Independent Roles for *EARLY FLOWERING 3* and *ZEITLUPE* in the Control of Circadian Timing, Hypocotyl Length, and Flowering Time[1]

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The circadian clock regulates many aspects of plant development, including hypocotyl elongation and photoperiodic induction of flowering. ZEITLUPE (ZTL) is a clock-related F-box protein, and altered *ZTL* expression causes fluence rate-dependent circadian period effects, and altered hypocotyl elongation and flowering time. EARLY FLOWERING 3 (ELF3) is a novel protein of unknown biochemical function. *elf3* mutations cause light-dependent circadian dysfunction, elongated hypocotyls, and early flowering. Although both genes affect similar processes, their relationship is unclear. Here we show that the effects of *ZTL* and *ELF3* on circadian clock function and early photomorphogenesis are additive. The long period of *ztl* mutations and ELF3 overexpressors are more severe than either alone. Dark-release experiments showing additivity in phase advances suggest that the arrthymicity caused by ZTL overexpression and that of the *elf3-1* mutation arise through independent pathways. A similar additive effect on hypocotyl elongation in red and blue light is also observed. In contrast, ELF3 and ZTL overexpressors act similarly to control flowering time in long days through the *CONSTANS/FLOWERING LOCUS T* (*CO/FT*) pathway. ZTL overexpression does not delay flowering through changes in *GIGANTEA* or *FLAVIN-BINDING*, *KELCH REPEAT*, *F-BOX* levels, but through a ZTL-mediated reduction in *CO* expression. In contrast, *ELF3* negatively regulates *CO*, *FT*, and *GIGANTEA* transcript levels, as the expression of all three genes is increased in *elf3-1*. The *elf3-1 co-1* double mutant flowers much earlier in long days than *co-1*, although *FT* message levels remain very low. These results show that *elf3-1* can derepress late flowering through a CO-independent mechanism. *ELF3* may act at more than one juncture, possibly posttranscriptionally.

Plant development is strongly affected by the light quality and intensity. *EARLY FLOWERING 3* (*ELF3*) and *ZEITLUPE* (*ZTL*) are two genes that function in light signaling to the plant. Although each was initially identified through very different genetic screens, analysis has subsequently shown that both strongly affect plant development and physiology in similar and also in contrasting ways.

The *elf3-1* mutation was identified in a screen for early flowering under short days (Zagotta et al., 1992, 1996). Subsequent analysis showed that *CONSTANS* (*CO*) expression was derepressed in *elf3* loss-of-function mutations, suggesting that ELF3 controls flowering by regulating *CO* (Suarez-Lopez et al., 2001). Early photomorphogenesis is also affected, with *elf3-1* plants showing long hypocotyls and petioles under red and blue light (Zagotta et al., 1996). Unexpectedly, *elf3* mutants are also defective in circadian clock function. Clock-regulated rhythms of leaf movement, hypocotyl elongation, and gene transcription become arrhythmic in *elf3* seedlings in constant light (LL; Hicks et al., 1996; Dowson-Day and Millar, 1999; Reed et al., 2000). In contrast, rhythms in *elf3* mutants persist in constant dark (DD; Hicks et al., 1996). These results and others, together with the observation that it can interact physically with phytochrome B (phyB), has lead to the notion that ELF3 is involved in the gating of photic input to the clock (McWatters et al., 2000; Covington et al., 2001; Liu et al., 2001). However, the molecular role of the protein remains obscure, and it is unclear whether all the light and photoperiod-dependent phenotypes derive from the defect in circadian function.

ZTL is the founding member of the three-member *ZTL* gene family, first identified as a long-period circadian clock mutant, *ztl-1* (Somers et al., 2000). Hypocotyl elongation in *ztl* mutants is hypersensitive to red light, though little affected in blue light. ZTL, LKP2 (LOV KELCH PROTEIN 2), and FKF1 (FLAVIN-BINDING, KELCH REPEAT, F-BOX) uniquely possess a light, oxygen, and voltage (LOV) domain at the N terminus, followed by an F-box domain and six carboxy-terminal kelch repeats (Somers, 2001, 2005). Fluence rate strongly affects period length in *ztl* mutants, implicating ZTL in the light input pathway

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to the clock (Somers et al., 2000). Flavin binding of the LOV domains of the phototropins and of the *Neurospora crassa* WHITE COLLAR-1 protein have implicated these polypeptides as blue-light photoreceptors (Briggs and Christie, 2002; Froehlich et al., 2002; He et al., 2002). Similarly, the bacterially expressed LOV domain of FKF1 can bind flavin mononucleotide in vitro (Cheng et al., 2003; Imaizumi et al., 2003) and can, in part, functionally substitute for the LOV domain in WHITE COLLAR-1 (Cheng et al., 2003). Similar results with the isolated ZTL and LKP2 LOV domains suggest that this gene family may constitute a novel type of blue-light photoreceptor. As an F-box protein, ZTL participates in an Skp/Cullin1/F-box-type E3 ligase and facilitates the proteasome-dependent proteolysis of the clock-associated protein TIMING OF CAB 1 (TOC1), targeting it for degradation via a proteasome-dependent pathway (Mas et al., 2003b; Han et al., 2004) The abundance of the ZTL protein itself is posttranscriptionally under circadian clock control, and its turnover is also proteasome dependent (Kim et al., 2003). Overexpression of ZTL down-regulates transcription of *CO* to delay flowering and lengthens hypocotyl (Somers et al., 2004).

Altered circadian period often correlates with abnormal hypocotyl growth and flowering time, and ELF3 and ZTL levels strongly affect these three processes. Here we investigate the genetic and physiological interactions between *ZTL* and *ELF3*. We show that the effects of *ZTL* and *ELF3* on circadian clock function and early photomorphogenesis are additive. In contrast, ELF3 and ZTL overexpressors act similarly to control flowering time, but through different mechanisms. ZTL overexpression delays flowering entirely through a ZTL-mediated reduction in *CO* expression. ELF3 acts more broadly, negatively regulating *CO*, *FLOWERING LOCUS T* (*FT*), and *GIGANTEA* (*GI*) transcript levels. Tests of *elf3-1 co* double mutants show that e*lf3-1* can derepress late flowering through a *CO*-independent mechanism. ELF3 appears to act at more than one juncture and possibly through a posttranscriptional mechanism.

RESULTS

Effects of Altered ZTL and ELF3 Expression on Free-Running Circadian Period

Previous reports have shown that reduced levels of ZTL lengthens the free-running period of circadian gene expression in continuous light and darkness, whereas increasing levels of ZTL dosage shorten the pace of the clock, to the point of arrhythmicity at very high levels of expression (Fig. 1A). The fluence rate dependence of period is also altered by ZTL expression level, indicating a role in modulating phototransduction to the oscillator (Somers et al., 2000, 2004).

The *elf3-1* mutation abolishes the rhythms of all circadian outputs tested, including *CAB2*:*LUCIFERASE* (*CAB*:*LUC*) expression (Fig. 1A), hypocotyl elongation, and cotyledon movement in LL, but has little effect on clock function in darkness (Hicks et al., 1996, 2001). Overexpression of ELF3 increases period length in LL, in support of the notion that, like ZTL, it normally acts to modulate the photosensitivity of the clock system (Covington et al., 2001). However, ELF3 appears to be a negative regulator of phototransduction, whereas ZTL acts positively, if the tendency toward arrhythmicity is interpreted as a hypersensitivity to light. We therefore examined the effects of coordinated changes in *ELF3* and *ZTL* expression on free-running period.

When the *elf3-1* mutation is combined with the *ztl-3* mutation (*ztl-3 elf3-1*) or the ZTL overexpressor (*elf3-1* ZTL OX), the free-running period of the clock-controlled *CAB2*::*LUC* reporter is arrhythmic, under either constant red light (Fig. 1A) or blue light (data not shown). These results are similar to those observed in the *elf3-1* single mutant (Fig. 1A), indicating that *elf3-1* is epistatic to ZTL activity.

Loss of rhythmicity can be difficult to interpret on its own, as it may mask a continued, underlying clock activity. Therefore, we also compared the phenotypes of the ELF3 overexpressor (ELF3 OX) and *ztl-1* single mutants with ELF3 OX *ztl-1* plants. When entrained seedlings were transferred to constant red light, the rhythms in the single mutants were robust, with the period of ELF3 OX seedlings close to wild type (24.7 ± 0.3 h) and *ztl-1* plants showing the expected long period (28.9 ± 0.6 h; Covington et al., 2001; Somers et al., 2004). However, the ELF3 OX *ztl-1* double mutant phenotype was more severe than either mutant alone, with period length longer than either single mutant (30.2 ± 0.6 h) and a severely reduced amplitude (Fig. 1B). Results were similar in blue light (data not shown). These data indicate an additive effect of the two genes on clock function.

Circadian cycling of the *CAB*:*LUC* reporter was also assessed in the various mutant combinations during a dark period extension into the 12-h subjective light period that occurs during standard light/dark (LD) entrainment (Fig. 2). This approach removes the light-dependent stimulation of *CAB*:*LUC* activity that normally occurs at lights on, and reports the phase of expression in each mutant combination as determined solely by the light-to-dark and dark-to-light transitions of the previous entrainment cycle. This protocol can also test for the phase of rhythmic activity in lines that are arrhythmic in LL but still cycle in darkness (e.g. *elf3-1*). Plants entrained in LD cycles were released into DD, and the phase of the first peak of each single and double mutant was recorded using the *CAB*::*LUC* reporter.

Relative to wild type, the first peak in darkness is phase advanced by 4 to 5 h in *elf3-1* (Fig. 2A; Reed et al., 2000) and ZTL OX plants (Fig. 2B). This is consistent with the extremely short period occasionally observed in LL in strong ZTL overexpressors (Somers et al., 2004; Fig. 1A) and suggests that the *elf3-1* mutation may similarly cause arrhythmicity in LL through extreme shortening of period. Similarly, the phase delays of 8 h in *ztl-1* Columbia (Col; Fig. 2C) and *ztl-3* (Fig. 2A) and 2 h in ELF3 OX (Fig. 2C) are consistent with

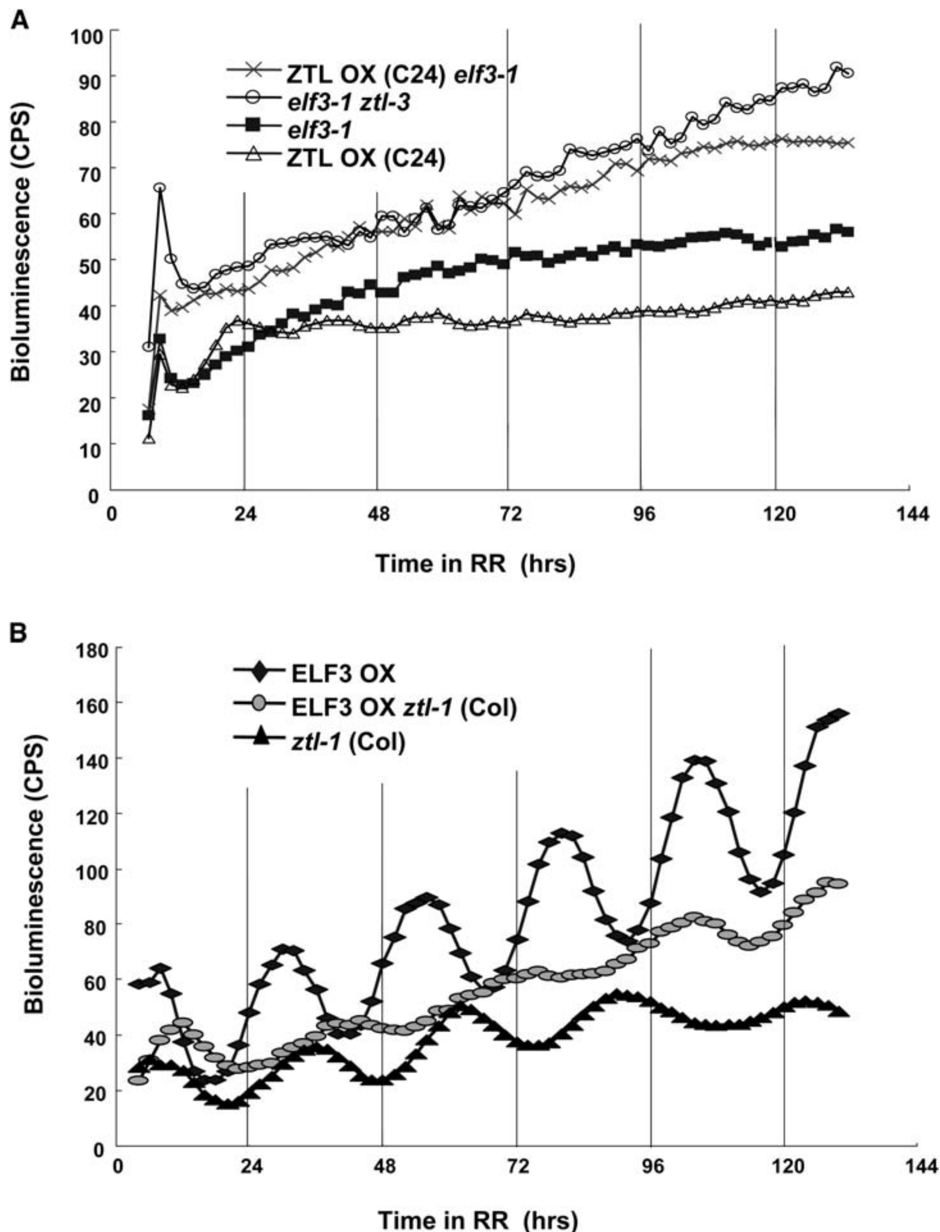

Figure 1. Effects of *ZTL* and *ELF3* expression levels on free-running period. A and B, Rhythmicity of *CAB*:*LUC* expression in *elf3-1*, ZTL OX (C24 ecotype), *elf3-1 ztl-3*, and *elf3-1* ZTL OX plants (A), and in ELF3 OX, *ztl-1* (Col), and ELF3 OX *ztl-1* double mutant plants (B). Plants were entrained in 12-h-white light /12-h-dark cycles and transferred to constant red light (RR; 20 μmol m^{-2} s^{-1}), and bioluminescence was monitored every 2 h for 4 to 5 d. Traces represent averages from at least 20 seedlings from two independent experiments.

the longer period observed in LL in these backgrounds. Interestingly, the ZTL OX *elf3-1* double mutant causes a reduction in peak amplitude but is clearly further phase advanced by 4 to 5 h relative to the two single mutants (Fig. 2B). Thus, despite the apparently similar arrhythmic phenotypes in LL, this analysis suggests that the two genes act separately on the same pathway, or in parallel on two independent pathways to affect circadian clock function.

The phase of *CAB*:*LUC* expression in the *elf3-1 ztl-3* double is similar to that of *elf3-1*alone (Fig. 2A), indicating that ELF3 is required for the strong phase delay observed in the absence of ZTL under these conditions. If ELF3 acts to suppress photic input to the clock, this appears to happen independently of the hyposensitivity to light caused by the loss of ZTL. Conversely, in the ELF3 OX *ztl-1* double mutant, ELF3 overexpression added little to the strong phase delay caused by *ztl-1* alone (Fig. 2C), although in LL ELF3 OX enhanced the effects of the absence of ZTL. Taken together, our data suggest that ZTL and ELF3 act to modulate clock activity largely independently of each other, with ELF3 acting to suppress photic input to the clock and ZTL acting to promote.

The Regulation of Hypocotyl Elongation by *ZTL* and *ELF3*

ZTL and ELF3 can each interact with phyB in vitro and in the yeast two-hybrid system, and both have

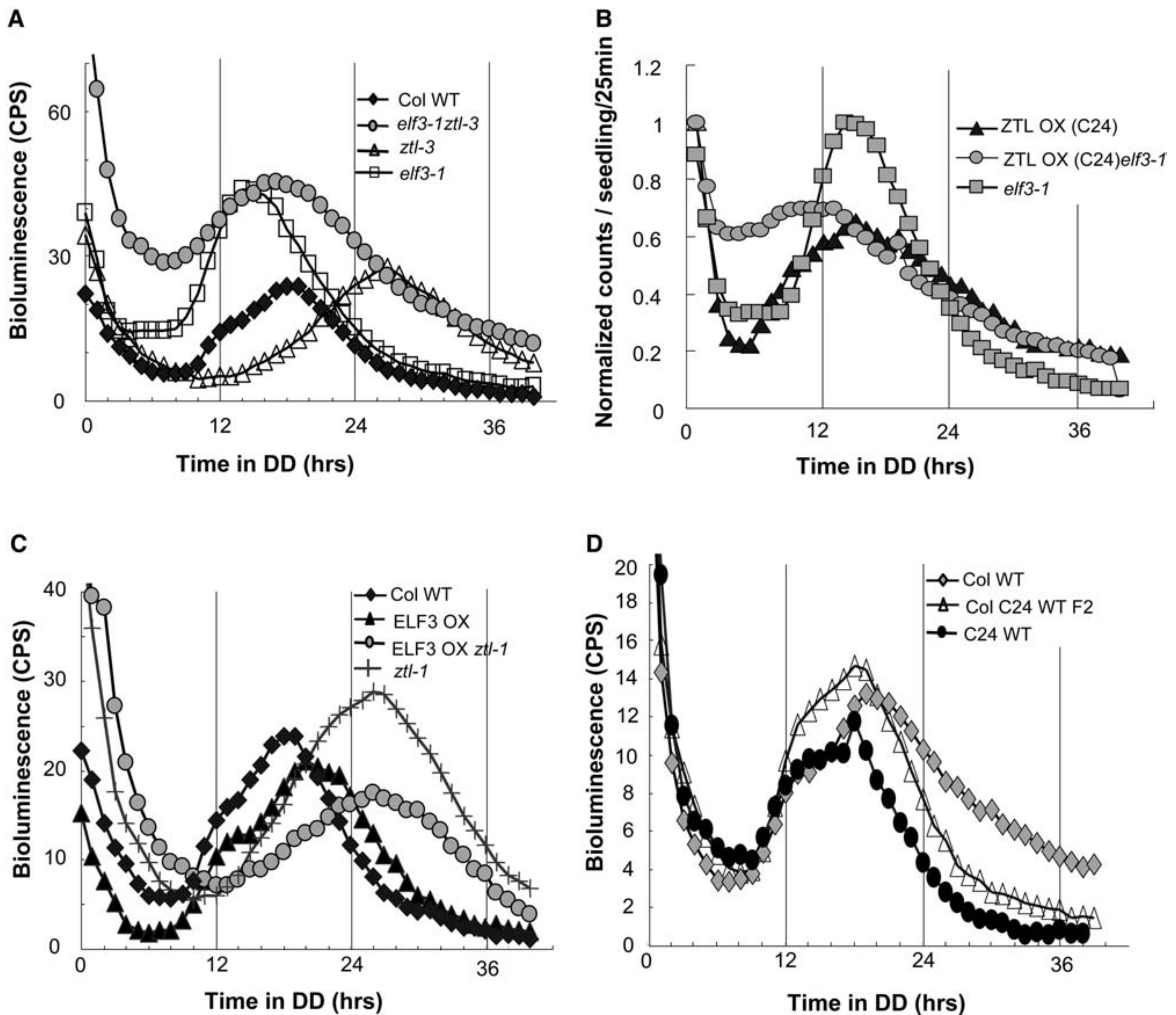

Figure 2. Effects of *ZTL* and *ELF3* expression levels on clock-controlled expression in extended dark. A to C, Timing of peak *CAB*:*LUC* expression in wild type (Col WT), *elf3-1*, *ztl-3*, and *elf3-1 ztl-3* plants (A); *elf3-1*, ZTL OX (C24), and *elf3-1* ZTL OX plants (B); and ELF3 OX, *ztl-1* (Col), and ELF3 OX *ztl-1* plants (C). D, Peak *CAB*:*LUC* expression in Col and C24 wild type and an F_2 population of a cross between Col and C24 are shown as controls. Seedlings were entrained in 12-h-light (white)/12-h-dark cycles, transferred to DD, and measured for luminescence expression every hour. Mean values (±SEM [A, C, and D]) and mean values normalized to mean expression level of 1 for each genotype (B) are shown.

been proposed to play a role in phyB-mediated signaling in early photomorphogenesis (Jarillo et al., 2001; Liu et al., 2001). To test whether *ELF3* and *ZTL* act in the same or different phytochrome-mediated signaling pathway during photomorphogenesis, we examined the fluence rate responsiveness of hypocotyl elongation in *ELF3* and *ZTL* mutant and overexpression lines.

In constant red light, the *ztl-3* mutant (Fig. 3B) and ELF3 OX (Fig. 3A) showed hypersensitivity to red light. In contrast, *elf3-1* and ZTL OX are both hyposensitive to red light at all intensities tested, with *elf3-1* slightly more effective at lengthening hypocotyl length (Fig. 3A). The *elf3-1 ztl-3* double mutant was intermediate in length compared to the single mutants. Interestingly, the *elf3-1 ztl-3* double mutant was more similar to *elf3-1* at high red light fluences, while being more similar to *ztl-3* at lower fluence rates (Fig. 3B). Similarly, the hypocotyl length of *elf3-1 ZTL OX* plants was longer than the two single mutants alone, under all intensities tested (Fig. 3A). These results show an additive effect of the two mutations, consistent with each gene acting independently to control hypocotyl elongation.

Arabidopsis (*Arabidopsis thaliana*) cryptochrome 1 is the major photoreceptor mediating blue-light inhibition of hypocotyl elongation. Although ZTL interaction with cryptochrome 1 has been shown in vitro

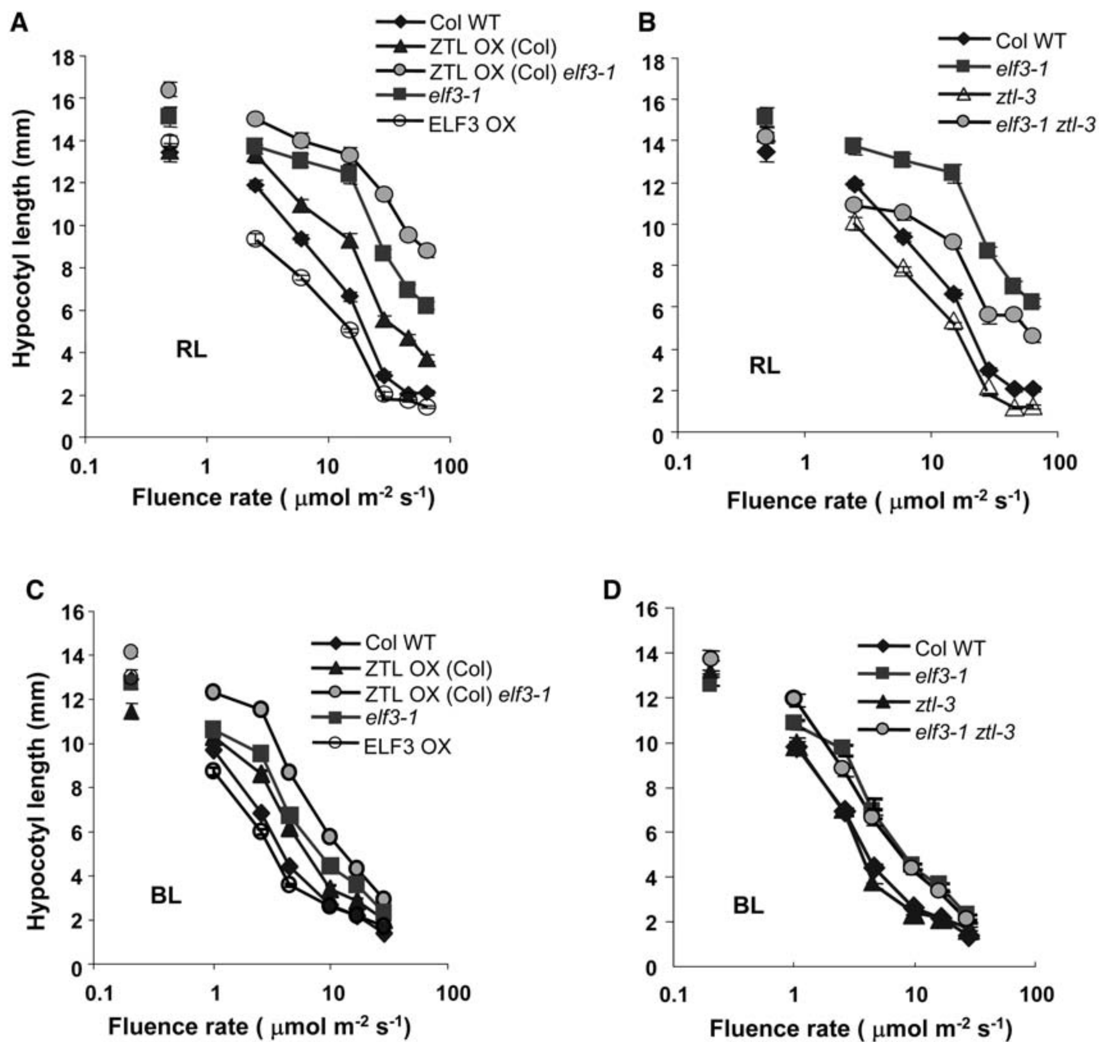

Figure 3. *ZTL* and *ELF3* control of hypocotyl growth during early photomorphogenesis. The wild-type (Col), *elf3-1*, *ztl-3*, ELF3 OX, ZTL OX (Col), *elf3-1 ztl-3*, and *elf3-1* ZTL OX plants were grown for 7 to 10 d under constant red light (RL; A and B) or constant blue light (BL; C and D) at the fluence rates indicated and measured for hypocotyl length. Unconnected data points show lengths for dark-grown seedlings. Values (mean hypocotyl length ± SEM) are representative of two trials; $n = 16$ to 25.

and in yeast two-hybrid tests, *ztl* loss-of-function mutations have no effect on blue light-mediated hypocotyl inhibition, and strong ZTL overexpression only modestly lengthens hypocotyl length (Somers et al., 2000, 2004). In contrast, *elf3-1* has long hypocotyls in constant blue light (Zagotta et al., 1996). To examine possible interactions between *ZTL* and *ELF3* in blue light-mediated hypocotyl inhibition, we tested the fluence rate responsiveness of different *ZTL* and *ELF3* mutant and overexpression combinations. Strong overexpression of ELF3 had little effect on hypocotyl length, very similar to the effects of the *ztl-3* mutation, over the entire range of fluence rates tested (Fig. 3, C and D). Both the *elf3-1* mutation and ZTL overexpression lengthen hypocotyls in blue light (Fig. 3C). The hypocotyl length of the *elf3-1 ztl-3* double mutant was very similar to hypocotyl length of the *elf3-1* single mutant itself (Fig. 3D), which would be expected if the effects of the two single mutants were simply additive. Similarly, the *elf3-1* ZTL OX showed hypocotyl lengths longer than each of single mutants alone (Fig. 3C). These data are consistent with an additive effect of each single mutant, suggesting that ZTL and ELF3 act in separate blue light-signaling pathways that converge to control hypocotyl elongation early plant development. Taken together, our results of testing under red and blue light support similar conclusions.

Interactions between *ZTL* and *ELF3* in the Control of Flowering Time

ZTL overexpression significantly delays flowering in long days, and this effect is strongly dependent on increasing ZTL dosage (Somers et al., 2004). *ztl* loss-of-function mutations have only a modest effect on flowering time, dependent on the ecotype (Fig. 4A; Somers et al., 2000, 2004). The effect on flowering time of changing ELF3 levels is more severe. *elf3-1* causes

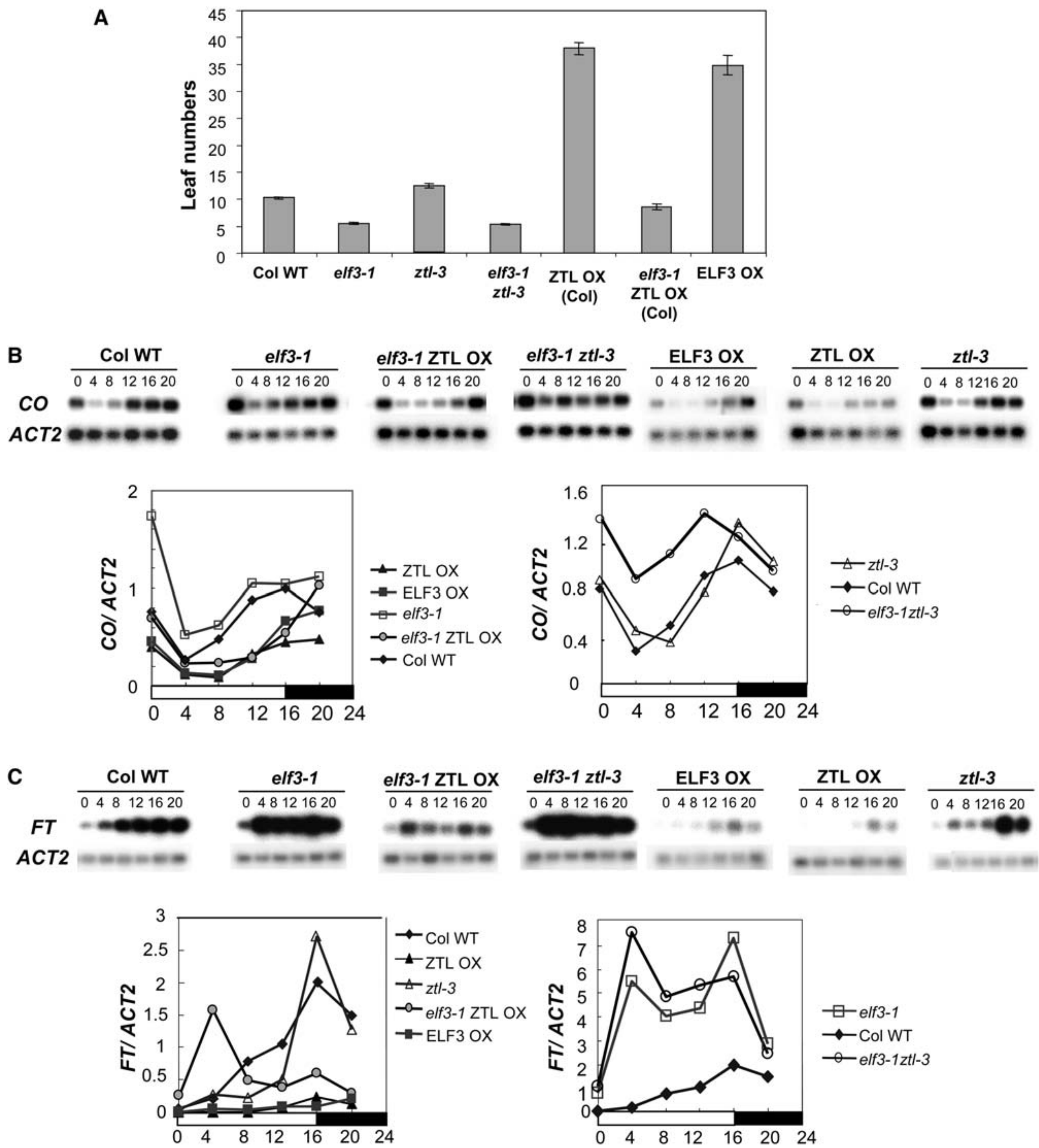

Figure 4. Control of flowering time by *ZTL* and *ELF3* expression levels. A, Total number of leaves (rosette + cauline) produced at flowering under long days (16 h light/ 8 h dark) in wild-type (Col), *elf3-1*, *ztl-3*, ELF3 OX, ZTL OX (Col), *elf3-1 ztl-3*, and *elf3-1* ZTL OX plants. Values (±SEM) are representative of two independent trials; $n = 13$ to 16. B and C, Expression levels of *CO* (B) and *FT* (C) transcripts under long days were determined by semiquantitative PCR for the lines described in A. Values are expressed relative to *ACTIN2* (*ACT2*) control. The same appropriate wild-type data are plotted in both sections of B and C to facilitate comparisons. White and black boxes represent light and dark periods, respectively. Data are representative of three independent trials.

significantly earlier flowering in short days and also shortens flowering time in long days (Hicks et al., 1996, 2001), whereas ELF3 overexpression greatly delays flowering time in long days (Fig. 4A; Liu et al., 2001). We tested whether these two genes act in the same or separate pathways to control flowering time under long days.

When placed in combination with *elf3-1*, both the *ztl-3* and ZTL OX lines flowered much earlier than the single mutants alone in long days. The *elf3-1 ztl-3* double mutant flowered the same as *elf3-1* alone, significantly earlier than the *ztl-3* single mutant (Fig. 4A). More strikingly, the *elf3-1* ZTL OX double mutant flowered slightly earlier than wild type, with approximately eight leaves, in contrast to the ZTL OX single mutant which flowered with about 40 leaves under long days. These results indicate that *elf3-1* is largely epistatic to *ZTL*, regardless of *ZTL* expression level (Fig. 4A).

The late flowering effects of ZTL overexpression occur through the strong reduction of *CO* and *FT* message levels (Fig. 4, B and C; Somers et al., 2004). *ztl-3* has little effect on either *CO* or *FT* levels in long days, relative to wild type, consistent with a wild-type flowering time (Fig. 4, B and C). The *elf3-1* mutation acts, in part through the same mechanism, to moderately elevate *CO* expression and greatly elevate *FT* transcript levels, leading to accelerated flowering (Suarez-Lopez et al., 2001; Fig. 4, B and C). Conversely, ELF3 overexpression causes a strong reduction in transcript levels of both genes, leading to strong delays in flowering in long days (Fig. 4, A–C).

To determine how *elf3-1* causes early flowering in the presence of high ZTL expression, we examined *CO* and *FT* transcript levels in the *ELF3* and *ZTL* double mutants under long days. *CO* and *FT* levels in the *elf3-1 ztl-3* double mutant were essentially the same as the *elf3-1* single mutant (Fig. 4, B and C). These results indicate that the acceleration of flowering in *elf3* mutants does not require *ZTL* protein. However, the *elf3-1* ZTL OX double mutant also showed reduced *CO* mRNA levels during the photoperiod, which is the critical time during which high *CO* expression activates *FT* transcription (Valverde et al., 2004). In this background, the level of *CO* transcript was as low or lower than wild type and *ztl-3* during the photoperiod, and almost as low as ZTL OX during this time. This was unexpected considering the much earlier flowering of *elf3-1* ZTL OX, relative to ZTL OX, and two to three leaves earlier than wild type (Fig. 4). We next examined the level of *FT* transcript in the *elf3-1* ZTL OX line. Surprisingly, FT levels were lower than wild type at all time points except during the early photoperiod (Fig. 4C). In this background, transcript levels of *FT* peaked early, 4 h after lights on, and then dropped to well below wild type for the rest of the photoperiod and throughout the short skotoperiod. This sharp peak, and then drop, in relative *FT* levels early in the photoperiod was observed in all biological (three) and technical (twice for each biological trial) repeats in this background (data not shown). Hence, high expression of ZTL is able to suppress the normally strong up-regulation of *FT* in the *elf3-1* single mutant, except during a short period early in the photoperiod.

Effects of *ELF3* and *ZTL* Mutations on *GI* and *FKF1* Expression

Mutations in *GI* and *FKF1* also delay flowering, and both have been proposed to up-regulate *CO* expression to control flowering (Suarez-Lopez et al., 2001; Imaizumi et al., 2003; Tseng et al., 2004). To examine whether ZTL regulation of *CO* expression occurs via changes in *GI* or *FKF1*expression, transcript levels of both genes were tested in ZTL OX and *ztl-3* backgrounds and compared to wild type. Neither overexpression nor absence of ZTL affected the mRNA levels of either gene very strongly, indicating that ZTL does not modulate *CO* or *FT* transcript levels by reducing *FKF1* or *GI* expression (Fig. 5, A and B).

We also tested the effects of *ELF3* absence and overexpression on *GI* and *FKF1* levels. *elf3-1* caused a consistently strong increase in *GI* levels at all time points during the photo- and skotoperiods, effectively eliminating the normal cyclic expression of GI (Fig. 5A). The absence or strong overexpression of ZTL had no consistent effect on this derepression of *GI* in the *elf3-1* background (Fig. 5A). ELF3 overexpression reduced *GI* transcript levels only modestly (to 60% to 70% of wild type at peak expression). *FKF1* expression in *elf3-1* is slightly increased at all time points under long days, but interestingly it remained cyclic, with peak expression at ZT 8. In contrast, *FKF1* transcripts in the ELF3 OX were essentially the same as wild type at all time points in long days. Consistent with the lack of effect of ZTL levels on *FKF1* expression, *FKF1* expression in the *elf3-1 ztl-3* and *elf3-1* ZTL OX lines was very similar to the *elf3-1* single mutant (Fig. 5B).

The moderate rise in *CO* expression in *elf3-1* (Fig. 4B) correlates with our observed moderate increase in *FKF1* expression in this background (Fig. 5B), consistent with *ELF3* acting through *FKF1* to affect *CO* expression. Recently a DOF transcription factor, CDF1, has been shown to repress *CO* expression. The F-box protein, FKF1, phase-specifically degrades this factor (Imaizumi et al., 2005). Hence, *ELF3* may also act on CDF1 expression or activity.

Taken together, these results indicate that ZTL overexpression does not delay flowering through changes in *GI* or *FKF1* message levels. Most likely late flowering arises through a ZTL-mediated reduction in *CO* expression, resulting in lower *FT* expression during the photoperiod. In contrast, *ELF3* may act through multiple pathways. It negatively regulates *CO*, *FT*, *GI*, and *FKF1* transcript levels, as the expression of all four genes is increased in *elf3-1*. Surprisingly, the *elf3-1 co-1* double mutant flowers much earlier in long days than *co-1*, though still later than the *elf3-1* single mutant and the wild type (Fig. 6A). These results suggest that *elf3-1* can derepress the inhibition of flowering caused by absence of *CO* through a *CO*-independent mechanism.

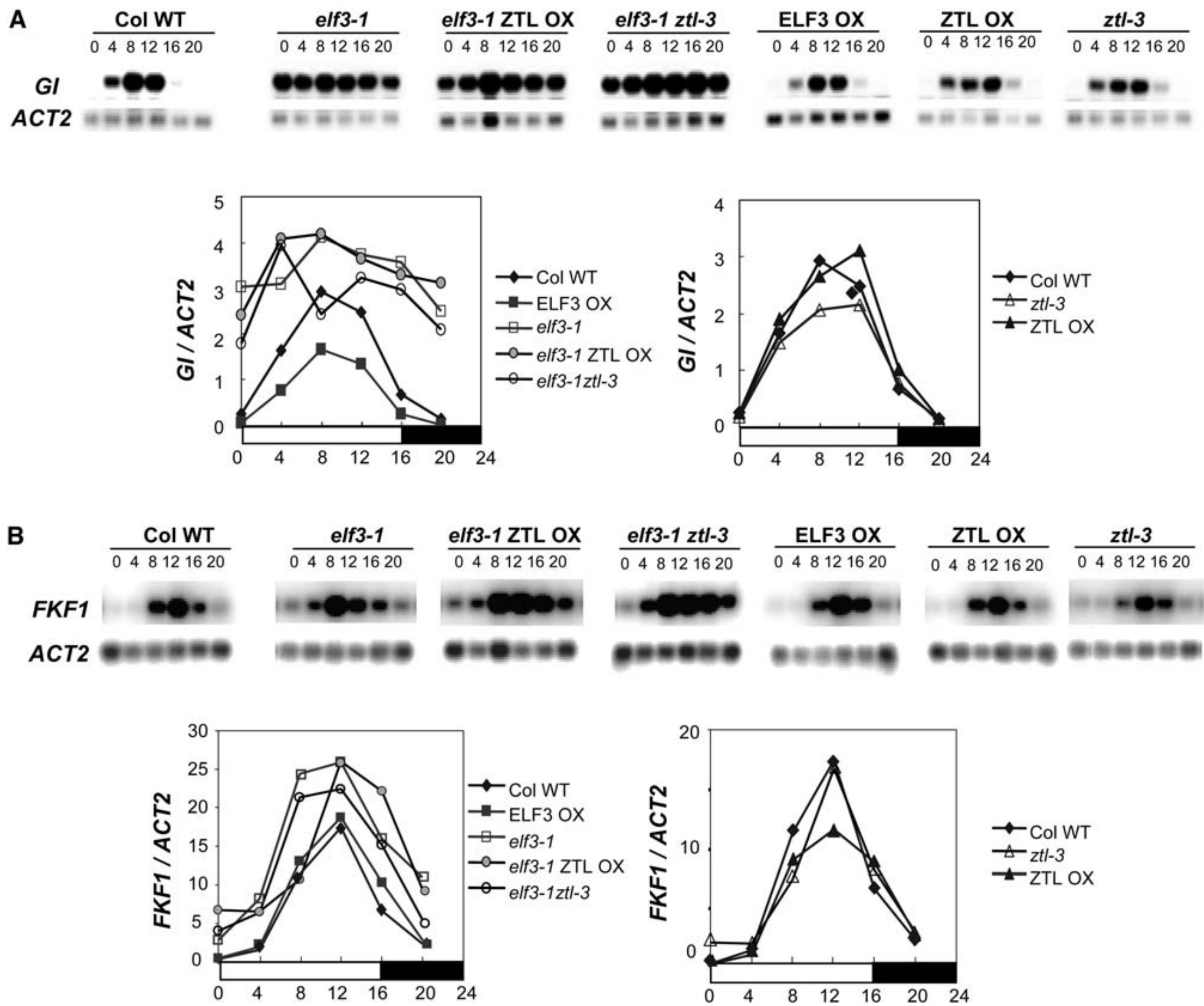

Figure 5. Effects of *ZTL* and *ELF3* expression levels on *GI* and *FKF1* expression. Expression levels of *GI* (A) and *FKF1* (B) transcripts under long days were determined by semiquantitative PCR for the same lines tested for flowering-time effects in Figure 4. Values are expressed relative to *ACTIN2* (*ACT2*) control. White and black boxes represent light and dark periods, respectively. Data are representative of three independent trials.

We tested whether *elf3-1* can act on FT expression by examining *FT* mRNA levels in the *elf3-1 co-1* double mutant. Quantitative reverse transcription (RT)-PCR was performed on four independent *elf3-1 co-1* segregants over a long-day time course and compared to wild type and *co-1* genotypes. Surprisingly, in all four double mutant isolates, *FT* message levels were markedly lower than wild type during the second half of the photoperiod and throughout the skotoperiod, and very similar to the *co-1* single mutant (Fig. 6B). During the first 8 h of the photoperiod, *FT* levels in only one of the *elf3-1 co-1* lines was near wild type, whereas *FT* expression in the remaining three double mutant lines were at or near *co-1* levels. Taken together, these data show that the *elf3-1* suppression of late flowering in *co-1* is not due to an increase in *FT* message levels.

Additionally, in light of new reports showing that *FD* acts together with *FT* to promote flowering (Abe et al., 2005; Wigge et al., 2005), we tested whether *FD* expression was elevated in the *elf3-1 co-1* double mutants, relative to wild type. Over a long-day time course, *FD* expression in the double mutants, *co-1* single mutant, and wild type were not significantly different at any point (data not shown), nor was there evidence of diurnal or LD variation in *FD* expression in any of the genetic backgrounds tested (data not shown; Abe et al., 2005).

DISCUSSION

Increasingly, many genes that control the function of the circadian oscillator are also being linked to the control of photomorphogenesis and to the timing of

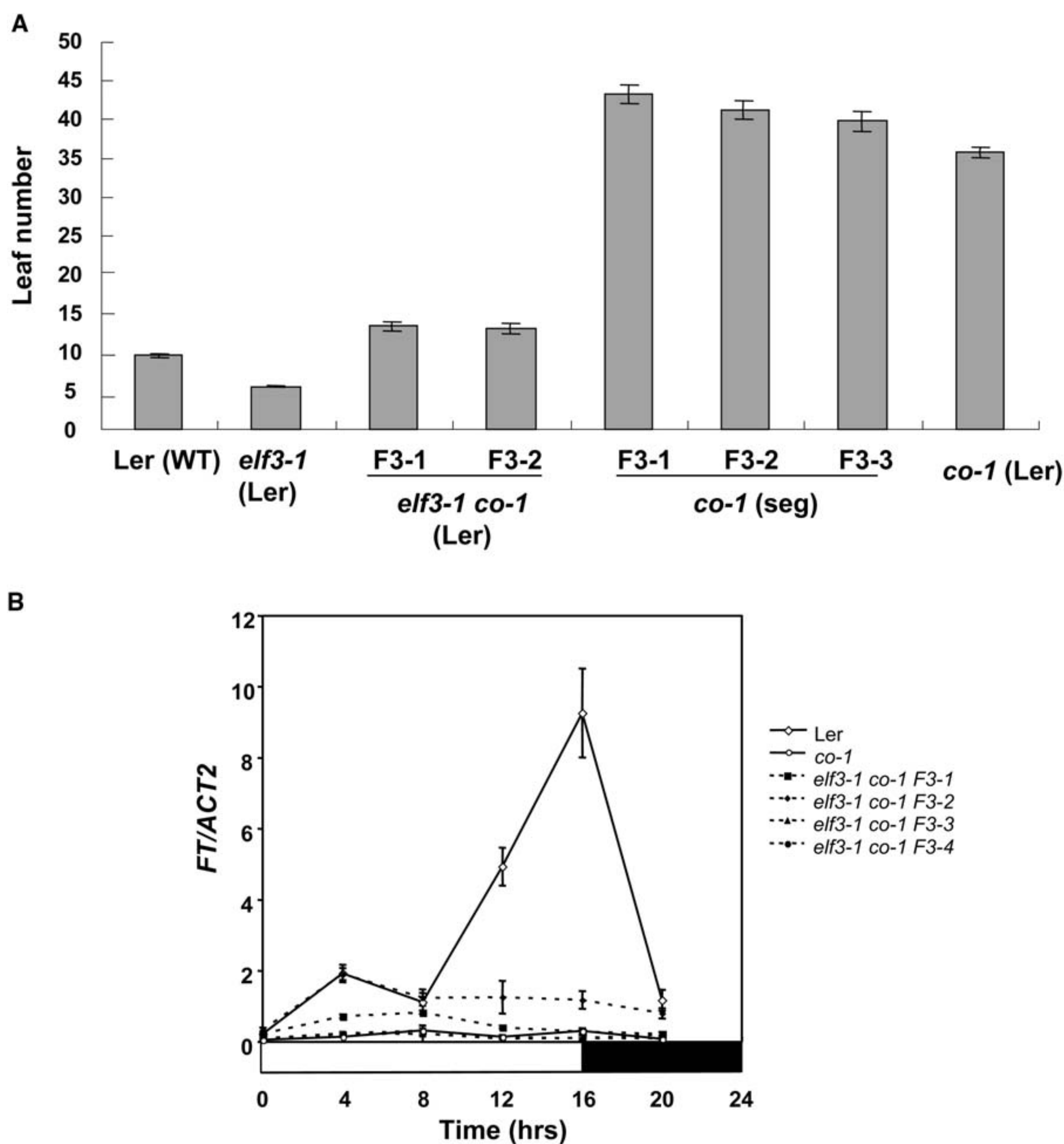

Figure 6. Effect of the *elf3-1 co-1* double mutant on flowering and *FT* expression. A, Total number of leaves (rosette + cauline; ±SEM) produced at flowering under long days (16 h light/ 8 h dark) in wild-type (L*er*), *elf3-1* (2 × introgressed into L*er*), *elf3-1 co-1*, *co-1* (L*er*), and *ELF3 co-1* segregants isolated from the *elf3-1* × *co-1* F_2 population (*co-1* [seg]). $n = 8$ to 20. B, Expression levels of *FT* transcripts under long days were determined by quantitative PCR for the lines described in A. Values are expressed relative to *ACTIN2* (*ACT2*) control. White and black boxes represent light and dark periods, respectively. Data are representative of two independent trials.

flowering. The molecular mechanisms that link clock function to these developmental phenomena are poorly understood. Here we have shown that for these three processes two genes, *ELF3* and *ZTL*, largely function independently of each other, rather than within the same pathway or biochemical complex. The extent of the effect of one gene, relative to the other, varies with the process, and in some cases the two act in opposition to each other. This has proved useful in unexpectedly revealing a more diverse role for ELF3 in the control of flowering.

ZTL and ELF3 Control Clock Function through Different Pathways

Previous evidence from single mutants and overexpressors of ZTL and ELF3 suggested that the genes act oppositely on clock function. Loss-of-function *elf3* mutations are similar to a strong ZTL overexpressor in that both cause circadian arrhythmicity in LL. Similarly, *ztl* mutants (e.g. *ztl-1* and *ztl-3*) and strong ELF3 overexpression lengthen circadian period. From these

results alone one could hypothesize that ELF3 negatively regulates ZTL activity, or vice versa. However, arrhythmicity persists in *elf3-1 ztl-3* double mutants, indicating that *elf3-1* does not abolish cycling through a derepression of ZTL levels. In addition, when examined in extended darkness, the phase of the first *CAB:luc* peak in the *elf3-1 ztl-3* double mutant falls later than in *elf3-1* but much earlier than *ztl-3* (Fig. 2A). This indicates that the two loss-of-function mutants can act, to a limited extent, to counter the effects of the other. Additionally, if *ELF3* affects period solely through *ZTL*, *ztl-1* should be epistatic to the effects of ELF3 OX. The strongly additive effect of the ELF3 OX *ztl-1* double mutant in LL suggests a convergence on period control from different pathways.

Similarly, if ZTL controls period through the repression of ELF3, the period of ZTL OX *elf3-1* plants should appear the same as *elf3-1* mutants. Instead, the phenotype in extended DD is more severe than either mutant alone (Fig. 2B), indicating additivity in their effects.

ZTL regulates the degradation of TOC1 and controls circadian period, at least in part, through this mechanism (Mas et al., 2003b). In contrast, the manner of ELF3 control of circadian period is still unknown. Our genetic interaction data suggests that ELF3 does not act to positively regulate *TOC1* expression, which could be expected of a gene that has the opposite phenotype of a negative regulator of *TOC1* (i.e. *ZTL*). If *ELF3* were a positive regulator of *TOC1*, *elf3-1 ztl-3* plants might be predicted to be near wild type in period, as the higher levels of TOC1 caused by a ZTL deficiency (Mas et al., 2003b) could be negated by the absence of ELF3. Instead, *elf3-1* arrhythmicity is epistatic to *ztl-3*. By similar reasoning, *ELF3 ZTL* double overexpressing plants could also be expected to antagonistically balance TOC1 levels to cause near wild-type period, but such plants are arrhythmic, similar to ZTL OX alone (data not shown). Additionally, *TOC1* transcription is increased in *elf3-1*, demonstrating a role for ELF3 in repressing *TOC1* expression (Alabadi et al., 2001).

ZTL and *ELF3* protein levels show rhythmic expression in LD, with similar phases of peak expression at or near dusk (approximately ZT 12–13, Liu et al., 2001; Kim et al., 2003). The intracellular location of both proteins also overlaps, with ELF3 (Liu et al., 2001) and ZTL (W.-Y. Kim and D.E. Somers, unpublished data) both present in the nucleus. Despite this temporal and spatial coincidence of expression, our data indicate that ELF3 and ZTL act additively, probably through different mechanisms and molecular partners, to control circadian function. Identification of molecular mechanism of ELF3 action will clarify this relationship.

ZTL and ELF3 in Hypocotyl Control

The manner of ELF3 control of hypocotyl length is unclear. In red light, *elf3* and *phyB* mutations act additively to control elongation, suggesting independent or partially redundant mechanisms. However, in vitro interactions between ELF3 and phyB

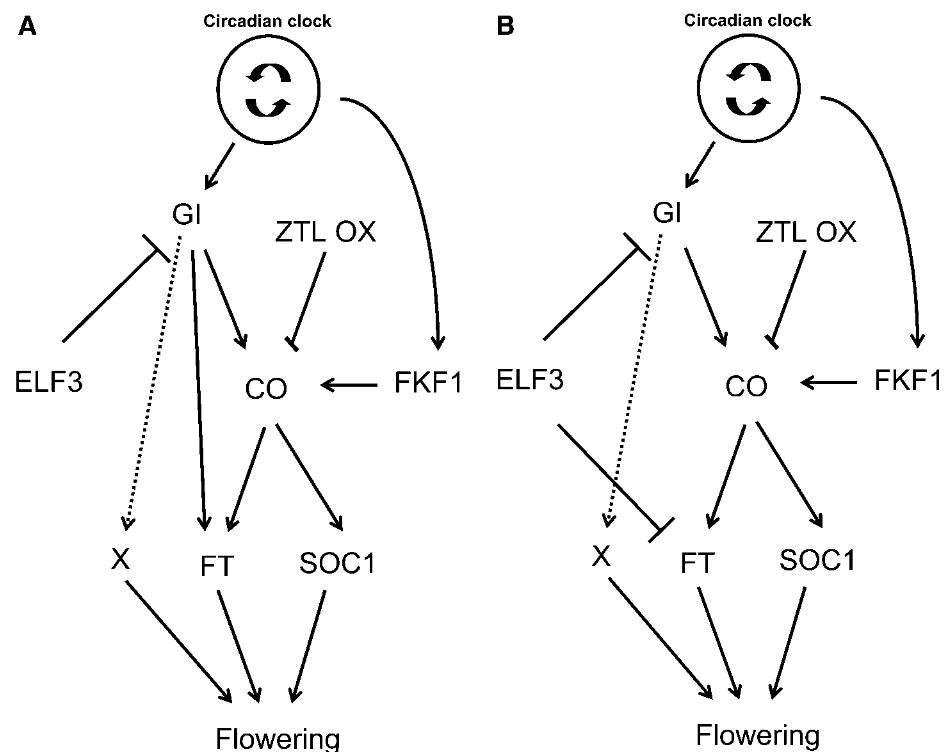

Figure 7. *ELF3* may regulate flowering through multiple flowering time-related genes in the *CO/FT* pathway. *ELF3* negatively regulates *GI*, *CO*, and *FT* expression; modest derepression of *FKF1* expression by *elf3-1* is not shown. Epistasis tests show *elf3-1* early flowering requires *GI* and *FT*, but not *CO*, indicating *ELF3* may regulate *CO/FT* expression via *GI* (Fig. 7A). The dotted line leading from GI to X (Mizoguchi et al., 2005) is supported by our data, but our results additionally suggest a posttranslational control of *FT* by *GI* (Fig. 7A), based on low *FT* levels in the *elf3-1 co-1* double mutant. Alternatively, this result plus high *FT* expression in the *elf3-1* ZTL OX background, when *CO* expression is wild type or lower, indicates *ELF3* may regulate *FT* expression posttranscriptionally (Fig. 7B). Other models are also possible; see text for more details.

demonstrate the potential for complex formation and indicate that in some circumstances they may act together (Reed et al., 2000; Liu et al., 2001). The long hypocotyl phenotype of the *elf3* mutants in blue light cannot be easily understood through a phy-based mechanism, suggesting that ELF3 acts downstream of where blue- and red-light photoreceptors intersect, or in a blue light-specific pathway, parallel to its role in red-light signaling.

The red- and blue-light hypocotyl length phenotypes of *ztl* mutants are similar to those observed in TOC1 overexpressors and *toc1* mutants (Mas et al., 2003a). This suggests that the effects we observe in manipulating ZTL levels are largely due to its effects on TOC1 levels. Since our double mutant analysis shows that ZTL acts independently of ELF3 in mediating photoinhibition of hypocotyl elongation, it follows that ELF3 probably acts through a mechanism separate from that of TOC1. However, until more is known of the primary action of ELF3 and TOC1, it is still not possible to know if the hypocotyl and clock effects of these two proteins are through the same or different mechanisms.

ELF3 Acts Multiply and Separately from ZTL to Control Flowering Time

The circadian clock regulates flowering through an output pathway that includes *CO* and *FT*. Both *ELF3* and ZTL OX affect flowering time by acting within the photoperiodic pathway. *ELF3* and ZTL OX can act as negative regulators upstream of *CO* and *FT* in this pathway, because ectopic expression of both genes negatively controls the abundance of *CO* and *FT* message levels. This relationship is unlike their effects on circadian period and hypocotyl length, where *ZTL* and *ELF3* act oppositely with respect to their dosage.

Although ZTL normally targets TOC1 to control circadian period, our data do not allow a positioning of TOC1 into a flowering time scheme. It appears that ZTL overexpression is not acting only through effects on TOC1 with respect to flowering time. If it did, *toc1* loss-of-function mutants should exhibit very late flowering, which they do not (Somers et al., 1998; Mas et al., 2003b). The molecular mechanism of ZTL OX in reducing *CO* message levels is currently unclear.

When overexpression of ZTL was paired with the absence of ELF3 (ZTL OX *elf3-1*), we observed a surprisingly high level of *FT* message early in the photoperiod that was not proportional to the low level of *CO* expression, when compared to wild type over the same time period (Fig. 4, B and C). The very high level of *FT* message normally seen in the *elf3-1* background was effectively suppressed throughout the photo- and skotoperiods by high ZTL expression, except during this short window of time. This suggests that ZTL-mediated suppression is limited during this early part of the day, allowing the derepressing effects of the *elf3* mutation to act and raise *FT* message levels. This action may occur posttranscriptionally, as the *CO* message levels in ZTL OX *elf3-1* are the same or lower than wild type, yet *FT* message levels are 4 to 5 times higher than wild type. This notion is further supported by the suppression of late flowering in *co-1* by *elf3-1*. In the *elf3-1 co-1* double mutant we found that *FT* message levels are maintained well below that of wild type during the late photoperiod, although flowering time is very similar to wild type. This suggests that the moderately high levels *CO* and very high levels of *FT* present in *elf3-1* contribute to, but are not solely responsible for, the early flowering phenotype. The *elf3-1 co-1* result is consistent with ELF3 acting posttranscriptionally to deactivate or destabilize *FT* protein. It is also possible that an additional factor, acting in parallel to or downstream of, the *CO/FT* pathway, is under *ELF3* or *GI* control (see below). Clearly, *ELF3* can act independently of *CO* to control flowering time.

Figure 7 illustrates how *ELF3* may act at more than one stage in the flowering time pathway. Chou and Yang (1999) have shown that *elf3-1 gi-1*and *elf3-1 ft* double mutants flower late in long days, indicating that *GI* and *FT* are required for the early flowering effects of *elf3-1*. However, our results indicate that *CO* is not required for *elf3-1* to promote early flowering, suggesting that *GI* can act independently of *CO* (Mizoguchi et al., 2005). Hence, *ELF3* may act solely through an effect on *GI* expression. Our observed increases in *CO* and *FT* expression in the *elf3-1* mutant could be indirect, through the derepression of *GI*. This model implies a direct regulation of *FT* by *GI*, in addition to its role in *CO* regulation (Fig. 7A). In addition, it would require a role for *GI* in posttranscriptional stabilization of *FT* protein. Alternatively, *GI* may act through a second route, in parallel with the *CO/FT* pathway (Fig. 7A), as recently suggested by Mizoguchi et al. (2005). In the *elf3-1 co-1* mutant, high levels of this factor, induced through the high levels of *GI*, could partially compensate for loss of signaling via the *CO/FT* pathway, resulting in earlier flowering. Alternatively, our results and those of Chou and Yang (1999) are consistent with ELF3 acting directly on *FT* protein, as noted earlier (Fig. 7B). These two models are not mutually exclusive, and others are also possible. Together, these results suggest a floral-induction pathway that is alternate or parallel to that through *CO*.

MATERIALS AND METHODS

Plant Materials

All mutations used in this study were in the Col-0 ecotype or C24 background of Arabidopsis (*Arabidopsis thaliana*), with the exception of *co-1*, which was in the Landsberg *erecta* (L*er*) ecotype (Putterill et al., 1995). The *ztl-1* (Col) line, *ztl-3* (Col), and ZTL OX (C24; ZTL overexpressor, C24 ecotype) were described previously (Somers et al., 2004), as was the ZTL overexpressor (ZTL OX [Col]; Han et al., 2004). The *elf3-1* mutation was identified using the primers 5′-TGTTGGTCAGTCTTCTCCGA-3′ and 5′-TCCCTACTGTCATTCAAGGG-3′, followed by digestion with *Hin*cII. The *ELF3* overexpression line (ELF3 OX) is as described previously (Covington et al., 2001).

Construction of Double Mutants

The plasmid pZP221-35S:ZTL-EGFP was transformed into *elf3-1* to generate ZTL OX *elf3-1* double mutants using standard techniques (Clough and Bent, 1998) and selected lines were confirmed via PCR-based scoring of transformants. Other double mutant combinations (*elf3-1 ztl-3* and ELF3 OX *ztl-1*) were obtained via crosses with the appropriate mutant in the appropriate ecotypic background, with the following exceptions: For imaging experiments, the ZTL OX (C24) *elf3-1* line is in a mixed background of C24 and Col ecotypes. This did not affect the arrhythmic phenotype described in Figure 1A, and the results of the period of F_2 seedlings of a cross between C24 wild type and Col wild type are shown in Figure 2D as a control for the results of Figure 2B. For the *elf3-1 co-1* double mutant, *elf3-1* was introgressed twice into the L*er* ecotype before mating with *co-1* mutants, resulting in a mixed background consisting predominantly of L*er*.

Plant Growth Conditions and Rhythm Analysis

Seedlings were grown on Murashige and Skoog medium (GIBCO BRL) + 3% Suc (0.8% agar) under 12-h-light/12-h-dark white fluorescent light (50–60 μmol m^{-2} s^{-1}) for 7 d, then sprayed with 3 mM luciferin (Biotium) before transfer to constant red light (peak wavelength 670 nm ± 15 nm half-peak bandwidth; Quantum Devices), blue light (Bili Blue [Interlectric] filtered through Rohm and Haas 2424 plexiglass 5 mm thick), or darkness and imaged 25 min every 2 h using a Peltier-cooled CCD slow-scan camera (Nightowl; Berthold Technologies). Postimaging luminescence quantitation used Win-Light software (Berthold Technologies). Period estimates were obtained using fast Fourier transform nonlinear least-squares analysis (Plautz et al., 1997). Mean period lengths and associated error metrics were variance weighted and are reported as standard error of the mean (SEM; Millar et al., 1995).

Hypocotyl Length Assays

Seeds were stratified in the dark at 4 C for 4 d, exposed to white light (70 μmol m^{-2} s^{-1}) for 1 to 2 h on Murashige and Skoog medium (GIBCO BRL) + 3% Suc (0.8% agar), then placed under the appropriate light quality and fluence rate (using varying layers of neutral density filters; Roscolux 397 [Rosco Laboratories]) for 7 to 10 d. Hypocotyl length was measured using SCION Image software.

Flowering-Time Analyses

Seeds were grown under long days (16 h light/8 h dark; 60–70 μmol m^{-2} s^{-1}) for 7 to 10 d on Murashige and Skoog medium (GIBCO BRL) + 3% Suc (0.8% agar), then transplanted to soil. Total number of rosette and cauline leaves were counted.

Gene Expression Analyses

Seedlings were grown for 7 d (70 μmol m^{-2} s^{-1} white fluorescent light) in 16-h-light/8-h-dark cycles and harvested on day 8 at the appropriate times. Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instruction. Except in Figure 6B, transcripts of *Actin2*, *CO*, *FT*, *FKF1*, and *GI* were quantified by RT-PCR, followed by DNA gel-blot analysis as described previously (Somers et al., 2004). The primer sequences and annealing temperatures used to amplify each gene are as follows: *CO*, 48°C, 5′-ACGCCATCAGCGAGTTCC-3′ and 5′-AAATGTATGCGTTATGGTTAATGG-3′; *FT*, 55°C, 5′-ACAACTGGAACAACCTTTGGCAATG-3′ and 5′-ACTACTATAGGCATCATCACCGTTCGTTACTCG-3′; *GI*, 52°C, 5′-CTGTCTTTCTCCGTTGTTTCACTGT-3′ and 5′-TCATTCCGTTCTTCTCTGTTGTTGG-3′; and *FKF1*, 55°C, 5′-GTCGTAACTGTCGATTCCTACA-3′ and 5′-ATCTCCAGTGTTCCAGTTATCT-3′. A portion of At3g18780 (*ACTIN2*; *ACT2*) was amplified using oligonucleotides 5′-AAAACCACTTACAGAGTTCGTTCG-3′ and 5′-GTTGAACGGAAGGGATTGAGAGT-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 empirically determined for each gene, and 18 cycles were used for *Actin2*; 22 cycles were used for *GI*, *FKF1*, and *FT*; and 23 cycles for *CO*. For Figure 6B, transcripts of *Actin2* and *FT* were measured by quantitative RT-PCR, essentially as previously described (Mockler et al., 2004). cDNAs were prepared from DNase-treated (Turbo DNAfree, Ambion) RNA samples using the Omniscript RT kit (Qiagen). Oligonucleotide primers were designed using PRIMER EXPRESS V2.0 software (Applied Biosystems) and were as follows: *FT*, 5′-CCTTTGGCAATGAGATTGTGTG-3′ and 5′-TTCCTGCAGTGGGACTTGG-3′; *FD*, 5′-TCCCGCGCTAGGAAACAG-3′ and 5′-CCTGCAAGTGAGCAACTTCAAG-3′; and *ACT2*, 5′-ACCTTTAACTCTCCCGCTATGTATGT-3′ and 5′-GGCACAGTGTGAGACACACCAT-3′. Expression level was calculated based on standard curves constructed for each primer set and normalized to *ACT2* in arbitrary units.

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