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ZEITLUPE Encodes a Novel Clock-Associated PAS Protein from *Arabidopsis*

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Summary

We have conducted genetic screens for period length mutants in *Arabidopsis* using a transgenic bioluminescence phenotype. This screen identified mutations at a locus, *ZEITLUPE* (*ZTL*), that lengthen the free-running period of clock-controlled gene transcription and cell expansion, and alter the timing of the daylength-dependent transition from vegetative to floral development. Map-based cloning of *ZTL* identified a novel 609 amino acid polypeptide consisting of an amino-terminal PAS domain, an F box and six carboxy-terminal kelch repeats. The PAS region is highly similar to the PAS domain of the *Arabidopsis* blue-light receptor *NPH1*, and the *Neurospora* circadian-associated protein WHITE COLLAR-1 (WC-1). The striking fluence rate-dependent effect of the *ztl* mutations suggests that ZTL plays a primary role in the photocontrol of circadian period in higher plants.

Introduction

The circadian clock is a timing mechanism that helps coordinate metabolism and physiology with the daily cycles of light and dark. The importance of this type of temporal regulation is substantiated by the near universal presence of a circadian system in eukaryotes (Dunlap, 1999) and in some prokaryotes (Golden et al., 1997). Heuristically, the circadian clock can be modeled as a central oscillator linked to the environment by an input signaling pathway and acting, through an output pathway, to confer rhythmicity on a wide range of processes.

Genetic screens for period length mutations have identified genes that appear to lie within the input pathway and the central oscillator (Dunlap, 1999). Studies of the temporal expression patterns of these loci have converged to support a molecular model of the central oscillator that is based on an autoregulatory negative feedback loop. In broad outline, this strategy employs negatively acting and positively acting factors that alternate in abundance by modulating their own gene expression (Dunlap, 1999). While the basic mechanism of a negative feedback loop has been conserved among all known systems, different individual components have often been recruited to fill a position in the loop.

Several types of transcription factors have been identified that function in circadian systems as positively acting factors. These include a bZIP transcription factor (VRILLE) in flies (Blau and Young, 1999), bHLH-PAS transcription factors (CLOCK, BMAL) in mammals and flies, and putative Zn finger PAS domain DNA binding proteins in *Neurospora* (WHITE COLLAR, WC-1; WHITE-COLLAR-2, WC-2) (Dunlap, 1999). In all cases the PAS domain facilitates the formation of heterodimers (CLOCK/BMAL) (Allada et al., 1998) or both hetero- and homodimers (WC1 and WC2) (Ballario et al., 1998). These positively acting factors play a crucial role as transcriptional activators of oscillator components, and then as targets of the same negatively acting factors whose transcription they promote. In plants, single myb domain DNA binding proteins (*CCA-1, LHY*) have been associated with clock function. Overexpression causes arrhythmicity; (*CCA-1, LHY*) and loss of function results in period shortening (*CCA-1*) (Schaffer et al., 1998; Wang and Tobin, 1998; Green and Tobin, 1999).

Protein kinases form a second class of clock-associated proteins. Mutations in *double-time*, a *Drosophila* locus that encodes a casein kinase 1ε, can alter the phosphorylation state of the key oscillator component, PERIOD (PER), to either lengthen or shorten period (Kloss et al., 1998; Price et al., 1998). Protein stability of oscillator components is likely to be to be a key parameter in determining period length and sustaining rhythmicity of the circadian clock. The phosphorylation state of PER strongly affects its stability, suggesting a mechanism by which *double-time* mutants alter period length in *Drosophila*. In *Arabidopsis*, a protein kinase CK2 has been identified as a CCA1-interacting protein that directly phosphorylates CCA1 in vitro, and overexpressing CK2 shortens period (Sugano et al., 1998, 1999).

The role of the flavoprotein, cryptochrome (CRY), in the circadian clock has developed differently among systems. In *Drosophila* (Emery et al., 1998; Stanewsky et al., 1998) and plants (Somers et al., 1998a), cryptochrome acts as a blue light circadian photoreceptor, consistent with its close homology to DNA photolyases (Cashmore et al., 1999). In *Drosophila* CRY forms a light-dependent physical association with the key negatively acting factor *timeless* (TIM), demonstrating a close relationship between photoperception and the oscillator (Ceriani et al., 1999). CRY1 and CRY2 in mouse are also intimately involved in negative feedback in the core oscillator, but act instead as transcriptional inhibitors of CLOCK-BMAL-activated transcription, and have no effect on light-induced expression (Okamura et al., 1999).

Circadian clocks are also comprised of novel genes that possess no previously characterized motifs. All three of the key elements of the circadian clock in cyanobacteria (the *kai* genes) are novel, as is the negatively acting *frequency* gene from *Neurospora* (Dunlap, 1999).

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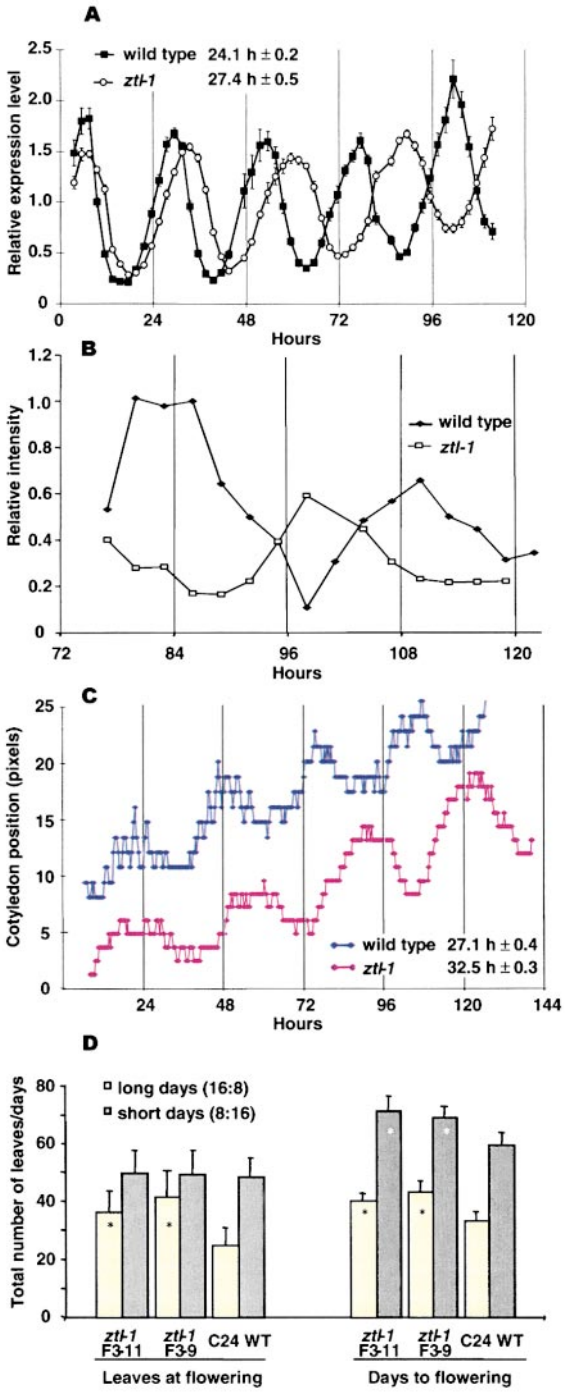

Figure 1. Mutations at *ZTL* Alter Multiple Circadian Outputs

(A) *ztl-1* lengthens the free-running rhythm of *CAB2::luciferase* expression in white light. Seedlings were entrained in white light/dark cycles (12 h:12 h) for 5 days before shifting to continuous white light (30 μmol m^{-2} s^{-1}) at time 0. Data (± SEM) are normalized to the mean luminescence value of the respective genotype over the length of the time course. Variance weighted mean period (± variance weighted SEM) for WT (n = 17) and *ztl-1* (n = 18) were calculated as described (Millar et al., 1995b; Plautz et al., 1997).

(B) *ztl-1* lengthens the free-running rhythm of *CCR2* mRNA abundance. Wild type and *ztl-1* plants were entrained as in (A) for 20 days, allowed to free-run in white light for 77 hr and harvested at 3 hr intervals over the time course indicated. RNA blots were hybridized with a *CCR2* DNA probe and quantitated relative to rRNA levels using an rDNA probe.

(C) *ztl-1* lengthens the free-running rhythm of cotyledon movement. Seedlings were entrained as in (A) for 7–9 days then shifted to continuous white light (17–22 μmol m^{-2} s^{-1}). Shown are representative traces selectively aligned according to their first peaks to illustrate the difference in free-running period. Values are period estimates of the respective traces obtained using an FFT-NLLS best fit algorithm (± 0.5 · CI) as described (Millar et al., 1995b; Plautz et al., 1997).

(D) *ztl-1* delays flowering. Total number of rosette and cauline leaves (± SD) produced by the end of flowering and the number of days to flowering (1 cm high bolt) under long days (16:8) and short days (8:16) for wild type (C24 ecotype) and two 5× backcrossed populations of *ztl-1*. * indicates mean value is significantly different from WT ($P < .001$); n = 15–21.

In *Drosophila*, TIM partners with PER through the PER PAS domain, but TIM itself shares no modular motif with other proteins of known function in the database, apart from clearly related homologs (Zylka et al., 1998). All of these components disrupt rhythmicity when absent and cycle at the mRNA and protein levels, suggesting they play roles in the central oscillator itself.

Our cloning and characterization of *ZEITLUPE (ZTL)* now identifies a novel class of molecule that is closely associated with the control of circadian period in plants. The predicted ZTL protein consists of a PAS-like LOV domain, an F box domain, and six kelch repeats. We observe strong light-dependent effects of *ZTL* mutations on period length, consistent with the presence of a domain (LOV) in this molecule responsible for flavin binding in known blue light photoreceptors. The F box motif is found in proteins that target specific substrates for proteolytic degradation, while kelch domains are likely to facilitate protein–protein interactions. Together, these motifs point to the possible identification of an element of a novel, light-regulated proteolytic system involved in the degradation of components of the circadian clock.

Results

Mutations at *ZTL* Alter a Wide Range of Clock-Controlled Processes

A search for period-altering mutations was conducted in *Arabidopsis thaliana* using a luminescence-based phenotype. Plants homozygous for the morning-phased clock-controlled reporter gene, *CAB2::luciferase* (*CAB2::luc*), were mutagenized and screened for individuals with altered periods of free-running luminescence rhythms (Millar et al., 1992, 1995a). An initial long period mutant was identified and mapped to the bottom of chromosome 5 (data not shown). The mutation at this locus (*ZEITLUPE*, *ZTL*; slow motion [G]) increases the free-running period of the luminescence rhythm in white light by more than 3 hr (Figure 1A). This mutation is codominant (data not shown) and has no significant effect on the amplitude of the rhythm (Figure 1A) or on the morphology or appearance of the plants (data not shown). A second allele (*ztl-2*) exhibits quantitatively similar phenotypes (data not shown). To determine how pervasively mutations at this locus affect the circadian clock, we examined a wider range of circadian phenotypes in the *ztl* background.

Table 1. Free-Running Period Estimates of Cotyledon and Leaf Movement Rhythms in Wild-Type and *ztl* Mutant Seedlings

	Genotype	Period (hr) ± SD (n)
Trial 1	wild type	28.1 ± 1.0 (9)
	ztl-1 (Bx2)[a]	30.4 ± 0.8 (8)
Trial 2	wild type	27.3 ± 0.7 (10)
	ztl-1 (Bx4)[a]	32.0 ± 1.3 (9)
	ztl-2 (Bx1)[a]	32.8 ± 1.4 (8)
Trial 3	wild type	28.4 ± 0.8 (10)
	ztl-1 (Bx5)[a]	32.3 ± 0.6 (7)
	ztl-2 (Bx1)[a]	34.1 ± 1.0 (19)

Plants were entrained in L/D cycles (12 hr : 12 hr) for 5 days, transferred to continuous light, and blade position recorded every 20 min for 7–9 days. Period estimates (variance-weighted mean ± variance-weighted SD) were obtained as described (Millar et al., 1995b; Plautz et al., 1997).
[a] Indicates extent of backcrossing.

CCR2 codes for a putative RNA binding protein and exhibits a robust circadian rhythm of mRNA abundance that peaks late in the subjective day (Carpenter et al., 1994). We tested the effect of the *ztl-1* mutation on the cycling of *CCR2* mRNA abundance to determine if a gene with peak expression differently phased from *CAB2* would be similarly affected. After 3 days in continuous light, peak expression of *CCR2* mRNA abundance in the *ztl-1* background was 9 to 12 hr out of phase with the peak levels in the wild type (WT) (Figure 1B). This likely results from a 3–4 hr phase delay per cycle, consistent with *ZTL* acting upstream of both *CAB2* and *CCR2* in the circadian control of their expression.

Circadian rhythms in plant organ movement have been demonstrated in a wide variety of plant species, and are largely based on rhythmic changes in cellular volume (Engelmann and Johnsson, 1998). To test the effects of the *ztl* mutations on circadian-regulated processes other than gene expression, we measured the free-running period of cotyledon and leaf movement rhythms in *Arabidopsis* seedlings. Period length in both of the *ztl* mutant backgrounds was increased by 4–5 hr, relative to WT (Figure 1C; Table 1). The slightly stronger effect of *ztl* mutations on the lengthening of the period of organ movement, compared to the *CAB2::luc* rhythm in white light (Table 1; Figure 1A) may result from the lower light intensities used in this assay (Figures 1A and 1C) and/or from differences in the light input pathway to the clock between mesophyll cells and petiolar cells.

Arabidopsis is a facultative long-day plant, flowering more rapidly in long days (16 hr light:8 hr dark) than in short days (8 hr light:16 hr dark). The control of the transition from vegetative to reproductive growth in plants is complex (Levy and Dean, 1998), but a role for the circadian clock in this photoperiodic process has been firmly established (Thomas and Vince-Prue, 1997). As measured by leaf production, *ztl-1* selectively affected only the rate of flowering in long days, producing ca. 50% more leaves prior to flowering than the WT. Days to flowering was significantly increased over WT by *ztl-1* in both long and short days (Figure 1D). These results are consistent with a role for *ZTL* within the photoperiodic timing system of *Arabidopsis* and, taken together with the effects on rhythms of gene expression and cell physiology, show that *ZTL* has a pervasive effect on the circadian system throughout development.

The Period Effects of *ztl-1* Are Strongly Fluence Rate Dependent

Previous studies have shown a log-linear relationship between light input and the pace of output rhythms (Aschoff, 1979; Somers et al., 1998a, 1998b). To investigate if *ZTL* plays a role in light input to the circadian system, we separately tested the effects of varying red light (RL) and blue light (BL) intensities to determine if the *ztl-1* phenotype is light quality and/or fluence rate dependent (Figure 2).

The close overlap of the RL and BL fluence rate response curves (FRCs) shows that the *ztl-1* mutation does not discriminate between red and blue light signaling to the clock (Figure 2). Similar results were obtained with *ztl-2* (data not shown). However, period length is strongly dependent on fluence rate under both light qualities. At the highest RL intensities period length in *ztl-1* is 2–3 hr longer than WT, and increases to nearly 10 hr over a two-order magnitude decrease in fluence rate (Figure 2). Similarly, over a 30-fold decrease in BL intensity, the difference between *ztl-1* and WT period increases from 4 hr to more than 8 hr (Figure 2).

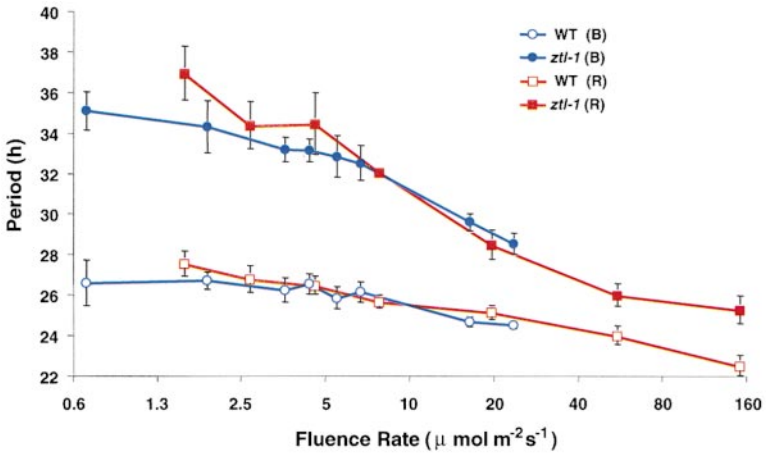

Figure 2. Period Length Effects of the *ztl-1* Mutation Are Fluence-Rate Dependent

Wild type (WT) and *ztl-1* seedlings were entrained for 5 days in white light/dark cycles (12 hr/12 hr), transferred to continuous red (R) or blue (B) light of the appropriate fluence rate and luminescence levels measured every 2 hr for 114 hr. Variance-weighted mean periods (± variance-weighted SD) were estimated from > 100 hr in free run (n = 16–21 seedlings/data point). Representative of three independent trials.

ZTL Mediates Light Signaling to the Circadian Clock with Little Effect on Photomorphogenesis

In addition to control by the circadian clock, the *CAB2* promoter is strongly and rapidly up-regulated by light. When dark-grown seedlings are given a short pulse of light and returned to darkness there is an acute rise in promoter activity followed by subsequent cycling of expression that is clock-regulated and independent of the magnitude of the acute peak (Figure 3A; Millar and Kay, 1996; Anderson et al., 1997; Somers et al., 1998b).

The extent of the acute peak in *ztl-1* is identical to WT, but the second peak occurs 6 hr later than WT (Figure 3A). In addition, over a 6- to 10-fold range of either RL or BL pulses, there were no consistent significant differences between *ztl-1* and WT in the extent of the rise or timing of the acute response (data not shown). These results show that *ZTL* acts independently of BL- and RL-mediated acute induction of *CAB2* expression, but is involved in controlling period length of the clock in etiolated, nonchlorophyllous seedlings as shown from the later phase of the second peak in expression.

Light-regulated control of hypocotyl extension involves phytochrome (phy) and cryptochrome (cry) photoreceptors to initiate light signaling within these cells (Casal and Mazzella, 1998; Neff and Chory, 1998). As deficiencies in these receptors also alter circadian period (Somers et al., 1998a), we wished to determine if ZTL acts prior to or after the putative branchpoint separating phototransduction to the clock from photomorphogenesis. Over a 20-fold decrease in BL fluence rate, hypocotyl length in *ztl-1* increased more than 3-fold and was nearly identical to WT at all intensities tested (Figure 3B). In contrast, under RL *ztl-1* was significantly shorter than WT over the entire fluence-rate range (Figure 3B). At most, length was reduced to 70% of WT and was most apparent at the highest intensities. Hypocotyl length in dark-grown plants was the same in the mutant and wild type, indicating that the differences were light dependent.

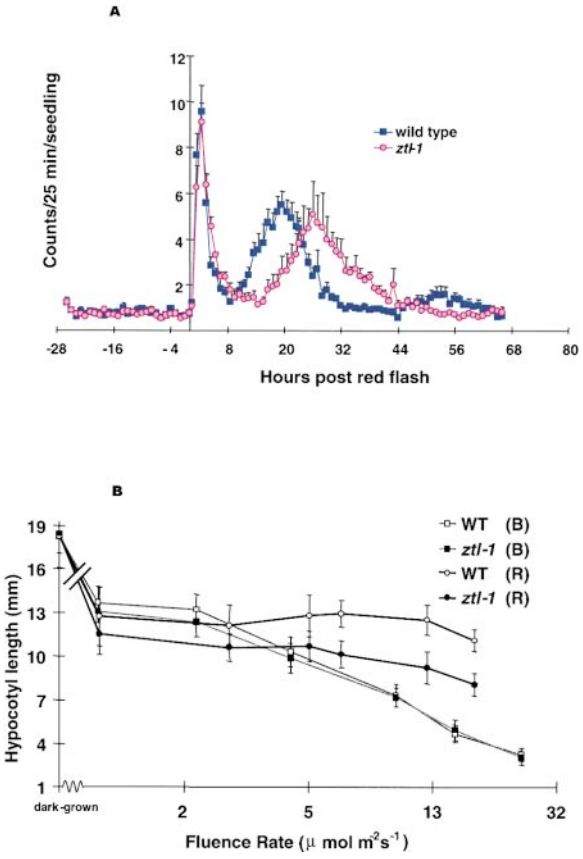

Figure 3. Effects of *ztl-1* on Light-Dependent Gene Expression and Photomorphogenesis

(A) *ztl-1* has no effect on the acute induction of *CAB2* expression in dark-grown seedlings. Plants were grown in darkness for 6 days prior to a 5 min RL pulse at T = 0. Mean luminescence/seedling (± SEM) was obtained from imaging 4 clusters of 15–30 seedlings. Representative of two trials.

(B) Effects of *ztl-1* on hypocotyl length in red light (R) and blue light (B). Wild type (WT) and *ztl-1* plants were grown for 10 days in continuous light under the light quality and fluence rate indicated. Values (± SD) are representative of three independent trials; n = 15–41/data point.

ZTL Defines a Novel Gene Family

ZTL was mapped to the bottom of chromosome 5 using commercially available CAPS and SSLPs markers (Research Genetics). High-resolution mapping based on period length proceeded using additional CAPS and SSLPs markers developed from genomic sequence made available from the Arabidopsis Genome Initiative (Kazusa DNA Research Institute). We identified two flanking SSLPs approximately 2 cM each from *ZTL* that were scored simultaneously in each of 1078 plants to obtain 81 recombinants (Figure 4A). From this pool of recombinants, we resolved an 18 kb region that included 3 predicted open reading frames (ORFs) (Figure 4A). Multipass sequencing of the central candidate ORF from both *ztl* mutant alleles identified single base substitutions in each that change an aspartate to an asparagine (Figure 4B). From these results we conclude that this gene codes for ZTL.

With this sequence an EST (accession no. N38582) was obtained from the Arabidopsis Biological Resource Center (ABRC). The DNA sequence of this EST predicts a 2.3 kb message, which corresponds well to a 2.4 kb band detected on RNA blots (data not shown). From these results we conclude that this EST corresponds to the full-length cDNA of *ZTL*. A species-wide database search with this sequence found two predicted ORFs within the *Arabidopsis* genome database that show full-length homology to *ZTL* (accession nos. AAC09038 and AAF16557), but none from nonplant species (Figure 5A). Both genes are expressed and designated *LKP2* (M. Wada, personal communication) and *FKF1* (accession no. AAF32298), respectively. The nucleotide sequence of *ZTL* from the Cape Verde Islands (Cvi) ecotype shows a single base difference in the Cvi locus that predicts a proline (P) to threonine (T) change at amino acid 35, near the beginning of the PAS-like LOV domain (Figure 4B).

ZTL is predicted to encode a novel 609 amino acid polypeptide with three distinct motifs (Figure 5A). Near the N terminus is a region with a high degree of similarity to a PAS-like domain (LOV) found in a group of blue-light photoresponsive molecules from *Arabidopsis (NPH1)*,

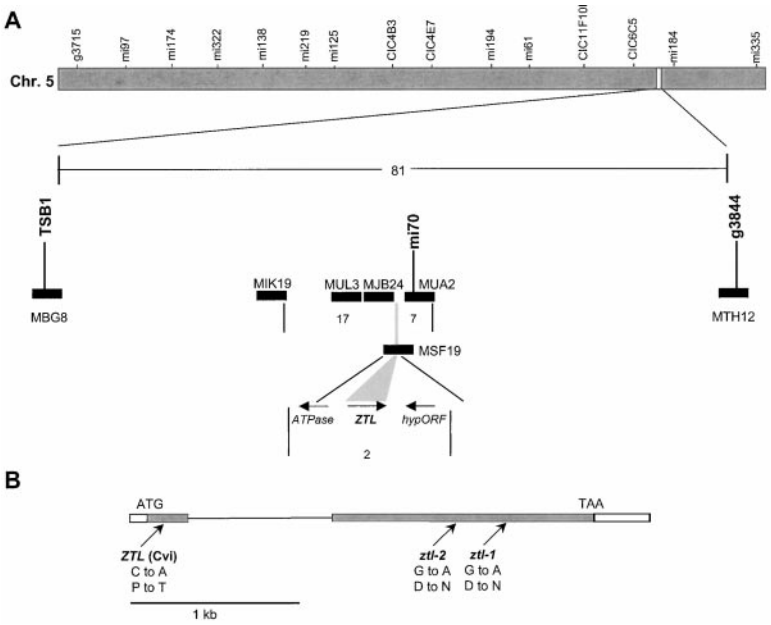

Figure 4. Map-Based Cloning and Gene Structure of *ZTL*

(A) Summary of the physical and genetic position of *ZTL*. The position of a 3–4 cM region on chromosome 5 that contains *ZTL* is shown flanked by the genetic markers TSB1 and g3844. Values indicate the number of recombinants identified between the two nearest PCR-based markers (black vertical lines) or between a marker and the *ztl*/*ZTL* phenotype (gray vertical line). P1 clones (Kazusa DNA Research Institute) from which useful PCR-based markers were derived are shown (black boxes) relative to each other and to established genetic markers. The direction of transcription of the *ZTL* ORF relative to a putative ATPase and a hypothetical protein (hypORF) is indicated (arrows).

(B) Gene structure of *ZTL* and position of known mutations and amino acid polymorphisms. The positions of the initiation (ATG) and stop (TAA) codons are shown in the context of the protein coding sequence (gray boxes), the intron (line) and untranslated regions (open boxes). The nucleotide and respective amino acid changes in the three known divergences from the wild type (C24 ecotype) are indicated. Cvi: Cape Verde Island ecotype.

Adiantum (PHY3), and *Neurospora (WC-1)* (Ballario et al., 1998; Nozue et al., 1998; Briggs and Huala, 1999; Christie et al., 1999) (Figure 5B). Over this region ZTL shares between 38% and 40% amino acid identity with both of the two PAS domains found in NPH1 and PHY3 and the single region in WC-1. This is very similar to the degree of identity (42%–43%) shared between the two PAS domains (LOV1 and LOV2) found within the NPH1/PHY3 class of molecules (Christie et al., 1999). In contrast, comparisons of the ZTL PAS domain with other members of the *ZTL* gene family show 73% (LKP2) and 76% (FKF1) identity. Interestingly, while the insertion of an 11 aa gap is required in the NPH1/PHY3 group to maintain optimal alignment (Figure 5B; positions 52–62), WC-1 aligns with 50% similarity to the ZTL family over that region.

The second motif in ZTL, an F box, lies ca. 45 aa downstream of the PAS domain (Figure 5A). The F box motif is present in a wide range of proteins and serves to recruit target proteins to the SCF class of E3 (ubiquitin ligase) ubiquitination complexes, in preparation for proteolysis (Patton et al., 1998). The first F box proteins were described as *cdc* mutants from yeast (Bai et al., 1996), but genes involved in flower development (*UFO*, Samach et al., 1999; *FIM*, Ingram et al., 1995), auxin responsiveness (*TIR*; Ruegger et al., 1998), and jasmonate signaling (*COI1*; Xie et al., 1998) have been cloned and identified as F box proteins in plants.

Alignment of the F box region from known plant F box proteins with the ZTL protein family shows very limited amino acid identity but a high degree of functional conservation (Figure 5C). Further comparison to a species-wide F box consensus motif (Patton et al., 1998) shows that the majority of key residues, and the spacing between them, are conserved in ZTL and the two paralogs (Figure 5C).

The third distinguishing feature of ZTL is six repeated kelch motifs that comprise the C-terminal 50% of the molecule (Figure 5D). The kelch motif was first described from *Drosophila* (Xue and Cooley, 1993) and occurs as 4–6 tandem repeats, with each repeat forming a four-stranded β sheet to create a conserved tertiary structure, a β propeller (Adams et al., 2000). With very little primary sequence conservation, this superfamily defines a structurally and functionally diverse group involved in determination of cell morphology and organization, gene expression, and cytoskeleton modulation (Adams et al., 2000).

The kelch motifs predicted for each member of the *ZTL* gene family match at 7 of 8 kelch consensus residues (Figure 5D). The alignment indicates how the sixth kelch motif might form from sequences preceding and concluding the other five repeats and consequently close the propeller structure (Adams et al., 2000). The mutations in *ztl-1* and *ztl-2* are both D-to-N changes that lie at conserved positions in the *ZTL* gene family in the third and first kelch repeat, respectively (Figure 5D).

ZTL Expression Is Independent of Light/Dark Cycles and the Circadian Clock

Some molecular components of all known circadian clocks, as well as phototransduction elements to the oscillator in certain systems, cycle with a 24 hr rhythm (Emery et al., 1998; Stanewsky et al., 1998; Dunlap, 1999). The strong light-dependent effect of *ztl-1* on period length suggests it may act within the light input pathway to the oscillator and we tested whether *ZTL* expression might itself be light or clock-regulated.

In both WT and *ztl-1* backgrounds there was no evidence of either light regulation of *ZTL* mRNA abundance (Figure 6), or of circadian regulation of transcript levels (data not shown). These results also show that the *ztl-1*

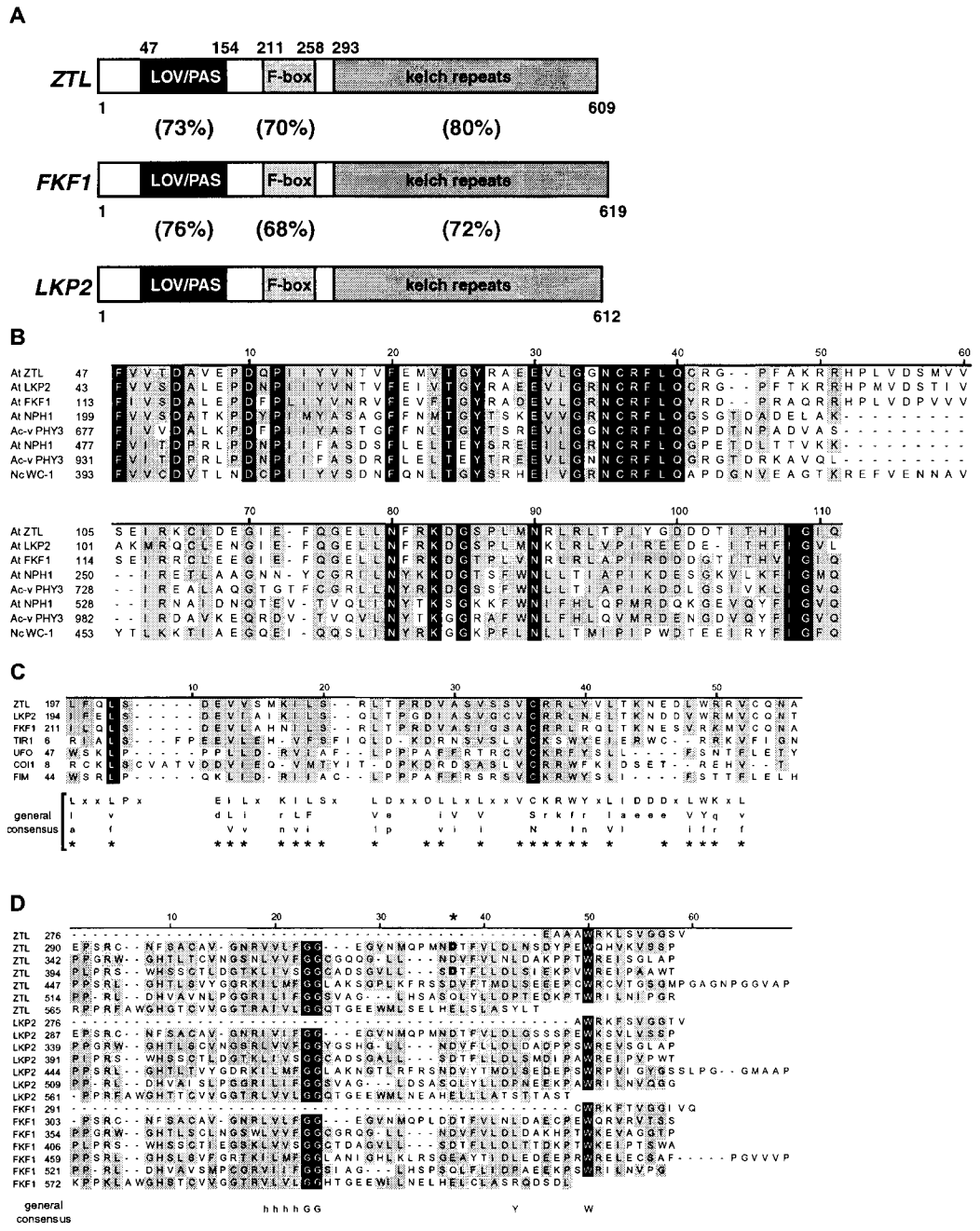

Figure 5. Predicted Amino Acid Sequence and Domain Alignments of ZTL

(A) Schematic alignment of *ZTL* (accession no. AF254413) with paralogs *FKF1* (accession no. AAF32298) and *LKP2* (M. Wada, personal communication). Percent amino acid identity of each paralog motif with ZTL is shown in parentheses.

(B) Alignment of the PAS domain of ZTL, LKP2, and FKF1 with select similar proteins. *Arabidopsis* NPH1 (At NPH1), a flavoprotein involved in phototropism; *Adiantum capillus-veneris* PHY3 (Ac-v PHY3), a fern phytochrome with limited similarity to NPH1; *Neurospora crassa* WC-1 (Nc WC-1), a regulator of blue-light and circadian clock responses. Duplicate entries result from the presence of two PAS domains in the respective protein, beginning at the residues indicated.

(C) Alignment of the F box domains identified from F box proteins known to be expressed in *Arabidopsis* and *Antirrhinum*. *UFO* (flower development; Samach et al., 1999); *TIR* (auxin responsiveness; Ruegger et al., 1998); *COI1* (jasmonate signaling; Xie et al., 1998); *FIM* (flower development; Ingram et al., 1995). The general consensus is derived from a select comparison of F box proteins drawn from eleven different organisms (modified from Patton et al., 1998). The most highly conserved residues are capitalized. * indicates a concordance of the majority of ZTL/LKP2/FKF1 residues at that position with the general consensus.

(D) Alignment of the 6 kelch domain–containing regions from ZTL, LKP2, and FKF1. Each repeat is numbered according to where the first residue of the respective region lies in the original polypeptide sequence. The positions of the two conserved aspartate (D) residues mutated in *ztl-1* and *ztl-2* are shown in bold below the asterisk. The general consensus is according to Adams et al. (2000); h: hydrophobic residue.

In all alignments identical amino acids are boxed in black; gray shading indicates functionally conservative substitutions in more than half the comparisons. All alignments were obtained using Clustal with some manual adjustments.

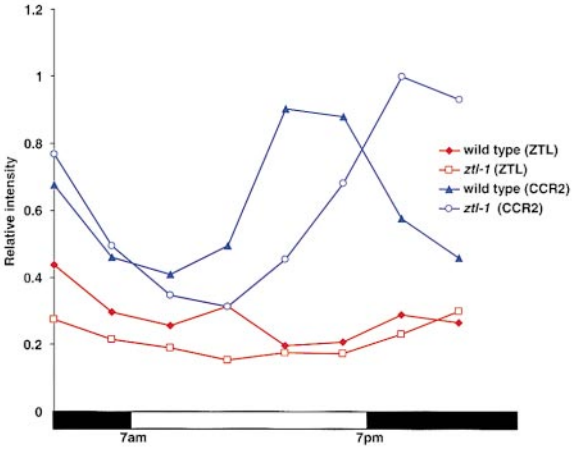

Figure 6. *ZTL* mRNA Abundance Is Not Light Regulated

Wild type and *ztl-1* plants were entrained in 12 hr:12 hr white light (open bar)/dark (black bars) cycles for 20 days after which tissue was harvested every 3 hr for 24 hr. RNA blots were hybridized with *CCR2* (CCR2) or *ZTL* (ZTL) DNA probes, and quantitated relative to rRNA levels using an rDNA probe.

mutation does not affect its own transcription or mRNA stability.

Discussion

ZTL Has a Functional Role in the Plant Circadian System throughout Development

Both *ztl* mutant alleles lengthen the free-running period of all the clock-controlled processes tested, with very little effect on other physiological and developmental processes in the plant. Gene transcription, as measured by the *CAB2::luciferase* reporter gene, and *CCR2* mRNA abundance both cycle with a significantly longer period than WT, indicating that rhythmic cellular and subcellular processes are controlled by ZTL protein. Organ movement rhythms, which arise from variations in cell volume as a result of coordinated changes in cell physiology, are similarly affected by *ztl* mutations. The *ztl-1* effect on flowering time also suggests that photoperiodic timing, a less direct measure of circadian clock function, is compromised by the mutation. Taken together (Figures 1A–1D), our results demonstrate that *ZTL* acts throughout the life of the plant to control the pace of the circadian clock.

ZTL Is Largely Dedicated to the Light Input Pathway to the Clock

Plants lacking discernible circadian cycling, through overexpression (Schaffer et al., 1998; Wang and Tobin, 1998) or loss-of-function mutations (Hicks et al., 1996), often possess unusually long hypocotyls. These effects are likely due to the loss of appropriate clock-controlled gating of rhythmic growth (Dowson-Day and Millar, 1999). The *ztl-1* mutation slightly enhances the sensitivity of the hypocotyl to RL-mediated growth inhibition, but has no effect in BL (Figure 3B). Since the effect of the *ztl* mutations on period is equally severe in RL and BL, it is unlikely that the hypocotyl phenotype is due to altered period length. One possible alternative is that a BL-activated and RL-activated state of ZTL may form under the respective light qualities, with the former specific for clock-related processes and the latter more broadly acting.

The *ztl-1* period phenotype is strongly light-dependent (Figure 2), yet even at the lowest fluence rates tested the mean level and amplitude of the luminescence rhythm is wild type (data not shown). This observation is confirmed by the wild-type acute response to light seen in *ztl-1* dark-grown plants (Figure 3A), and supports the notion that ZTL acts only on the clock-controlled component of gene transcription. Whereas individual photoreceptors act over a limited range of fluence rates (Somers et al., 1998a), the *ztl* mutations affect period at high and low fluences of RL and BL suggesting that *ZTL* acts downstream of a convergence of photoreceptors. Alternatively, ZTL may be a novel photoreceptor or act within a novel phototransduction pathway(s).

The relative shape of the FRC may indicate the position of a clock component within the system. The slopes of the FRCs of *toc1-1*, a short period mutant (Somers et al., 1998a), and *ztl-1* differ markedly. Over decreasing RL intensity, period in *ztl-1* increases from 3 hr to 10 hr longer than WT (Figure 2). Similar differences between WT and mutant FRCs were observed in tests with photoreceptor mutants and overexpressors (Somers et al., 1998a). In contrast, over a similar decrease in RL intensity, the *toc1-1* mutant retains a consistently 2–3 hr shorter period than WT, meaning that the slope of the FRC remains parallel to WT (Somers et al., 1998b). These observations are consistent with the notion that qualitative changes in components within a signaling cascade will result in non-linear changes in output as input signal varies linearly. This places *ZTL*, like the phytochromes and cryptochromes, within a light signaling pathway, and *TOC1* either at the terminus of a light input chain or within the central oscillator itself (Somers et al., 1998b).

ZTL May Function within a Novel Light-Dependent Signaling Pathway to the Clock

The PAS domain is the only protein motif that is shared commonly among the characterized circadian clocks in eukaryotes (Dunlap, 1999). It has been found in an increasing number of signaling proteins (Taylor and Zhulin, 1999), facilitating protein–protein interactions in some polypeptides (Huang et al., 1993) and cofactor binding in others (Gong et al., 1998; Pellequer et al., 1998). To date, only the former function had been verified for the PAS-containing proteins involved in circadian clocks.

The strong light dependence of the *ztl-1* period phenotype coupled with the close sequence similarity of the ZTL PAS motif to the PAS-like LOV domains in NPH1, PHY3, and WC-1 strongly suggests that ZTL may be a novel BL photoreceptor. All three molecules are BL photoreceptors or involved in BL phototransduction. NPH1 (phototropin; Christie et al., 1999) contains two LOV domains and is a blue light–activated autophosphorylating serine/threonine protein kinase responsible for photoperception in plant phototropism (Briggs and Huala, 1999). The C terminus of PHY3 is very similar to

NPH1 and is fused to an N-terminal phytochrome-like region, creating a novel RL/BL receptor (Nozue et al., 1998). WHITE COLLAR-1 (WC-1), a Zn finger transcription factor from *Neurospora* involved in BL signaling to the circadian clock (Crosthwaite et al., 1997), contains a single LOV domain and may be a BL photoreceptor (Talora et al., 1999). LOV domains mediate homodimerization (Ballario et al., 1998) and flavin binding (Christie et al., 1999), though it is not known whether these features are mutually exclusive.

Since the absorption spectra of flavin and the action spectrum of flavin binding proteins are most pronounced in BL, the equally strong effect of *ztl-1* in RL alone is intriguing (Figure 2). However, coaction among different photoreceptor classes is well-known in plants (Mohr, 1994; Casal and Mazzella, 1998; Neff and Chory, 1998). It is possible that ZTL can be activated by a component of a RL signaling pathway in addition to direct activation by BL, with either state sufficient to effect changes in the circadian system. Alternatively, the ZTL PAS domain may only facilitate protein–protein interactions, and ZTL is a downstream component of convergent BL and RL transduction chains.

ZTL Implicates Ubiquitin-Dependent Proteolysis Coupled to the Control of Circadian Period

The F box and kelch domains, which comprise the C-terminal two-thirds of ZTL (Figures 5C and 5D), may act together to define the function of the ZTL protein. The F box is a highly degenerate hydrophobic sequence of ca. 40 amino acids found in proteins that recruit specific substrates to a core ubiquitin ligase complex (SCF) for ubiquitination and subsequent proteolytic degradation (Craig and Tyers, 1999). The interaction partner of the F box is SkP1, which is the anchor by which the F box protein associates with the SCF complex. The wide range of F box proteins identified from yeast (Craig and Tyers, 1999), metazoans (Winston et al., 1999; Regan-Reimann et al., 1999), and plants (Ingram et al., 1995; Ruegger et al., 1998; Xie et al., 1998; Samach et al., 1999) are involved in cell cycle regulation, transcriptional control of metabolism and development, and hormonal (auxin and jasmonate) responses (Patton et al., 1998; Craig and Tyers, 1999).

Substrate specificity comes from additional motifs within the F box protein, often lying C-terminal to the F box (Patton et al., 1998). In ZTL this position is occupied by six kelch repeats that terminate the protein (Figures 5A and 5D). Kelch repeats are predicted to form a β propeller from individual four-strand β sheets. Although relatively few binding partners of kelch repeat proteins have been identified, their role in the determination of cell morphology and organization, in gene expression and as enzymes and actin-associated proteins suggests a diversity of molecular interactions (Adams et al., 2000). The importance of these motifs to ZTL function is highlighted by the remarkable similarity of the two *ztl* mutations. Both are D-to-N changes in identically positioned residues within the first and third kelch repeats (Figure 5D). It is likely that these are loss-of-function hypomorphic mutations since the *fkf1* deletion phenotypes are similar to those in *ztl-1* (see Nelson et al., 2000 [this issue of *Cell*]). As well, the *ztl-1* FRC resembles those from *phyA* and *cry1* loss-of-function mutants (Somers et al., 1998a). If ZTL functions as other known F box proteins, then these mutations may diminish a protein–protein interaction between ZTL and a clock-related substrate that is normally targeted for proteolytic degradation. Increased amount of this substrate could decrease the pace of the clock directly by repressing positive activators in the cycle.

Conclusions

With the recognition that the molecular composition of the circadian oscillator involves negative feedback of gene products on gene expression, cyclic changes in protein abundance have become part of the definition of the circadian clock. It is not surprising, then, to find that circadian period can be affected by mutations in an F box protein that may be part of a proteolytic pathway. The novel addition of a PAS\LOV domain to this motif brings to the molecule the potential of facilitating direct light-regulated proteolysis of oscillator components.

Evidence that substrate recognition by F box proteins is strictly phosphorylation dependent (Craig and Tyers, 1999) fits well with the known role of phosphorylation in the function of the circadian clock (Edery et al., 1994; Zeng et al., 1996; Dunlap, 1999). The half-life of PER in *Drosophila* depends on the phosphorylation state, and mutations in a casein kinase 1ε (*double-time*) effects phosphorylation of PER and alters period length (Kloss et al., 1998; Price et al., 1998). The stabilizing interaction partner of PER, TIM, also undergoes time-dependent phosphorylation and light-dependent degradation via the proteosome–ubiquitin pathway (Naidoo et al., 1999). This could therefore represent a conserved theme in regulation of clock protein levels.

Phosphorylation may play a role in the plant circadian clock as well. In *Arabidopsis*, protein kinase CK2 phosphorylates CCA-1, a cyclically expressed myb-related transcription factor that shortens circadian period when absent and flattens cycling when overexpressed (Sugano et al., 1998; Wang and Tobin, 1998; Green and Tobin, 1999). This same kinase, when overexpressed, also shortens period and alters flowering time (Sugano et al., 1999). No dedicated components of a phosphorylation-dependent degradation system have been identified in the fly or plant systems, but candidate substrates appear readily available.

The light-independent steady-state expression of *ZTL* mRNA suggests that *ZTL* is not part of an autoregulatory feedback loop, but acts as an important ancillary component in the maintenance of the circadian cycle. However, the protein may be light labile or regulated independently of message levels, or it may be present constitutively but act rhythmically if only activity is light/clock dependent. We are developing additional reagents to address these questions.

The cloning and characterization of *ZTL* adds a novel component to the list of molecular factors that influence clock function, and broadens the class of elements to be considered in understanding the construction of circadian cycling systems. Importantly, the PAS domain again arises as the consistent theme for conservation of functional domains in clock systems from plant and fungi to man.

Experimental Procedures

Plant Growth Conditions and Period Length Assays

ztl-1 was originally isolated from an EMS-mutagenized population (C24 ecotype) carrying the *CAB2::luciferase* reporter gene (Millar et al., 1995a). *ztl-2* was identified from further screens of the same EMS-mutagenized population and exhibited phenotypes similar to *ztl-1* in all cases. Measurements of period length in continuous white light (50–60 μmol m^{-2} s^{-1}) and red and blue light fluence-rate experiments were conducted at 22°C as previously described (Somers et al., 1998b). Dark-grown seedlings were imaged in 15–30 seedling clusters as previously described (Anderson et al., 1997). For leaf movement analysis, plants were entrained for 5–6 days in light/dark cycles (12 hr:12 hr), transferred to continuous light (17–20 μmol m^{-2} s^{-1}), and cotyledon position recorded every 20 min as described in Hicks et al. (1996). Period estimates were obtained according to Plautz et al. (1997) and Millar et al. (1995b).

Hypocotyl Analyses

Seeds were stratified in the dark at 4°C for 6 days, exposed to white light (30 μmol m^{-2} s^{-1}) for 15–30 min and returned to darkness for 24 hr before placement under the appropriate light quality and fluence rate for 7–10 days. Hypocotyl length was measured with a ruler.

Flowering Time Analyses

Seeds were stratified on agar plates and grown in white fluorescent light under either long days (LD) (16 hr light: 8 hr dark) (100–120 μmol m^{-2} s^{-1}) or short days (SD) (8 hr light: 16 hr dark) (100–120 μmol m^{-2} s^{-1}) for 7–10 days after which they were transplanted to soil. The total number of rosette and cauline leaves were recorded as the measure of developmental time to flowering.

RNA Gel Blot Analyses

RNA was extracted from 21-day-old seedlings using the Rneasy Plant Mini Kit following the manufacturer's protocols (Qiagen Inc., CA). Total RNA (10 μg/lane) was fractionated on 1.2% formaldehyde gels and blotted onto NitroPlus supported nitrocellulose (MSI, MA). A 568 bp PCR fragment from the *ZTL* EST (accession no. N38582) was produced using the following primers (MSF3a-F: 5′ TCG ATG CTA AGC CGC CTA CT 3′, and MSF3-R: 5′ GGC GGT CTT CCT GGA ATG TT 3′), and was random prime labeled using [^{32}P]dCTP. The CCR2 and rDNA probes were labeled as previously described (Kreps and Simon, 1997). Hybridizations were performed using ExpressHyb Hybridization Solution (Clontech, CA), and washes were done at a final stringency of 65°C in 0.2× SSC/0.1% SDS. Quantitation of the Northern blots was done on a Phosphorimager and analysis of the images performed using ImageQuant Software (Molecular Dynamics, CA).

Isolation and Analysis of Recombinant F2 Plants

ztl-1 (C24 ecotype) was crossed to wild-type Columbia (Col-1) and long period ($>$ 26 hr) plants were identified in the F2 population by estimating the free-running period of each plant after 4 days in continuous light, or by assessing relative peak luminescence during a 24 hr imaging window after 3 days in continuous light. Genotypes were confirmed in the F3 progeny. Commercially available PCR-based markers (SSLPs and CAPS) (Research Genetics) were used to map *ztl-1* to the bottom of chromosome 5. Additional SSLPs and CAPS markers were developed using publicly available *Arabidopsis* genomic sequence (Kazusa DNA Research Institute; http://www.kazusa.or.jp/kaos/).

DNA Sequence Determinations

All DNA sequence determinations of mutant and ecotype alleles of *ZTL* were obtained from 500–1000 base PCR amplicons treated with shrimp alkaline phosphatase (Boehringer Mannheim) and Exonuclease I (Boehringer Mannheim) prior to chain termination sequencing by standard fluorescent procedures on an ABI 377.

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