synthase; this provided confirmation that sorbitol is involved in the mobility of boron (Patrick H. Brown, UC Davis, USA).

Several papers described screens to identify traits relating to plant nutrition in wild accessions, old cultivars, land races and modern genotypes. The development of markers for these traits and the usefulness of marker-assisted breeding were also discussed. Differences in the approach used by physiologists and plant breeders surfaced repeatedly and led to vigorous discussions. The convivial atmosphere played a large part in generating these active discussions, and fulfilled one of the aims of the symposium series: to ensure effective communication between pure and applied plant nutritionists and plant breeders. In essence, plant breeders like it 'simple, crude and cheap', with an emphasis on field selection, particularly in relation to yield. By contrast, plant physiologists are reductionists, i.e. more interested in mechanistic details of the processes. More meetings of this kind, in particular involving large numbers of geneticists and plant breeders, will be vital to bring the two camps together and to optimize nutrition for plant yield and quality.

Sally Smith*
The Centre for Plant Root Symbiosis and Dept of Soil Science, The University of Adelaide, SA 5064, Australia

Andrew Smith
The Centre for Plant Root Symbiosis and Dept of Botany, The University of Adelaide, SA 5064, Australia

*Author for correspondence
(tel +61 8 8303 7210; fax +61 8 8303 6511; e-mail ssmith@waite.adelaide.edu.au)

It's about time: putative components of an *Arabidopsis* circadian clock

Circadian rhythms are a widespread biological phenomenon and have been described for both prokaryotes and eukaryotes. The rhythms are characterized by period lengths of approximately 24 h, and the normal environmental cycles of light and temperature provide temporal information that entrains (resets) the biological clock. Within the laboratory it is possible to deprive organisms of environmental time cues, and it is the persistence of circadian rhythms under these constant conditions that demonstrates the endogenous nature of the biological clock.

The central oscillator

For many years, the goal of research in this field has been to identify components of the circadian central oscillator. Mutant alleles of the *period* locus of *Drosophila* were first identified in 1971, and additional mutations that affect fundamental properties of the clock and confer altered period length or arrhythmicity have since been isolated from a diverse range of organisms, including cyanobacteria, *Neurospora* and, of course, *Arabidopsis*[1]. Mutational analyses are most advanced in *Drosophila* and *Neurospora*, where the characterization of the genes identified by these mutations has yielded a model of the central oscillator as a negative feedback loop in which rhythmic transcription of key clock genes is inhibited by the nuclear accumulation of the protein products of these genes[1,2]. A spate of recent publications has filled in critical gaps in the description of this negative feedback loop (Fig. 1) and has illustrated striking conservation, both at the level of loop function and primary protein sequence motifs, of clock components from *Drosophila*, *Neurospora* and mammals[3,4].

The plant clock

What then of plant clock components? In spite of the historical importance of plants to the scientific study of circadian rhythms[5], the molecular components of plant clocks remain unknown and we cannot yet predict the extent to which plant clocks will resemble the fungal, animal or cyanobacterial equivalents. In *Arabidopsis*, a genetic screen based on alterations in rhythmic expression of a luciferase (*luc*) transgene driven by regulatory elements of a light harvesting chlorophyll *a*/*b* binding protein gene (*CAB2*, also known as *Lhcb1*1*) has revealed a series of *timing of CAB* (*toc*) mutations that disrupt clock function[6], but as yet, none of the genes responsible have been cloned. The *early flowering 3* (*elf3*) mutant was identified on the basis of a daylength-insensitive early flowering phenotype. The mutant *elf3* exhibits conditional arrhythmicity of both leaf movement and *CAB* gene expression in continuous light, but shows normal clock function in continuous dark[7]. This has been interpreted as evidence that *ELF3* encodes a component of a light input pathway as opposed to a component of a central oscillator. Overexpression in transgenic *Arabidopsis* of a clock-regulated glycine-rich RNA-binding protein, GRP7 (also known as CCR2), blocks the oscillation in mRNA abundance of *GRP7* and the closely related *GRP8* (also known as *CCR1*), but does not affect other circadian oscillations. This suggests that GRP7 is a key component of a slave (non-self-sustaining) oscillator, but not of a central oscillator[8].

However, the long wait for plant central oscillator components may finally be over. Two recent studies published in *Cell*, describe a pair of putative components of an *Arabidopsis* clock[9,10]. In Elaine Tobin's lab, *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) was first identified as an MYB-related transcription factor that binds to a region of the *Arabidopsis CAB1* (*Lhcb1*3*) promoter necessary for phytochrome responsiveness[11]. The binding target of CCA1 (consensus AA[A/C]AATCT) in the *CAB1* promoter is closely related to a region of the *CAB2* promoter that is sufficient to confer circadian transcription on a *luc* reporter gene[12], and CCA1 also binds to this 36 bp clock regulatory region[11]. If CCA1 really is the transcriptional activator responsible for circadian transcription of the *CAB* genes, then one might expect CCA1 abundance to oscillate also. Indeed, both *CCA1* mRNA and protein exhibit circadian oscillations in abundance, and the peak in CCA1 protein concentration precedes the peak in *CAB* transcription[10]. A circadian oscillation in CCA1 binding-activity might also be predicted, and data addressing this issue are eagerly awaited.

Manipulating the expression of *CCA1*

Elaine Tobin's lab sought to perturb *CAB* gene transcription in transgenic plants by either under- or over-expressing *CCA1*. Expression of antisense *CCA1* mRNA in transgenic plants reduces phytochrome-mediated induction of *CAB1*, confirming the *in vivo* role of CCA1 as a transcription activator[11]. The effects of underexpression of CCA1 on circadian rhythms have not been determined. However, transgenic lines overexpressing CCA1 (CCA1-ox) are arrhythmic in *CAB* transcription, indicating that CCA1 abundance limits *CAB* transcription and that constitutive CCA1 expression is sufficient to yield constitutive *CAB* transcription. This is a satisfying result, indicating that CCA1 is important for circadian regulation of *CAB* transcription. The phenotype of the CCA1-ox plants is pleiotropic: CCA1-ox plants lose circadian

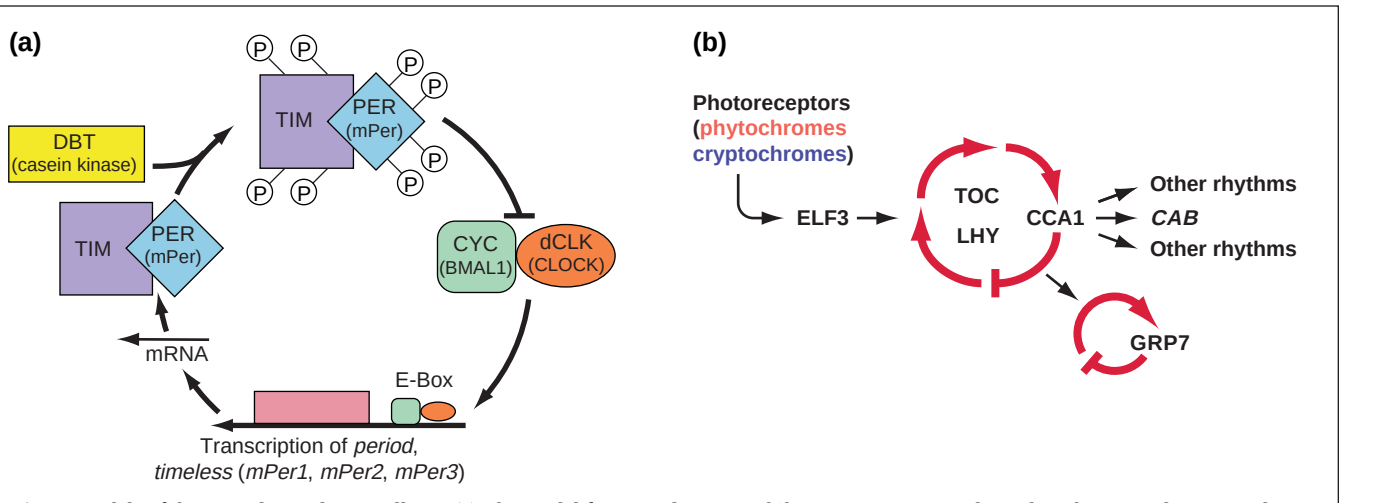

Fig. 1. Models of the central circadian oscillator. (a) The model for animals. *Drosophila* components are indicated, with mammalian equivalents indicated in parentheses. P indicates period and timeless phosphorylation. (b) A model illustrating the conceptual framework of a plant central oscillator, and input and output pathways. The positions of the various components are speculative. Single arrows can represent multiple steps in a pathway. The GRP7 slave oscillator is indicated as a non-self-sustaining loop dependent upon input from the central oscillator for continued oscillation. Abbreviations: TIM, timeless; PER, period; DBT, *Drosophila* double-time; dCLK, *Drosophila* clock; ELF3, early flowering 3; LHY, late elongated hypocotyl; CCA1, circadian clock associated 1; CYC, cycle; BMAL1, brain and muscle arnt-like protein 1; CAB, chlorophyll a/b binding protein; TOC, timing of CAB; GRP7, glycine-rich RNA-binding protein 7.

regulation of other mRNAs including *CAT3* and *GRP7*, which oscillate 180° out of phase with *CAB* genes; the plants are also arrhythmic in leaf movement and late flowering. Clearly, CCA1 does much more than simply activate *CAB* gene transcription. Critically, CCA1 overexpression suppresses expression of the endogenous *CCA1* gene, indicating that CCA1 negatively autoregulates. Taken together, these results implicate CCA1 as a key component of a circadian oscillator.

Late flowering mutants

In an independent approach, George Coupland's lab used a screen for late flowering mutations (in an *Arabidopsis* population carrying the maize *Ac–Ds* transposon system) to identify *late elongated hypocotyl* (*lhy*)[9]. The mutant phenotype co-segregates with a single *Ds* insertion; LHY was cloned by virtue of the molecular *Ds* tag. The *Ds* element employed carries an outwardly directed promoter (CaMV 35S) that is known to induce dominant mutations through activation of adjacent genes. Indeed, *lhy* is inherited as a dominant mutation in which the *Ds* element is inserted upstream of *LHY*, resulting in constitutive high-level expression[9]. Thus, the *Ds*-tagged *lhy* mutants are functionally LHY overexpressors.

The most prominent feature of the deduced amino acid sequence of LHY is a single 47-residue region related to the helix–loop–helix DNA-binding domain of the MYB family[9]. This region is 87% identical to the corresponding region in CCA1, and is essential for DNA-binding in CCA1 (Ref. 11). The similarity between LHY and CCA1 extends beyond the MYB domain, and there are three other regions, each of 20–25 amino acids, which have at least 80% identity. Members of the MYB superfamily are found in all eukaryotes, and animal haploid genomes typically include 1–3 MYB genes. In plants, the MYB family has expanded dramatically: the *Arabidopsis* haploid genome includes over 80 known MYB genes that are involved in many distinct processes[13]. However, the MYB domain is repeated in almost all MYB superfamily members, whereas CCA1 and LHY have only a single copy of the MYB motif.

Are CCA1 and LHY functionally redundant?

The phenotype of plants carrying the dominant *lhy* allele is pleiotropic and similar to the phenotype of CCA1-ox plants. In addition to the delay in flowering, both exhibit elongated hypocotyls, and as with *CCA1*, *LHY* mRNA abundance oscillates with a circadian rhythm in wild-type plants, but is arrhythmic in *lhy* mutants. Expression of other clock-regulated genes, including a *cab:luc* fusion transgene and *grp7* is arrhythmic in *lhy* mutants. Similarly, the rhythm in leaf movement is lost. Thus, constitutive overexpression of *LHY*, as of *CCA1*, disrupts multiple rhythms. The fact that overexpression of either *CCA1* or *LHY* simultaneously disrupts circadian clock function and delays flowering, provides genetic confirmation of the critical role of the circadian clock in the photoperiodic determination of flower initiation that has long been inferred from physiological experiments[14].

Overexpression of either *CCA1* or *LHY* confers similar phenotypes: are CCA1 and LHY functionally redundant? This can best be addressed through analysis of loss-of-function alleles for each gene and, particularly, through construction of a mutant plant carrying loss-of-function alleles for both genes. Nonetheless, it seems unlikely that CCA1 and LHY are completely redundant, as there are subtle differences between the LHY overexpressor and CCA1-ox phenotypes. In *lhy* mutants, endogenous *LHY* transcript abundance is arrhythmic but at a level intermediate between peak and trough levels of the wild-type oscillation. However, CCA1 overexpression almost totally represses endogenous *CCA1* transcript accumulation and also represses *LHY* transcript accumulation to trough levels. It will be interesting to assess *CCA1* transcript oscillations in *lhy* mutants.

Role of phosphorylation

The key clock components period (PER) and timeless (TIM) in *Drosophila* and frequency (FRQ) in *Neurospora*, are differentially phosphorylated throughout the circadian cycle[2–4], and a mutation in the *Drosophila double-time* (*dbt*) gene, which encodes a casein kinase I activity responsible for the phosphorylation of period, results in arrhythmicity[15,16]. Is there a role for phosphorylation in the activity of CCA1 or LHY? Recently, Elaine Tobin's lab performed a yeast two-hybrid screen for *Arabidopsis* proteins that interact with CCA1 and identified a regulatory β subunit of casein kinase II (CK2), CKB3 (Ref. 17). Further *in vitro* experiments showed that CCA1 interacts with two other CK2 β subunits, CKB1 and CKB2, and with two CK2 catalytic α subunits, CKA1 and CKA2. In addition, CK2, as well as a CK2-like activity from *Arabidopsis* whole-cell extracts, phosphorylates CCA1 *in vitro*, although this phosphorylation did not affect the ability of recombinant CCA1 to bind to its

DNA target in electrophoretic mobility shift assays. However, the treatment of plant extracts with protein phosphatase abolished the formation of the major CCA1–DNA complex, as did treatment of the extracts with CK2 inhibitors. This clearly implicates phosphorylation of CCA1 by the serine-threonine kinase activity of CK2 in the *in vivo* regulation of CCA1 activity[17]. It will be important to monitor CK2 activity as well as the phosphorylation state of CCA1 throughout the circadian cycle and in response to phase-shifting stimuli; and the potential effects of phosphorylation on LHY.

Future prospects

Are CCA1 and LHY genuine clock components? Quite possibly, although several experimental predictions must be fulfilled before CCA1 and LHY are elevated to that pantheon. Overexpression of either CCA1 or LHY results in arrhythmicity, satisfying one important criterion. Loss-of-function alleles of clock components from *Drosophila* and *Neurospora* stop the clock[1,2], making the analysis of loss of function a critical test for both CCA1 and LHY. Pulses of CCA1 or LHY should shift the phase of the clock to that normally specified by the induced level of CCA1 or LHY during their normal daily oscillations. However, it is known that output pathways can provide input to oscillators, and that input pathways may themselves be under circadian control. Consequently, it is quite difficult to establish unequivocally that a particular gene encodes a component of a central oscillator (for detailed discussion, see Ref. 18). Certainly CCA1 is a critical player in the output pathway that governs circadian regulation of *CAB* genes. If CCA1 also proves to be a component of the central oscillator, it will provide a dramatic example of multiple tasking within the circadian system.

The properties of CCA1 and LHY make them serious contenders as plant clock components. Could it be that MYB proteins will be the plant functional analogs of the ubiquitous PAS proteins [e.g. TIM, clock and cycle (brain and muscle arnt-like protein 1)] in animal and fungal clocks[1–4]? Ultimately, we may need to wait for a fuller description of the complete negative-feedback loop in plants before we can be certain. Many key questions remain to be answered. For example, what is the effect of LHY overexpression on the oscillation in CCA1? The *elf3* mutation eliminates *LHY* mRNA oscillations in continuous light[9]. Is the arrhythmicity of *elf3* (Ref. 7) entirely due to this effect on *LHY*? If so, this implies that *lhy* loss-of-function alleles should confer arrhythmicity which argues against redundancy between LHY and CCA1. What are the phenotypes conferred by *lhy* or *cca1* loss-of-function mutations and what is the phenotype of the double mutant homozygous for loss of function of *lhy* and *cca1*? Also, what are other targets of CCA1 and LHY, and what are the regulators of CCA1 and LHY mRNAs that confer their circadian oscillations?

Conclusion

These recent studies illustrate a marvelous convergence. Elaine Tobin's lab set out to study light regulation of gene expression and George Coupland's lab set out to study photoperiodic initiation of flowering, yet both have converged on the circadian clock and, quite possibly, have identified components of a central oscillator. These reports were published 269 years after de Mairan first established that a circadian rhythm (leaf movement in *Mimosa*) was endogenous[19], and provide a first glimpse into the molecular mechanism of a plant circadian oscillator. One might say, it's about time.

Acknowledgements

My thanks to Tom Jack and Mary Lou Guerinot for comments on the manuscript. Work in my lab is supported by a National Science Foundation grant (MCB 9723482).

C. Robertson McClung
Dept Biological Sciences, Dartmouth College, Hanover, NH 03755-3576, USA
(tel +1 603 646 3940; fax +1 603 646 1347; e-mail mcclung@dartmouth.edu)

References

1 Dunlap, J.C. (1996) Genetic and molecular analysis of circadian rhythms, *Annu. Rev. Genet.* 30, 579–601
2 Young, M.W. (1998) The molecular control of circadian behavioral rhythms and their entrainment in *Drosophila*, *Annu. Rev. Biochem.* 67, 135–152
3 Dunlap, J. (1998) An end in the beginning, *Science* 280, 1548–1549
4 Reppert, S.M. (1998) A clockwork explosion! *Neuron* 21, 1–4
5 Bünning, E. (1960) Opening address: biological clocks, in *Cold Spring Harbor Symposia on Quantitative Biology* XXV, pp. 1–9, Cold Spring Harbor Laboratory Press
6 Millar, A.J. *et al.* (1995) Circadian clock mutants in *Arabidopsis* identified by luciferase imaging, *Science* 267, 1161–1163
7 Hicks, K.A. *et al.* (1996) Conditional circadian dysfunction of the *Arabidopsis early-flowering 3* mutant, *Science* 274, 790–792
8 Heintzen, C. *et al.* (1997) *At*GRP7, a nuclear RNA-binding protein as a component of a circadian-regulated negative feedback loop in *Arabidopsis thaliana*, *Proc. Natl. Acad. Sci. U. S. A.* 94, 8515–8520
9 Schaffer, R. *et al.* (1998) *LATE ELONGATED HYPOCOTYL*, an *Arabidopsis* gene encoding a MYB transcription factor, regulates circadian rhythmicity and photoperiodic responses, *Cell* 93, 1219–1229
10 Wang, Z-Y. and Tobin, E.M. (1998) Constitutive expression of the *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) gene disrupts circadian rhythms and suppresses its own expression, *Cell* 93, 1207–1217
11 Wang, Z-Y. *et al.* (1997) A MYB-related transcription factor is involved in the phytochrome regulation of an *Arabidopsis Lhcb* gene, *Plant Cell* 9, 491–507
12 Carré, I.A. and Kay, S.A. (1995) Multiple DNA-protein complexes at a circadian-regulated promoter element, *Plant Cell* 7, 2039–2051
13 Romero, I. *et al.* (1998) More than 80 *R2R3-MYB* regulatory genes in the genome of *Arabidopsis thaliana*, *Plant J.* 14, 273–284
14 Lumsden, P.J. and Millar, A.J., eds (1998) *Biological Rhythms and Photoperiodism in Plants*, Bios Scientific Publishers
15 Kloss, B. *et al.* (1998) The *Drosophila* clock gene *double-time* encodes a protein closely related to human casein kinase Ie, *Cell* 97, 97–107
16 Price, J.L. *et al.* (1998) *double-time* is a novel *Drosophila* clock gene that regulates PERIOD protein accumulation, *Cell* 94, 83–95
17 Sugano, S. *et al.* (1998) Protein kinase CK2 interacts with and phosphorylates the *Arabidopsis* circadian clock-associated 1 protein, *Proc. Natl. Acad. Sci. U. S. A.* 95, 11020–11025
18 Roenneberg, T. and Merrow, M. (1998) Molecular circadian oscillators: an alternative hypothesis, *J. Biol. Rhythms* 13, 167–179
19 de Mairan, J. (1729) Observation botanique, *Hist. Acad. Roy. Sci.* 35–36