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ELF3 Modulates Resetting of the Circadian Clock in Arabidopsis

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The Arabidopsis *early flowering 3* (*elf3*) mutation causes arrhythmic circadian output in continuous light, but there is some evidence of clock function in darkness. Here, we show conclusively that normal circadian function occurs with no alteration of period length in *elf3* mutants in dark conditions and that the light-dependent arrhythmia observed in *elf3* mutants is pleiotropic on multiple outputs normally expressed at different times of day. Plants overexpressing ELF3 have an increased period length in both constant blue and red light; furthermore, etiolated ELF3-overexpressing seedlings exhibit a decreased acute *CAB2* response after a red light pulse, whereas the null mutant is hypersensitive to acute induction. This finding suggests that ELF3 negatively regulates light input to both the clock and its outputs. To determine whether ELF3's action is phase dependent, we examined clock resetting by using light pulses and constructed phase response curves. Absence of ELF3 activity causes a significant alteration of the phase response curve during the subjective night, and constitutive overexpression of ELF3 results in decreased sensitivity to the resetting stimulus, suggesting that ELF3 antagonizes light input to the clock during the night. The phase of ELF3 function correlates with its peak expression levels in the subjective night. ELF3 action, therefore, represents a mechanism by which the oscillator modulates light resetting.

INTRODUCTION

Organisms exhibit daily biological rhythms in many physiological processes. These overt rhythms proceed even in the absence of external environmental cues and are thought to be regulated by an internal biochemical oscillator, the circadian clock. The circadian clock allows organisms to anticipate changes in their environment, such as the onset of dawn and dusk, thereby coordinating temporal phases of physiology with the external environment. In plants, the circadian clock organizes a wide array of daily rhythms, including the expression of genes involved in photosynthesis, sugar metabolism, nutrient assimilation, stomatal opening, and leaf movements. The oscillator possesses a resetting mechanism to receive timing cues from the environment. Resetting of the clock by light enables the oscillator to maintain a stable phase relationship with the external photoperiod and to detect gradual shifts in the onset of dawn as seasons change. The oscillator and the light-resetting mechanism work in concert to measure photoperiod and thereby coordinate photoperiod-dependent events such as floral induction.

The *early flowering3-1* (*elf3-1*) mutation in Arabidopsis was identified in a screen for mutants with altered flowering time phenotypes (Zagotta et al., 1992). The mutant flowers early in both short and long photoperiods compared with the wild type (Zagotta et al., 1996). Previous analyses of overt circadian rhythms in the mutant suggested a gross defect in oscillator function and/or regulation. Clock-regulated rhythms of leaf movement, hypocotyl cell elongation, and transcription from the *chlorophyll A/B–binding protein 2* gene (*CAB2*) promoter become arrhythmic in *elf3-1* mutant seedlings under free-running conditions of constant light (LL; Hicks et al., 1996; Reed et al., 2000). A weaker allele, *elf3-7*, which has a less severe flowering time phenotype than does the *elf3-1* allele, also exhibits similar arrhythmia in clock outputs under LL (Reed et al., 2000). In constant dark (DD), however, *elf3* mutants exhibit some signs of clock function (Hicks et al., 1996). Therefore, it was proposed that ELF3 mediates an interaction between light and the circadian clock and that defects in ELF3 function lead to light-dependent arrhythmia. Recent reports support this hypothesis,

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suggesting that ELF3 interacts physically with a photoreceptor (Liu et al., 2001) and acts as a *zeitnehmer* for light signaling to the circadian clock (McWatters et al., 2000).

In Arabidopsis, the interaction between light and the circadian clock is dependent on both the fluence rate and the quality of light. For diurnal organisms with a functional clock in LL, there exists an inverse relationship between light intensity and free-running period length: increasing light intensity causes a shortening of period length of the overt rhythm (Aschoff's rule; see Aschoff, 1979), resulting in a classic fluence rate response curve (FRC). Such FRCs can be constructed for different wavelengths of light. In Arabidopsis, two major classes of photoreceptors, phytochromes and cryptochromes, mediate red and blue light photoreception, respectively (Somers et al., 1998a). Blue light and red light FRCs, along with genetic analyses, have suggested the involvement of different photoreceptors under different light quality and fluence rates in signaling to the clock (Millar et al., 1995; Devlin and Kay, 2000). For example, mutants lacking phytochrome B (phyB) exhibit a specific deficiency in response to high fluence rates of red light for shortening of period length, and transgenic plants overexpressing phyB have period lengths shorter than those of wild-type plants (Millar et al., 1995). Such FRC analyses have been used to place period-affecting mutants in either the light input pathway or the oscillator (Somers et al., 1998b, 2000).

Another variable for the interaction between light input and the circadian clock occurs in a phase-dependent manner. Although light is a strong resetting stimulus for the circadian oscillator, the same amount of light administered as a bright pulse at different phases of the circadian cycle may cause phase resetting of different magnitudes and/or directions (Johnson, 1992). Such phase shifts can be plotted as a function of the circadian phase of the stimuli to make a phase response curve (PRC). Typically, PRCs of circadian oscillators for light pulses exhibit phase delays in the early subjective night and phase advances in the late subjective night, with little phase resetting occurring during the subjective day (Johnson, 1998). Regulation of the magnitude and direction of phase resetting allows the organism to maintain a stable phase relationship between the circadian oscillator and the external light environment. The shape of the PRC reflects the strength of the resetting stimulus. For example, lower doses of light pulses during subjective night produce smaller phase shifts than do those caused by stronger light pulses (Johnson, 1992). Therefore, the shape of the PRC can be used to measure the sensitivity of the oscillator to light input at different phases.

However, the fundamental requirement for using PRCs as a tool to determine light inputs is an assay of robust circadian rhythmicity that persists in DD, which allows the effects of light pulses on phase resetting to be measured. In Arabidopsis, most of the overt rhythms used to study clock function for an extended time, such as rhythmic transcription from the *CAB2* promoter, do not persist in DD (Johnson, 1998). This dilemma was overcome recently with the use of transgenic plants containing a luciferase transcriptional reporter driven by the *CCR2* (for *COLD CIRCADIAN REGULATED 2*) promoter, which exhibits robust bioluminescence cycling under free-running conditions of both LL and DD (Strayer et al., 2000).

An additional interaction between the oscillator and light signaling in Arabidopsis occurs at the output levels. Some clock-regulated genes respond to light pulses by means of an acute phytochrome-mediated induction of transcription (Karlin-Neumann et al., 1988). This acute induction is followed by circadian cycling that persists for one or two cycles. The magnitude of acute induction varies with the time of day, suggesting possible gating by the circadian clock (Millar and Kay, 1996). It is not known conclusively whether the light input pathway to the clock participates in the gating of the acute response. Mutants that affect light input, such as *elf3*, should prove useful in characterizing this pathway.

The *ELF3* gene was cloned recently and found to code for a novel protein with no significant sequence similarity to characterized proteins in the existing public databases. The *elf3-1* allele has a nonsense mutation in the third of four exons (Hicks et al., 2001). This mutant accumulates neither *ELF3* transcript nor protein product; therefore, it is likely a null allele (Hicks et al., 2001; Liu et al., 2001). To determine the role of ELF3 in the Arabidopsis circadian system, we characterized the expression pattern of *ELF3* and analyzed the effects of the misexpression of ELF3 on circadian rhythms and light responses. Furthermore, we used the *ccr2*::*luc* reporter to determine conclusively the effect of *elf3* mutations on oscillator function in the absence of light.

RESULTS

ELF3 Expression Is Regulated by the Circadian Clock

To determine ELF3's role in the circadian system and to gauge the influence of the clock and light input on ELF3, we defined *ELF3*'s transcriptional expression by using noninvasive imaging of plants containing a fusion construct of the *ELF3* promoter and the firefly luciferase coding region, as pioneered with *cab2*::*luc* (Millar et al., 1992).

Bioluminescence from *elf3*::*luc* in the wild-type background maintained a robust circadian rhythm after transfer to LL conditions (Figure 1A). In LL, *elf3*::*luc* expression peaked at ~16 hr circadian time and had an antiphasic relationship with the expression of the morning-phased reporter, *cab2*::*luc*. Under free-running conditions of constant darkness (DD), cycling of *elf3*::*luc* persisted with a similar phase (Figure 1D). Although *elf3*::*luc* expression was less robust after a few days in DD than in LL, a weak *elf3*::*luc* rhythm was detectable in these dark-adapted plants up to 6 days after transfer to dark. The cycling exhibited by *elf3*::*luc* was consistent with that observed in RNase protection as-

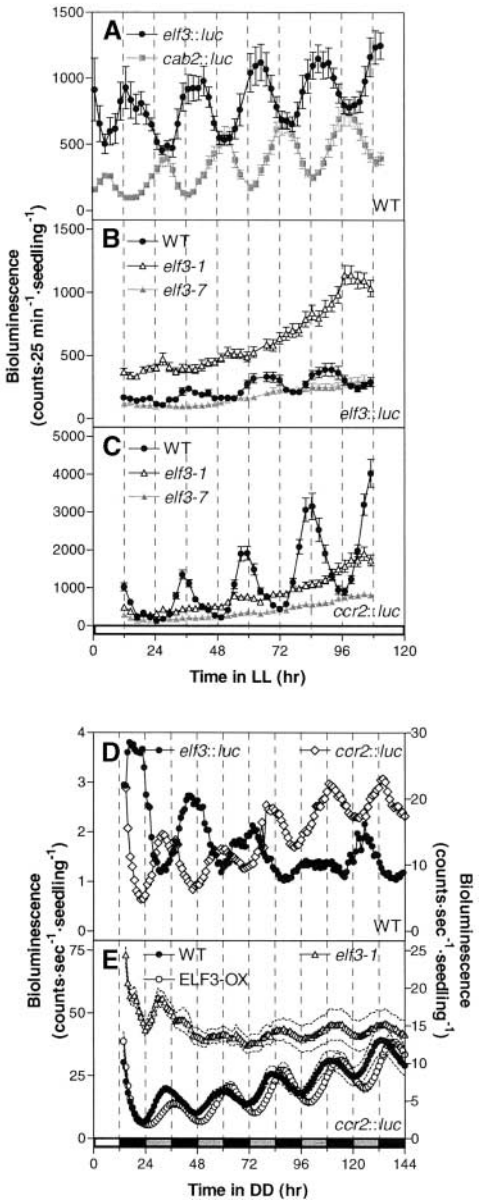

Figure 1. *ELF3* Transcription Is Regulated by the Circadian Clock.

Seedlings were grown under 12-hr-light (white fluorescent; 50 to 60 μmol·m^{-2}·sec^{-1})/12-hr-dark cycles for 6 days before transfer to LL (time 0) or DD (time 12). Shaded bars indicate subjective day and night. WT, wild type.

(A) *elf3*::*luc* expression is rhythmic in continuous white light. Mean bioluminescence traces (±SE) from wild-type *elf3*::*luc* (closed circles; n = 5) and *cab2*::*luc* (gray squares; n = 6) seedlings are plotted.

(B) *elf3* mutations cause arrhythmic expression of *elf3*::*luc* in LL. Mean *elf3*::*luc* bioluminescence traces (±SE) from wild-type (closed circles; n = 7), *elf3-1* (open triangles; n = 12), and *elf3-7* (closed gray triangles; n = 9) seedlings are plotted.

(C) *elf3* mutations lead to arrhythmic *ccr2*::*luc* expression in LL. Mean *ccr2*::*luc* bioluminescence traces (±SE) from wild-type (closed circles; n = 12), *elf3-1* (open triangles; n = 12), and *elf3-7* (closed gray triangles; n = 12) are plotted.

(D) *elf3*::*luc* expression is rhythmic in DD. Representative bioluminescence traces from individual wild-type *elf3*::*luc* (closed circles) and *ccr2*::*luc* (open diamonds) seedlings are plotted. Data from *elf3*::*luc* plants are plotted on the left *y* axis, and *ccr2*::*luc* data are plotted on the right *y* axis.

(E) *ccr2*::*luc* rhythms are robust in DD, and period length is unaffected by ELF3 levels. Mean *ccr2*::*luc* bioluminescence traces from wild-type (closed circles; n = 27), *elf3-1* (open triangles; n = 27), and ELF3-OX (open circles; n = 27) are plotted. Data from wild-type and ELF3-OX plants are plotted on the left *y* axis, and *elf3-1* data are plotted on the right *y* axis.

says (Hicks et al., 2001) and protein gel blot analyses of nuclear proteins (Liu et al., 2001). Also, the phase of *elf3*::*luc* expression is roughly equivalent to the predicted phase of ELF3 function on the basis of the phase at which the oscillator arrests in the *elf3* mutants in LL (McWatters et al., 2000).

elf3 Mutations Cause Conditional Arrhythmia of Both Day- and Night-Phased Circadian Markers

Circadian expression of ELF3 may define a clock output that regulates a subset of clock-controlled genes with a similar phase of expression. Lesions in *ELF3* have been shown to cause arrhythmic expression of the morning-specific circadian clock–regulated genes *CAB2* and *LHY* (Hicks et al., 1996; Schaffer et al., 1998). To determine whether ELF3 acts directly upstream of genes expressed in the morning in a promoter-specific manner, we tested the effect of *elf3* mutations on clock-controlled genes that were expressed at different phases. Expression of both *elf3*::*luc* (Figure 1B) and *ccr2*::*luc* (Figure 1C; peaks at ~12 hr circadian time) became arrhythmic when the *elf3* mutants were placed in LL.

ELF3 Is Not a Classic Component of the Central Oscillator

Both the circadian rhythm of *ELF3* expression and the global effects of mutations at the *ELF3* locus on multiple circadian outputs suggest a role for ELF3 in the central oscillator or in the light input pathway to the clock. If it is a component of the oscillator, ELF3's action should persist in the absence of light, as has been shown for the oscillator mutant *toc1* (Somers et al., 1998b). However, if it is an input component, the phenotypes of the *elf3* mutation should be restricted to LL conditions only. Although the expression of *cab2*::*luc* in etiolated seedlings after a red light pulse showed some sign of clock function, it was difficult to assess the pace of the oscillator from the low amplitude and the rapidly damping *cab2*::*luc* rhythm under the free-running conditions of DD.

However, using the *ccr2*::*luc* reporter, we were able to show that circadian regulation in *elf3-1* mutants persisted for several days under DD conditions (Figure 1E). Although the amplitude of *ccr2*::*luc* rhythm in DD was affected by the loss of *ELF3*, neither the phase nor the period length of the rhythm in the mutant (period length [τ] = 25.41 ± 0.28 hr [variance-weighted mean ± variance-weighted SE] as estimated by fast Fourier transformation–non-linear least squares [FFT-NLLS] analysis; n = 17) was statistically different (using Student's two-tailed heteroscedastic t test) from that of the wild-type rhythm (τ = 25.07 ± 0.09 hr; n = 23).

The persistence of rhythmic expression of a clock-controlled gene in DD suggests the presence of a functional clock in the *elf3-1* mutant in DD. However, it is possible that cycling ELF3 action is essential for functioning of the clock under LL; therefore, it may act as an essential clock component under LL conditions. If this were true, constitutive expression of ELF3 would disrupt clock function in LL, leading to arrhythmia. To test this hypothesis, we made use of plants constitutively overexpressing a genomic copy of *ELF3* driven by a strong viral promoter (the 35S promoter from *cauliflower mosaic virus*). Overexpression of ELF3 protein was verified by protein gel blot analysis (Liu et al., 2001). Several circadian markers were used to assay the state of the circadian system in ELF3-overexpressing (ELF3-OX) plants. Transcription of *ELF3* and *CCA1* (for *CIRCADIAN CLOCK ASSOCIATED 1*, a myb-related factor; Wang and Tobin, 1998), which encode gene products believed to be closely associated with clock function, continued to cycle with a circadian rhythm in ELF3-OX plants under LL (Figure 2A). Also unaffected by ELF3 overexpression was the rhythmic transcription of two circadian clock–regulated output genes, *CAB2* and *CCR2* (Carpenter et al., 1994; Kreps and Simon, 1997) (Figure 2B). We checked for cycling expression of clock outputs in ELF3-OX seedlings under DD and found that all of the circadian reporters assayed continued to cycle: *ccr2*::*luc* (Figure 1E), *cab2*::*luc* (data not shown), and *elf3*::*luc* (data not shown). The persistence of overt rhythms in plants constitutively expressing ELF3 excluded the possibility that ELF3 cycling is required for oscillator function. It appears that ELF3 expression alone is sufficient for a functional clock in both LL and DD.

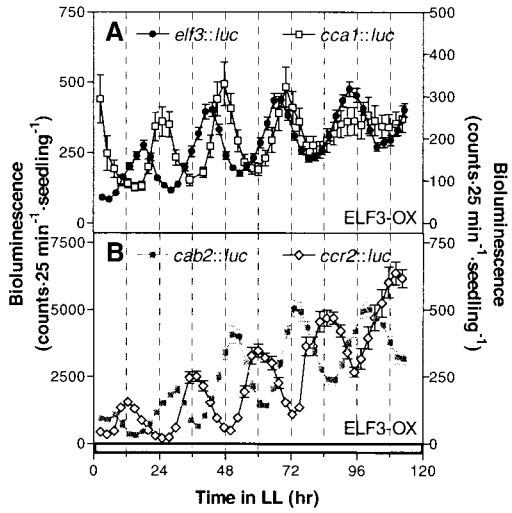

Figure 2. The Circadian Clock Remains Intact in Seedlings Overexpressing ELF3.

Luminescence in seedlings constitutively overexpressing a genomic copy of ELF3 under the control of the *cauliflower mosaic virus* 35S promoter (ELF3-OX) was determined as described in Figure 1 and Methods. After entrainment in 12-hr-light/12-hr-dark cycles, plants were transferred to continuous red light (~200 μmol·m^{-2}·sec^{-1}) (time 0).

(A) Expression of genes closely associated with circadian function, *elf3*::*luc* (closed circles; n = 36) and *cca1*::*luc* (open squares; n = 9), is rhythmic in ELF3-OX seedlings. Data from *elf3*::*luc* plants are plotted on the left y axis, and *cca1*::*luc* data are plotted on the right y axis.

(B) Expression of the circadian output genes *cab2*::*luc* (closed gray squares; n = 14) and *ccr2*::*luc* (open diamonds; n = 10) is rhythmic in ELF3-OX seedlings. Data from *cab2*::*luc* plants are plotted on the left y axis, and *ccr2*::*luc* data are plotted on the right y axis.

Error bars indicate ±SE.

Overexpression of ELF3 Lengthens Circadian Period Length in a Light-Dependent Manner

Although circadian rhythms were robust in ELF3-OX plants, we observed that in constant white light, the period length of rhythms in ELF3-OX plants (26.41 ± 0.18 hr for *cab2*::*luc* in ~70 μmol·m^{-2}·sec^{-1}) was actually longer than it was in wild-type plants (24.28 ± 0.15 hr), suggesting a change in the pace of the central oscillator. However, under DD, in which robust *ccr2*::*luc* rhythms persisted, overexpression of ELF3 did not alter period length (τ = 25.10 ± 0.10 hr in ELF3-OX [n = 27]; τ = 25.07 ± 0.09 hr in the wild type [n = 23]). Rather, the phase of *ccr2*::*luc* expression after transfer to DD was delayed by ~2 hr. This delay likely was an artifact of the ELF3-OX clock running slowly during the last light period before the seedlings were transferred from light/dark entrainment to DD.

To evaluate ELF3's role as an input to the clock, we took advantage of Aschoff's rule and constructed FRCs by measuring the period length of the clock in wild-type and ELF3-OX plants over a range of red light and blue light fluence rates. At medium to high fluence rates of red light, overexpression of ELF3 extended the period length of *cab2*::*luc* (Figure 3A). In continuous blue light, however, the period length of *cab2*::*luc* (Figure 3B) was increased in ELF3-OX plants regardless of fluence rate. Similar effects were seen on the period length of *elf3*::*luc* in ELF3-OX plants (data not shown). These effects on period length indicate that the *ELF3* gene product is involved in the repression of light input to the circadian oscillator.

ELF3 Attenuates Light Induction of a Circadian Output

elf3 null mutants are hypersensitive to the phytochrome-mediated acute induction of *cab2*::*luc* (Anderson et al., 1997), suggesting that ELF3 also may antagonize the light induction of light-responsive clock outputs. ELF3-OX seedlings, therefore, should have decreased induction and/or increased decay kinetics of *cab2*::*luc* expression after a light pulse. Etiolated seedlings were given a 2-min red light pulse, and bioluminescence rhythms from *cab2*::*luc* were followed for several hours (Figure 4A). Consistent with previous reports, *cab2*::*luc* expression in *elf3-1* seedlings increased nearly 12-fold compared with that of the preflash level, whereas the wild-type seedlings showed only an approximately sixfold increase. Although the acute induction was decreased in ELF3-OX seedlings (approximately fourfold induction), the difference in induction levels between the wild type and ELF3-OX was not statistically significant. Interestingly, the decay kinetics of the acute induction also differed among the three genotypes. In both *elf3-1* and wild-type seedlings, *cab2*::*luc* expression continued to increase for ~3 hr after light treatment, whereas in ELF3-OX seedlings, expression of *cab2*::*luc* peaked at least 1 hr earlier. Furthermore, in the ELF3-OX seedlings, the decrease after the acute induction was faster and to a more reduced level than was that in the wild type. *elf3-1* exhibited the slowest decay kinetics.

The acute response addressed above was followed several hours later by long-period oscillations in *cab2*::*luc* expression, a response typically attributed to regulation by the circadian clock. *elf3-1* null mutants display the acute, phytochrome-mediated response but are defective in the subsequent circadian regulation (Anderson et al., 1997). Although the weak allele, *elf3-7*, like the null mutant, was arrhythmic in LL, we wanted to determine whether enough ELF3 activity was present in *elf3-7* to allow cycling of *cab2*::*luc* after a light pulse. Indeed, induction in etiolated *elf3-7* seedlings was similar to that in the wild type for both the acute and circadian responses (Figure 4B).

ELF3 Regulates Light Resetting of the Clock

ELF3's involvement in both blue light and red light signaling to the clock and in the induction of outputs suggests that its action may lie very close to the oscillator rather than in directly antagonizing a photoreceptor. Because ELF3 appears to be an integral component of light input to the clock and its own expression cycles, it likely gates both light input to the clock and acute induction of the circadian outputs by restricting the light sensitivity of these pathways in the evening. Therefore, it may be expected that ELF3 is involved in light resetting of the clock.

We probed the role of ELF3 in light resetting of the clock by measuring the phase changes of *ccr2*::*luc* expression after light pulses were given to wild-type, *elf3-1* null, and

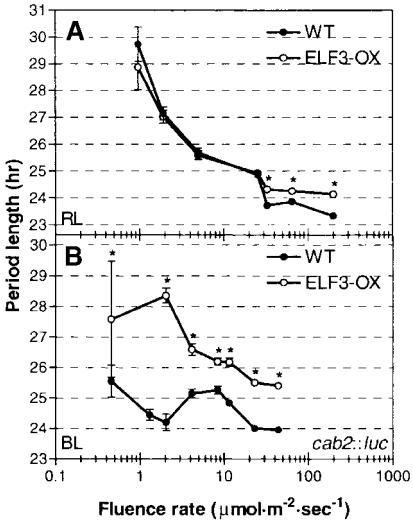

Figure 3. ELF3 Overexpression Lengthens the Period of the Circadian Clock in Red Light and Blue Light.

After entrainment, wild-type (WT) and ELF3-OX seedlings were transferred to continuous red light or blue light at specific fluence rates, and luminescence was determined as described in Figure 1 and Methods. Variance-weighted means (± variance-weighted SE) after >112 hr in free-running conditions are presented in FRCs. Asterisks, $P < 0.01$ (Student's two-tailed heteroscedastic *t* test).

(A) Effect of red light fluence rate on free-running period of *cab2*::*luc* expression in wild-type (closed circles; $n = 18, 18, 18, 18, 17, 16,$ and 18) and ELF3-OX (open circles; $n = 16, 18, 18, 18, 13, 11,$ and 36) seedlings.

(B) Effect of blue light fluence rate on free-running period of *cab2*::*luc* expression in wild-type (closed circles; $n = 14, 13, 12, 18, 22, 18, 18,$ and 17) and ELF3-OX (open circles; $n = 6, 17, 17, 18, 18, 18,$ and 18) seedlings.

ELF3-OX plants in DD. Red light and blue light PRCs (Figures 5A and 5B, respectively) showed that wild-type plants exhibited little resetting in the subjective morning, when light pulses resulted in small phase advances. Later in the subjective day, the light stimuli elicited delays that continued to increase in magnitude. The greatest phase shift was during the subjective night, when large delays became large advances at what is termed the breakpoint. Near dawn, the small phase advances observed in the early morning returned. Although the overall shapes of both red light and blue light PRCs were similar, they differed in the magnitude of their largest phase resetting. Red light produced up to ~7-hr phase delays and ~10-hr phase advances, whereas blue light produced up to ~9-hr phase delays and ~6-hr phase advances. ELF3-OX plants experienced smaller phase shifts than did wild-type plants. The effects of ELF3 overexpression on light pulse entrainment of the clock were most pronounced in the red light PRC; at most, ELF3-OX plants experienced ~6 hr of phase delays or advances. Blue

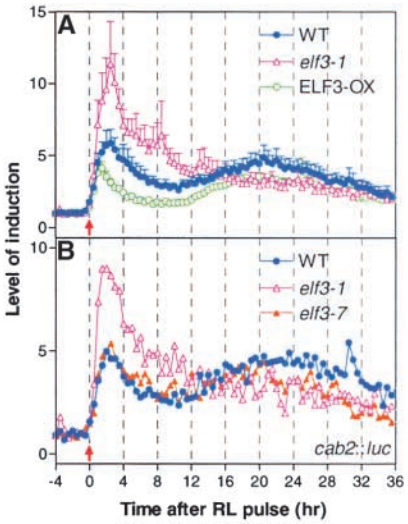

Figure 4. ELF3 Attenuates Phytochrome-Mediated Induction of Circadian Outputs.

Luminescence from clusters of ~50 etiolated *cab2*::*luc* seedlings was measured before and after a 2-min red light (RL) pulse (~25 μmol·m^{-2}·sec^{-1} at time 0; red arrow). Preflash levels were normalized to 1 so that induction levels could be evaluated and compared.
(A) ELF3 levels affect the magnitude of *cab2*::*luc* acute induction. Normalized mean *cab2*::*luc* bioluminescence traces (±SE) from the wild type (WT; blue circles; $n = 3$), *elf3-1* (open purple triangles; $n = 4$), and ELF3-OX (open green circles; $n = 4$) are plotted.
(B) Complete phytochrome response is present in the *elf3-7* weak allele. Representative individual traces from the wild type (blue circles), *elf3-1* (open purple triangles), and *elf3-7* (closed orange triangles) are plotted.

light pulses given to ELF3-OX plants also elicited smaller delays than they did in wild-type plants.

In response to both red light and blue light pulses, the breakpoint observed in wild-type PRCs was abolished by ELF3-OX: the transition from delay to advance was continuous and more gradual (Figures 5A and 5B). *elf3-1* seedlings, which lack ELF3 protein, showed comparable phase delays and advances during the subjective day. However, during subjective night, when *ELF3* expression would normally peak, light-pulsed *elf3-1* null mutants either experienced larger than wild-type phase shifts or became arrhythmic in *ccr2*::*luc* expression. This effect was more pronounced with red light pulses than with blue light pulses. Likewise, the conditional arrhythmia of *elf3* mutants was more prevalent in continuous red light than blue light (Hicks et al., 1996). When administered in early to mid subjective night, red light pulses induced arrhythmia in most of the mutant seedlings; circadian rhythms were detected in only 9% compared with 69% in the wild type during the same phase and 47% in *elf3-1* during other phases. Blue light pulses, however, produced increasingly larger phase delays, up to 11 hr, in subjective night before a light pulse caused arrhythmia (none of 21 *elf3-1* plants were rhythmic, whereas four of 21 wild-type plants and 18 of 21 ELF3-OX plants were rhythmic during this phase).

DISCUSSION

ELF3 Is a Circadian Clock–Regulated Negative Component of Light Signaling

Expression of *ELF3* is clock regulated, and ELF3 feeds back to antagonize both light signaling to the clock and light-mediated acute induction of circadian outputs (see the model in Figure 6). By altering the levels of ELF3 so that expression is greatest at night, the plant is able to restrict the sensitivity of its clock to light signals during the night. This gating of light input to the oscillator results in an efficient clock that is resistant to transitory fluctuations in light levels at night—levels that are present in nature but do not correspond with dawn/dusk (i.e., change in cloud cover or stellar/lunar illumination).

ELF3 is not the first example of a circadian output involved in light signaling. White collar 1 (WC-1) is regulated by the Neurospora clock in a post-transcriptional manner. WC-1 is believed to feed back as an activator of *frequency* (*frq*; a Neurospora oscillator component) and clock-controlled genes as well as to mediate the light induction of *frq*, clock-controlled genes, and itself (Lee et al., 2000). ELF3 and WC-1 are negative and positive regulators of light signaling in their respective circadian systems. The opposite functions of these cycling proteins may indicate either that they accomplish a similar goal in distinct manners or that in each of these organisms there is a pair of proteins with opposing functions that work together to properly regulate light signaling. Indeed, the circadian regulation of phyB (Bognar et al., 1999) and other photoreceptors, including cryptochromes (Harmer et al., 2000), has been demonstrated in Arabidopsis, supporting the possibility of rhythmically expressed positive and negative regulators of light signaling in this system. Although protein levels of phyB appear not to cycle (Bognar et al., 1999), post-translational circadian regulation of photoreceptors may occur. For example, transcription of *SPA1*, a negative regulator of phyA signaling (Hoeker et al., 1999), is regulated by the circadian clock (Harmer et al., 2000). Proteins such as ELF3 and SPA1 may be responsible for the circadian regulation of photoreceptor activity. It is hypothesized that such feedback loops contribute to robust oscillations and stable output (Lee et al., 2000).

Although most of the circadian phenotypes linked to misexpression of ELF3 are light dependent, a dark phenotype was observed consistently. The amplitude of circadian rhythms was decreased in the absence of ELF3. Therefore, ELF3 may function independent of light signaling to pro-

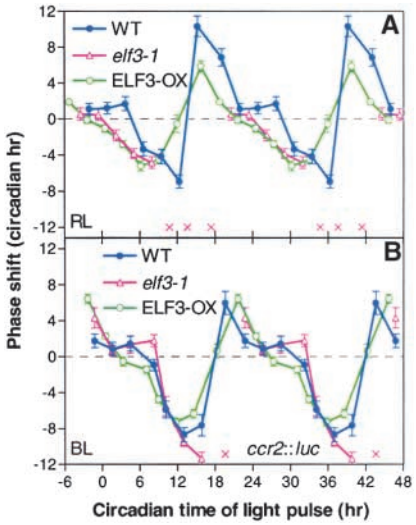

Figure 5. ELF3 Levels Affect Sensitivity of the Clock to Resetting by Light.

PRCs for the wild type (WT; blue circles), *elf3-1* (open purple triangles), and ELF3-OX (open green circles) constructed by plotting phase shifts of *ccr2*::*luc* expression (± pooled SE; circadian hour) elicited by hour-long light pulses are plotted against the circadian times at which the light pulses were administered. Phase advances are plotted as positive values, and delays are plotted as negative values. Circadian times at which a light pulse induces arrhythmicity in *elf3-1* seedlings are indicated by purple ×'s above the *x* axis. PRCs have been double plotted to aid in identification of the circadian features.
(A) Red light PRC.
(B) Blue light PRC.

mote more robust oscillations by maintaining normal oscillator amplitude.

The conditional arrhythmia in LL and the decreased amplitude in DD of circadian outputs in *elf3* mutants could be accounted for if ELF3 were part of the clock. However, three lines of evidence suggest that ELF3 does not function as a central component of the molecular oscillator: (1) circadian output from *elf3* mutants is rhythmic in DD; (2) overexpression of ELF3 does not abolish rhythmicity of any outputs assayed in free-running conditions; and (3) in DD, period length is independent of ELF3 levels. Furthermore, the persistence of *elf3*::*luc* cycling in ELF3-OX seedlings suggests that ELF3 is not a component of a slave oscillator responsible for its own rhythmic expression.

ELF3 Acts in the Light Input Pathway to the Clock

Both red light and blue light FRCs of ELF3-OX plants are different from that of the wild type, suggesting a role of ELF3 in general light input to the clock. Under blue light, overexpression of ELF3 caused period lengthening over a wide range of fluence rates, similar to blue light FRCs of plants deficient in both CRY1 and CRY2 function. However, the hypocotyl elongation response of ELF3-OX plants under blue light was similar to that of the wild-type plants (X. Liu and R. Wagner, unpublished results), suggesting that ELF3 is not involved in the cryptochrome-mediated blue light inhibition of hypocotyl elongation. Under continuous red light of high fluence rate, ELF3-OX lengthens the free-running period length in a manner similar to the loss of functional phyB (Somers et al., 1998a). Again, the ELF3-OX plants do not exhibit the hypocotyl length response typical of phyB mutants; rather, hypocotyl elongation is hypersensitive to red light in ELF3-OX seedlings (Liu et al., 2001). Under low fluence rates of red light, ELF3-OX did not affect the period length of *cab2*::*luc* expression. Either ELF3 acts independent of the phyA signaling pathway to the clock or the fluence rate is too small to be affected by overexpression of ELF3. In general, overexpression of ELF3 restricted light signaling to the clock and resulted in longer period length under continuous light. The predicted hypocotyl phenotypes based on FRC comparisons, however, were not observed for ELF3-OX seedlings. ELF3's role in the regulation of hypocotyl elongation remains unclear.

Phytochrome-Mediated Acute Induction of Circadian Outputs

The circadian clock represses the phytochrome-mediated response to discrete light pulses in a phase-specific manner, resulting in the gating of induction such that outputs (e.g., *cab2*::*luc*) are less sensitive to light during the subjective night (Millar and Kay, 1996). For this type of gating to exist, a circadian system must generate rhythmic expression of gene products that allow and/or inhibit induction. In Arabidopsis, phytochromes are the major positive factors, whereas ELF3 is a negative regulator of this type of induction. Altering phytochrome or ELF3 levels changes the magnitude

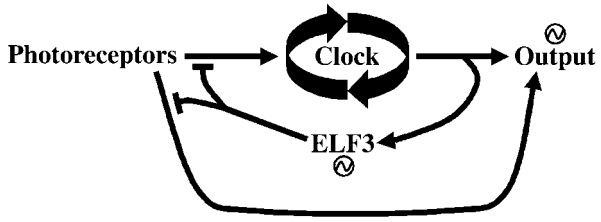

Figure 6. Model of the Role of ELF3 in the Plant Circadian System.

ELF3 has a unique role within the circadian system of Arabidopsis. Circadian regulation of *ELF3* expression enables the oscillator to control the light sensitivity of both clock resetting and the induction of circadian outputs in a phase-specific manner. This is a prime example of a gating mechanism in which a circadian output feeds back to regulate input pathways.

of light induction of *cab2*::*luc* in etiolated seedlings: the more phytochrome or the less ELF3, the greater the induction after a light pulse.

Compared with the wild type, acute induction of *cab2*::*luc* was diminished in ELF3-OX (Figure 4A), *phyA*, and *phyB* etiolated seedlings (Anderson et al., 1997). The second expression peak, ~20 hr after the light pulse, was normal in *phyA* but remained low in *phyB* and ELF3-OX seedlings (Anderson et al., 1997; see also Figure 4A). These findings suggest that ELF3 may attenuate the phyB-mediated induction of circadian outputs, but they do not rule out involvement in the phyA pathway. If high levels of ELF3 are capable of completely blocking signaling through the phytochromes that ELF3 antagonizes, induction in seedlings deficient in those phytochromes should resemble that in ELF3-OX seedlings. The *phyA phyB* double mutant exhibits extremely low levels of induction after a light pulse (Anderson et al., 1997). This suggests that ELF3 may not act in the phyA pathway unless the inhibition by ELF3 is not absolute. In etiolated PHYA-OX seedlings, the active form of phyA, PfrA, is induced fourfold at 2 hr after a light pulse and maintains a twofold induction for up to 50 hr compared with the preflash levels (Anderson et al., 1997). Although PfrA levels are increased and fairly constant, *cab2*::*luc* expression cycles with increased amplitude, indicating that a rhythmic, negative regulator is acting on phyA induction of circadian outputs. This factor is either ELF3 or a protein with a similar function, perhaps SPA1. A light pulse experiment with *elf3-1* seedlings deficient in one or more phytochromes could specify in which pathways ELF3 acts. *elf3-1* seedlings would lose their hypersensitivity to light if the phytochrome(s) antagonized by ELF3 was absent.

This hypersensitivity of etiolated *elf3* null seedlings is accompanied by the absence of successive peaks in *cab2*::*luc* expression after a light pulse (Anderson et al., 1997; see also Figures 4A and 4B). The presence of increased levels of the active form of phyA after a light pulse in etiolated PHYA-OX seedlings and the inherent stability of the active form of phyB suggest that light-pulsed etiolated seedlings experience the equivalent of constant dim illumination because the phytochrome pathways continue to be active. This persistence of phytochrome signaling may be the cause of arrhythmic *cab2*::*luc* expression after a light pulse in *elf3-1*. However, *elf3-7* seedlings appear to have enough ELF3 activity to repress *cab2*::*luc* expression and maintain a circadian rhythm. If this is the case, there may be a fluence threshold below which outputs in the *elf3-7* mutant retain circadian regulation in LL.

ELF3 Modulates Resetting of the Clock by Light

Period length under continuous light is the net effect of advances and delays produced by light input to the clock at different phases; therefore, it does not reveal the phase-specific interactions between the oscillator and the light input. Clock-regulated expression of *ELF3* and its inhibitory effects on light input suggest that ELF3's effects on light input may be dependent on the phase of the oscillator, with maximum effects at phases of peak ELF3 expression and minimum effects when ELF3 levels are low. PRCs were helpful in addressing this question.

For Drosophila, the shape of the PRC is determined mostly by the oscillator component TIMELESS (TIM). Phase shifts are caused by light-induced degradation of the TIM protein. The magnitude and direction of phase resetting by light pulses can be explained by the levels of TIM and its subcellular localization, respectively. In Neurospora, light pulses cause phase shifting by inducing transcription of FRQ rather than by accelerating its degradation (Crosthwaite et al., 1995). The overall shape of the Neurospora PRC is determined by FRQ transcript level, with the greatest phase shifts occurring when FRQ transcript levels are minimal (Crosthwaite et al., 1995). These examples present two potentially different modes of resetting by light: acceleration of protein degradation and induction of transcription. Because ELF3 levels affect light resetting of the clock and ELF3 attenuates light-mediated induction of circadian outputs and, perhaps, the oscillator component(s), the Arabidopsis clock may be reset by light-mediated transcriptional induction of the oscillator component(s).

The Arabidopsis clock continues to run in DD in both *elf3-1* and ELF3-OX seedlings, as indicated by *ccr2*::*luc* rhythms. Therefore, it is not surprising that PRCs from each of these genotypes have an overall shape similar to that of the wild type. The magnitude of phase shifts, however, changed in the mutant backgrounds. In response to pulses of red light or blue light during subjective night, wild-type seedlings produced large delays and advances with a clear breakpoint in between (Figure 6). Such PRCs usually are produced when the light pulses are saturating. Interestingly, the phase of the breakpoint coincided with the peak expression of *ELF3*. In ELF3-OX seedlings, reduced phase shifts were elicited by pulses in subjective night. Such topology of PRCs is usually associated with subsaturating light pulses (Pittendrigh, 1981; Johnson, 1992), suggesting that ELF3 antagonizes the light-resetting mechanism.

Deficiency of ELF3, as in *elf3-1* plants, also had a significant effect during the subjective night. Light pulses on wild-type seedlings usually produce large phase shifts of up to 10 hr during this period. With the progression of subjective night, light pulses elicited increasingly larger phase shifts in the absence of ELF3. However, at the phase when ELF3 expression was maximal and light pulses produced the largest phase shifts in the wild type, a light pulse caused arrhythmia in most of the *elf3-1* plants. As with ELF3-OX plants, the effect of red light pulses on *elf3-1* plants was more severe than that of blue light pulses (see discontinuity in *elf3-1* PRCs in Figure 5); likewise, the conditional arrhythmia of *elf3* mutants is more prevalent in continuous red light than blue light (Hicks et al., 1996). This phenomenon of the suppression of circadian rhythmicity by critical light pulses that

drive the oscillator permanently or transiently to the steady state (i.e., singularity) has been described for *Drosophila pseudoobscura*, mammals, and plants (Leloup and Goldbeter, 2000). The inhibitory effect of ELF3 on light input to the clock seems to serve as a safeguard mechanism against such severe light-induced perturbations of the oscillator. By increasing the intensity of the light pulse stimulus, it may be possible to suppress circadian rhythmicity in wild-type Arabidopsis.

Differences in the shapes of blue light and red light PRCs in the wild type may be due to independent action of the two signaling pathways in light pulse entrainment of the oscillator. This is corroborated by differences in the PRCs of *elf3-1* seedlings. Furthermore, under LL conditions, red light fluence rate showed a clear inverse relationship with free-running period length, as expected for a circadian light-signaling mechanism in a diurnal organism. However, the blue light FRC lacks a clear inverse relationship with free-running period length (Somers et al., 1998a). The two pathways may be acting on separate oscillator components or on the same oscillator component but with different kinetics of resetting.

Alterations in FRCs in ELF3-OX seedlings and altered PRCs in both ELF3-OX and *elf3-1* seedlings clearly demonstrate a genetic interaction between ELF3 and both blue light and red light input to the clock. ELF3 reduces the effectiveness of light in signaling and in resetting the clock. There are two possibilities for the mechanism by which ELF3 modulates light resetting. ELF3 may act simply as a valve outside the clock to regulate the flux of light. Alternatively, it may act directly on the oscillator and regulate its light sensitivity. The second mechanism is more likely. The reduced amplitude of *CCR2* cycling in *elf3-1* compared with that in the wild type is indicative of ELF3's role in maintaining the amplitude of the oscillator. However, the normal period length of both *elf3-1* and ELF3-OX in DD suggests that ELF3 may not be essential in setting the pace of the oscillator in the absence of light input. Unless the function of ELF3 is post-translationally circadian regulated (e.g., rhythmic modification of activity by phosphorylation), the evidence of normal clock functioning in ELF3-OX seedlings in the absence of light suggests that the mere presence of ELF3, not its cycling, is necessary for its role in maintaining a robust oscillation. However, large changes in the shape of the PRCs for both ELF3-OX and *elf3-1* plants during the subjective night suggest a role in maintaining oscillator progression during the night phase of the photoperiod. Such a role is consistent with ELF3's peak phase of expression.

Summary

Negative feedback loops are a central theme in circadian biology. We have shown that ELF3 feeds back to the clock in a rhythmic manner to negatively regulate light input to the oscillator and acute induction of circadian outputs during the night. Furthermore, *elf3* is the only mutation shown to make a circadian system highly susceptible to suppression of circadian rhythms by a critical light pulse.

METHODS

Mutant Alleles and Heterozygous Lines

The *elf3-1* mutation is a C-to-T transition in exon 3 that results in early termination. The *elf3-7* mutation is a G-to-A transition in the exon 1 donor splice site that is predicted to result in an in-frame deletion of eight amino acids (K. Hicks and R. Wagner, unpublished results). ELF3-OX plants, which constitutively overexpress *ELF3* genomic sequence, are described by Liu et al. (2001). In all experiments, the wild-type control is Columbia (Col-0).

Construction of the *elf3*::Ω::*luc* Reporter Construct

The 1.9-kb HindIII restriction fragment of the *ELF3* promoter, just upstream of the *ELF3* gene, was inserted into pPZP221, a T-DNA transformation vector used as a *promoter*::*luciferase* reporter system. pPZP221 is composed of an omega translational enhancer followed by the coding region from the firefly luciferase gene and the nopaline synthase termination sequence (Millar and Kay, 1996).

Creation of Transgenic Lines

Arabidopsis thaliana Col-0 seedlings were transformed via the *Agrobacterium tumefaciens*–mediated process; vacuum infiltration was used for the overexpression lines. T1 seed were surface sterilized and sown on Petri dishes containing half-strength Murashige and Skoog (1962) medium, 0.5% (w/v) sucrose, 0.8% (w/v) agar, and antibiotic. Transgenic *elf3*::*luc* T1 seed were selected on 75 mg/mL gentamycin. T2 seedlings were evaluated in bioluminescence assays (see below). The most robust lines with a single site of insertion (as judged by Mendelian segregation) and with luciferase activity faithful to the RNase protection data were chosen for further experiments.

Bioluminescence Assays

Seed for rhythm analyses were grown on 1 × Murashige and Skoog/0.8% agar medium with 3% (w/v) sucrose and imaged as described previously (Millar et al., 1995). However, expression of *ccr2*::*luc* in constant darkness (DD) was measured and analyzed using the Night Owl imaging system with WinLight software (Perkin-Elmer), whereas *elf3*::*luc* expression in DD was measured using the Packard TopCount multiplate scintillation counter as described by Strayer et al. (2000). Transgenic *cab2*::*luc* (Millar et al., 1992) had been introgressed into Col-0 before crossing with plants of various *elf3* mutant backgrounds. The other circadian markers used were constructed originally in the Columbia ecotype. Period lengths for fluence response curves were measured as described previously (Somers et al., 1998b), and period estimates were obtained according to Plautz et al. (1997) and Millar et al. (1995). Dark-grown etiolated seedlings were imaged in clusters of ~50 seedlings as described previously (Anderson et al., 1997). To depict the level of induction after a light

pulse, counts from etiolated seedlings were normalized by setting prepulse levels to 1.

Phase Response Curves

Seedlings were grown as described above. After 5 days in light/dark cycles, seedlings were transferred to DD, and imaging of bioluminescence from *ccr2*::*luc* expression was initiated with the Night Owl imaging system. Beginning after 1 full day in DD, individual plates of seedlings were exposed to 1-hr light pulses at ~3-hr intervals for ~24 hr. Red light pulses were 40 $\mu mol \cdot m^{-2} \cdot sec^{-1}$, and blue light pulses were 25 $\mu mol \cdot m^{-2} \cdot sec^{-1}$. The period and phase of rhythms after the light pulses were estimated (Millar et al., 1995; Plautz et al., 1997) and converted to circadian time. These phase estimates of different pulsed populations were averaged to determine the variance-weighted mean and compared with those from nonpulsed control seedlings to evaluate the magnitude and direction of the phase shifts. The pooled standard error was calculated using the following equation:

$$\text{pooled SE} = \sqrt{[([\text{vwSD}_p{}^2 \cdot (n_p - 1) + \text{vwSD}_n{}^2 \cdot (n_n - 1)]/ [n_p + n_n - 2]) \cdot (1/n_p + 1/n_n)]},$$

where n_p and n_n are the sample sizes and vwSD_p and vwSD_n are the variance-weighted standard deviations for pulsed and nonpulsed populations, respectively. For the *elf3-1* phase response curves (PRCs), some phase shifts are not plotted due to arrhythmia after the light pulse. In these cases, circadian rhythmicity was observed after the light pulse in only 0 to four of 21 to 27 *elf3-1* plants. Rhythmicity was scored for traces with FFT-NLLS phase estimates between 22 and 30 hr that were represented faithfully by their respective fits.

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