Clock proteins: **Turned over after hours?**

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Light sensitivity and the involvement of unstable proteins are key features of circadian clocks. Both photoreception and ubiquitin conjugation may be associated with nuclear regulators encoded by genes recently identified in *Arabidopsis*.

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Classical scholars interested by the rhythmic movements of some plants' leaves made the first records of daily biological rhythms. The mechanism underlying their 24-hour timing is now known as the circadian system or circadian clock. Recent studies have uncovered three members of a small protein family that control development and may be novel components of the clock in the model plant *Arabidopsis thaliana* ([1,2] and M. Wada, personal communication in [1]). Each protein has three domains that have been implicated in protein–protein interactions and more besides. One domain can bind a flavin chromophore in photoreceptor proteins and another targets proteins for degradation [1,2]. With such rich relationships, these proteins should go far.

The circadian clocks of plants and almost all other eukaryotes behave very similarly: all have a rhythmic period close to 24 hours and can be reset by changes in ambient light. Studies of mutant insects, fungi, cyanobacteria and rodents with altered or absent timing have identified several genes involved in negative feedback loops, through which a few proteins rhythmically regulate the transcription of their cognate genes [3]. The loops are necessary — though perhaps not sufficient — for normal timing, and their responses to light explain the resetting of rhythms in the organism. Their detailed organisation and gene sequences are not obviously conserved among these taxa. Genes that may function in a similar feedback system have been identified in *Arabidopsis* in the past five years [4]. The recent identification of the three related *Arabidopsis* genes — *ZTL*, for *ZEITLUPE* or slow motion [1], *FKF*, which encodes a 'flavin-binding kelch repeat F-box' protein [2], and *LKP2*, which encodes an 'LOV domain kelch protein' (M. Wada, personal communication in [1]) — reflects a rapid convergence of several experimental approaches, which is pleasing both personally and scientifically.

Routes around the clock

Clock genetics in higher plants was uneventful until the 1990s. Then, staying up late in the lab, Nagy and Kay in Chua's group serendipitously found that the circadian clock controlled transcription from the chlorophyll *a/b*-binding protein (*CAB*) promoter, which was a workhorse of plant molecular biology. Kay proposed to find clock mutant plants, based upon their mis-timed *CAB* transcription. The other proposed ingredients were a bioluminescent firefly luciferase (*luc*) reporter gene, which might allow *CAB:luc* transgenic plants to glow rhythmically, and a photon-counting camera (initially located in Singapore). We identified our first circadian clock mutants in the latter half of 1992, by screening mutagenised *CAB:luc* plants for mis-timed luminescence [5]. The Kay team [1] has now used map-based cloning to identify a mutant allele of *ZTL* in one of the long-period mutants and to show that the short-period mutant *toc1* identifies another new family of plant regulatory proteins [6].

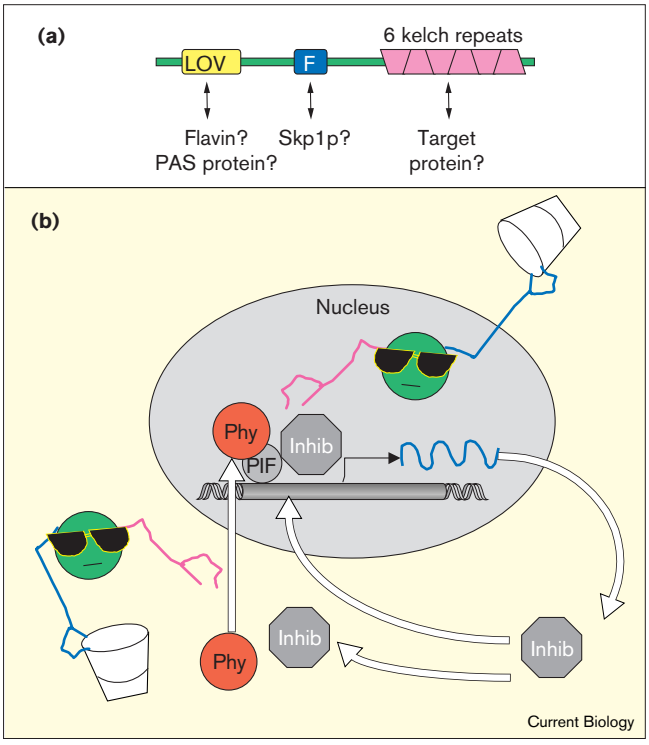
Clock mutants of *Arabidopsis* are now arriving like buses. The circadian clock is a genome-wide regulator that regulates many processes, such as flowering and the growth of the seedling stem or hypocotyl. Genetic screens for mutants that mis-regulate any of these processes may therefore recover clock-associated genes. Targeted screens are not even required, because long-hypocotyl seedlings and plants with altered flowering time — like the *fkf* mutant [2] — stand out in any genetic screen in *Arabidopsis*. Biochemical or molecular screening for proteins that regulate gene expression can likewise uncover clock proteins, if the clock controls the gene of interest. The 'circadian clock associated' (CCA1) protein was identified by its binding to the *CAB* promoter, but itself turns out to be rhythmically expressed and involved in a clock-like negative feedback loop [4]. Further clock genes are likely to be identified from the characterisation of other regulators, just as the *toc1* gene was recently identified by homology of its protein product to bacterial response regulators [7] and by binding to a hormone-regulated transcription factor [8].

As the *Arabidopsis* genome sequence rolled out in 1999, the third member of the *ZTL* family, *LKP2*, was identified by its homology of part of its protein product to the LOV domain (M. Wada, personal communication in [1]). This domain is found in some photoreceptors, in addition to a motley group of proteins including redox sensors, ion channels — the LOV acronym refers to 'light, oxygen and voltage' — and clock-associated proteins. LOV is one of three intriguing sequence motifs that link this gene family to a web of protein regulators.

Sequence relationships

The LOV sequence near the amino terminus of ZTL (Figure 1a) is closely related to the LOV domains of two

Figure 1



(a) Domain structure of ZTL family proteins. The PAS-like LOV domain (yellow), the F box (blue) and six kelch repeats (pink) are shown, together with their potential interaction targets. The LOV domain may bind a flavin chromophore or other proteins (perhaps photoreceptors). In some yeast proteins, the F box mediates interaction with Skp1p, resulting in assembly of an E3 ubiquitination complex and consequent multi-ubiquitination of target proteins. Targets of the ZTL family might be brought into the complex by interaction with the kelch domains. **(b)** The ZTL proteins may act as light-sensitive adaptors, either nuclear or cytoplasmic. The colours and postulated functions of the kelch domains (pink) and F box (blue) are in (a). The multiple complexes of the ubiquitination and proteasomal degradation machinery are shown as wastebins. Potential targets for degradation include phytochrome photoreceptors (Phy) and inhibitors (Inhib) of phytochrome translocation or of light-regulated gene activation (similar negative elements exist in clock-associated feedback loops in other species).

blue-light photoreceptors — *Arabidopsis* phototropin and an unusual fern phytochrome — and the single LOV domain of the fungal *white collar 1* (*wc-1*) product. The photoreceptors' LOV domains bind the blue-light-absorbing flavin chromophore [9]. Wc-1 is a transcription factor that mediates blue-light responses and its LOV domain was shown to be required for dimerisation of the protein. Dimerisation is also an important function of PAS domains — a motif closely related to LOV — of non-photoreceptor proteins that are critical in the insect and mammalian clock feedback loops. So might all clocks include a LOV/PAS protein, suggestive of an ancestral mechanism? One possible scenario would replace the early photoreceptor function of the LOV domain with LOV-mediated binding to separate photoreceptor proteins — phytochromes, for example, which have PAS domains.

The central portion of these proteins shows sequence homology to the F box (Figure 1a) [10]. The F box was originally identified in yeast cell-cycle proteins by its interaction with Skp1p, a component of E3 ubiquitination complexes. F boxes are present in a large family of eukaryotic proteins that are thought to act as substrate-specific adaptors, promoting the conjugation of ubiquitin to substrate proteins that are then degraded by the proteasome. Accordingly, F-box proteins carry various protein interaction domains, some including so-called kelch repeats. Previously identified plant F-box proteins are implicated in floral development and hormone signalling, and may also control protein degradation. The key function of F-box proteins is to broker a tripartite protein interaction, however, and this ability could be adopted to mediate processes other than proteolysis [10].

The carboxyl half of each ZTL family protein contains six kelch repeats (Figure 1a). In proteins whose structure has been determined, each kelch repeat has been found to form a blade of the squat, cylindrical structure known as a beta-propeller [11]. The superfamily of beta-propeller proteins is diverse and the propeller has often to mediate interaction with other proteins. The *ztl* mutations substitute a conserved residue in the first and third kelch repeats, indicating that the kelch domain is important for *ZTL* function. Given their combination of sequence motifs, the idea that ZTL proteins have a function in degrading target regulatory proteins is attractive, but raises some major questions. Would such a degradation function be active throughout the circadian cycle? Does light affect its activity? What proteins are the targets and in which subcellular compartment do they reside?

Functional puzzles

Protein turnover should *a priori* be crucial for a clock that involves rhythmic protein accumulation, but as yet no F-box proteins have been specifically implicated in other species' clocks. In *Drosophila*, for example, the feedback protein Timeless is phosphorylated by the Doubletime protein kinase [3], prior to light-dependent proteolysis by the ubiquitin–proteasome system [12]. This system is essential for timing, because *doubletime* null mutant flies are arhythmic. The ZTL family might promote the turnover of proteins of a similar negative feedback loop (Figure 1b). The loss-of-function *ztl* mutation lengthens the period of the clock by up to 10 hours in low light levels but by only 3 hours under high light [1]. The long period might be expected if rhythmically active proteins are slow to degrade, but the effect is evidently less severe than in *doubletime* mutant fruitflies. Degradation might be promoted by light, which partially rescues the mutant phenotype. Blue and red light shorten the period equally

in *ztl* mutants [1], suggesting that ZTL is not a specific blue-light photoreceptor, but functions downstream of multiple photoreceptor pathways. The *fkf* mutation, in contrast, affects the rhythmic waveform of the varying *CCA1* and *CAB* RNA levels, but not their period [2].

Phototransduction components might be affected by the *ztl* and *fkf* mutations, as the mutant plants have short hypocotyls and late flowering phenotypes suggestive of phototransduction defects. But the clock itself also affects these traits and the light activation of *CAB* is normal in *ztl* mutants, again suggesting that ZTL and FKF may not be directly involved in phototransduction. The generation of multiply-mutant plants may be required to resolve these issues, because the expression patterns of the *ZTL* gene family overlap and their products may be to some extent redundant. *FKF* and *ZTL* RNAs are present in most organs throughout plant development [2]. *ZTL* is expressed arhythmically and without obvious light responses [1] but *FKF* expression is evening-specific and light-activated [2]. Do increasing light levels partially rescue *ztl* mutants by increasing *FKF* expression? Several clock-associated functions are carried out by the products of multi-gene families in *Arabidopsis* — there are five phytochrome genes and at least seven *CCA1*-like proteins, for example — so such regulatory interactions might be common.

Nuclear solutions?

Cellular and molecular data, in contrast, tend to simplify the picture. The phytochrome B photoreceptor has been shown to translocate into the nucleus, where it interacts with the DNA-binding protein PIF3 [13]. PIF3 can bind with activated phytochrome to light-regulated promoter sequences, including the *CCA1* promoter [14]. CCA1 and related proteins bind to the promoters of *CAB* and other clock-regulated genes, so the light signal may pass from photoreceptor to light-regulated promoters in two steps at most. The list of potential targets for the ZTL family may be correspondingly short.

Enter regulated proteolysis. A second transcription factor, HY5, also binds and activates transcription from light-responsive promoters. A recent study [15] suggests that HY5 is targeted for proteasomal degradation by COP1, a WD40 protein that may function as an E3 ubiquitin ligase. Mutations of the *COP1* gene cause a much more severe phenotype than single mutations of the *ZTL* family genes. If both systems control the stability of nuclear phototransduction proteins, it will be extremely interesting to find how their specificity differs, particularly if COP1 operates slowly and the ZTL family proteins invoke a more rapid or transient degradation.

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