THE ROYAL SOCIETY

doi 10.1098/rstb.2001.0965

Clock-associated genes in *Arabidopsis*: a family affair

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The identification of components of the plant circadian clock has been advanced recently with the success of two forward genetics approaches. The *ZEITLUPE* and *TOC1* loci were cloned and each was found to be part of two separate, larger gene families with intriguing domain structures. The ZTL family of proteins contains a subclass of the PAS domain coupled to an F box and kelch motifs, suggesting that they play a role in a novel light-regulated ubiquitination mechanism. TOCl shares similarity to the receiver domain of the well-known two-component phosphorelay signalling systems, combined with a strong similarity to a region of the CONSTANS transcription factor, which is involved in controlling flowering time. When added to the repertoire of previously identified clock-associated genes, it is clear that both similarities and differences with other circadian systems will emerge in the coming years.

Keywords: circadian timing; *Arabidopsis*; *ZEITLUPE*; *TOC1*; *APRR1*; gene family

1. INTRODUCTION

The recent cloning of two period-affecting loci from *Arabidopsis* (*ZEITLUPE* (*ZTL*) and *TOC1*) has added new players to the cast of characters known to contribute to circadian cycling in plants. Each is related to two separate and diverse classes of proteins with known functions in both animal and plant systems, but with no previous associations with the circadian clock. Furthermore, each is a representative of two separate families of closely related genes in *Arabidopsis*, which previously had no known function. Incorporating the members of both gene families into the still unclear picture of the plant circadian system will be a major challenge for the next few years. This review attempts to put these recent developments into perspective.

2. THE *ZTL* GENE FAMILY: *ZTL*, *FKF1* AND *LKP2*

The three members of the *ZTL* family share a remarkably high degree of protein sequence identity. A 70–80% amino-acid identity extends evenly along the entire protein, with regions of strongest dissimilarity found only at the extreme N-terminus and in interdomain regions (Nelson *et al.* 2000; Somers *et al.* 2000). *ZTL* (also referred to as *LKP1* (Kiyosue & Wada 2000)) and *FKF1* were identified initially from distinctive mutant phenotypes, whereas *LKP2* (also referred to as *FKL1* (Nelson *et al.* 2000)) is known only from genomic sequence similarity and from unpublished gene expression data (M. Wada, personal communication; S. Kay, personal communication). The focus here will be on the potential roles of *ZTL* and *FKF1* in the plant circadian clock.

(a) *Mutant phenotypes*

(i) *ZTL*

The identification of the *ZTL* locus came from a screen for period-length mutants using a non-invasive luminescence-based assay (Millar *et al.* 1995). One codominant mutant allele (*ztl-1*) was recovered that lengthens the free-running period in white light from 24.5 h to 27.5 h. Period is similarly lengthened under high-intensity red light (RL) or blue light (BL), and increases to 8 or 9 h longer than wild-type (WT) when plants are free-run at low light intensities. Further characterization showed that the clock-regulated expression of two differently phased genes (*CAB2* and *CCR2*) is similarly affected by *ztl-1*, linking their circadian cycling at the nuclear level to at least one common element. Leaf-movement rhythms, probably a manifestation of differential clock-regulated cell expansion, are also longer than WT. Flowering time in *ztl-1* is significantly later than WT in long days, but similar to WT in short days, consistent with an altered circadian timing (Putterill, this issue). Taken together these results indicate that *ztl-1* acts pervasively on a wide range of clock-controlled phenotypes in plants (Somers *et al.* 2000).

Less clearly connected to clock function is the shortened hypocotyl length that the *ztl-1* mutant displays under continuous RL. When linked to circadian clock function, previous reports have shown that hypocotyls are longer than WT in arrhythmic backgrounds (*cca1*, *lhy*, *elf3-1*) (Hicks *et al.* 1996; Schaffer *et al.* 1998; Wang & Tobin 1998), but the correlation between longer period and shorter hypocotyl in *ztl-1* is unprecedented. To confound further a simple explanation, hypocotyl elongation in the *ztl-1* mutant is WT when grown under BL, despite the strong light-dependent effect of the mutation on period length in both RL and BL. Clearly, *ZTL* plays at least partially differing roles in the phototransduction pathways controlling cell expansion and circadian period.

One caution in the interpretation of the *ztl-1* phenotype lies in the uncertain nature of the mutation. The mRNA abundance of *ztl-1* is normal, although the level of *ztl-1* protein is unknown. The strong lengthening of period at low light intensities, relative to WT, is similar to that seen

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in some phytochrome (PHY) and cryptochrome (CRY) null mutants (Somers *et al.* 1998*a*), suggesting that *ztl-1* may be acting to diminish the strength of light signalling to the clock. The phenotype of the *ztl-2* mutation is very similar to *ztl-1*, and both arise from single amino-acid changes in two highly conserved domains. Although based on the domain structure of the protein (see figure 1) the general mode of action of *ZTL* has been surmised, the actual partners in this process are still unknown. Hence, the known phenotypes are consistent with *ztl-1* acting as either a gain-of-function or loss-of-function allele. Constitutive overexpression of *ZTL* does not help resolve this question: the late flowering and long hypocotyls (Kiyosue & Wada 2000; Nelson *et al.* 2000; D. E. Somers, unpublished data) of overexpressing lines are not easily reconciled with the late flowering and WT (or short in RL) hypocotyl phenotype of *ztl-1*. The identification of a null mutant could help end much of this uncertainty.

(ii) *FKF1*

FKF1 (flavin-binding, kelch repeat, F box) is a closely related homologue of *ZTL*. A deletion mutant (*fkf1*) was originally identified as causing late flowering only in long days (Nelson *et al.* 2000), very much like *ztl-1*. As with *ztl-1*, *fkf1* shows short hypocotyls in RL, but a similar shortening is also seen in BL. Circadian period length is unaffected by this mutation, although there may be a slight effect on the phasing of certain clock-controlled transcripts. The *fkf1* mutation results from a 65 kb deletion, and the mutant phenotypes are rescued by transgenic complementation with *FKF1*. A direct comparison between *ZTL* and *FKF1* gene function is hindered by the absence of equivalent alleles for the two genes. Although both genes appear to play some role in controlling flowering time, it is unclear whether they act through the same pathway or even the same mechanism.

(b) *Predicted protein structure*

The highly conserved predicted protein structure of the *ZTL* gene family is striking in the way that three previously identified protein motifs have been assembled into a unique combination of domains (figure 1). These motifs, the PAS–LOV domain, the F box and the kelch repeat, have been extensively studied in the context of better-characterized proteins. Consideration of this previous work can now aid in suggesting a role for the ZTL protein family in the circadian clock and in light-signalling pathways in general.

(i) *PAS–LOV domain*

Near the N-terminus of the ZTL family proteins lies a 110 amino-acid region that bears strong similarity to the recently described LOV domain. The LOV domain was originally coined to describe two closely similar regions in the *NON-PHOTOTROPIC HYPOCOTYL* (*NPH1*) gene, that codes for a protein involved in BL-induced phototropism in plants (Huala *et al.* 1997). This region was defined based on similarity to a number of prokaryotic and eukaryotic proteins involved in light, oxygen and voltage sensing (hence *LOV* (Huala *et al.* 1997)). Upon further sequence comparisons it has become clear that the LOV domain is a distinct subgroup of the more general PAS motif. This domain was originally defined by motifs found in three proteins: the *Drosophila* clock protein period (PER), the vertebrate aryl hydrocarbon receptor nuclear translocator (ARNT), and *Drosophila* single-minded protein (SIM) (Taylor & Zhulin 1999). With its greater expansion into a superfamily, the PAS domain has become difficult to characterize based solely on primary amino-acid sequence. In a cross-kingdom, multiple alignment of more than 300 PAS domains from more than 200 proteins only two glycines are retained as specifically named residues in the emergent consensus sequence (Taylor & Zhulin 1999). Despite this lack of primary sequence conservation the superimposition of the three-dimensional models of three PAS domains, based on the crystal structures from three unrelated proteins (PYP, HERG and FixL), reveals a remarkable structural similarity shared among these proteins (Pellequer *et al.* 1999). One contributing factor to this appears to be the conservation among the three of a common core of *ca.* 20 non-polar residues that occupy structurally equivalent sites (Perutz *et al.* 1999) (figure 1*a*). These participate in the formation of a pocket or fold created by a β-sheet flanked by α-helices (Pellequer *et al.* 1999). In some cases this pocket is occupied by a small molecule (haem in FixL and 4-hydroxycinnamoyl in PYP), or in other molecules (e.g. ARNT) this region may be available to accommodate large, internally positioned, hydrophobic residues (Pellequer *et al.* 1998).

This superfamily can be divided into two fundamental classes: those involved in mediating protein–protein interactions and those more primarily involved in environmental sensing (Crews & Fan 1999). Until the identification of the *ztl-1* mutation, only the *Neurospora* WC-1 protein connected environmental input with circadian cycling (Crosthwaite *et al.* 1997). All other PAS-containing clock proteins lie within the first class, with the PAS regions most often facilitating heterodimer formation between transcription factors (Dunlap 1999). Here I shall refer to the PAS regions of the ZTL family and a second group of signalling molecules as the PAS–LOV domain to emphasize their distinct relationship.

The eukaryotic proteins with PAS motifs most similar to the ZTL PAS–LOV domain also share in common a role in BL signalling (figure 1*a*). The *white collar-1* (*wc-1*) mutants in *Neurospora* are blind to all BL-mediated processes, including light-mediated resetting of circadian cycling (Ballario *et al.* 1996; Crosthwaite *et al.* 1997). WC-1 is a putative transcription factor, possessing one PAS–LOV, one PAS domain, and a zinc-finger DNA-binding motif. Three different single amino-acid mutations in the LOV domain eliminate BL sensing in WC-1 (Ballario *et al.* 1998) and two of these residues are identical or conserved in the ZTL family.

A second related protein is NPH1 (phototropin), a BL-activated serine–threonine protein kinase involved in the primary events of BL-mediated phototropism in plants (Christie *et al.* 1998). NPH1 has two PAS–LOV domains and both can be successfully aligned with the PAS fold when the crystal structure of the voltage-dependent K channel HERG protein is used as a template (Salomon *et al.* 2000). Briggs and co-workers have shown that flavin mononucleotide is non-covalently bound by the NPH1 PAS–LOV domains and probably occupies the volume

created by the hydrophobic core of the PAS domain (Christie *et al.* 1999). There is a high degree of primary sequence conservation (*ca.* 40% identical) between the ZTL, NPH1 and WC-1 PAS–LOV domains, which includes most of the key hydrophobic residues conserved in the PAS domain (figure 1*a*). These parallels, together with the marked effect of the *ztl-1* mutation on BL signalling to the clock, strongly suggest that ZTL also binds a flavin. However, *ztl-1* also affects hypocotyl elongation and circadian period in RL. This suggests that either ZTL family proteins have a much wider absorption spectrum than other members of the PAS–LOV class, or that RL signalling through ZTL–FKF1 is first transduced via other RL-absorbing photoreceptors, such as the PHYs. However, until chromophore binding to ZTL–FKF1 is demonstrated, it is still possible that the PAS–LOV domain primarily facilitates protein–protein interactions in these proteins. Additionally, since the WC-1 PAS–LOV domain can homodimerize *in vitro* (Ballario *et al.* 1998), flavin binding and dimerization may not be mutually exclusive.

(ii) *The F box and kelch domains*

The remaining portion of the ZTL–FKF1 protein is likely to function as a biochemical unit. Evidence for this comes largely from the extensive characterization of the Skp1–cdc 53–F-box (SCF) ubiquitin ligase complex. This multimeric assembly is the primary means by which proteins targeted for proteolytic degradation by the 26S proteasome are ubiquitinated (see figure 1*b*). A key component of this complex is the F box protein, a group of very diverse proteins that share only a single conserved region (the F box) of *ca.* 45 amino acids. Through interaction with the Skp1 protein within the SCF complex, the F box provides the means by which proteins destined to be targeted for proteolytic degradation are anchored to the ligase assembly, thus facilitating their proper positioning for ubiquitin attachment. Although originally identified as cell-cycle mutants in yeast, F box proteins are involved in a wide range of developmental and signalling processes in plants and animals (Craig & Tyers 1999; Willems *et al.* 1999; Xiao & Jang 2000). We have recently confirmed that the ZTL F box region can indeed function as expected by showing interaction with an *Arabidopsis* Skp1 homologue (ASK1) in yeast two-hybrid interaction tests (M. Mason and D. E. Somers, unpublished data).

Much of the specificity of the SCF system derives from residues C-terminal to the F box, which interact with the protein target and probably aid in its final positioning in the SCF complex (Schulman *et al.* 2000). Typically these protein-interaction regions consist of WD40 domains and leucine-rich repeats (LRR) (see later figure 3*b*) (Adams *et al.* 2000). Similar domains have been identified in most of the nearly 40 F box proteins that have been identified in the *Arabidopsis* genome. Of these, the ZTL F box family is unique in containing a series of kelch repeats in the position of the potential protein-interaction domain (Xiao & Jang 2000). Kelch-containing proteins are found widely in animal systems, invariably positioned in the polypeptide to facilitate protein–protein contacts. Each kelch repeat forms a four-stranded β-sheet that assembles with the remaining repeats to create a β-propeller, similar to the three-dimensional structure formed by WD40 motifs (Adams *et al.* 2000) (figure 1*c*). Interestingly, both known ZTL mutations (*ztl-1* and *ztl-2*) contain single aspartate (D) to asparagine (N) amino-acid changes in the third and first kelch repeats, respectively. The phenotypes of both alleles are quantitatively similar (Somers *et al.* 2000), strengthening the notion that this domain is critical to ZTL function.

(c) *ZTL*: in vivo *function?*

Current evidence suggests that neither *ZTL* nor *FKF1* is a core element (state variables) of the plant circadian clock (Somers 1999). Although *FKF1* message cycles robustly, deletion of this gene has no effect on circadian period (Nelson *et al.* 2000). Conversely, *ZTL* message levels do not oscillate and are unaffected even by light–dark cycles (Somers *et al.* 2000). Most probably, the role of ZTL is ancillary to the primary feedback loops that are presumed to constitute the plant circadian system (Somers 1999). However, given the strong sequence similarity and lack of equivalent mutant alleles, it is possible that there is partial redundancy among the family members. This question will only be fully addressed by examining double and triple null-mutant combinations.

Taking together both the mutant phenotypes and the predicted domain structure of ZTL, we can broadly sketch an outline of how ZTL might interface with the circadian cycle. One model involves the ZTL F box protein in a light-dependent interaction with a substrate or substrates whose concentrations or activities directly affect circadian period. This association could come about through a photoinduced conformational change in ZTL, resulting in recognition of the appropriate substrate via the kelch repeats. Once transported to the SCF complex, the target substrate would be ubiquitinated and removed from the cycle via the 26S proteasome (figure 2).

This scheme is only one of many possible interactions between light, ZTL and the circadian cycle. Other possible scenarios include: (i) light-dependent nuclear localization (or exclusion) of ZTL protein; (ii) light-induced ZTL degradation (or stabilization); and (iii) light-dependent association (or dissociation) of ZTL with an SCF complex. Recent work using GFP::ZTL gene fusions have provided hints to support the first case (Kiyosue & Wada 2000). Since components of the proteasome have been detected in both the cytoplasm and the nucleus, the first and third possibilities could be linked. However, none excludes the other, and photic input may impinge on the plant clock through more than one pathway. For example, disruption of PHY and CRY phototransduction also lengthens free-running period (Somers *et al.* 1998*a*; Devlin & Kay 2000). The physical association between PHYA and the CRYs (Ahmad *et al.* 1998) and between PHYB and CRY2 (Mas *et al.* 2000) indicates that in some circumstances different photoreceptor classes may be tightly associated into 'photosomes', and signal transduction to the clock might proceed within complex protein assemblies that include photoreceptors. ZTL could participate in these complexes, targeting even the photoreceptors themselves for destruction. Alternatively, ZTL signalling to the clock could occur via a parallel pathway, and double-mutant analyses should help discriminate between these alternatives.

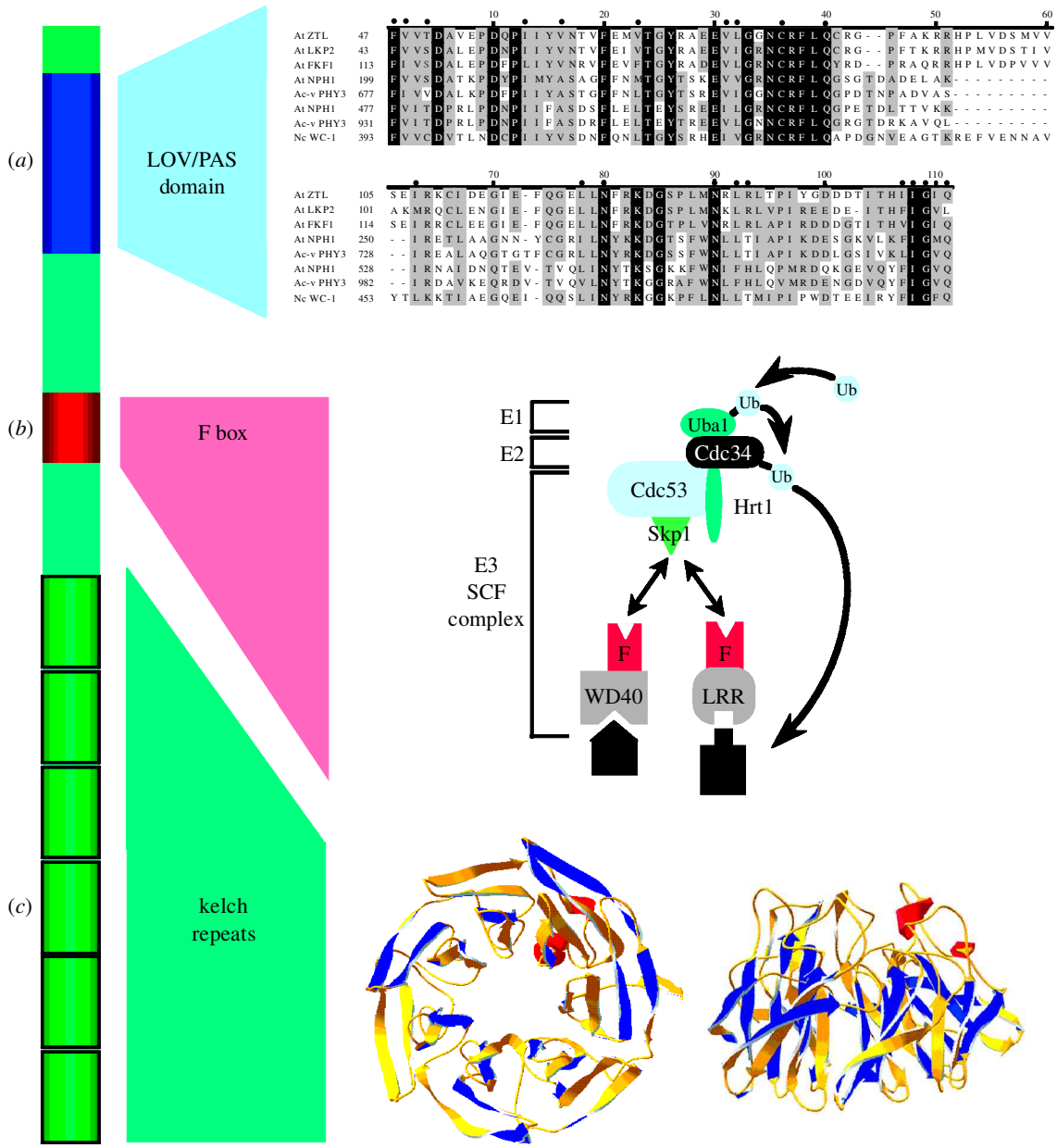

Figure 1. The relationship of the ZTL family protein motifs to previously known processes and structures associated with those domains. (*a*) Alignment of the ZTL family PAS–LOV domain with PAS–LOV domains from *NPH1*, a structurally similar molecule from a fern (*Adiantum*; *PHY3)* and the *Neurospora* BL-signalling protein *WC-1* (modified from Somers *et al*. 2000). (•) Indicates positions corresponding to the occurrence of conserved internal hydrophobic residues in crystallized PAS proteins, which are considered important for PAS pocket formation (Perutz *et al*. 1999). Identical residues are boxed in black; functionally conserved residues are shaded grey. (*b*) Schematic representation of the role of an F box protein in the ubiquitin ligation pathway. Ub, ubiquitin; E1, ubiquitin-activating enzyme (Uba1); E2, ubiquitin-conjugating enzyme (Cdc34); E3, ubiquitin ligase complex in which Cdc53, Hrt1 and Skp1 form a scaffold for the docking of various F box proteins (F-WD40, F-LRR) and their associated substrates (black) with Skp1. All nomenclature follows that of *Saccharomyces cerevisiae* according to Deshaies (1999). (*c*) End-on (left) and side view (right) of the β-propeller structure formed by the seven kelch repeats of galactose oxidase. See text (§2b) for details.

All previous work has shown that target recognition by F box proteins is strictly phosphorylation-dependent (Deshaies 1999). Hence, in the above schemes the substrate concentration, and consequently circadian period, could depend both on the light-dependent activation state of ZTL and on the phosphorylation state of the substrate(s). This is analogous to the relationship between light, TIM and PER in the *Drosophila* clock. There, phosphorylation of PER by *doubletime* (*dbt*), a casein kinase 1ε, destabilizes PER abundance, but heterodimeric association between PER and TIM acts to stabilize PER levels and facilitates nuclear import of the pair. TIM protein levels, in turn, are rapidly depleted by light, probably through direct interaction between the light-activated CRY photoreceptor and TIM (Ceriani *et al*. 1999; Young 2000; Rosato & Kyriacou, this issue). This degradation is preceded by phosphorylation and is proteasome-mediated (Naidoo *et al*. 1999). No F box

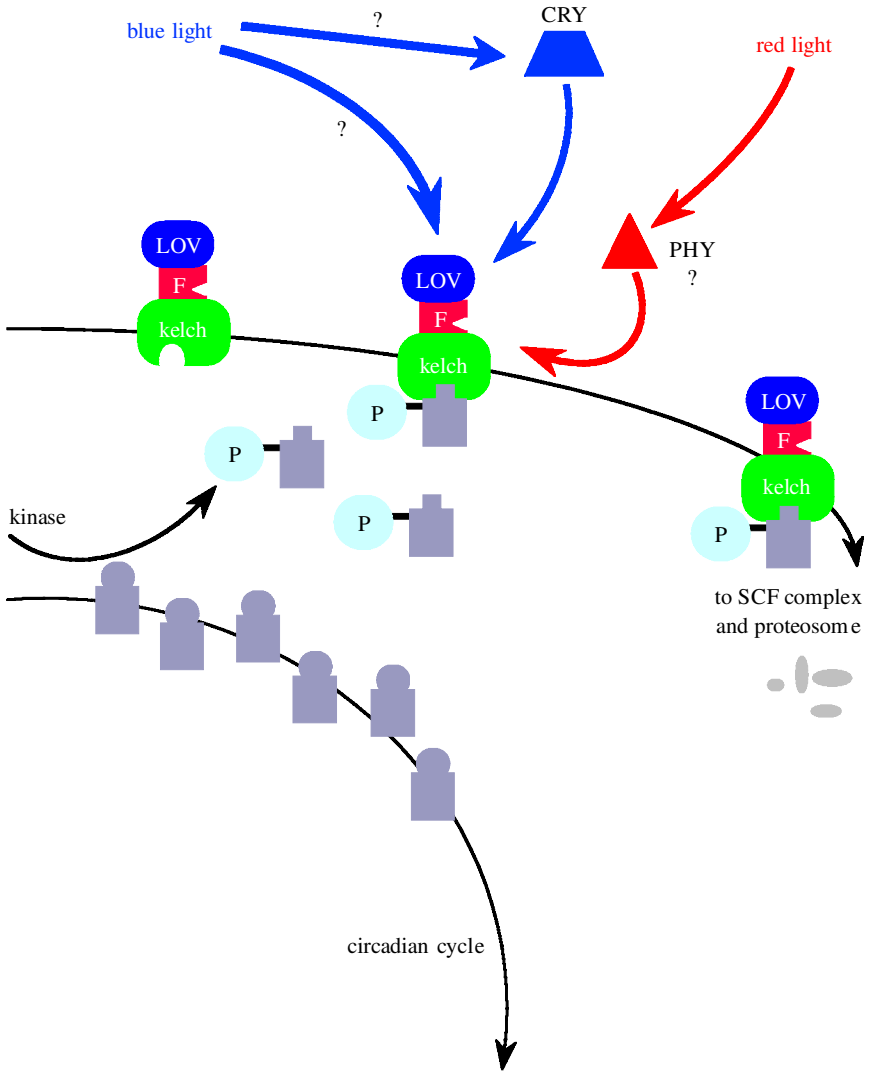

Figure 2. One working hypothesis of the proposed role of ZTL in light-activated ubiquitination of clock-associated substrates. ZTL is activated by BL or RL either by direct light absorption or indirectly, by other phototransduction elements (e.g. CRY and PHY). The light-modified conformation of ZTL can now interact with period-determining factor(s) (grey forms) of the circadian cycle. Interaction may depend on substrate phosphorylation (P). Association of the ZTL–substrate complex with the SCF complex results in substrate ubiquitination and ultimate destruction via the proteasome (grey ovals: degraded protein). The role of ZTL in setting circadian period would be directly correlated to its effect on the active concentration of the targeted substrates.

protein has yet been identified as an element of the circadian system in flies.

In plants, ZTL is poised to play the role of both a photoreceptor and F box protein. CCA-1, a myb transcription factor which shortens period when absent and causes arrhythmicity when overexpressed, can be phosphorylated by protein kinase CK2, which itself shortens period when overexpressed in plants (Sugano *et al.* 1998, 1999; Wang & Tobin 1998; Green & Tobin 1999). Clearly this story is far from complete, but a picture is emerging in which similar ends may be attained through remarkable parallels—and divergences.

3. THE *TOC1–APRR1* GENE FAMILY: *TOC1–APRR1*, *APRR3*, *APRR5*, *APRR7* AND *TL1–APRR9*

(a) *Mutant phenotypes*

(i) *TOC1–APRR1*

The first indication of any involvement of these family members with circadian period came from a single mutant recovered from same screen that identified *ztl-1* (Millar *et al.* 1995). The codominant *toc1-1* mutation shortens the circadian cycling of the *CAB2::luciferase* reporter from 24.5 h to *ca.* 21 h. Stomatal and leaf-movement rhythms are similarly shortened, and flowering time is significantly earlier than WT only in short days (8 L:16 D) (Somers *et al.* 1998*b*). The nearly equally rapid flowering of *toc1-1* in long (16 L:8 D) and short (8 L:16 D) days can be corrected when the length of the environmental light–dark cycle (normally 24 h) is adjusted to more closely match the 21 h period of the endogenous clock of *toc1-1*. Hence, photoperiod responsiveness is restored and *toc1-1* plants flower late when grown under a 7 L:14 D regime, but still flower early under 14 L:7 D. This result highlights the importance of coordinating endogenous timing with environmental cycles and clearly links photoperiodic timing of flowering to the circadian clock (Strayer *et al.* 2000). *TOC1–APRR1* appears integral to this process.

Unlike with *ZTL*, there is no indication that *TOC1–APRR1* is involved in a phototransduction pathway to

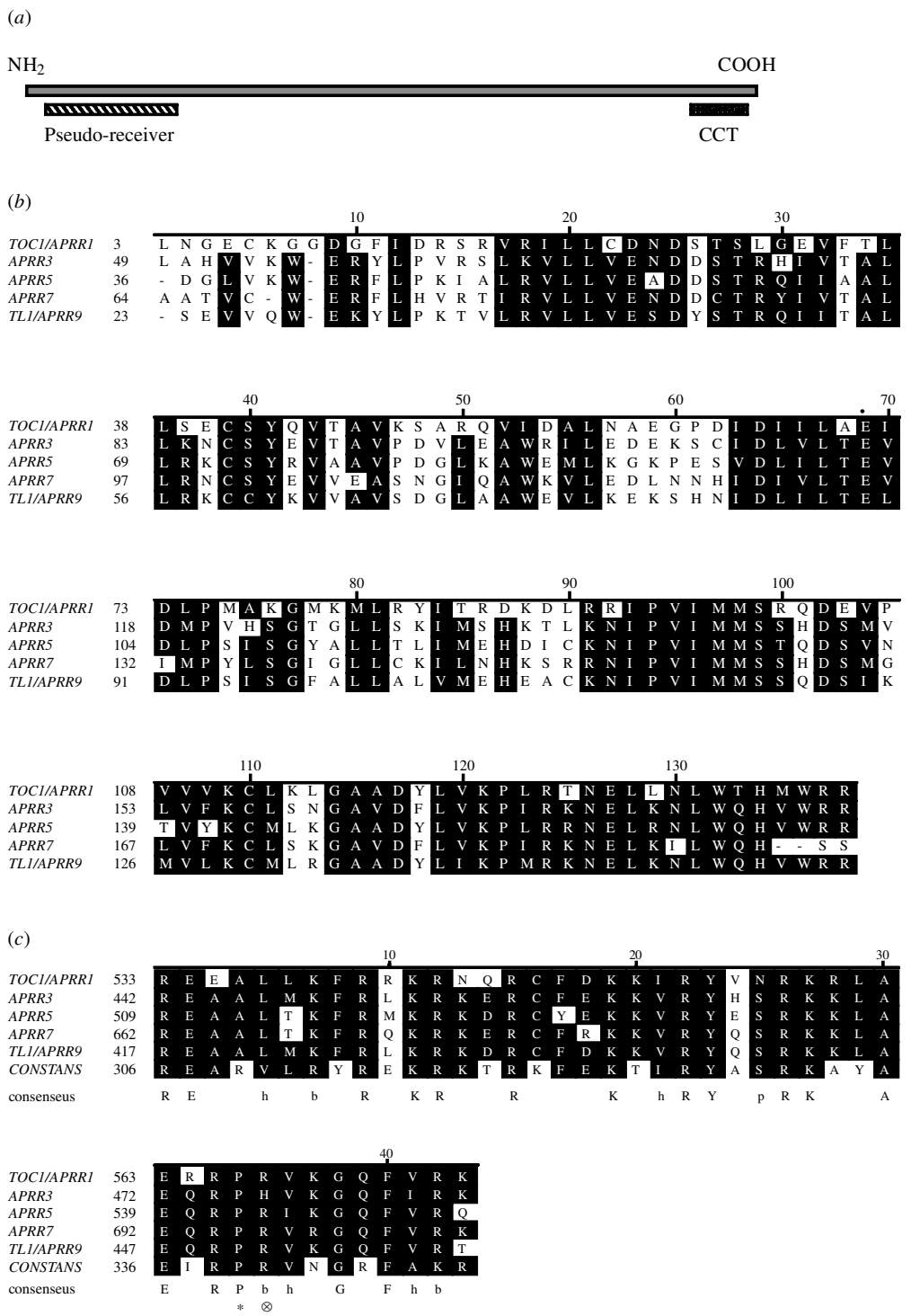

Figure 3. TOC1–APRR1 domain alignments. (*a*) Schematic representation of the positions of the pseudo-receiver and CCT domains present in all members of the *TOC1–APRR1* gene family. The length of each protein varies among the family members but the relative position of the two domains remains the same in each. (*b*) Alignment of the amino-acid sequence of the pseudo-receiver domains of the TOC1–APRR1 family. The position of the glutamate (E) that occupies the site of the aspartate (D) phosphoryl acceptor in two-component receivers is noted (•). (*c*) Alignment of the TOC1–APRR1 family and CONSTANS CCT domains. (*, ⊗) indicate site of *co-5* and *co-7* mutations, respectively. In (*b*, *c*), reverse contrast regions denote positions where four or more residues are functionally conserved. Consensus: h, hydrophobic; b, basic; p, polar and single-letter amino-acid abbreviations.

the clock. Over a wide range of light intensities the period of *toc1-1* remains uniformly 3 h shorter than WT (Somers *et al.* 1998*b*) and is similarly shortened in constant darkness (Strayer *et al.* 2000). Consistent with the putative nuclear localization signal present near the C-terminus, *TOC1–APPR1*::green fluorescent protein fusions are constitutively localized to the nucleus (Makino *et al.* 2000; Strayer *et al.* 2000). There are no known effects of *toc1* mutations on hypocotyl length or other photomorphogenic processes, suggesting that this

gene is truly dedicated to the control of circadian timing.

Additionally, *TOC1–APRR1* message levels undergo robust circadian cyclings, which are shortened in *toc1* mutant backgrounds. This feedback effect on self-expression fulfils one of the canonical features of a state variable of the clock, but much more information is needed to place *TOC1–APRR1* in the central oscillator. For example, RNA expression data suggest that neither of the two *TOC1* mutant alleles is null. A non-redundant oscillator component should cause arrhythmicity when absent or held at constant levels. Identification of a knockout mutant and overexpression data will help position *TOC1* in the circadian system.

(b) *Predicted domain structure*

Compared with the ZTL protein family, an alignment of the predicted amino-acid sequence of the five members of the TOC1–APRR1 group shows a much more restricted range of sequence similarity. One region near the N-terminus and a second near the C-terminus are conserved among the five proteins, separated by dissimilar sequences of variable length (figure 3*a*). The region of most striking interest is the N-terminal pseudo-receiver motif, which is highly similar to the receiver domain found in two-component signalling systems. From prokaryotes to higher plants, many receptor–ligand signal-transduction chains initiate activity upon ligand-induced receptor phosphorylation. Subsequent signal transduction proceeds by multistep, sequential phospho-transfers that alternate between histidine and aspartate residues (H-to-D) along a chain of one or more intermediates. The phosphorelay process terminates on the final receiver domain which effects the relevant action (D'Agostino & Kieber 1999; Urao *et al.* 2000). While ethylene sensing was the first recognized example of a phosphorelay signalling system in plants, cytokinin signalling and osmosensing also rely on two-component signalling, and a large number of unassigned two-component homologues have been identified in the *Arabidopsis* genome (Urao *et al.* 2000).

The critical difference between the classic receiver domain and the pseudo-receiver motif found in the TOC1–APRR1 family is the marked absence of a conserved aspartate residue universally present in true receiver domains (figure 3*b*). This residue, which serves as the terminal phosphoryl acceptor, is occupied by a glutamate (E) in all family members (Matsushika *et al.* 2000; Strayer *et al.* 2000). Interestingly, a directed D-to-E change at this site in response regulators very often leads to a constitutively active molecule, with the glutamate presumably mimicking the action of a phosphorylated aspartate (Klose *et al.* 1993; Makino *et al.* 2000). The *toc1-2* splice-site mutation occurs in the pseudo-receiver domain (Strayer *et al.* 2000).

The second region of sequence conservation lies near the C-terminus (figure 3*a*,*c*). This 45 amino-acid stretch is rich in basic residues and shares a high degree of amino-acid similarity with a motif found in the *CONSTANS* (*CO*) gene family (Makino *et al.* 2000); Strayer *et al.* 2000). *CONSTANS* and *CONSTANS-LIKE* (*COL*) genes appear to encode zinc-finger transcription factors, and the CONSTANS protein promotes flowering in *Arabidopsis* (Putterill *et al.* 1995). When the *TOC1–APRR1* CCT (CONSTANS, CONSTANS-LIKE and TOC1) domains are aligned with the *CO* CCT domain, the six gene consensus is rich in basic arginine and lysine residues (figure 3*c*) and includes a putative nuclear localization motif. Additionally, point mutations at two other positions correspond to *co* mutants (*co-5*, *co-7*) which affect flowering time (Robert *et al.* 1998; Strayer *et al.* 2000). A yeast two-hybrid screen for interactors with ABI3, a major regulator of *Arabidopsis* embryo development, recovered TOC1–APRR1 (Kurup *et al.* 2000). This interaction was specific for the CCT domain and introduction of the two *co* mutations diminished reporter gene activity. The *toc1-1* point mutation also lies in the CCT domain (Strayer *et al.* 2000).

To date, only mutations in *TOC1–APRR1* have been reported, and the intense interest in this gene family comes from the clear importance of *TOC1* in the circadian system coupled with the very intriguing circadian expression patterns displayed by all members of the family.

(c) *The* TOC1–APRR1 *family: expression characteristics*

The role, if any, of the remaining four family members in the circadian clock is unclear, as they are only known from their expression patterns. Together with *TOC1–APRR1*, they each display robust circadian cycling of transcript abundance with each peak sequentially offset from the previous and next by nearly equal time-intervals. The peak expression of all members occurs during the subjective day, with *TL1–APRR9* peaking soon after dawn (*ca.* ZT3) followed by *APRR7*, *APRR5* and *APRR3* spaced *ca.* 2–3 h apart. *TOC1–APPR1* peak expression comes last in the series, nearest subjective dusk (Matsushika *et al.* 2000; Strayer *et al.* 2000) (figure 4). With the exception of *TOC1–APPR1*, most of the expression of the other family members occurs during the photoperiod even under short days (8 L:16 D). *TOC1–APRR1* mRNA levels show a minor peak in the middle of the skotoperiod in a 12 L:12 D cycle, which increases in amplitude during short days (Matsushika *et al.* 2000; Strayer *et al.* 2000). The protein expression patterns are still unknown for all of these genes.

(d) *The* TOC1–APRR1 *family:* in vivo *function?*

The question of knowing what time it is comes down to establishing an internal timing reference mechanism that is itself tied to one or more environmental cues (Somers 1999). Mizuno and co-workers (Matsushika *et al.* 2000) propose that the relative expression levels of the suite of five *TOC1–APRR1* family members could provide the basis for such a self-referential mechanism. By simultaneously assessing the relative expression levels of all five genes at a given time, the plant could uniquely mark that point in the circadian cycle by virtue of the sequentially staggered peaks of expression. This could provide the basis for understanding how order in a nearly continuous gradient of differently phased gene expression can be sustained (Harmer *et al.* 2000). Although the mechanism by which such a measurement system operates is far from clear, TOC1–APPR1 interacts in the two-hybrid assay with a myc-related nuclear protein that contains a bHLH motif very similar to phytochrome interacting factor 3 (PIF3)

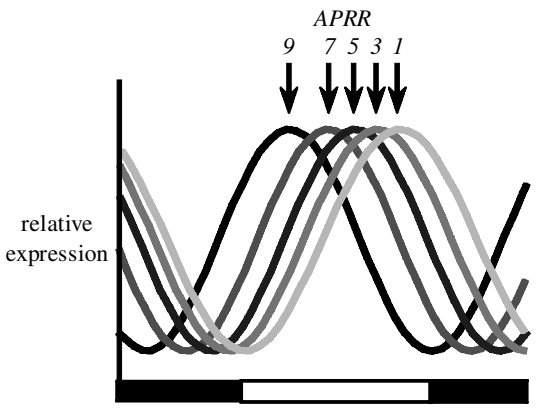

Figure 4. Highly idealized expression pattern of the mRNA levels of the *TOC1–APRR1* gene family under a 12 L (white bar):12 D (black bar) cycle. The peak expression times are similar to that reported for the respective gene indicated (Matsushika *et al*. 2000), but the overall waveforms are more variable. Based on Matsushika *et al*. (2000).

(Makino *et al*. 2000). PIF3 interacts with PHYB and binds the *CCA1* promoter, the latter protein known to cause arrhythmicity when overexpressed. Similar PIF3-binding sites are present in the *TL1–APPR9* promoter (Matsushika *et al*. 2000). These intriguing associations hint at what direction the next set of experiments may go.

The above model predicts that the loss (knockout) of any one of the family members should so offset the timing scheme that the phase of all clock-controlled outputs is altered. A corollary to this view is that the *TOC1–APRR1* family members do not *a priori* need to participate in the primary mechanism and sustenance of the circadian clock itself. As a group, these could simply be part of the phase-determining mechanism that links overt rhythms to the central oscillator. Hence, knockouts would not affect period length. However, since the *toc1-1* and *toc1-2* mutations clearly alter the free-running period, this simple scheme is flawed. The *TOC1–APRR1* expression pattern is the only one of the group that shows marked differences under different photoperiods, and it stands most separate from the other family members in a phylogenetic analysis (Matsushika *et al*. 2000). Possibly, the role of this protein differs fundamentally from those of the others in the group.

The circadian phenotype of *toc1-1*, together with the closely staggered temporal spacing of the peak expression of the five *TOC1–APRR1* family members, makes their inclusion into the primary clockwork in plants appear attractive. However, it is clear that further elimination and modification of each family member will be necessary to conclusively place them as a group into the *Arabidopsis* circadian system.

4. CONCLUSIONS

The emergence of the *ZTL* and *TOC1-APRR1* gene families as probable key players in the plant clock brings to the fore a theme that has been percolating in the background for some time now, that is the role of families of closely related proteins as key players in the plant circadian system. Plants recruit two CRYs and four or more PHYs to mediate light input to the clock; fruitflies use a single CRY and an opsin. *CCA1* and *LHY*, both clearly involved in the plant circadian clock (Schaffer *et al*. 1998; Wang & Tobin 1998), are members of a much larger myb family, the *REVEILLE* genes, which exhibit circadian cycling (Andersson *et al*. 1999). By comparison, the two bHLH–PAS transcription factors in the fly and mammalian clock act as a single heterodimer (Reppert & Weaver 2000). Now the three-member *ZTL* family and the five-member *TOC1–APRR1* gene family must be factored into the plant circadian equation. Possibly, this multiplicity of factors is simply a safeguarding redundancy to ensure circadian clock function. Alternatively, each family may serve as a reservoir of possibilities, with different members recruited as necessary to make subtle alterations in the face of the unpredictable environmental changes to which sessile organisms must adjust. In any case, plants clearly have devised a new variation on the theme of 'an autoregulatory negative feedback loop'.

I would like to thank Steve Kay (The Scripps Research Institute, La Jolla, CA, USA) and Masamitsu Wada (Tokyo Metropolitan University) for sharing unpublished results.

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