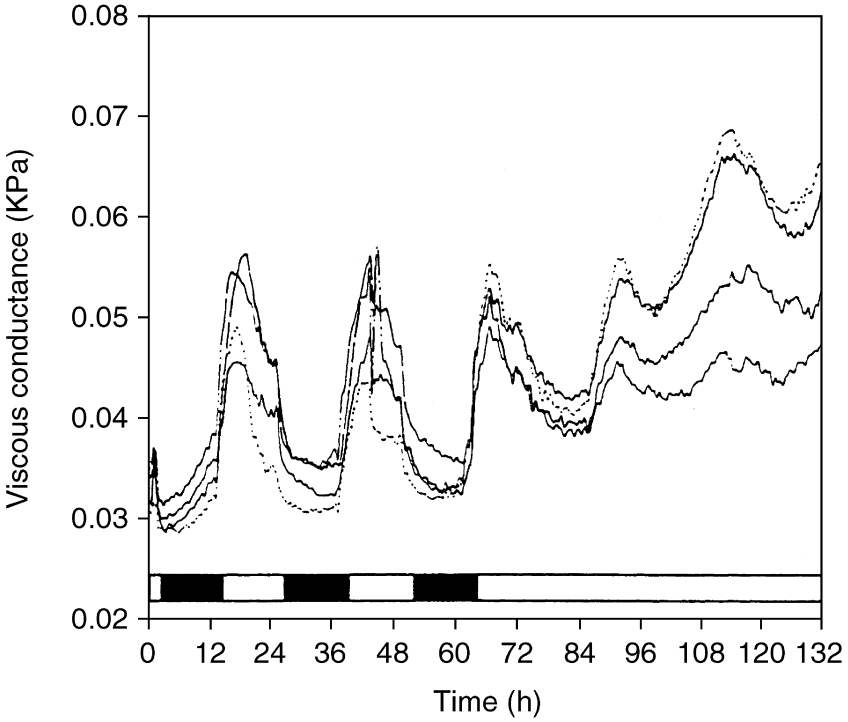

Fig. 9 Diurnal- and circadian-regulated stomatal movements in *Arabidopsis* (C24). Each trace represents measurements taken from a single leaf of an individual plant. Stomatal movements were detected by measuring the conductance of leaves to air flow, high viscous conductances were measured when stomata opened and low conductances when stomata closed. The light regime is represented by the open (lights on) and closed (lights off) boxes. Reproduced from (Webb, 1998).

the responses of stomata (Table 1). Therefore, there is an output from the clock that rhythmically permits and prevents other signalling pathways from impacting on the stomatal opening mechanisms.

An example of the circadian gating of the stomatal response to an extracellular signal is the rhythmic alteration in stomatal sensitivity to light (Darwin, 1898; Mansfield & Heath, 1963; Mansfield & Heath, 1964; Martin & Meidner, 1971; Snaith & Mansfield, 1985; Gorton *et al.*, 1993). The magnitude and kinetics of the acute response of stomata to white, red and blue light depends on the phase in the circadian cycle at which the light signal is applied. Sensitivity to white, red and blue light peaks in the early to middle part of the subjective day. Furthermore, light signals are less effective in opening stomata during subjective night compared to subjective day. Similarly, dark is most effective at closing stomata during subjective night (Martin & Meidner, 1971).

The circadian clock gates the responses of stomata to many extracellular stimuli in addition to light. Circadian gating limits the ability of some signals (e.g. IAA, K^+, anions, fusiccocin) to open stomata in subjective night (Snaith & Mansfield, 1985; Snaith & Mansfield, 1986) and reduces the effectiveness of closing signals (e.g. ABA) in the early to middle part of the subjective day (Correia *et al.*, 1995).

(b) Entraining stomatal rhythms The phase of stomatal circadian rhythms is probably set by the timing of both the lights on and the lights off signals during entrainment (Mansfield & Heath, 1963; Mansfield & Heath, 1964; Heath, 1984; Holmes & Klein, 1986). However, the mechanisms by which the light signals entrain stomatal circadian behaviour are not understood. Blue light perception for stomatal opening is via PHOT1 and PHOT2, redundant blue-light photoreceptors with serine/throenine kinase activity (Kinoshita *et al.*, 2001). Blue light probably activates the PHOT1 and PHOT2 serine/throenine kinase, which in turn increases guard cell $[Ca^{2+}]_i$, this is followed by a Ca^{2+}-dependent activation of the plasma membrane H^+-ATPase via phosphorylation and 14-3–3 protein interactions leading to stomatal opening (Kinoshita *et al.*, 2001; Kinoshita & Shimazaki, 2002). Zeaxanthin has been proposed to be a blue light receptor for stomatal opening but the absence of blue light-induced stomatal opening in *phot1–5/phot2–1* double mutants argues against this hypothesis (Kinoshita *et al.*, 2001; Zeiger *et al.*, 2002). Red light is probably absorbed by chlorophyll (except in the orchid Paphiopedeilum, which is unusual because the guard cells do not have chloroplasts and exceptionally red-light-induced stomatal opening in this species appears to involve phytochrome) (Zeiger *et al.*, 2002). It is unclear whether PHOT1, PHOT2 and chlorophyll act as the photoreceptors for circadian entrainment of stomata, or whether as in other circadian responses circadian entrainment is via the phytochromes and cryptochromes. It was originally thought that phytochromes may be involved in regulating the amplitude of the rhythm of stomatal movements but not the phase (Holmes & Klein, 1986). However, recent data suggest that this hypothesis requires re-examination. An *out of phase 1* (*oop1*) mutant of *Arabidopsis* has been identified by a mutant screen using rhythmic sensitivity to SO_2, a toxic gas,

to identify circadian mutations (Salome *et al.*, 2002). Plants show rhythmic sensitivity to SO_2, which might be correlated with the rhythmic movements of stomata that restrict entry of the gas to the mesophyll. The *oop1* mutation alters circadian phase in leaf movement, CO_2 assimilation and *LHCB* transcription but period is unaltered. *oop1* is a nonsense mutation of *PHYB* resulting in a truncated protein and therefore implicates PHYB in setting circadian phase of multiple circadian responses (Salome *et al.*, 2002). Unfortunately, the effect of *oop1* on stomatal rhythms has not been measured directly. Nevertheless, the nature of the screen by which *oop1* was identified suggests that PHYB is involved in setting the circadian phase of stomata.

(c) Cellular mechanisms underlying circadian stomatal behaviour Detailed models describing the cellular mechanisms that bring about stomatal movements in response to extracellular stimuli have been proposed (Assmann, 1993; Webb *et al.*, 1996b; Leckie *et al.*, 1998; Schroeder *et al.*, 2001). Briefly, stomatal opening is bought about by the activation of a plasma membrane H^+-ATPase. ATP driven H^+efflux hyperpolarizes the plasma membrane (to around –200 mV) activating a hyperpolarization-activated K^+-influx channel in the plasma membrane. H^+-efflux also generates an electrochemical gradient for K^+ uptake. K^+ is accumulated in the vacuole along with Cl^- and malate^{2-}, which act as counter ions. Cl^- is probably taken up by symport with protons, whilst malate is produced by the fixation of CO_2 by phosphoenolpyruvate carboxylase (PEPC). The accumulation of these osmotica in the vacuole results in an osmotic gradient for water uptake. As water is taken up, there is a large increase in turgor of the guard cells. As a result of the radial thickening of the cell wall, the guard cells push apart and the stoma opens.

Stomatal closure caused by ABA, for example, is brought about the co-ordinate efflux of the osmotica and water. Efflux at the plasma membrane is caused by depolarisation to around –60 mV, this activates a depolarisation-activated K^+-efflux channel. Depolarisation of the plasma membrane is a Ca^{2+}-dependent event caused primarily by the activation of slow anion efflux channels. There also is a depolarising influx of Ca^{2+} via a hyperpolarization-activated Ca^{2+} channel which elevates $[Ca^{2+}]_i$ and leads to inhibition of the H^+-ATPase and activation of the anion channels. The net effect of the events at the plasma membrane is depolarisation of the membrane, anion and K^+ efflux and loss of water from the cell. Malate content also is reduced by glycolysis and gluconeogensis. The sequence of events at the tonoplast are less well understood. However, it is clear that elevated $[Ca^{2+}]_i$ activates a number of Ca^{2+}-dependent transport activities that result in efflux of anions and cations from the vacuole (MacRobbie, 1998). K^+ ions may leave the vacuole via the slow vacuolar and the vacuolar potassium channels in the tonoplast. Ligand-gated Ca^{2+} channels activated by Ins(1,4,5)P3, cADPR and inositol hexakisphosphate are also in the tonoplast (Schroeder *et al.*, 2001). It appears that fluxes of K^+ and anions also underlie circadian stomatal movements (Snaith & Mansfield, 1985; Snaith & Mansfield, 1986).

Alterations of $[Ca^{2+}]_i$ are central to the regulation of stomatal behaviour and it is tempting to speculate that circadian oscillations of $[Ca^{2+}]_i$ regulate the circadian fluxes of ions that bring about circadian stomatal movements It is particularly tempting to suggest such a role for Ca^{2+} in the circadian regulation of guard cells because Ca^{2+} acts as a second messenger in the guard cell transduction chains activated by extracellular signals which promote both stomatal opening (auxins (Irving *et al.*, 1992) and blue light (Kinoshita *et al.*, 2001)) and stomatal closure (abscisic acid (McAinsh *et al.*, 1990; Schroeder & Hagiwara, 1990; Irving *et al.*, 1992; Schroeder *et al.*, 2001), CO_2 (Webb *et al.*, 1996a), extracellular calcium [(Gilroy *et al.*, 1991; McAinsh *et al.*, 1995) and oxidative stress (McAinsh *et al.*, 1996)]. Additionally, $[Ca^{2+}]_i$ regulates many of the channels that catalyse the ion fluxes that underlie circadian stomatal movements (Snaith & Mansfield, 1986; Schroeder *et al.*, 2001).

Recent data support a role for Ca^{2+}-based signalling pathways in the circadian regulation of stomata (Jung *et al.*, 2002). Reduction in the cellular concentration of phosphatidylinositol 3 phosphate (PtdIns3P) and phosphatidylinositol 4 phosphate (PtdIns4P) by inhibition of phosphatidylinositol kinase activity or by over expression in the guard cell of PtdIns3P- and PtdIns4P-binding proteins, reduced stomatal opening in the light and dark during the early part of the photoperiod (Jung *et al.*, 2002). The opening of stomata in detached epidermis in the light or dark during the early part of the photoperiod may be partially caused by the circadian clock. Phosphoinositide metabolism is important in guard cell Ca^{2+}-signalling and inhibition of PtdIns kinase activity inhibited ABA-induced increases in $[Ca^{2+}]_i$ in the guard cell (Jung *et al.*, 2002). Thus, there is a possible link between Ca^{2+}-signalling mechanisms and the circadian regulation of stomata.

3. Circadian regulation of root physiology

The circadian clock regulates multiple transport mechanisms in the root (Henzler *et al.*, 1999; Clarkson *et al.*, 2000). Diurnal changes in root hydraulic conductivity are coincident with diurnal changes in aquaporin transcript abundance in *Lotus japonicus*. The peak of root hydraulic conductivity, occurs 6–8 h into the photoperiod and is preceded 2–4 h earlier by a peak in aquaporin transcript abundance. It is likely that the circadian clock is regulating aquaporin abundance in the endodermis and/or the stele to modulate the permeability of cell membranes to water, resulting in increased root hydraulic conductivity in the middle of the photoperiod (Henzler *et al.*, 1999). Similarly, the root pressure oscillates with a circadian period for several cycles, even when the root is excised. The changes in root pressure are caused by circadian regulation of solute pumping in to the xylem and demonstrates regulation of ion transporters in the root by the circadian

clock (Henzler *et al.*, 1999). Thus, the clock modulates the permeability of root cells to water (possibly altering the number of water channels in the plasma membrane) and also modulates solute concentration in the xylem. This results in a circadian-regulated alteration in the driving force for water transport in the plant.

4. Circadian regulation of Crassulacean acid metabolism

Another important area of the circadian regulation of plant physiology is the circadian regulation of gas exchange during Crassulacean acid metabolism (CAM). Outlined below is some of the major features of the circadian regulation of CAM. The physiology and biochemistry of CAM was the subject of a recent special edition of *Functional Plant Biology* (2002, volume 29, issue 6). Refer to the excellent articles contained within for detail and source references.

CAM is a metabolic adaptation to low internal leaf CO_2, which typically arises in terrestrial plants as a result of drought. In CAM plants, PEPC is part of a CO_2 concentrating mechanism (Hartwell *et al.*, 2002; Taybi *et al.*, 2002; Wyka & Lüttge, 2003). The essential feature of CAM is the temporal separation of CO_2 fixation in to four phases. During the night, the stomata open and CO_2 is fixed by PEPC. The products of PEPC activity, malate or citrate, are stored in the vacuole (Phase I). Opening of stomata at night to allow CO_2 fixation is an adaptation to conserve water in arid environments. At dawn, PEPC and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) are transiently active at the same time, causing a burst of CO_2 uptake (Phase II). During the day, the malate stored during Phase I crosses the tonoplast and is decarboxylated in the cytosol to release CO_2, which is refixed by Rubisco (Phase III). During this period, the stomata close because decarboxylation elevates the CO_2 concentration in the leaf. Sometimes towards the end of the day, if sufficient water is available, when the vacuolar malate supply is exhausted the stomata reopen and atmospheric CO_2 is fixed directly by Rubisco (Phase IV). To prevent futile cycles of carbon, the activities of PEPC and Rubisco are temporally separated such that PEPC functions mostly at night and Rubisco during the day.

The correct functioning of CAM requires the co-ordinated temporal-regulation of PEPC and Rubisco activity, malate transport across the tonoplast, malate decarboxylation, stomatal movements and many other metabolic processes. The circadian clock contributes to the temporal regulation of CAM, though metabolic, hormonal and environmental factors can over ride circadian control (Hartwell *et al.*, 2002; Taybi *et al.*, 2002). In particular, the circadian clock regulates the activity of PEPC (Hartwell *et al.*, 2002), the expression of PEPC kinase, leaf malate content (Wyka & Lüttge, 2003), stomatal movements (see section IV.2), the expression of genes involved in the degradation of starch during the night (Dodd *et al.*, 2003) and Rubisco activity in LL (Dodd *et al.*, 2003; Wyka & Lüttge, 2003).

The circadian clock regulates PEPC activity via PEPC kinase, which reversibly phosphorylates PEPC during the night phase reducing the sensitivity of PEPC to inhibition by malate. The expression of PEPC kinase is under circadian control via a dephosphorylation-sensitive pathway (Hartwell *et al.*, 2002). PEPC kinase is an unusual kinase in that it appears to lack regulatory domains. The regulation of PEPC kinase activity appears to be totally at the level of gene expression. PEPC is dephosphorylated by a protein phosphatase 2 A (Nimmo, 2000; Hartwell *et al.*, 2002), however, the contribution of this activity to circadian control of PEPC is unclear.

It has been proposed that the circadian clock controls malate transport across the tonoplast and that the circadian regulation of PEPC kinase and consequently PEPC are downstream consequences of alterations in the cytosolic malate concentration (Nimmo, 2000). Other responses, such as the circadian regulation of Rubisco activity, may operate independently of malate (Wyka & Lüttge, 2003). The apparent independent circadian-regulation of PEPC and Rubisco activities might support a role for the presence of multiple oscillators operating in CAM plants, some of which may be self-regulating feedback loops (Wyka & Lüttge, 2003). However, at least some CAM responses are likely to be controlled by a central oscillator similar to the *LHY/CCA1/TOC1* oscillator in *Arabidopsis* because homologues of the major clock genes, *CCA1/LHY* and *TOC1* and also *ZTL* and *ELF4* are present in the CAM plant *Mesembryanthemum crystallinum* L. (J. Hartwell, pers. comm.). Intriguingly, CAM induction appears to occur via a Ca^{2+}-based signalling pathway but it is not known if Ca^{2+}-signalling is involved in the circadian regulation of CAM (Taybi *et al.*, 2002).

V. The benefits of the circadian regulation of physiology

The ubiquitous nature of the circadian regulation of plant physiology and the persistence of circadian behaviour throughout evolution suggest that there is adaptive advantage to circadian regulation. Recent evidence suggests that the circadian clock might increase reproductive fitness of plants. Plants in which the oscillator is disrupted by *CCA1* over expression were less viable in very short days (4 h light/20 h dark) than the wild type (Green *et al.*, 2002)

It is thought that the clock increases reproductive fitness by acting as an internal chronometer to inform of external time and to measure also the duration of external time. In plants, this is the likely role of the clock in photoperiodism. This ensures flower formation in synchrony with other members of the species and during the season favourable to that species. The clock allows the plant to coordinate physiological, developmental and metabolic activities in a specific phase relationship to the environmental cycle. Importantly, circadian regulation allows the control of physiology to be predictive

rather than merely reactive. The most obvious examples of this is the coordinate readiness of the photosynthetic and stomatal apparatus in C_3 plants before dawn (Johnson *et al.*, 1998).

Less clear are the quantifiable physiological benefits that lead to the observed increase in reproductive fitness. Previously, I proposed that the circadian control of stomatal movements increases the water use efficiency (WUE) of the plant (assimilation/evaporation) (Webb, 1998). In C3 plants, the circadian clock promotes stomatal opening in the morning during the early part of the photoperiod when solar radiation is high but air temperature is not. In the afternoon, when air temperatures and water loss from evapotranspiration might be expected to be high, the circadian clock promotes stomatal closure. Closure is also promoted in the dark period when photosynthesis does not occur. This will tend to increase photosynthesis whilst limiting water loss and thereby increases WUE.

The circadian control of stomatal physiology might increase WUE in three ways that could not be achieved by the regulation of stomatal function by environmental and physiological signals alone (Webb, 1998): firstly, the circadian oscillator exerts predictive control over stomatal physiology, both altering the aperture of the stomatal pore and reducing the lag phase between perception and response to light/dark signals. The endogenous rhythm ensures that guard cells anticipate dawn and dusk and circadian gating increases the sensitivity of the stomata to light signals at dawn, resulting in rapid opening following a lights on signal. Similarly stomata predict dusk and start to close many hours before the onset of darkness, reducing water loss. Secondly, circadian control ensures that stomatal physiology is co-ordinated with other physiological and metabolic responses, such as photosynthesis. Loss of synchronisation of assimilation and conductance would greatly reduce WUE. Lastly, circadian gating might limit responses to extracellular signals that may be inappropriate to the phase of the cycle. For example, stomatal opening in response to IAA in the leaf would increase water loss and thus reduce WUE. However, this is prevented by the circadian gating that reduces stomatal sensitivity to IAA during the night (Snaith & Mansfield, 1985).

A modelling approach has been used to quantify the effects of circadian rhythms in the field on the gas exchange of *Saururus cermuns* L., a wetlands perennial (Williams & Gorton, 1998). That investigation was unable to identify a significant benefit of the circadian regulation of photosynthesis and stomatal conductance. This may have been due, in part, to attempting to model subtle effects from a small data set obtained from one species (Williams & Gorton, 1998). My laboratory is also investigating the physiological benefit of circadian regulation of gas exchange by comparing WUE in 'clock-stopped' circadian mutants and wild type of *Arabidopsis*. Our data suggest that the circadian clock does increase instantaneous WUE under some conditions (A.N. Dodd *et al.*, unpublished).

VI. Future prospects

Traditionally, the circadian regulation of physiology has been measured at a whole plant or organ level. Studies at cellular or subcellular resolution have been hampered by the incompatibility of invasive cell physiology techniques with the long time scales required to measure circadian behaviour. These problems are compounded by the inaccessibility of many cell types in the organs. The development of noninvasive cellular physiology tools and the ability to study plant cells, such as guard cells and pulvini extensor and flexor cells, in isolation from the rest of the plant provide opportunities to probe circadian behaviour at cellular resolution. Particular opportunities are provided by the continued development of recombinant reporters of cellular activity, such as aequorin and cameleon green fluorescent proteins, another recombinant reporter of Ca^{2+}. Doubtless, new fluorescent and bioluminescent reporters of cellular processes will provide opportunities for visualising circadian regulation of cellular physiology in a noninvasive manner. One technology that will rapidly advance the understanding of circadian cell physiology is the use of fluorescence energy transfer to visualise temporal regulation of protein–protein interactions.

The regulation of ion transport is a central theme in the circadian regulation of plant physiology. Ironically, this has proved the most difficult aspect of the circadian regulation of physiology to investigate. The tools for measuring ion fluxes are typically invasive and technically demanding. 'Classical' ion flux measurement tools include impalement of cells with ion selective electrodes, patch-clamp analysis of ion channel activities in wall-less protoplasts, introduction of fluorescent ion indicators into the cytosol by microinjection or other harsh dye loading protocols (Webb *et al.*, 1996b) and prolonged exposure to harmful irradiation to excite fluorescent indicators. Radioactive tracers can act as a noninvasive tool for investigating ion flux but to observe the regulation of ion transport at a cellular level of resolution requires isolation of the cell type of interest from the rest of the plant (Snaith & Mansfield, 1986). Novel noninvasive tools for measuring multiple ion fluxes such as multi electrode ion flux estimation (MIFE™, University of Tasmania, Australia) promise advances in understanding the circadian regulation of physiology (Shabala *et al.*, 1997).

Forward and reverse genetics, genomics, transcriptomics and proteomics provide powerful tools to investigate the circadian regulation of physiology in a noninvasive manner. Comparisons of the circadian regulation of cell and whole plant physiology in wild type and mutant backgrounds have the potential to position genetic lesions with respect to other components of the circadian signalling cascade. This represents an important aid to describe the hierarchy of the circadian signalling networks and cellular mechanisms underlying the circadian regulation of physiology.

Mathematical modelling is another approach that will become more widely used to understand the circadian regulation of

physiology. Sufficient molecular information is now available to enable mathematical modeling of the plant clock (Andrew J. Millar, pers. comm.), similar to ongoing computational studies of the central oscillator mechanism in animals (Goldbeter, 2002). Progress will be rapid because of the sharing of software tools and data using the Internet. Mathematical modelling based on sound experimentation is essential for comprehension of oscillations because of the complex and nonlinear interactions in potentially very large and multivariate data sets (Goldbeter, 2002). Importantly, many of the mathematical tools that have been developed for modelling and understanding stimulus-induced oscillations of $[Ca^{2+}]_i$ are appropriate for investigating other rhythmic biological behaviour including circadian rhythms (Goldbeter, 2002; Schuster *et al*., 2002). This suggests that the circadian regulation of $[Ca^{2+}]_i$ represents an attractive model for investigating the circadian regulation of physiology.

VIII. Conclusions

Advances in understanding circadian biology are being made rapidly. Principle areas of current interest are the mechanisms of photoreception and transduction, the identity and organisation of oscillator components, the hierarchy of the output signalling pathway and the nature of the cellular processes regulated by the circadian clock. Coupled with this mechanistic understanding, there is an urgent need to investigate the quantitative advantages of the circadian regulation of physiology and to determine how the clock increases reproductive fitness. Genetic resources will be coupled to bioinformatic, modelling and noninvasive physiological tools to address these fundamental questions.

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