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The F-Box Protein ZEITLUPE Confers Dosage-Dependent Control on the Circadian Clock, Photomorphogenesis, and Flowering Time [W]

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As an F-box protein, ZEITLUPE (ZTL) is involved in targeting one or more substrates for ubiquitination and degradation via the proteasome. The initial characterization of ZTL suggested a function limited largely to the regulation of the circadian clock. Here, we show a considerably broader role for ZTL in the control of circadian period and photomorphogenesis. Using a ZTL-specific antibody, we quantitated and characterized a ZTL dosage series that ranges from a null mutation to a strong ZTL overexpressor. In the dark, *ztl* null mutations lengthen circadian period, and overexpression causes arrhythmicity, suggesting a more comprehensive role for this protein in the clock than previously suspected. In the light, circadian period becomes increasingly shorter at higher levels of ZTL, to the point of arrhythmicity. By contrast, hypocotyl length increases and flowering time is delayed in direct proportion to the level of ZTL. We propose a novel testable mechanism by which circadian period and amplitude may act together to gate phytochrome B–mediated suppression of hypocotyl. We also demonstrate that ZTL-dependent delay of flowering is mediated through decreases in *CONSTANS* and *FLOWERING LOCUS T* message levels, thus directly linking proteasome-dependent proteolysis to flowering.

INTRODUCTION

The circadian clock controls the diurnal amplitude and phasing of a wide variety of rhythmic phenomena in plants. The range of processes (e.g., flowering time, stomatal opening, leaf movement, hypocotyl expansion, and gene expression) and the level of control (transcriptional, posttranscriptional, and posttranslational) indicate the extent to which the clock permeates all aspects of plant development (Harmer et al., 2000, 2001; McClung, 2001). The core molecular organization of the circadian system has been defined in a number of model organisms outside of plants and consists of a transcription–translation negative feedback loop. Although such a loop, in principle, can be sustained with only two components and a sufficient time delay, in practice the molecular nature of the oscillator is more elaborate, with multicomponent interlocking feedback loops as the emergent model in more than one system (Young and Kay, 2001).

In addition to the basic timekeeping function of the central oscillator, the system also requires a signal transduction input pathway that connects the environmental light or temperature cycles to the pacemaker, a process known as entrainment. Similarly, a link from the entrained oscillator to the overt outputs is required to impose the appropriate phase of expression on the processes that are actually being controlled by the clock. Molecular analyses of many of the circadian model systems have shown that such a simple linear flow from input to oscillator to output is not a realistic view. In some circumstances the oscillator may control the sensitivity of the input pathway, potentially through the circadian modulation of photoreceptor expression (Toth et al., 2001; Sharrock and Clack, 2002; Cheng et al., 2003; Millar, 2003; Roenneberg and Merrow, 2003).

A wide range of gene products has become associated with the plant circadian clock, largely through screens for period length and flowering time mutants (Barak et al., 2000; McClung et al., 2002). The MYB-related transcription factors CCA1 and LHY have emerged as likely key elements in the pacemaker, insofar as their joint loss results in rapidly damped oscillations in continuous light (LL) (Alabadi et al., 2002; Mizoguchi et al., 2002). However, these are members of a much larger, closely related family that may also contribute to the sustenance of rhythmicity (Carré and Kim, 2002). A second important class of five genes, the *TOC1/PRR* family, is defined by some shared but incomplete similarities with receiver domain elements of the two-component response regulator system and by a second sequence that is found in the *CONSTANS* (*CO*) transcription factor, involved in photoperiodic control of flowering (Matsushika et al., 2000; Strayer et al., 2000; Somers, 2001). The founding members of these two gene families, *LHY/CCA1* and *TOC1*, are proposed to form an autoregulatory feedback loop, whereby *TOC1* expression in the late day and early night promotes *LHY/CCA1* transcription. The subsequent rise of *LHY/CCA1* transcript and protein levels during early and midday acts to repress TOC1 activity through direct binding to elements of the *TOC1* promoter (Alabadi et al., 2001). This promotive/repressive cycle

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incorporates time lags of many hours to culminate in a 24-h rhythm and is clearly only a framework onto which the other period-affecting factors must be incorporated. These other genes include those of unknown function (e.g., *GI*, Park et al., 1999; *ELF3*, Hicks et al., 2001; *ELF4*, Doyle et al., 2002; *SRR1*, Staiger et al., 2003) and those with pleiotropic phenotypes and likely broadly based functions, like *DET1* (involved in chromatin remodeling; Benvenuto et al., 2002; Schroeder et al., 2002) and *COP1* (involved in proteasome-dependent degradation; Schwechheimer and Deng, 2000).

By contrast, the red and blue photoreceptors are a well-defined class of proteins that also affect circadian period and phase. Loss of phytochrome B (phyB) and phyA lengthens period over discrete ranges of red light fluence rates, and the other three phytochromes make minor contributions to period control (Somers et al., 1998; Devlin and Kay, 2000). In addition, both the absence and overexpression of phyB can result in phase advances, depending on the light quality (Hall et al., 2002; Salome et al., 2002). Mutations in *CRY1* and *CRY2* alone have slight effects on period under blue light, but the double mutant lengthens period much more severely and over a wide fluence range. These results indicate that together they control much of the blue light input to the clock, though phyA is also important at low fluence rates (Somers et al., 1998; Devlin and Kay, 2000).

A third class of proteins, the three-member *ZTL* gene family, combines elements of a photoreceptor with domains involved in proteasome-dependent proteolysis. ZEITLUPE (ZTL) is the best characterized of the group, and the N terminus contains a LOV domain, which bears close similarity to the flavin binding region of the Arabidopsis (*Arabidopsis thaliana*) photropins and the Neurospora (*Neurospora crassa*) *WHITE COLLAR-1* (*WC-1*) protein. Both of these proteins are dedicated (Arabidopsis) or generic (Neurospora) blue light photoreceptors, and the LOV domains are the primary photosensing regions (Briggs and Christie, 2002; Froehlich et al., 2002; He et al., 2002). The remainder of ZTL consists of an F-box and six kelch repeats. These two domains act together to bring target proteins into a larger Skp1-Cullin-F-box (SCF) complex (L. Han and D.E. Somers, unpublished data) for ubiquitination and subsequent proteasome-dependent degradation (Deshaies, 1999; Vierstra, 2003). As an unusual N-terminal extension that is not normally present on F-box proteins, the LOV domain carries the potential of conferring light-dependent activity on ZTL. The interaction of ZTL with portions of phyB and cryptochrome 1 in the yeast two-hybrid assay (Jarillo et al., 2001) and the fluence rate–dependent effects of *ztl* mutations on period both support this notion (Somers et al., 2000).

Here, we describe how circadian period and hypocotyl expansion are closely tied to the level of ZTL protein. Our results demonstrate the close linkage between photomorphogenesis and the photocontrol of the pace of the circadian system and the pivotal role ZTL plays in these interrelated processes. We establish a relationship between phyB and ZTL in the control of hypocotyl expansion and position ZTL within the *CO/FLOWERING LOCUS T* (*FT*)-mediated pathway of photoperiodic control of flowering. We also show a function for ZTL in the dark, suggesting a more comprehensive role for this protein in the control of circadian period than previously suspected.

RESULTS

ZTL Protein Levels Control Circadian Period

To better understand the role of ZTL in the control of circadian period, we characterized new *ztl* mutant alleles and examined the effects of additional genomic and cDNA copies of the locus in planta. The previously described *ztl-1* and *ztl-2* alleles confer similar long-period phenotypes under all conditions tested, but the severity of these mutations is unclear. Both are as a result of separate but similar single amino acid substitutions within two of six kelch domains (Somers et al., 2000). Mutant *ztl-1 and ztl-2* protein levels are near wild-type levels, suggesting these polypeptides may be partially functional (Figures 1A and 1B). We subsequently identified a T-DNA insertion line that lacks detectable *ZTL* mRNA (Jarillo et al., 2001) and *ZTL* protein (Figure 1A;

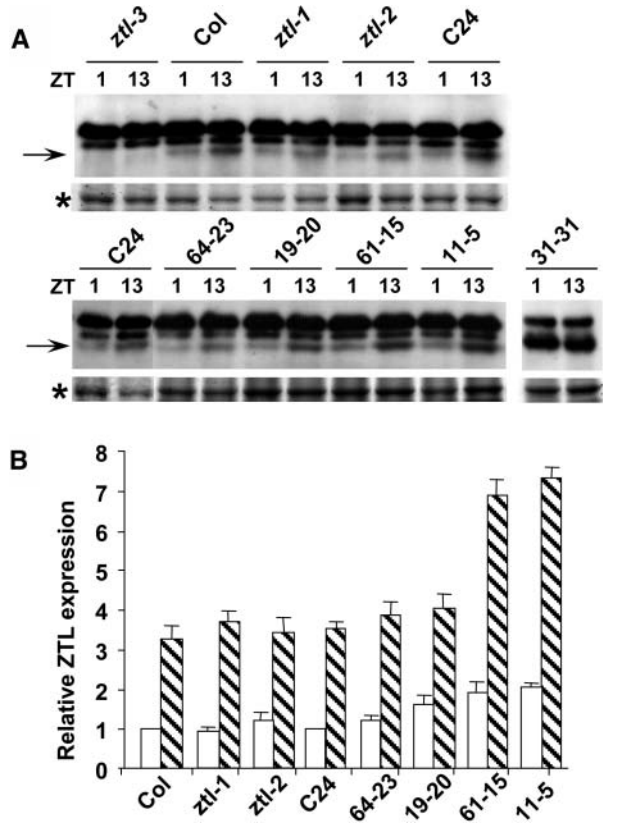

Figure 1. Graded Series of ZTL Protein Expression.

(A) Immunoblots showing ZTL levels (arrows) at times of minimum (zeitgeber time 1 [ZT1]) and maximum (ZT13) accumulation (W.Y. Kim et al., 2003) in eight independent mutant and enhanced expression lines. Two nonspecific bands lie just above the ZTL-specific band. Representative region of Coomassie blue–stained gel of protein loadings equal those used for the immunoblots is shown below (*).

(B) Quantitation of ZTL levels in **(A)** at ZT1 and ZT13, adjusted for loading differences (*) and normalized to the appropriate wild-type (Col or C24) levels at ZT1. Mean values (±SE) from three independent trials. Very high expresser (31-31; saturated signal) and *ztl-3* (no signal) omitted in this analysis.

Table 1. Estimates of Free-Running Period of *CAB2*:*luc* and *CCR2*:*luc* Expression in *ztl-1* and *ztl-3* Backgrounds

	CAB2:*luc*		*CCR2*:*luc*		
Genotype	Period (±SD) RR	Period (±SD) BB	Period (±SD) RR	Period (±SD) BB	Period (±SD) DD
ztl-1	29.0 ± 0.7	28.9 ± 0.4	28.8 ± 0.3	29.3 ± 0.3	34.9 ± 1.8
ztl-3	29.7 ± 0.4	29.1 ± 0.6	28.8 ± 0.4	29.1 ± 0.8	32.7 ± 2.8
C24 WT	25.5 ± 0.5	24.2 ± 0.5	NA	NA	NA
Col WT	25.1 ± 0.4	24.7 ± 0.5	23.8 ± 0.2	24.2 ± 0.2	25.9 ± 1.1

Plants were entrained for 6 d in 12-h-light (white)/12-h-dark cycles, then imaged in RR (20 μmol·m^{-2}·s^{-1}) or BB (20 μmol·m^{-2}·s^{-1}) for 5 d. Variance-weighted period and standard deviation estimates obtained as described previously (Millar et al., 1995; Plautz et al., 1997). NA, not applicable. All *CCR2*:*luc* lines are in Col background.

W.Y. Kim et al., 2003). Using a clock-regulated *CAB*:*luciferase* (*luc*) reporter, this knock-out allele (*ztl-3*) shows a long-period phenotype identical to *ztl-1* under a range of fluence rates and under all light qualities tested (continuous red light [RR] and continuous blue light [BB]) (Table 1, Figures 2A and 2B). A second, independent reporter of circadian period, *COLD CIRCADIAN RHYTHM–RNA BINDING2* (*CCR2*):*luc* (Strayer et al., 2000), is similarly affected by both alleles under red and blue light (Table 1). Both reporters show a 4- to 5-h lengthening of period in both alleles relative to the wild type under either RR or BB. The amplitude and waveform (*CAB*:*luc*) of *ztl-1* and *ztl-3* homozygous lines are also identical (Figure 3).

Because *ztl-1* is codominant (data not shown) and stably expresses mutant *ztl-1* protein, it is possible that the *ztl-1* long period derives from a mechanistically different process than does the long period in *ztl-3*. To address this question, we tested the *ztl-1*/*ztl-3* transheterozygote, reasoning that if *ztl-1* acts as a dosage-dependent dominant negative interactor within a protein complex, a halving of *ztl-1* protein levels might reduce the phenotype severity. In fact, the period of the transheterozygote (*ztl-1*/*ztl-3*) is indistinguishable from both the *ztl-1*and *ztl-3* homozygotes (Figure 3). These results show that the *ztl-1* point mutation effectively eliminates ZTL protein function, although ZTL levels are unaffected (Figure 1). The *ztl-1* mutation increases the sensitivity of circadian period to light intensity, particularly at low fluence rates (Figures 2A and 2B; Somers et al., 2000). Consistent with the evidence that *ztl-1* is a null mutation, the fluence rate responsiveness of *ztl-3* and *ztl-1* are indistinguishable at all red and blue light fluence rates tested (Figures 2A and 2B).

We also tested the effects of increased ZTL protein levels on circadian period. Additional genomic copies of *ZTL* or expression cassettes of the *ZTL* cDNA under the control of the constitutive 35S promoter of the *Cauliflower mosaic virus* (35S:*ZTL*) were transformed into the wild-type and *ztl-1* backgrounds. Numerous independently transformed lines expressing ZTL at above wild-type levels were identified, and five were chosen for further characterization (Figures 1A, 1B, and 4). Because ZTL is expressed throughout the plant and in almost all cell types (Kiyosue and Wada, 2000), the effects we observe through 35S promoter–driven overexpression are likely only as a result of increased protein abundance and not of spatially ectopic expression.

Overexpression of ZTL shortened period from 2 to 6 h and caused arrhythmicity at the highest levels (Figure 4). There was little effect on *CAB*:*luc* expression level, though there is a slight trend toward reduced amplitude with increased ZTL expression (Figure 4). Those lines with periods only slightly shorter than the wild type (lines 64-23 and 19-20; period [τ] = 23 h versus 25.5 h) resulted from the expression of an additional genomic copy of *ZTL* expressed in the *ztl-1* background, and ZTL protein is expressed at levels detectably higher than *ztl-1* (Figures 1A and 1B). One of the second class of even shorter period lines (5 to 6 h less than the wild-type τ = 20 h; line 61-15) also arose this way, presumably as a result of T-DNA insertion site position effects. The second of the very short period lines (11-5; τ = 20 h) expressed at least one additional genomic copy of *ZTL* in the wild-type background. All four lines still exhibit a diurnal variation in ZTL level under light/dark cycles (Figures 1A and 1B), as reported previously for the wild type (W.Y. Kim et al., 2003), indicating that the posttranscriptional control of ZTL can occur at levels elevated above the wild type.

The strongest ZTL overexpressor (31-31), derived from the addition of a single *35S*:*ZTL* expression cassette (data not shown) in the wild-type background, usually resulted in arrhythmicity in blue and red light at all fluence rates tested (see Supplemental Figure 1 online). However, in some instances we observed numerous individuals with very short period (τ = 13.1 h ± 0.6 [SD] h; n = 8) and low amplitude cycling (Figures 4A and 4B). The relative amplitude errors were consistently markedly higher (>0.6) than those of the other enhanced expression lines (Figure 4B), indicating less robust rhythmicity (Dowson-Day and Millar, 1999; Mas et al., 2003a). Despite this low frequency occurrence, it is consistent with the trend toward shorter periods with higher levels of ZTL. At this very high expression level, ZTL abundance does not detectably vary over the circadian time course (Figure 1A). Together with the *ztl* mutants and the wild type, these results show the extreme sensitivity of circadian period to ZTL levels.

In contrast with the increased sensitivity to fluence rate evident in the *ztl-1* and *ztl-3* backgrounds, higher steady state ZTL levels show little fluence rate responsiveness, and period is almost independent of light intensity (Figures 2A and 2B). These results suggest that high levels of ZTL circumvent or saturate the normal photocontrol of circadian period.

ZTL Affects Circadian Period Independent of Light

We and others have suggested that ZTL activity is light dependent (Somers et al., 2000; Schultz et al., 2001). This has

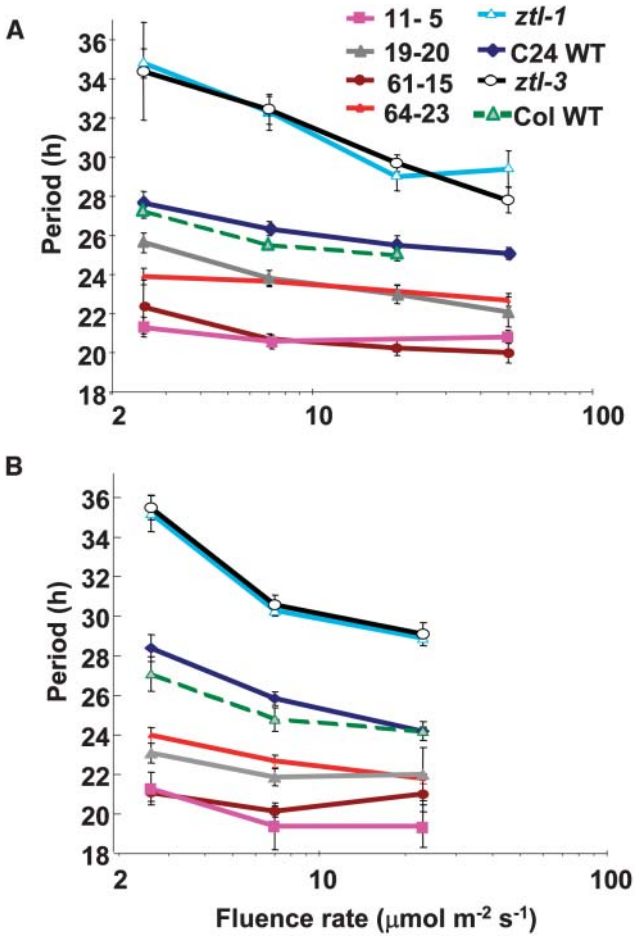

Figure 2. Fluence Rate Dependence of Circadian Period Is Controlled by *ZTL* Protein Level.

Eight transgenic lines expressing the *CAB*:*luc* reporter and different levels of ZTL (Figure 1) were entrained under 12-h-light/12-h-dark cycles for 7 d, transferred to RR **(A)** or BB **(B)** of the appropriate fluence rate, and luminescence levels measured every 2 h for 5 d. Variance-weighted mean periods (±variance-weighted SD) were determined according to Plautz et al. (1997). Each datum is a mean of 4 to 26 seedlings.

been inferred from the strong effect of *ztl* mutations on the fluence rate–response curve and from the observed interaction between ZTL and phyB and cryptochrome 1 in two-hybrid interaction assays (Jarillo et al., 2001). In addition, the ZTL LOV domain bears 40% amino acid identity to the LOV domains of the plant phototropin and the Neurospora WC-1 proteins, both known to act as blue light photoreceptors (Briggs and Christie, 2002; Froehlich et al., 2002; He et al., 2002). We therefore tested the effect of *ztl* mutations and ZTL overexpression on circadian period in darkness. Plants entrained in 12-h/12-h light/dark (LD) cycles were released into constant darkness (DD), and circadian period followed with the clock-regulated *CCR2*:*luc* reporter (Strayer et al., 2000). *ztl-1* and *ztl-3* mutations lengthen free-running period by 7 to 9 h relative to the wild type (Table 1, Figure 5). The extent of this period lengthening is similar to that seen under low fluence RR and BB using the *CAB*:*luc* reporter (Table 1, Figures 2A and 2B). This trend of period lengthening as fluence rate diminishes to the longest period in DD supports the notion of the same or very similar circadian system operating in both LL and DD. These results also suggest that the same circadian oscillator controls the two reporters or that ZTL is an element common to both systems. Consistent with this notion, strong overexpression of ZTL (line 31-31) caused arrhythmicity of the CCR2:*luc* reporter in DD (data not shown). These results also show that ZTL can affect circadian period in the absence of light.

ZTL-Mediated Control of Hypocotyl Expansion

Loss of function or aberrant expression of diverse elements of the plant circadian system can cause light-dependent changes in hypocotyl length (Hicks et al., 1996; Schaffer et al., 1998; Wang and Tobin, 1998; Mas et al., 2003a). The *ztl-1* mutation causes hypersensitivity to red light (Somers et al., 2000). We extended this finding by examining the effects of extreme changes in *ZTL* expression, either complete absence or strong overexpression, on hypocotyl growth. Figure 6 shows that high levels of ZTL cause significant hypocotyl lengthening at all fluence rates and light qualities examined. The strongest effect occurs under red light, where the highest intensities double hypocotyl length in the ZTL OX line relative to the wild type (Figure 6A). Under blue light, where the *ztl-1* mutation has little effect, there is a slight hypocotyl lengthening in the strong overexpressor relative to the wild type, and it is consistently slightly longer over the entire fluence range (Figure 6B). These results confirm and extend previous reports (Kiyosue and Wada, 2000; Nelson et al., 2000). We also examined the *ztl-3* null mutant and found fluence rate and light quality relationships similar to that of *ztl-1* (Figures 6C and 6D). *ztl-3* only effects a hypersensitivity to red light, confirming our earlier conclusion that the *ztl-1* mutation causes loss of ZTL function.

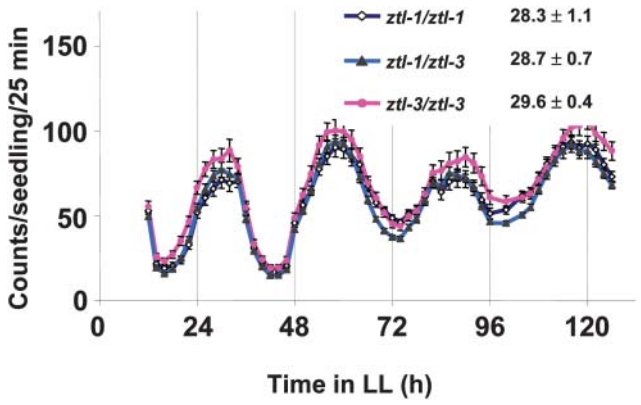

Figure 3. Phenotypic Equivalence of a *ztl* Protein Positive Mutant (*ztl-1*) and an Insertional Knockout Mutant (*ztl-3*).

Plants were entrained in 12-h-light/12-h-dark cycles, transferred to BB (20 μmol m^{-2} s^{-1}), and assayed for *CAB*:*luc* expression (luminescence). Variance-weighted period (h) ±SE determined as described in Figure 2.

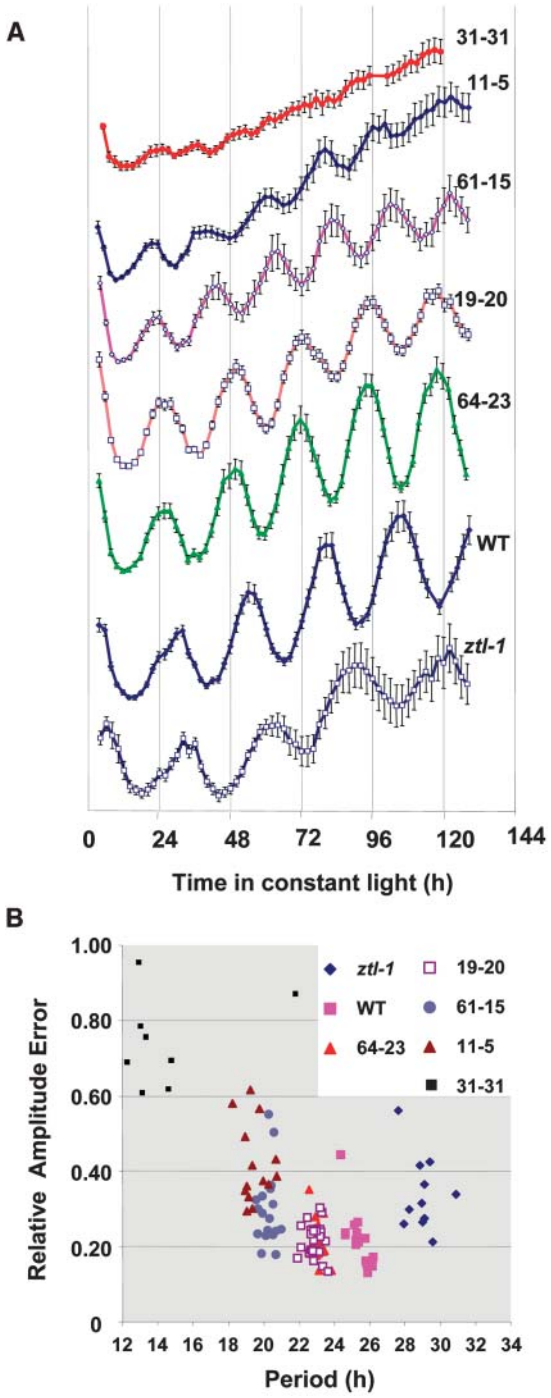

Figure 4. ZTL Level Controls Waveform and Amplitude of Circadian Period.

(A) Representative traces of luminescence expression (*CAB:luc*) from a set of seven lines expressing decreasing levels (top to bottom) of ZTL. Plants were entrained in 12-h-light (white)/12-h-dark cycles and transferred to RR (20 μmol m^{-2} s^{-1}). Traces (±SE) are positionally staggered for clarity; luminescence levels in all lines are closely similar. See text for details.

(B) Period estimates for each line plotted against their relative amplitude error. The full 116-h time course (beginning with ZT 12) was used for all period estimates, except for a 60-h window (from 12 to 72 h in RR) for line 31-31.

We further tested the suite of different ZTL overexpressors to more closely examine the relationship between ZTL levels and hypocotyl length. The four lines with increasingly shortened free-running period (Figures 1 and 3) showed a similar relationship relative to each other and the wild type, with higher ZTL protein levels correlating with increasingly longer hypocotyls under all fluence rates tested (Figure 6E). These results show an inverse correlation between free-running period and hypocotyl length, suggesting that the long-hypocotyl phenotypes of previously known arrhythmic mutants (e.g., *elf3-1*, *lhy-1*, and *cca1*) do not arise from arrhythmicity per se.

We then addressed the mechanism of how increased ZTL levels increase hypocotyl length. The long hypocotyl of the strong overexpressor (31-31) in red light is similar to the long-hypocotyl phenotype of the *phyB* mutant. Line 31-31 has wild-type levels of phyB, and the *phyB* mutant has wild-type levels of ZTL (data not shown). We tested whether the phyB signaling system might be compromised by high steady state levels of ZTL, thus lengthening hypocotyl, by performing end-of-day far-red (EODFR) assays. Plants were grown in 10-h-light/14-h-dark cycles and given 15 min of far-red light (FR) at the end of the photoperiod during the last 4 d. Control plants received no end-of-day treatment. As reported previously (Robson et al., 1993), the *phyB* mutant showed no additional elongation as a result of the FR treatment, which converts the active, inhibitory phyB (Pfr) into the inactive Pr form, thereby allowing increased hypocotyl elongation during the subsequent dark period (Figure 7). By contrast, the *ztl-1* and *ztl-3* mutants and their respective wild-type backgrounds showed similar twofold doublings in hypocotyl length in response to the FR treatment. The two ZTL-enhanced expression lines, ZTL OX (ecotype Columbia [Col]) and 31-31 (C24), also showed strong responses to the FR treatment (~60% increase). These data indicate that the long hypocotyl in these lines is not because of an interference of phyB signaling by ZTL.

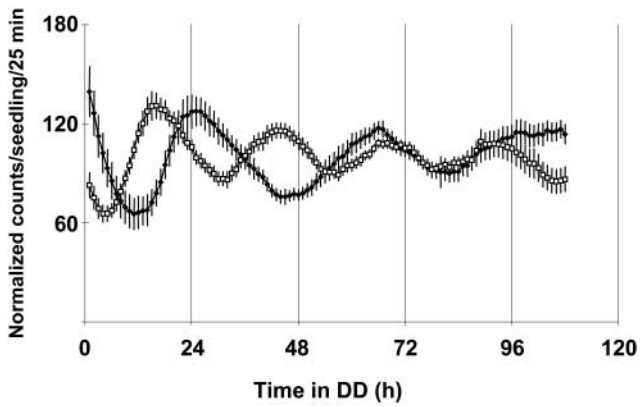

Figure 5. ZTL Controls Circadian Period in the Dark.

Wild-type (Col; open squares) and *ztl-1* (Col; closed diamonds) seedlings expressing the *CCR2:luc* reporter were entrained in 12-h-light (white)/12-h-dark cycles, transferred to DD, and measured for luminescence expression every hour. Mean values (±SE) normalized to mean expression level of 100 for each genotype.

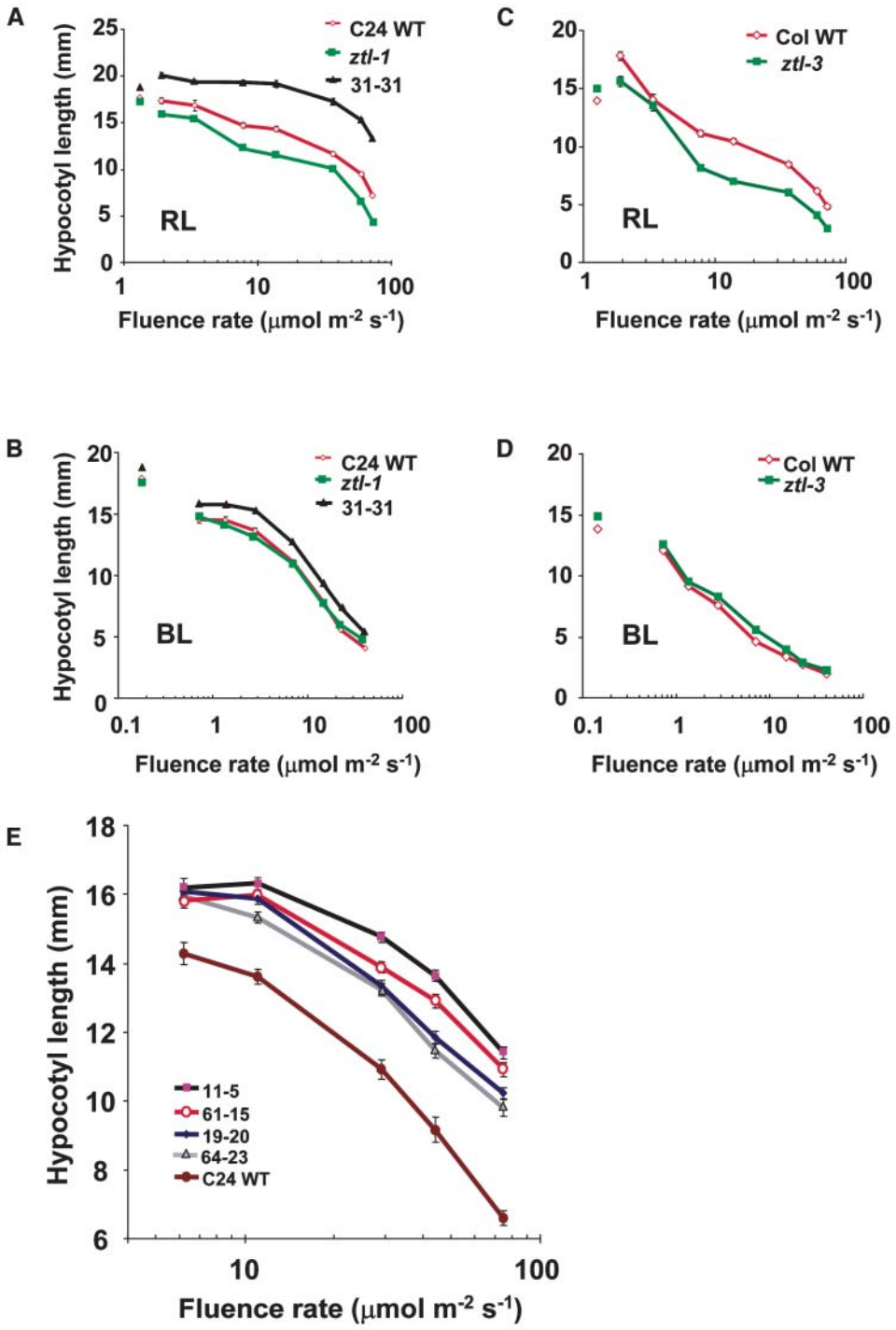

Figure 6. ZTL-Mediated Control of Seedling Hypocotyl Length during Photomorphogenesis.

The wild type (C24), *ztl-1*, and a strong ZTL overexpressor (31-31) (**[A]** and **[B]**) and wild-type (Col) and *ztl-3* (**[C]** and **[D]**) plants were grown for 10 d under RR (**[A]** and **[C]**) or BB (**[B]** and **[D]**) at the fluence rates indicated and measured for hypocotyl length. Unconnected data points show lengths for dark-grown seedlings. Wild-type (C24) and four ZTL-enhanced expression lines (**[E]**; see Figure 1) were grown for 10 d under RR at the fluence rates indicated. Values (mean hypocotyl length ±SE) are representative of two trials; $n = 16$ to 29.

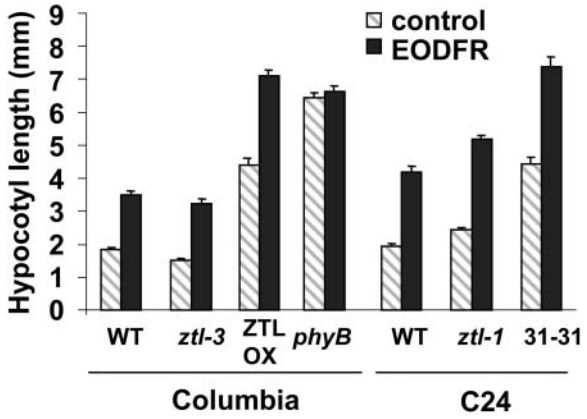

Figure 7. PhyB Control of Hypocotyl Length Is Not Disrupted by Reduced or Enhanced ZTL Levels.

Seeds from two ecotypes (Col and C24) with wild-type (WT; *phyB*), reduced (*ztl-1* and *ztl-3*), and enhanced (ZTL OX and 31-31) expression levels of ZTL were germinated in LL for 2 d, then transferred to a 10-h-light/14-h-dark cycle for 4 d without (control) or with (EODFR) the addition of 15 min of FR at the beginning of the dark period. Values (mean hypocotyl length ±SE) are representative of three trials; $n = 8$ to 23.

ZTL Represses *CO* and *FT* Expression and Delays Flowering

Previously we described a modest delay in flowering under long days (16 h light/8 h dark) caused by the *ztl-1* mutant in the C24 background (Somers et al., 2000). Introgression of *ztl-1* into the Col background and a test of the *ztl-3* (Col) mutation show that loss of ZTL function in the Col ecotype has no effect on flowering time under long days (Figure 8A). This result suggests that the slight delay evident in the C24 background is a result of one or more allele-specific differences between the C24 and Col ecotypes in the genetic interactors with ZTL. By contrast, strong overexpression of ZTL in the C24 background (Figure 8A, line 31-31) and Col background (Kiyosue and Wada, 2000; Nelson et al., 2000; data not shown) significantly delays flowering time in long days, to the extent that the plants flower equally late under short and long days. Interestingly, the series of transgenic lines with increasingly higher levels of ZTL show correspondingly later flowering in long days (Figure 8A). Lines 61-15 and 11-5 express ZTL at levels much below that of line 31-31 (Figure 1A), but all three lines show very similarly delayed flowering. These results suggest that there is a threshold level of ZTL above which there is no additional effect on flowering time.

Transcript levels of two genes, *CO* and *FT*, are positively correlated with the photoperiodic control of flowering time in Arabidopsis (Suarez-Lopez et al., 2001; Yanovsky and Kay, 2002). In particular, high or low levels of *FT* transcript during the photoperiod correlate well with earlier or later flowering, respectively. We therefore tested the effects of high ZTL levels on the temporal expression pattern of *CO* and *FT* to determine whether late flowering arises through a *CO*/*FT*-mediated pathway. *CO* and *FT* message levels were determined every 4 h in the wild type (C24) and ZTL high overexpressing plants (line 31-31) grown under long days (16 h light/8 h dark). Constitutively high expression of ZTL strongly suppressed the expression level of *CO* and *FT* at nearly all time points, regardless of illumination (Figure 8B). *CO* levels in 31-31 were reduced threefold to fourfold over much of the time course, and *FT* levels were reduced >20-fold relative to peak expression in the wild type (Figure 8B). Identical results were obtained in the Col background (data not shown). These data are consistent with the notion that ZTL can suppress flowering time by inhibiting *CO* and *FT* expression. This likely occurs by ZTL acting upstream within a signaling pathway that controls photoperiodic flowering via a *CO*/*FT*-mediated mechanism.

ZTL Controls the Phase of Clock-Associated Components

To investigate how ZTL affects circadian period at the molecular level, we measured the effect of different levels of ZTL on the expression of two putative clock components, *CCA1* and *TOC1*. Under white light entrainment (12 h light/12 h dark), the three *ztl* mutations (*ztl-1*, *ztl-2*, and *ztl-3*) caused a strong reduction in peak *CCA1* mRNA levels and a slight delay in the rise to the early morning peak of *CCA1* expression (Figure 9A). The *ztl* mutations also strongly reduced peak CCA1 levels (Figure 9D). Strong ZTL overexpression (line 31-31) reduced *CCA1* message levels by half, caused a slightly earlier rise in *CCA1* message accumulation, and broadened the profile of the expression peak overall. This anticipation of lights on suggests that high levels of ZTL expression are not sufficient to stop the clock under LD cycles, despite *CAB:luc* arrhythmicity in extended LL and DD.

During the second day in LL, the *ztl* mutations delayed the peak phase of *CCA1* message accumulation, consistent with the period lengthening effects of the mutations (Figure 9A). *CCA1* message levels were not detectably lower than in the wild type, unlike the significant reduction seen soon after lights on during entrainment. However, peak levels of CCA1 in *ztl-1* and *ztl-3* backgrounds remained lower than the wild type (Figure 9D) but were similar to (*ztl-1*) or higher than (*ztl-3*) the low peak levels observed in the mutants in LD (Figure 9D and data not shown). This difference between *CCA1* transcript and protein levels in LD compared with LL in the mutants suggests some level of positive posttranscriptional control of CCA1 levels by ZTL in LL. In line 31-31, *CCA1* message cycled out of phase with the wild type, probably as a result of a strong phase advance (Figure 9A). The *ZTL* dosage differences had very similar effects on *LHY* message levels and patterns in LD and LL (data not shown).

TOC1 transcript levels normally peak in early subjective night, with clearly reduced levels early in the subjective day (Figure 9B, see Supplemental Figure 2 online; Strayer et al., 2000). The three long period *ztl* mutations caused the expected phase delay of the *TOC1* trough in LD (Figure 9B). ZTL overexpression still allowed strong cycling of *TOC1* transcript in LD and caused a slight phase advance in peak *TOC1* message levels, with no detectable effect on the level of peak and trough accumulation (Figure 9B). In LL, *TOC1* peak transcript levels were phase delayed in the mutant backgrounds, and overall amplitude was somewhat reduced. ZTL overexpression had little effect on *TOC1* transcript accumulation and caused a phase advance

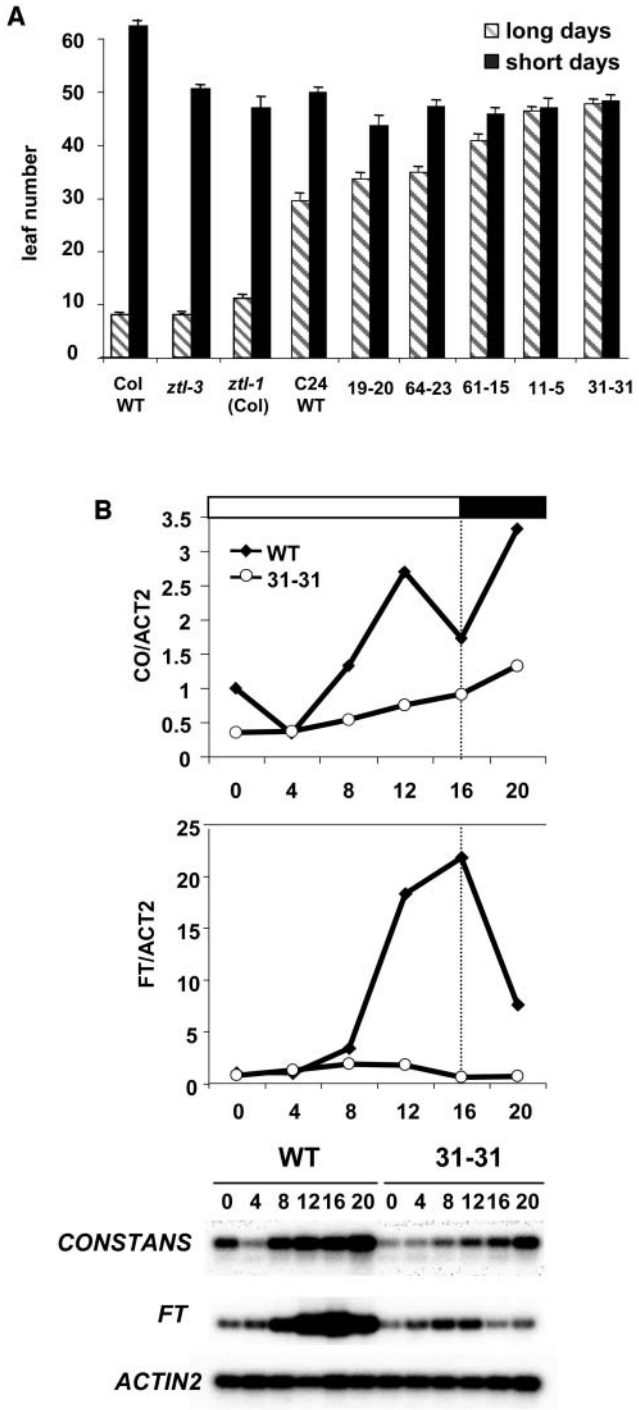

Figure 8. High ZTL Levels Delay Flowering and Alter *CO* and *FT* Expression.

(A) Total number of leaves (rosette + cauline) produced at flowering under long days (16 h light/ 8 h dark) and short days (8 h light/16 h dark) in wild-type (C24, Col), *ztl* mutant (*ztl-3* [Col] and *ztl-1*[Col]), and overexpressor (31-31) backgrounds. Values (±SE) are representative of two trials; $n = 10$ to 16.

(B) Expression levels of *CO* and *FT* transcripts under 16 h light/8 h dark were determined by semiquantitative PCR for the wild type (C24) and a ZTL overexpressor (31-31) every 4 h. Values are expressed relative to *ACT2* control. Open and closed boxes represent light and dark periods, respectively. Data representative of two independent trials.

consistent with period shortening (Figure 9B, see Supplemental Figure 2 online).

Together, these results show that ZTL likely controls period by affecting the phasing of peak transcript levels of two key components of the Arabidopsis circadian clock. However, *CCA1* mRNA and protein levels were markedly reduced (Figure 9, see Supplemental Figure 2 online), and though the long periods of the *ztl* mutants are opposite to the short periods seen in *cca1* loss-of-function mutants (Green and Tobin, 1999), ZTL control of CCA1 levels may affect period indirectly.

Arrhythmicity in the *elf3* mutant is a result of the absence of ELF3; overexpression of *ELF3* causes only modest lengthening of period (Hicks et al., 1996; Covington et al., 2001). We tested whether period phenotypes caused by differences in ZTL abundance may be through changes in *ELF3* expression levels. In LD, *ELF3* transcript levels were rhythmic with peak expression during early night (Figure 9C, see Supplemental Figure 2 online; Hicks et al., 2001). *ztl* mutations cause a slight delay in peak *ELF3* expression but have no detectable effects on *ELF3* transcript levels or amplitude. In LL, *ELF3* mRNA is still weakly cyclic in the wild type, much less so in the mutants, but expression levels do not differ markedly (Figure 9C, see Supplemental Figure 2 online). Significantly, ZTL overexpression has no marked effect on *ELF3* transcript accumulation in either LD or LL (Figures 9C). These results suggest that the arrhythmicity seen in the ZTL OX is not because of changes in *ELF3* mRNA abundance.

DISCUSSION

ZTL Levels Control Circadian Period

Through a combination of null mutations and graded levels of enhanced expression, we have shown that ZTL can modulate circadian period over a >20-h range. At the long period extreme, *ztl* null mutants lengthen period to ~35 h in the dark, 9 h longer than the wild type. Free-running periods as short as 13 h were observed in strong ZTL overexpressors in the light. Except for line 31-31, the absolute level of ZTL increase over the wild type was only on the order of twofold or less (Figure 1B). The relatively strong effect of these small changes raises the question of the significance of the normal threefold to fourfold diurnal variation in ZTL in the wild type (Figure 1; W.Y. Kim et al., 2003). If ZTL activity remained proportional to its abundance over this range, large variations in period would be expected. In addition, ZTL cycling damps normally to high levels in extended LL in the wild type (W.Y. Kim et al., 2003) with little change in free-running period. These data suggest that ZTL activity is somehow temporally and/or spatially restricted during the circadian cycle. This could be achieved through posttranslational modifications and/or sequestering into regions or complexes of inactivity.

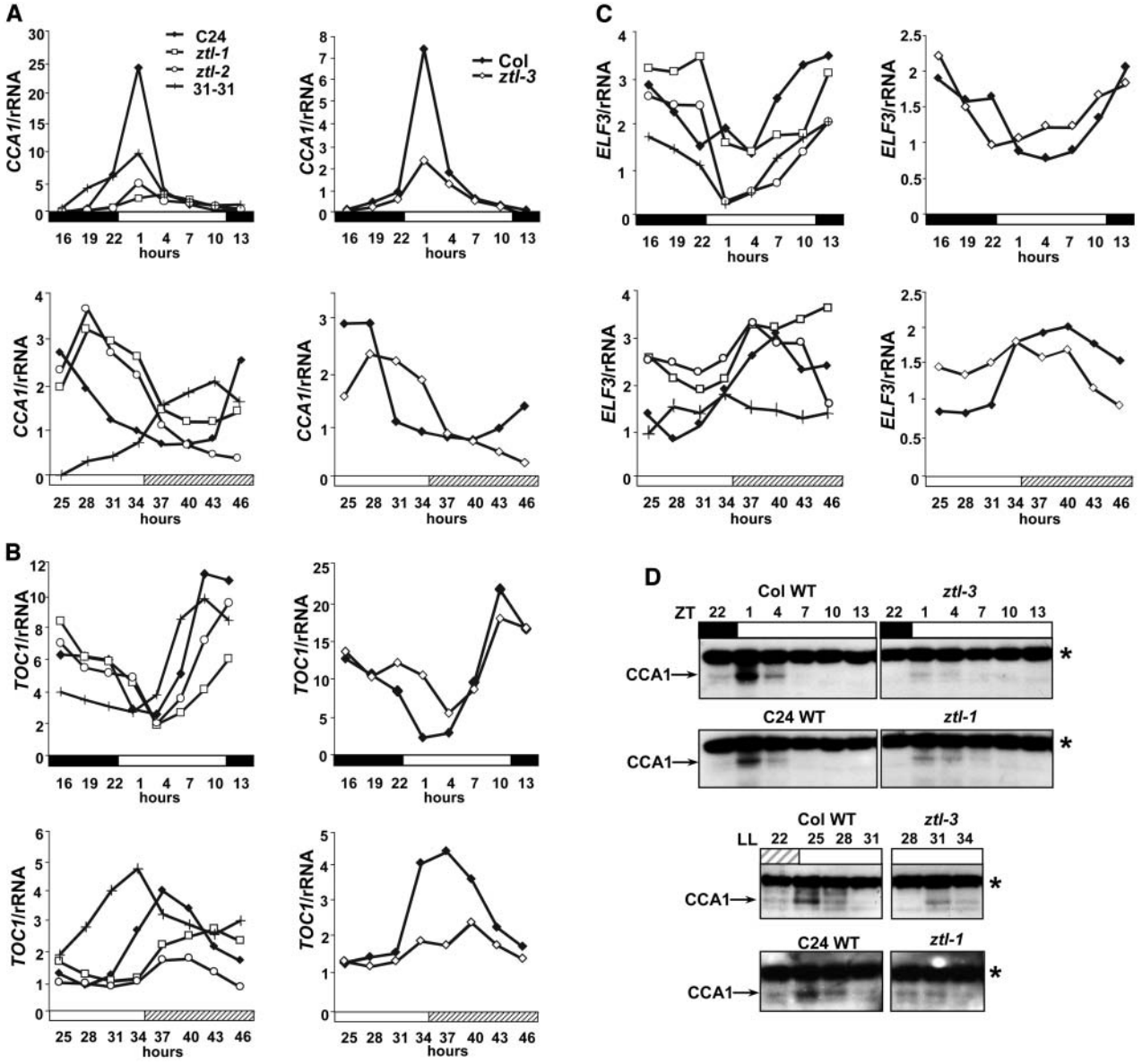

Figure 9. ZTL Levels Alter Cycling of Circadian-Regulated Genes in LD and LL.

Wild-type (C24 and Col), *ztl-1*, *ztl-2*, *ztl-3*, and overexpressor line 31-31 plants were grown for 10 d in 12-h-light/12-h-dark cycles and sampled every 3 h for LD or transferred to LL and harvested every 3 h (beginning with ZT25) for LL. The cycling of *CCA1* **(A)**, *TOC1* **(B)**, and *ELF3* **(C)** was analyzed by RNA gel blotting. Values are expressed relative to rRNA loading control. CCA1 protein **(D)** detected with anti-CCA1 antibody under LD (ZT; hours) and LL (hours in LL). Asterisks indicate nonspecific band cross-reacting with anti-CCA1 antibody. Open and closed boxes indicate light and dark period, respectively, and cross-hatched boxes indicate subjective light.

The ZTL dosage series also demonstrates that one way to achieve arrhythmicity is through increasing period to the point of cycling loss. The extremely short period (~13 h) occasionally observed in line 31-31 suggests a possible upper limit to the pace of the oscillator in Arabidopsis. Until more is known about the molecular interactors of the oscillator and their properties, the explanation for this limit remains obscure. However, our findings raise the question of whether arrhythmicity attained through increasingly shorter periods is fundamentally different from arrhythmicity attained through increasingly longer periods. Comparative analysis of graded series of enhanced expressers of other period-controlling loci may be revealing. Such an expression series for CCA1 has previously been reported but remains unanalyzed for period differences (Wang and Tobin, 1998).

ZTL Turnover Is Independent of Functional Activity

The *ztl-2* and *ztl-1* mutations are identically positioned D-to-N exchanges in the first and third kelch repeats, respectively (Somers et al., 2000). Kelch domains are found in a wide range of proteins, usually in tandem arrays of 4 to 7 repeats. Based on the crystal structure of the seven-kelch domain from galactose oxidase, it is likely that the ZTL kelch domain folds into a six-bladed whorl of four-stranded β-sheets to form a β-propeller. This structure provides an extensive surface available for protein–protein interactions (Adams et al., 2000) and is likely at least part of the substrate interaction domain of ZTL. The long-period phenotype of the protein-positive *ztl-1* and *ztl-2* mutants is identical to the *ztl-3* null mutant, indicating the essential role of the kelch domain in effecting ZTL activity.

Some F-box proteins are autocatalytically targeted for ubiquitination within their own SCF complex (Galan and Peter, 1999). We have previously shown that ZTL is degraded via the proteasome in a phase-specific manner, although the degradation pathway is not known (W.Y. Kim et al., 2003). Our results now show that a fully functional ZTL is not necessary for this regulation, as both mutant proteins show a diurnal regulation in abundance similar to the wild type. These data also suggest that the ZTL–substrate interaction is likely not responsible for the observed variation in ZTL abundance, although it is formally possible that *ztl-1* and *ztl-2* still bind substrate(s) nonproductively.

ZTL, the Clock, and Photomorphogenesis

To date, there have been no reports of a single component of the circadian system that, when disrupted, leaves seedling photomorphogenesis unaffected. The close correlation between ZTL levels, period length, and hypocotyl expansion reinforces the notion that many elements of phototransduction pathways are shared and interconnected.

Increasingly shorter periods resulted from higher levels of ZTL, which correlated with increasingly longer hypocotyls. The higher ZTL levels also diminished the amplitude of the luminescence rhythms (Figure 4). Two possible ways to reduce amplitude is to raise the troughs toward the peaks or reduce peak expression downward to trough levels. With this in mind, our data may usefully be viewed in the context of a previous report describing the timing of the permissive and restrictive phases of hypocotyl expansion in LL (Dowson-Day and Millar, 1999). This group showed that the phase of maximal hypocotyl elongation (peak) occurs near subjective dusk, and greatest growth arrest (trough) occurs at subjective dawn. If the peak corresponds to the permissive (expansion) portion of the cycle, then longer hypocotyls will occur with increasingly shorter periods if trough levels have risen toward peak levels. During each cycle the plants spend more time in the expansion phase than in the arrest phase, and hypocotyls elongate more relative to the wild type. Other clock mutants with similarly short periods but with short hypocotyls may cause peak levels to fall toward the troughs, resulting in more time during a cycle spent in the restrictive growth phase. *cca1* and *lhy* loss-of-function mutants, with short periods and hypocotyls that are wild type (Mizoguchi et al., 2002) or hypersensitive to light (Mas et al., 2003a), are of this class. Interestingly, *CCA1/LHY* shows peak expression in the early subjective day (during the time of normal growth arrest), whereas ZTL peaks during the early subjective night (time of maximal hypocotyl expansion).

The above interpretation is consistent with our finding that phyB-mediated suppression of hypocotyl still operates in ZTL overexpressors (Figure 7). It also explains why *ztl-1* and line 31-31 have similar hypocotyl lengths in the dark, where phy is inactive. If gating of phyB-mediated suppression is the sole function of clock-controlled hypocotyl extension, then our model predicts that the *phyB* mutant will be epistatic to ZTL overexpressors. We are currently testing this hypothesis using double mutants.

ZTL and Flowering Time

A molecular link has recently been established between photoperiod, gene expression, and flowering time. The regulation of *FT* expression by *CO* and light is fundamental to the floral transition in the long-day plant Arabidopsis, in which high levels of *FT* message during the photoperiod correlate with more rapid flowering (Yanovsky and Kay, 2003). The late-flowering phenotype of the strong ZTL overexpressor (31-31) phenocopies one class of late-flowering mutants in causing a strong delay of flowering under long days but no effect under short days (Mouradov et al., 2002). Significantly, *ft* and *co* mutants are among that group. Here, we have shown that enhanced ZTL accumulation causes late flowering, most likely through a strong depression in the levels of *CO* and *FT*. Under long days, there is still some variation in *CO* abundance, consistent with the diurnal variation we found in *CCA1*, *TOC1*, and *ELF3* levels (Figure 9). However, this much-reduced accumulation of *CO* is apparently not adequate to induce sufficient *FT* expression. Interestingly, line 31-31 also flowers late in LL, where we observed rapid damping of clock-regulated promoter activity and message accumulation. These results then suggest that the absence of appropriate phasing relative to an LD cycle is not a factor in the delayed flowering seen in 31-31.

We cannot presently distinguish whether ZTL affects *CO*/*FT* expression via its effect on circadian period or whether its regulation of *CO*/*FT* is through a clock-independent mechanism. The very similar late-flowering phenotype of line 11-5, which retains short-period cycling, and line 31-31, which is largely arrhythmic, suggests the latter case. Tests of other arrhythmic mutants, both late flowering (*lhy-1*) and early flowering (*elf3-1*), also support this notion (Suarez-Lopez et al., 2001). Like line 31-31, both *lhy-1* and *elf3-1* are arrhythmic in LL, and both exhibit their respective late or early flowering when grown under LL. However, *lhy-1* shows reduced levels of *FT* message and *elf3-1* enhances FT levels (Suarez-Lopez et al., 2001). Both results are consistent with the model of *FT*-mediated regulation of flowering time but also suggest that a functional clock is not required and that these genes control flowering through a different mechanism. Alternatively, the circadian system may be stopped at different positions or phases in the cycle in these mutants, effectively locking different sets of genes into on or off positions, resulting in different flowering times (Carré, 1998).

Because the *ZTL* family members are closely related, it is possible that abnormally high levels of ZTL result in its ectopic intercalation into an LKP2- or FKF1-dependent mechanism of flowering time control. However, insofar as an *fkf1* deletion also delays flowering (Nelson et al., 2000), a more complete analysis of overexpression and loss-of-function mutations of the three family members is needed.

ZTL and Clock-Associated Components

By examining the effects of ZTL loss and overexpression on *CCA1*, *TOC1*, and *ELF3* expression, we have shown that ZTL does not control circadian period through sustained changes in the transcription or transcript stability of these genes. However, the phasing of all three transcripts is affected in LD and LL, indicating that clock activity has been affected despite the normal expression levels of these transcripts. These results emphasize the role of ZTL as a component of an SCF complex (L. Han and D.E. Somers, unpublished data), whereby ZTL acts to degrade one or more protein components of the circadian system. Apart from the observation noted below, the degradation targets of ZTL do not appear to be transcription factors that directly regulate these three genes.

An exception to the above is the strongly reduced level of *CCA1* transcript and protein in the *ztl* mutants soon after dawn. This transient change suggests that the light-dependent upregulation of *CCA1* requires ZTL. Under sustained LL, *CCA1* mRNA abundance remains at wild-type levels in the *ztl* mutants and overexpressor, although CCA1 protein levels remain below wild type in the mutants in LL. This suggests that ZTL participates in at least two functions in the light: one controlling an acute response of *CCA1*/*LHY* induction to light and a second involving the maintenance of circadian period in LL. It is not clear how the two roles are related because the reduced levels of CCA1 in LL should shorten period (Green and Tobin, 1999), if that is the only effect of ZTL loss on the clock.

CCA1 and LHY have been attractive candidate substrates for ZTL. Protein levels of both are strongly rhythmic and cycle out of phase with ZTL protein abundance (Wang and Tobin, 1998; J.Y. Kim et al., 2003; W.Y. Kim et al., 2003). This relationship fits well a simple model whereby low levels of LHY and CCA1 occur through their proteolysis at times of peak ZTL abundance/activity. Times of low ZTL abundance would then allow reaccumulation of the two proteins. However, our results indicate that CCA1 is not a substrate for degradation by ZTL. Whereas loss of ZTL should cause an increase in the accumulation of the degradation target, CCA1 levels are instead significantly reduced in the *ztl* mutant backgrounds. In addition, in line 31-31 CCA1 levels damp high in LL (L. Han and D.E. Somers, unpublished data). The concomitant reduction of *CCA1* message and protein levels in the *ztl* mutants also indicates that ZTL affects *CCA1* gene expression at the transcriptional level, possibly through effects on a component of the *CCA1* transcriptional activation complex. Most likely this target is a negative regulator of *CCA1* expression, although not necessarily a direct substrate of ZTL. The recent report of TOC1 as one likely degradation target of ZTL (Mas et al., 2003b) does not explain these *CCA1* expression results. A current model proposing TOC1 as a positive regulator of *CCA1* expression (Alabadi et al., 2001) would predict that *CCA1* message and protein levels should rise in the *ztl* mutant backgrounds, as higher TOC1 levels are maintained. The opposite is observed, suggesting that the current model is incomplete.

Neither does it appear that the drops in *CCA1*/*LHY* levels are directly responsible for the *ztl* mutant phenotype. Single gene and double mutant *cca1* and *lhy* plants have shorter periods (Schaffer et al., 1998; Wang and Tobin, 1998; Alabadi et al., 2002; Mizoguchi et al., 2002), the opposite of the *ztl* mutants. Clearly, a more complex set of interactions are involved in period control.

Perspective

The phytochromes and cryptochromes are true light-activated photoreceptors, as evidenced by the absence of aberrant hypocotyl or circadian phenotypes in the mutants in the dark and the fluence rate dependence of these phenotypes in the light (Somers et al., 1998; Devlin and Kay, 2000). Although recent reports indicate that the ZTL LOV domain can bind flavin and undergo light-dependent changes in absorption (Imaizumi et al., 2003), the effect of the mutants and overexpressors on period in both light and dark suggests that ZTL acts as more than a typical photoreceptor. It may complex with one or more known photoreceptors under some circumstances (e.g., red, blue, or white light) and target one or more proteins for light-dependent degradation. Under other conditions (e.g., dark), it may act independently or partner with a second complex to facilitate proteolysis of a separate set of substrates (Mas et al., 2003b). The existence of multiple substrates for a single F-box protein is well established (Craig and Tyers, 1999; Jackson et al., 2000). Presumably for ZTL, under all conditions at least some of the targeted polypeptides are involved in the control of the pace of the oscillator. Hence, unlike the on/off light switch nature of the phytochromes, ZTL may act more like a Y-valve, redirecting the ubiquitinating potential of the participating SCF complex in one direction or another, depending on the light environment.

METHODS

Plasmid Construction and Plant Material

The PCR-generated full-length *ZTL* cDNA sequence was cloned downstream of the double *Cauliflower mosaic virus* 35S promoter (pRTL2; Restrepo et al., 1990) into unique NcoI and KpnI restriction sites using the pZL1 plasmid clone 220G18 (ABRC) as the template and the primers 5′-GAATTTCCATGGAGTGGGACAGTG-3′ and 5′-ATACAT-AGGTACCCTTTGGTTACGTG-3′ to introduce the appropriate restriction sites (underlined) in the resulting amplicon. The native *ZTL* gene was cloned from the BAC plasmid (pBeloBAC11) clone T4D6 (ABRC) using the primers 5′-ATCATTCTAGAGGTGCTTGTTGGAAG-3′ and 5′-TTCTAGA-GAGGAAGGTGCTAGTGTC-3′ to generate a 5577-bp product that includes 2379 bp upstream of the ATG and 541 bp downstream of the stop codon. This product and the 35S promoter–driven cDNA were subcloned into the binary vector pZP221 (Hajdukiewicz et al., 1994) and transformed into Arabidopsis via Agrobacterium-mediated transformation (Clough and Bent, 1998). The *ztl-1* and *ztl-2* alleles (C24 ecotype) were identified from an ethyl methanesulfonate–mutagenized population as described previously (Somers et al., 2000). The *ztl-1* (Col) line derived from a three-generation introgression of the original *ztl-1* mutant into the

Col ecotype. The *ztl-3* (Col) contains a T-DNA insertion at amino acid 440 and is an independent characterization of a mutant described previously (Jarillo et al., 2001).

Plant Growth Conditions and Rhythm Analysis

Seedlings for luminescence analysis were grown on MS (GIBCO BRL, Cleveland, OH) + 3% sucrose (0.8% agar) under 12-h-light/12-h-dark white fluorescent light (50 to 60 μmol m^{-2} s^{-1}) for 5 to 7 d, then sprayed with 3 mM luciferin (Biotium, Hayward, CA) before transfer to constant red light (peak wavelength 670 nm ± 15 nm half-peak bandwidth; Quantum Devices, Barneveld, WI), blue light (Bili Blue [Interlectric, Warren PA] filtered through Rohm and Haas 2424 plexiglass [Philadelphia, PA] 5 mm thick), or darkness and imaged 25 min every 2 h using a Peltier-cooled CCD slow scan camera (Nightowl; Berthold Technologies, Bad Wildbad, Germany). Postimaging luminescence quantitation used WinLight software (Berthold Technologies). Period estimates were obtained using fast Fourier transform nonlinear least-squares analysis (Plautz et al., 1997), with period estimates falling between 15 and 45 h taken to be within the circadian range, except where noted. Mean period lengths and associated error metrics were variance weighted (Millar et al., 1995). Relative amplitude error (range 0 to 1.0) was used to assess individual rhythm robustness, with values close to 0 indicating robust cycling and values at or near 1 indicating a rhythm with an error value as large as the amplitude itself (not statistically significant) (Plautz et al., 1997). Relative amplitude error values >0.6 were taken as unreliably weakly rhythmic and not included in reporting mean period length, except where noted.

Hypocotyl Length Assays

Seeds were stratified on MS (GIBCO BRL) + 3% sucrose (0.8% agar) for 3 d, exposed to white light (30 μmol m^{-2} s^{-1}) for 1 to 2 h, then placed under the appropriate light quality and fluence rate (using varying layers of neutral density filters; Roscolux 397 [Rosco Laboratories, Stamford, CT]) for 7 to 10 d. Hypocotyl length was measured by a ruler or using SCION Image software (Frederick, MD).

EODFR Response

Seeds were sterilized and plated on medium containing MS salt (GIBCO) (0.8% agar). After 4-d stratification, seeds were germinated under continuous white fluorescent light (100 μmol m^{-2} s^{-1}) for 2 d, then grown under a 10-h-light/14-h-dark photoperiod with or without 15 min of FR (peak wavelength 735 nm ±15 nm half-peak bandwidth; Quantum Devices) given at the beginning of darkness, for each of 4 d. Hypocotyl lengths were measured at the beginning of the seventh photoperiod (SCION Image software).

Flowering Time Analyses

Seeds were stratified on MS (GIBCO BRL) + 3% sucrose (0.8% agar) for 3 d then grown under long days (16 h light/8 h dark; 70 to 100 μmol m^{-2} s^{-1}) or short days (8 h light/16 h dark; 70 to 100 μmol m^{-2} s^{-1}) for 7 to 10 d, after which they were transplanted to soil. Total number of rosette and cauline leaves were counted.

Gene Expression Analyses

RNA Gel Blot Analyses

RNA extractions and RNA gel blot analyses were performed as described previously (W.Y. Kim et al., 2003). Equivalent lane loadings were obtained by ethidium bromide staining of total RNA. The *ELF3* full-length cDNA was radioactively labeled according to W.Y. Kim et al. (2003) and used to detect *ELF3* message.

Immunoblotting

For ZTL detection, extracts were prepared from 12-d-old Arabidopsis seedlings. Supernatant protein concentration was determined (Bio-Rad, Hercules, CA), concentrated by trichloroacetic acid precipitation, and resuspended in urea/SDS loading buffer (40 mM Tris-Cl, pH 6.8, 8 M urea, 5% SDS, 1 mM EDTA, and 2% 2-mercaptoethanol) to a final concentration of 10 μg/μL, separated by SDS-PAGE (8%), and subjected to immunoblot analysis as described previously (W.Y. Kim et al., 2003). For CCA1 detection, ground tissue was resuspended in extraction buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, 3 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 5 μg/mL leupeptin, 1 μg/mL aprotinin, 1 μg/mL pepstatin, and 5 μg/mL chymostatin) by gentle vortexing and clarified by centrifugation at 14,000*g* for 10 min at 4°C. Protein concentration was determined (Rc/Dc reagent; Bio-Rad). Forty micrograms of protein per lane was fractionated by SDS-PAGE (8%) and transferred to polyvinylidene fluoride membrane (Bio-Rad). Immunoblotting and immunodetection were performed using a polyclonal antibody (Cocalico, Reamstown, PA) raised to the C-terminal region (N-terminal deletion of the first 136 amino acids) of CCA1. The immunoblots were incubated (2 h) with affinity-purified primary antibody diluted 1:1000 in 1.6% nonfat dry milk in TBS (10 mM Tris-Cl, pH 8.0, and 150 mM NaCl), washed with 1× TBS (three times), probed with diluted (1:3000) anti-rabbit secondary antibody (Amersham, Buckinghamshire, UK) for 1.5 h, and developed by enhanced chemiluminescence (Amersham). The CCA1-specific band was identified by comparison of wild-type protein extracts to those from a *cca1* null line (Green and Tobin, 1999). A single-band difference of the correct predicted size was identified.

RT-PCR

Seedlings were grown in 16-h-light/8-h-dark cycles for 7 d (70 μmol m^{-2} s^{-1} white fluorescent light), and seedlings were collected on day 8 at the appropriate times. RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) and quantified by spectrophotometer. Transcripts of *CO* and *FT* were quantified by reverse transcription (RT)–PCR, followed by DNA gel blot analysis as described previously (Yanovsky and Kay, 2002), with the following modifications: RTs were performed using SuperScript II RT (Invitrogen), oligo$(dT)_{12\text{-}18}$ (Invitrogen), and 4 μg of total RNA according to manufacturer's instructions. The RT reactions were diluted fivefold, and 2 μL of the dilutions was used for PCR. The primer sequences and annealing temperatures used to amplify *CO* and *FT* fragments are as follows: *CO*, 48°C, 5′-ACGCCATCAGCGAGTTCC-3′ and 5′-AAATGTATGCGTTATGGTTAATGG-3′; and *FT*, 60°C, 5′-ACAACTGGAACAACCTTTGGCAATG-3′ and 5′-ACTACTATAGGCATCATCACCGTTCGTTACTCG-3′. A portion of At3g18780 (*ACTIN 2*; *ACT2*) was amplified using oligonucleotides 5′-AAAACCACTTACAGAGTTCGTTCG-3′ and 5′-GTTGAA CGGAAGGGATTGAGAGT-3′ with the annealing temperature of 55°C and used as an internal control to normalize the amount of cDNA. The exponential range of amplification was determined for each gene, and 19 cycles were used for *ACT2* and 23 cycles were used for *CO* and *FT*. Four microliters of 20-μL PCR products were separated on 2% agarose gel and transferred to Hybond-XL nylon membrane (Amersham). The PCR fragments of each gene were radioactively labeled and used as probes for DNA gel blot hybridization. DNA gel blot hybridizations were performed at 65°C overnight in phosphate buffer (0.5 M $NaHPO_4$, pH 7.2, 7% SDS, 1 mM EDTA, and 100 μg/mL of herring sperm DNA), followed by two washes in 2× SSC containing 0.1% SDS and two washes in 0.2× SSC containing 0.1% SDS

at 65°C. Images were quantified using PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

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REFERENCES

Adams, J., Kelso, R., and Cooley, L. (2000). The kelch repeat superfamily of proteins: Propellers of cell function. Trends Cell Biol. **10,** 17–24.

Alabadi, D., Oyama, T., Yanovsky, M.J., Harmon, F.G., Mas, P., and Kay, S.A. (2001). Reciprocal regulation between TOC1 and LHY/CCA1 within the Arabidopsis circadian clock. Science **293,** 880–883.

Alabadi, D., Yanovsky, M.J., Mas, P., Harmer, S.L., and Kay, S.A. (2002). Critical role for CCA1 and LHY in maintaining circadian rhythmicity in Arabidopsis. Curr. Biol. **12,** 757–761.

Barak, S., Tobin, E.M., Andronis, C., Sugano, S., and Green, R.M. (2000). All in good time: The Arabidopsis circadian clock. Trends Plant Sci. **5,** 517–522.

Benvenuto, G., Formiggini, F., Laflamme, P., Malakhov, M., and Bowler, C. (2002). The photomorphogenesis regulator DET1 binds the amino-terminal tail of histone H2B in a nucleosome context. Curr. Biol. **12,** 1529–1534.

Briggs, W.R., and Christie, J.M. (2002). Phototropins 1 and 2: Versatile plant blue-light receptors. Trends Plant Sci. **7,** 204–210.

Carré, I.A. (1998). Genetic dissection of the photoperiod-sensing mechanism in the long-day plant *Arabidopsis thaliana*. In Biological Rhythms and Photoperiodism in Plants, P.J. Lumsden and A.J. Millar, eds (Oxford, UK: BIOS Scientific Publishers), pp. 257–269.

Carré, I.A., and Kim, J.Y. (2002). MYB transcription factors in the Arabidopsis circadian clock. J. Exp. Bot. **53,** 1551–1557.

Cheng, P., Yang, Y., Wang, L., He, Q., and Liu, Y. (2003). WHITE COLLAR-1, a multifunctional Neurospora protein involved in the circadian feedback loops, light sensing, and transcription repression of wc-2. J. Biol. Chem. **278,** 3801–3808.

Clough, S.J., and Bent, A.F. (1998). Floral dip: A simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J. **16,** 735–743.

Covington, M.F., Panda, S., Liu, X.L., Strayer, C.A., Wagner, D.R., and Kay, S.A. (2001). Elf3 modulates resetting of the circadian clock in Arabidopsis. Plant Cell **13,** 1305–1316.

Craig, K.L., and Tyers, M. (1999). The F-box: A new motif for ubiquitin dependent proteolysis in cell cycle regulation and signal transduction. Prog. Biophys. Mol. Biol. **72,** 299–328.

Deshaies, R.J. (1999). SCF and Cullin/Ring H2-based ubiquitin ligases. Annu. Rev. Cell Dev. Biol. **15,** 435–467.

Devlin, P.F., and Kay, S.A. (2000). Cryptochromes are required for phytochrome signaling to the circadian clock but not for rhythmicity. Plant Cell **12,** 2499–2510.

Dowson-Day, M.J., and Millar, A.J. (1999). Circadian dysfunction causes aberrant hypocotyl elongation patterns in Arabidopsis. Plant J. **17,** 63–71.

Doyle, M.R., Davis, S.J., Bastow, R.M., McWatters, H.G., Kozma-Bognar, L., Nagy, F., Millar, A.J., and Amasino, R.M. (2002). The ELF4 gene controls circadian rhythms and flowering time in Arabidopsis thaliana. Nature **419,** 74–77.

Froehlich, A.C., Liu, Y., Loros, J.J., and Dunlap, J.C. (2002). White Collar-1, a circadian blue light photoreceptor, binding to the frequency promoter. Science **297,** 815–819.

Galan, J.M., and Peter, M. (1999). Ubiquitin-dependent degradation of multiple F-box proteins by an autocatalytic mechanism. Proc. Natl. Acad. Sci. USA **96,** 9124–9129.

Green, R.M., and Tobin, E.M. (1999). Loss of the circadian clock-associated protein 1 in Arabidopsis results in altered clock-regulated gene expression. Proc. Natl. Acad. Sci. USA **96,** 4176–4179.

Hajdukiewicz, P., Svab, Z., and Maliga, P. (1994). The small, versatile pPZP family of Agrobacterium binary vectors for plant transformation. Plant Mol. Biol. **25,** 989–994.

Hall, A., Kozma-Bognar, L., Bastow, R.M., Nagy, F., and Millar, A.J. (2002). Distinct regulation of CAB and PHYB gene expression by similar circadian clocks. Plant J. **32,** 529–537.

Harmer, S.L., Hogenesch, J.B., Straume, M., Chang, H.S., Han, B., Zhu, T., Wang, X., Kreps, J.A., and Kay, S.A. (2000). Orchestrated transcription of key pathways in Arabidopsis by the circadian clock. Science **290,** 2110–2113.

Harmer, S.L., Panda, S., and Kay, S.A. (2001). Molecular bases of circadian rhythms. Annu. Rev. Cell Dev. Biol. **17,** 215–253.

He, Q., Cheng, P., Yang, Y., Wang, L., Gardner, K.H., and Liu, Y. (2002). White Collar-1, a DNA binding transcription factor and a light sensor. Science **297,** 840–843.

Hicks, K.A., Albertson, T.M., and Wagner, D.R. (2001). Early flowering3 encodes a novel protein that regulates circadian clock function and flowering in Arabidopsis. Plant Cell **13,** 1281–1292.

Hicks, K.A., Millar, A.J., Carré, I.A., Somers, D.E., Straume, M., Meeks-Wagner, R., and Kay, S.A. (1996). Conditional circadian dysfunction of the Arabidopsis early-flowering 3 mutant. Science **274,** 790–792.

Imaizumi, T., Tran, H.G., Swartz, T.E., Briggs, W.R., and Kay, S.A. (2003). FKF1 is essential for photoperiodic-specific light signalling in Arabidopsis. Nature **426,** 302–306.

Jackson, P.K., Eldridge, A.G., Freed, E., Furstenthal, L., Hsu, J.Y., Kaiser, B.K., and Reimann, J.D. (2000). The lore of the RINGs: Substrate recognition and catalysis by ubiquitin ligases. Trends Cell Biol. **10,** 429–439.

Jarillo, J.A., Capel, J., Tang, R.H., Yang, H.Q., Alonso, J.M., Ecker, J.R., and Cashmore, A.R. (2001). An Arabidopsis circadian clock component interacts with both CRY1 and phyB. Nature **410,** 487–490.

Kim, W.Y., Geng, R., and Somers, D.E. (2003). Circadian phase-specific degradation of the F-box protein ZTL is mediated by the proteasome. Proc. Natl. Acad. Sci. USA **100,** 4933–4938.

Kim, J.Y., Song, H.R., Taylor, B.L., and Carré, I.A. (2003). Light-regulated translation mediates gated induction of the Arabidopsis clock protein LHY. EMBO J. **22,** 935–944.

Kiyosue, T., and Wada, M. (2000). LKP1 (LOV kelch protein 1): A factor involved in the regulation of flowering time in Arabidopsis. Plant J. **23,** 807–815.

Mas, P., Alabadi, D., Yanovsky, M.J., Oyama, T., and Kay, S.A. (2003a). Dual role of TOC1 in the control of circadian and photomorphogenic responses in Arabidopsis. Plant Cell **15,** 223–236.

Mas, P., Kim, W.Y., Somers, D.E., and Kay, S.A. (2003b). Targeted degradation of TOC1 by ZTL modulates circadian function in Arabidopsis thaliana. Nature **426,** 567–570.

Matsushika, A., Makino, S., Kojima, M., and Mizuno, T. (2000). Circadian waves of expression of the *APRR1/TOC1* family of pseudo-

response regulators in *Arabidopsis thaliana*: Insight into the plant circadian clock. Plant Cell Physiol. **41,** 1002–1012.

McClung, C.R. (2001). Circadian rhythms in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. **52,** 139–162.

McClung, C.R., Salome, P.A., and Michael, T.P. (2002). The Arabidopsis circadian system. In The Arabidopsis Book, C.R. Somerville and E.M. Meyerowitz, eds (Rockville, MD: American Society of Plant Biologists), doi/10.1199/tab.0044, http://www.aspb.org/publications/arabidopsis/.

Millar, A.J. (2003). A suite of photoreceptors entrains the plant circadian clock. J. Biol. Rhythms **18,** 217–226.

Millar, A.J., Carré, I.A., Strayer, C.A., Chua, N.-H., and Kay, S.A. (1995). Circadian clock mutants in *Arabidopsis* identified by luciferase imaging. Science **267,** 1161–1163.

Mizoguchi, T., Wheatley, K., Hanzawa, Y., Wright, L., Mizoguchi, M., Song, H.R., Carré, I.A., and Coupland, G. (2002). LHY and CCA1 are partially redundant genes required to maintain circadian rhythms in Arabidopsis. Dev. Cell **2,** 629–641.

Mouradov, A., Cremer, F., and Coupland, G. (2002). Control of flowering time: Interacting pathways as a basis for diversity. Plant Cell **14** (suppl.), S111–S130.

Nelson, D.C., Lasswell, J., Rogg, L.E., Cohen, M.A., and Bartel, B. (2000). FKF1, a clock-controlled gene that regulates the transition to flowering in Arabidopsis. Cell **101,** 331–340.

Park, D., Somers, D.E., Kim, Y., Choy, Y., Lim, H., Soh, M., Kim, H., Kay, S.A., and Nam, H.G. (1999). Control of circadian rhythms and photoperiodic flowering by the Arabidopsis GIGANTEA gene. Science **285,** 1579–1582.

Plautz, J.D., Straume, M., Stanewsky, R., Jamison, C.F., Brandes, C., Dowse, H., Hall, J.C., and Kay, S.A. (1997). Quantitative analysis of Drosophila period gene transcription in living animals. J. Biol. Rhythms **12,** 204–217.

Restrepo, M.A., Freed, D.D., and Carrington, J.C. (1990). Nuclear transport of plant potyviral proteins. Plant Cell **2,** 987–998.

Robson, P., Whitelam, G.C., and Smith, H. (1993). Selected components of the shade-avoidance syndrome are displayed in a normal manner in mutants of Arabidopsis thaliana and Brassica rapa deficient in phytochrome B. Plant Physiol. **102,** 1179–1184.

Roenneberg, T., and Merrow, M. (2003). The network of time: Understanding the molecular circadian system. Curr. Biol. **13,** R198–R207.

Salome, P.A., Michael, T.P., Kearns, E.V., Fett-Neto, A.G., Sharrock, R.A., and McClung, C.R. (2002). The out of phase 1 mutant defines a role for PHYB in circadian phase control in Arabidopsis. Plant Physiol. **129,** 1674–1685.

Schaffer, R., Ramsay, N., Samach, A., Corden, S., Putterill, J., Carré, I.A., and Coupland, G. (1998). The late elongated hypocotyl mutation of Arabidopsis disrupts circadian rhythms and the photoperiodic control of flowering. Cell **93,** 1219–1229.

Schroeder, D.F., Gahrtz, M., Maxwell, B.B., Cook, R.K., Kan, J.M., Alonso, J.M., Ecker, J.R., and Chory, J. (2002). De-etiolated 1 and damaged DNA binding protein 1 interact to regulate Arabidopsis photomorphogenesis. Curr. Biol. **12,** 1462–1472.

Schultz, T.F., Kiyosue, T., Yanovsky, M., Wada, M., and Kay, S.A. (2001). A role for LKP2 in the circadian clock of Arabidopsis. Plant Cell **13,** 2659–2670.

Schwechheimer, C., and Deng, X.W. (2000). The COP/DET/FUS proteins—regulators of eukaryotic growth and development. Semin. Cell Dev. Biol. **11,** 495–503.

Sharrock, R.A., and Clack, T. (2002). Patterns of expression and normalized levels of the five Arabidopsis phytochromes. Plant Physiol. **130,** 442–456.

Somers, D.E. (2001). Clock-associated genes in Arabidopsis: A family affair. Philos. Trans. R. Soc. Lond. B Biol. Sci. **356,** 1745–1753.

Somers, D.E., Devlin, P.F., and Kay, S.A. (1998). Phytochromes and cryptochromes in the entrainment of the Arabidopsis circadian clock. Science **282,** 1488–1490.

Somers, D.E., Schultz, T.F., Milnamow, M., and Kay, S.A. (2000). *ZEITLUPE* encodes a novel clock-associated PAS protein from *Arabidopsis.* Cell **101,** 319–329.

Staiger, D., Allenbach, L., Salathia, N., Fiechter, V., Davis, S.J., Millar, A.J., Chory, J., and Fankhauser, C. (2003). The Arabidopsis SRR1 gene mediates phyB signaling and is required for normal circadian clock function. Genes Dev. **17,** 256–268.

Strayer, C., Oyama, T., Schultz, T.F., Raman, R., Somers, D.E., Mas, P., Panda, S., Kreps, J.A., and Kay, S.A. (2000). Cloning of the Arabidopsis clock gene TOC1, an autoregulatory response regulator homolog. Science **289,** 768–771.

Suarez-Lopez, P., Wheatley, K., Robson, F., Onouchi, H., Valverde, F., and Coupland, G. (2001). CONSTANS mediates between the circadian clock and the control of flowering in Arabidopsis. Nature **410,** 1116–1120.

Toth, R., Kevei, E., Hall, A., Millar, A.J., Nagy, F., and Kozma-Bognar, L. (2001). Circadian clock-regulated expression of phytochrome and cryptochrome genes in Arabidopsis. Plant Physiol. **127,** 1607–1616.

Vierstra, R.D. (2003). The ubiquitin/26S proteasome pathway, the complex last chapter in the life of many plant proteins. Trends Plant Sci. **8,** 135–142.

Wang, Z.Y., and Tobin, E.M. (1998). Constitutive expression of the *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) gene disrupts circadian rhythms and suppresses its own expression. Cell **93,** 1207–1217.

Yanovsky, M.J., and Kay, S.A. (2002). Molecular basis of seasonal time measurement in Arabidopsis. Nature **419,** 308–312.

Yanovsky, M.J., and Kay, S.A. (2003). Living by the calendar: How plants know when to flower. Natl. Rev. Mol. Cell Biol. **4,** 265–275.

Young, M.W., and Kay, S.A. (2001). Time zones: A comparative genetics of circadian clocks. Nat. Rev Genet **2,** 702–715.