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ELF4 Is Required for Oscillatory Properties of the Circadian Clock1[W]

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Circadian clocks are required to coordinate metabolism and physiology with daily changes in the environment. Such clocks have several distinctive features, including a free-running rhythm of approximately 24 h and the ability to entrain to both light or temperature cycles (zeitgebers). We have previously characterized the early flowering4 (ELF4) locus of Arabidopsis (Arabidopsis thaliana) as being important for robust rhythms. Here, it is shown that ELF4 is necessary for at least two core clock functions: entrainment to an environmental cycle and rhythm sustainability under constant conditions. We show that elf4 demonstrates clock input defects in light responsiveness and in circadian gating. Rhythmicity in elf4 could be driven by an environmental cycle, but an increased sensitivity to light means the circadian system of elf4 plants does not entrain normally. Expression of putative core clock genes and outputs were characterized in various ELF4 backgrounds to establish the molecular network of action. ELF4 was found to be intimately associated with the circadian clock-associated1 (CCA1)/long elongated hypocotyl (LHY)-timing of cab expression1 (TOC1) feedback loop because, under free run, ELF4 is required to regulate the expression of CCA1 and TOC1 and, further, elf4 is locked in the evening phase of this feedback loop. ELF4, therefore, can be considered a component of the central CCA1/LHY-TOC1 feedback loop in the plant circadian clock.

Many organisms have evolved circadian clocks to facilitate optimal timing of rhythmic behaviors. Plants use an endogenous oscillator and predictable signals from the environment to anticipate changes in circadian time. Key outputs controlled by the clock include the timing of germination, optimization of photosynthetic processes relative to the time of day, and floral transition. Each of these has been shown to be crucial for plant fitness (Green et al., 2002; Dodd et al., 2005). In recent years, several molecular components associated with the plant clock have been identified. Most of these components are themselves circadian regulated, with peak expression of each phased to occur at a specific time of day. For example, the MYB-related transcription factors, CIRCADIAN CLOCK-ASSOCIATED1 (CCA1) and LONG ELONGATED HYOCOTYL (LHY); Schaffer et al., 1998; Wang and Tobin, 1998), are morning-specific genes, both acting in a feedback loop on the pseudoresponse regulator TIMING OF CAB EXPRESSION1 (TOC1), which peaks in the evening (Strayer et al., 2000; Alabadi et al., 2001; Mas et al., 2003). This transcription-translation feedback loop has been placed at the core of the Arabidopsis (Arabidopsis thaliana) clock (Alabadi et al., 2001). The original single-loop model was recently extended to incorporate additional loops (Farre et al., 2005; Locke et al., 2005, 2006; Salome and McClung, 2005a; Zeilinger et al., 2006). Beyond this core, the wider plant circadian system constitutes a complex network of multiple and interconnected pathways, many of which feed back on each other, controlling responses to light, temperature, and day length. These features are poorly understood.

Previously, we identified early flowering4 (elf4) from Arabidopsis and showed that ELF4 is important for circadian precision and normal clock function (Doyle et al., 2002). The elf4 loss-of-function mutation attenuated...
free-running rhythmicity in all clock outputs tested and this included components believed to make up the central clock machinery (Doyle et al., 2002; Kikis et al., 2005; this article). Circadian specificity of ELF4 within the clock was only partially defined with these studies.

Light signals perceived by photoreceptors, including phytochromes and cryptochromes (Lin, 2002; Nagy and Schafer, 2002; Quail, 2002), are the most important environmental inputs to the plant circadian clock (Ni, 2005). Photoperception allows entrainment of the clock to dawn and dusk cues, allowing correct phasing of the various clock-controlled genes and pathways (Salome and McClung, 2005b). Clock control of light-signaling pathways is critical for photoperiodic regulation of various clock-controlled genes and pathways (Salome and McClung, 2005). Photoperception allows entrainment of the clock to downstream red-light perception (Khanna et al., 2005). Accordingly, we tested elf4-1 mutants and ELF4 overexpression (ELF4-ox) lines for alterations in detecting light input signals and/or diurnal processing of information (ELF4-ox construction is described below). elf4-1 seedlings had a mild hypocotyl elongation phenotype under a range of fluences of red light because elf4-1 appeared hyposensitive to red-light repression of elongation growth (Fig. 1A). We thus confirm previous work by the Quail group (Khanna et al., 2003). Interestingly, ELF4-ox lines were indistinguishable from wild type under these assay conditions. Thus, if ELF4 is a component of proper red-light perception, then it is not a genetically limiting factor for the repression of hypocotyls by light.

A gating assay was conducted to test whether the red-light defects in elf4-1 were in part due to alterations in circadian processing of light information. For this, wild-type and elf4-1 plants harboring the CAB2: luciferase (LUC) marker were entrained to 12/12 LD cycles and replicate samples were placed into continuous darkness (DD). From subjective dark (zeitgeber time [ZT] = 12, noted here as at the start of the Fig. 1B graph; transfer time = 0) at 2-h intervals, a set of replicate samples was given a 5-min pulse of red light and the acute response of light activation of CAB2:LUC induction was assayed. As reported previously for white-light pulses (McWatters et al., 2000), we could confirm that wild-type Arabidopsis has a gated response of CAB2 induction (Fig. 1B). The response to light was at a maximum during the early part of the subjective day, around the time when the plants anticipated the transition of dark to light (subjective dawn; time 12 h in Fig. 1B), indicating the gate is open during the (subjective) day but closed during the (subjective) night. The elf4-1 mutant showed defects in its response to red-light treatments over the course of the entire experiment, but most especially during subjective night (times 0–12 h and 24–36 h in Fig. 1B). In elf4-1 mutants, the gate was open during subjective night (Fig. 1B) when elf4-1 displayed high activation of CAB2 in response to the light pulse. These plants have increased sensitivity to light at night relative to wild type and thus elf4 is a partial gating mutant. Red-light perception in the elf4-1 mutant is thus altered, at least in part, because of an underlying clock defect that affects the gating of this red-light response pathway.

RESULTS

Hypo- and Hypermorphic Red-Light Signaling in elf4 Plants

Under natural 24-h days, the LD rhythm defines the diurnal environment. However, signaling through light input pathways in plants is itself a clock-controlled process, being gated by so-called zeitnehmer functions, one of which requires ELF3 (McWatters et al., 2000). Previous reports on ELF4 characterization have supported ELF4 action in a phytochrome B-dependent pathway of red-light perception (Khanna et al., 2003). Accordingly, we tested elf4-1 mutants and ELF4 overexpression (ELF4-ox) lines for alterations in detecting light input signals and/or diurnal processing of information (ELF4-ox construction is described below). elf4-1 seedlings had a mild hypocotyl elongation phenotype under a range of fluences of red light because elf4-1 appeared hyposensitive to red-light repression of elongation growth (Fig. 1A). We thus confirm previous work by the Quail group (Khanna et al., 2003). Interestingly, ELF4-ox lines were indistinguishable from wild type under these assay conditions. Thus, if ELF4 is a component of proper red-light perception, then it is not a genetically limiting factor for the repression of hypocotyls by light.

elf4-1 Mutants Arrest Their Clock in the Evening

It was noted that, after transfer to constant conditions following exposure to LD cycles, elf4-1 mutant plants displayed weak rhythmicity on the first day (Doyle et al., 2002). This could mean that the oscillator was, upon transfer to constant conditions, running
down rather than stopping instantly. To understand the kinetics of the elf4 oscillator, we undertook an assay of oscillator behavior following the transfer from entraining conditions to DD. Seedlings harboring the CAB2:LUC reporter were entrained to 8/16 LD cycles and then transferred to DD at dusk (ZT8). At 3-h intervals from 1 h after the light-to-dark transition, a 5-min red-light pulse was given to replicate plates of seedlings and luminescence was measured over the next 48 h. This light pulse is not sufficient to reset the clock in wild type (Millar et al., 1992; McWatters et al., 2000; Covington et al., 2001; Hall et al., 2002), but it does induce a circadian peak of CAB2 activity, the timing of which is under circadian control in wild-type plants (Millar and Kay, 1996).

Until 32 h after the last dawn (i.e. subjective dusk for these plants previously entrained to 8/16 LD), the timing of the peak in elf4 seedlings was indistinguishable from that of wild-type plants (Fig. 2A). However, the two sets of seedlings responded differently to pulses given at or 36 h after the last dawn (t test; P < 0.05): Wild-type seedlings continued to show circadian control, but the peak of CAB2:LUC in elf4-1 occurred about 30 h after the pulse, regardless of when the pulse was given (Fig. 2A). Thus, the circadian clock in elf4 runs down at the end of the first subjective day in DD to a point where it is strongly reset by even a brief light pulse. We interpret this as although rhythmicity can be driven by a light zeitgeber in elf4, ELF4 is needed to sustain clock activity beyond the end of the first subjective day in DD.

Characterization of ELF4-ox Plants

We previously concluded based on loss-of-function studies that ELF4 is both a repressor of the floral transition and required to sustain normal clock function (Doyle et al., 2002). Because ELF4 expression is normally rhythmic, plants overexpressing ELF4 (ELF4-ox) under the control of the constitutive cauliflower mosaic virus 35S promoter (Supplemental Fig. S1A) were tested to see whether rhythmicity of transcription was required for ELF4 function. We confirmed that elf4-1 was partially insensitive to photoperiod (early flowering in long days [t test; P < 0.01] and short days [t test; P < 0.001]). In contrast, ELF4-ox lines were only late flowering under inductive (long-day) photoperiods (t test; P < 0.001). Under the noninductive conditions of short days, ELF4-ox plants showed no additional delay in flowering (t test; P = 0.21; Fig. 3, A and B). This finding confirms that ELF4 is a floral repressor that works to coordinate the floral transition as part of the photoperiod pathway.

Because elf4-1 is a severe clock mutant under light or in darkness, it was reasoned that ELF4-ox lines should also show circadian alterations. Three independent transgenic lines were tested for alterations in circadian leaf movement rhythms. All lines showed an increased free-running period under LL (Table I; Supplemental Fig. S1). These results were confirmed for molecular rhythms of ELF4-ox plants harboring the morning CAB2:LUC and the evening CCR2:LUC reporters (Table I; Fig. 3, C–F). These lines also had rhythms with longer periods under LL after entrainment to LD cycles (Fig. 3, C and D). In darkness, ELF4-ox peaked later than wild type most significantly for the evening marker CCR2:LUC (Fig. 3, E and F). Thus, ELF4 modulates rhythmicity of multiple clock outputs. Here, we define based on these misexpression studies that ELF4 is a strong genetic repressor of clock periodicity.

Entrainment to LD Cycles Is Altered in elf4-1 Mutants

The gating assay (Fig. 1B) showed us that elf4-1 plants display greater sensitivity to light than wild type. CCA1 and CAB2 are both under clock control and normally rise during the late night with peak at or shortly after dawn, respectively. They are also regulated directly by light. CCR2 expression is also clock controlled, but is less directly affected by light (Suarez-Lopez et al., 2001; Kim et al., 2003), unlike CAB2 or CCA1. We measured CCA1, CAB2, and CCR2 expression

![Figure 1](image-url)
via LUC reporter activity in long- and short-day LD cycles to compare the effects of clock and light control on these genes.

In elf4-1 under long or short days, there was a strong reduction in the rising of gene expression during darkness and, instead, there was an abrupt increase in CCA1:LUC and CAB2:LUC expression immediately following lights on (Fig. 4), again implying an increase in light sensitivity in these plants relative to wild type. This suggests that the ability of the elf4-1 mutant to anticipate dawn was attenuated, extending the possibility that entrainment of the oscillator is altered in elf4-1. In contrast, ELF4-ox correctly anticipated the coming lights on before photic signals were present (Fig. 4). We interpret this as a strong suggestion that, whereas ELF4 is essential for normal entrainment to light, rhythmic accumulation of ELF4 transcript is not.

The transcription of CCR2 cycles, with a trough in the day and a peak in the night, is similar to the phase angle of ELF4 (Fig. 6A). Under short days, only a marginal rhythm is seen for CCR2:LUC in elf4-1; however, a weak rhythm that apparently is able to anticipate dusk is seen in long photoperiod conditions (Fig. 4, E and F), suggesting that the slave oscillator of CCR2 (Heintzen et al., 1997) still runs under these conditions even in the elf4-1 mutant. Again, the same phase of the CCR2 peak was seen in ELF4-ox plants compared to wild type (Fig. 4, E and F), reinforcing our earlier proposition that, whereas ELF4 is necessary for correct entrainment of plants, rhythmic ELF4 expression is not.

To further refine our understanding of clock resetting and ELF4’s role in this entrainment process, we measured the time taken by wild type, elf4-1, and ELF4-ox seedlings harboring CCR2:LUC to reentrain to a 12/12 LD cycle following the inversion of day and night (equivalent to jumping across 12 time zones instantaneously). The rapid change in light regime induces jet lag because the circadian clock is no longer in its correct orientation with respect to the environmental cycle. This protocol is similar to that used to define entrainment defects in cca1 and lhy mutants (Kim et al., 2003; Fig. 2). Under this regime, the timing of peak CCR2:LUC activity, relative to the lights-out signal, was nearly restored in elf4-1 within the first day (Fig. 2B). In contrast, the wild-type line did not display near-normal timing of the peak in CCR2 expression until the second day. Thus, elf4 resets faster than the wild type.

To understand the preliminary events that led to rapid clock resetting in elf4-1 relative to wild type, we repeated the assay with the three genotypes expressing LUC under the control of CCA1 or LHY promoters, respectively. This showed that the morning peak of CCA1 and LHY in wild type occurred when plants expected dawn (e.g. after time 48 for CCA1 [Fig. 2C] or time 72 for LHY [Fig. 2D]), although this point was now in darkness because of the extended night. Wild-type plants exhibited little response to the lights on that occurred at subjective dusk (listed as time 60 for CCA1 [Fig. 2C] and time 84 for LHY [Fig. 2D]), relative to the original entraining cycle (Fig. 2, C and D). These

Figure 2. The elf4 clock runs for 1 d and stops at subjective dusk (approximately time 32 h). A, Time to peak of CAB2:LUC activity in dark-adapted elf4-1 seedlings after red-light pulse treatment. Seedlings were entrained in 8/16 LD cycles and then transferred to darkness at dusk (ZT8). Time of pulse is shown as hours since last dawn; 5 min of red light were given at 3-h intervals from time 9. Error bars represent SEM. The experiment was repeated twice. B to D, Normalized CCR2:LUC, CCA1:LUC, and CCR2:LUC profiles of elf4-1 and ELF4-ox seedlings, compared to the wild type, before and after exposure to jet lag (an extended night of 24 h long) under LD cycles. White bars indicate light intervals and gray bars indicate darkness.
results can be explained by gated repression of light activation of these genes during the subjective night, similar to that shown for CAB2 in wild-type seedlings (Fig. 1B). In contrast, the peak of LUC activity in elf4-1 was much reduced after time 48, but the relative increase in gene induction in response to lights on at time 60 was much greater. This is consistent with the defective gating found in this mutant in which the gate for light responsiveness is open during subjective night. The light induction of CCA1 and LHY in elf4-1 is the likely cause of its rapid clock resetting.

ELF4-ox plants also exhibited accelerated clock resetting of CCR2:LUC relative to wild type. However, expression of CCA1:LUC and LHY:LUC in ELF4-ox matched that of wild type between time 36 and 72 for CCA1 (Fig. 2C) and time 48 and 96 for LHY (Fig. 2D), implying that the resetting behavior here is not likely to be due to changes in the gating of light responsiveness. Instead, we suggest it may be due to the longer endogenous period allowing easier resetting via a single-phase delay.

**Temperature Entrainment Defect in elf4**

Temperature cycles can rescue rhythmicity of the elf3 mutant, which acts to gate light input, in a subsequent interval of constant temperature (McWatters et al., 2000). elf4-1 was therefore tested against wild-type plants for rescue of rhythmicity following exposure to warm-cold cycles. As before, entrainment to LD cycles failed to rescue subsequent free-running rhythmicity for all markers tested (CAB2, CCA1, or CCR2); all these reporters were arrhythmic in elf4-1 populations grown under LL (Fig. 5, A, C, and E). In the absence of photoperiods, rhythmicity in elf4 for all three reporters could be driven in warm-cold cycles (Fig. 5, B, D, and F). Thus, the elf4 mutant can perceive ambient temperature cues. However, once the temperature cycle was discontinued, free-running rhythmicity was extremely weak beyond the first day in constant temperature for CAB2, CCA1, or CCR2 in elf4-1 (Fig. 5, B, D, and F). As expected, the control wild-type plants were robustly rhythmic under these conditions.

**Timing of ELF4 Action**

ELF4 is required for robust rhythmicity and for a normal response to LD cycles. To aid the understanding of ELF4’s role in the circadian signaling network,
molecular expression phenotypes of core clock genes were measured in various ELF4 genotypic backgrounds. Luminescence rhythms were measured in wild-type plants expressing ELF4:LUC under LL after entrainment under LD cycles. Compared to the evening marker CCR2, ELF4:LUC generated a rhythm with peak expression in the middle of the night (Fig. 6A). We compare this to our analysis on the ELF4 transcript under 12/12 LD photoperiods. There, we found peak expression at dusk (ZT12; Fig. 6G); we have previously shown that ELF4 transcript levels are clock controlled and peak in the evening and that ELF4 expression is affected by photoperiod (Doyle et al., 2002). Taken together, all of these results support an evening-to-night function of ELF4 action and illustrate that the precise timing of the ELF4 peak is influenced by the presence and/or duration of a photoperiod.

As expected, ELF4:LUC activity in elf4 was arrhythmic (Fig. 6B), as was that of CCR2:LUC expression, in agreement with our previous reports. Rhythmicity in the elf4-1 mutant could be rescued by restoring ELF4 expression with the ELF4:ELF4LUC construct (Fig. 6B). Like plants constitutively overexpressing ELF4, these plants had a long-period phenotype. Thus, ELF4 regulation appears to be primarily transcriptional and ELF4 activity is potentially dose dependent even under the control of its own promoter.

The elf4 mutant phenotype includes low transcription of the morning clock gene CCA1 (we confirmed our previous LUC data regarding CCA1 expression [Doyle et al., 2002] by direct analysis of its RNA [Supplemental Fig. S2A]). In contrast, CCA1:LUC rhythms were increased in amplitude and had a long period in plants that constitutively overexpressed ELF4 (Table I; Fig. 6C; Supplemental Fig. S3A). It is thus likely that ELF4 is a limiting factor in CCA1 induction. Also, in the elf4-1 mutant, LHY:LUC was repressed to a very low level and was arrhythmic (Fig. 6D), as was LHY transcript expression (Supplemental Fig. S2B), similar to the findings for CCA1 and LHY expression reported previously (Doyle et al., 2002; Kikis et al., 2005). Again, ELF4-ox plants displayed long-period LHY:LUC rhythms (Table I; Fig. 6D; Supplemental Fig. S3B). Thus, ELF4 is likely to control activation of both morning acting clock genes, CCA1 and LHY.

The current model of the CCA1/LHY-TOC1 loop (Alabadi et al., 2001; Locke et al., 2005) predicts an increase in TOC1 expression wherever there is given low CCA1 and LHY expression. Expression of TOC1: LUC in the null elf4-1 allele followed this prediction, being expressed arrhythmically and at a higher level in elf4-1 than wild-type seedlings free-running under LL conditions (Fig. 6E; Supplemental Fig. S3C). This finding was confirmed by examining TOC1 transcript expression in elf4-1 directly by real-time reverse transcription-PCR (Supplemental Fig. S2C); TOC1 expression was high and became arrhythmic within 24 h of the transfer to LL. In addition, we found that ELF4 transcription in the toc1 mutant was rhythmic, with an early phased peak (Fig. 6G), similar to the phase of ELF4 expression in the cca1 lhy double mutant (Kikis et al., 2005). Rhythmicity of TOC1:LUC was maintained in lines overexpressing ELF4, but there was reduced amplitude; we observed wild-type levels of TOC1 transcript in ELF4-ox (data not shown). As anticipated, TOC1:LUC rhythms displayed a long-period response in ELF4-ox (Table I; Fig. 6F; Supplemental Fig. S3D). Taken together, these last results suggest strongly that ELF4 is necessary for the feedback loop controlling rhythmicity of CCA1, LHY, and TOC1, where it acts at night to promote CCA1/LHY expression and thus indirectly repress TOC1.

**DISCUSSION**

Our data illustrate that elf4-1 plants have a range of deficiencies in their circadian responses to light, photoperiod, and temperature. Importantly, such plants do not display sustained rhythmicity in the absence of environmental signals. Misexpression studies of ELF4 further confirm an important clock function for this gene. Analysis of gene expression of key components of the plant clock (CCA1, LHY, and TOC1) and targeted assays to define the abrogated rhythm in elf4-1 revealed that the central circadian feedback loop in elf4 was locked into the evening phase. However, constitutive overexpression of ELF4 does not produce arrhythmia, but acts to delay the clock, causing a long-period phenotype seen across a range of assays. Plants overexpressing ELF4 exhibited robust rhythms of clock gene expression and these lines were able to respond to photoperiods, for example, flowering earlier in long days than short days. These results showed that ELF4 is essential for free-running circadian rhythms.

Here, we have presented evidence that expression of the various clock outputs is strongly affected by the LD zeitgeber in the elf4-1 mutant (Figs. 2 and 4). elf4-1
plants were found to show more rapid reentrainment following a change in the zeitgeber phase (Fig. 2, B–D), which indicates that the clock is reset more rapidly in these mutants than in the wild type. This is probably due to ELF4’s role of gating light input to the clock. The gate in elf4-1 plants never fully closes (Fig. 1B); hence, these plants are more sensitive to photic cues due to increased activity of the light-signaling pathway. Increased light sensitivity is also seen in the pattern of CAB2:LUC and CCA1:LUC expression in elf4 mutants (Fig. 4). In the absence of a zeitgeber, elf4-1 does not show robust free-running rhythms in any of the various hands of the clock (Figs. 5 and 6). Regardless of how the clock is assayed, the elf4-1 mutant is weakly rhythmic or arrhythmic under LL and DD, regardless of the previous entrainment protocol. We thus conclude that ELF4 is required for entrainment.

Most importantly, the putative clock components CCA1, LHY, and TOC1 are virtually arrhythmic after the first 24 h in LL in an elf4-1 background, implying that this feedback loop cannot continue to cycle in the absence of ELF4. We have shown that CCA1 and LHY levels are both low in the elf4-1 mutant, whereas TOC1 is high—strong circumstantial evidence that ELF4 acts to promote the former while repressing the latter (Fig. 6; Supplemental Fig. S2). This evidence leads to the conclusion that ELF4 is essential for correct clock function in Arabidopsis and that, in the absence of ELF4, the clock will stop after a single cycle. ELF4 transcription is rhythmic, with a peak during the early night, coinciding with the point at which the clock arrests in elf4-1, implying that ELF4 acts at this point of the 24-h cycle (Fig. 2A). A recent study by Quail and colleagues reported that TOC1 expression was unchanged in another elf4 mutant allele (elf4-101, a T-DNA insertion in the Columbia background; Kikis et al., 2005). A difference between elf4 alleles or genetic backgrounds might account for the discrepancy between their study and ours. However, a more plausible explanation is that experimental protocols differed widely. Our results show that the elf4-1 mutant has residual rhythmicity for 1 d following entrainment. In the earlier study, dark-grown seedlings were assayed for TOC1 levels immediately after 24 h under constant red light (Kikis et al., 2005).

Figure 4. Morning gene expression (CCA1:LUC, CAB2:LUC) is less affected than expression of an evening-specific gene (CCR2:LUC). Luminescence profiles of elf4-1 and ELF4-ox kept under entraining conditions, 8/16 LD short day (left) and 16/8 LD long day (right). A and B, CCA1:LUC. C and D, CAB2:LUC. E and F, CCR2:LUC. Gray blocks indicate night time. Error bars represent sem.
a short period upon the output rhythms (Somers et al., 1998; Alabadi et al., 2002). ELF4 transcription remains rhythmic in both the **toc1** mutant (Fig. 6G) and the **cca1 lhy** double mutant (Kikis et al., 2005); in each case, **ELF4** expression has an early phase. Thus, the relationship between **ELF4** and other clock genes appears asymmetric: **ELF4** is required for rhythmicity of other clock-associated genes, but they are not required for **ELF4** rhythms to exist, although they do drive the correct phase of **ELF4**.

We have shown that, although the **CCA1/LHY-TOC1** feedback loop is stalled in the evening phase in **elf4-1**, the clock has full oscillatory function in **ELF4**-ox, which shows robust rhythmicity of gene expression (Table I; Figs. 5 and 6), and this line is able to distinguish between long and short days for the purpose of controlling flowering time. However, the long-period phenotype and later flowering under long days of **ELF4**-ox plants highlights the notion that the level of **ELF4** expression calibrates circadian period. We have previously observed that **ELF4** levels are extremely low and lose rhythmicity in wild-type plants grown in extended darkness (Doyle et al., 2002), yet the **CCA1/LHY-TOC1** feedback loop continues in wild-type plants under these conditions. These two observations lead us to suggest that, although transcription of **ELF4** is normally rhythmic (due to exposure to natural LD and temperature cycles), and the presence of **ELF4** is sufficient to drive this loop, rhythmic **ELF4** transcription is not necessary for the clock to sustain oscillatory function.

A previous report on an **elf4** mutant allele that demonstrated arrhythmicity of the **CCA1/LHY-TOC1** feedback loop in dark-grown seedlings indicated that **ELF4** was required for light activation of this loop (Kikis et al., 2005). We show here that **ELF4** is required to sustain this loop under LL (Figs. 1, A and B, and 6). Taken together, these observations indicate that **ELF4** is necessary to start the clock, sustain it under constant conditions, and enable it to entrain to a zeitgeber. These conclusions considerably extend the earlier model that placed **ELF4** in a light input loop with **CCA1** and **LHY** (Kikis et al., 2005).

We suggest that **ELF4** functions to convert an hourglass into a clock. Without **ELF4**, the **CCA1/LHY-TOC1** feedback loop can be turned over by an environmental **Figure 5.** Temperature entrainment defects in **elf4-1**. A, C, and E, Seedlings were entrained to LD cycles and then transferred to LL. B, D, and F, One set of LD-entrained plants was subsequently given temperature cycles for 3 d (12:12 warm/cool [WC]) and then released into LL and constant temperature for 3 d. A and B, **CAB2:**LUC. C and D, **CCA1:**LUC. E and F, **CCR2:**LUC. White bars indicate free run under LL. A, C, and E, Gray bars indicate subjective night. B, D, and F, Red blocks indicate 24°C (daytime). Blue blocks indicate 18°C (nighttime). Red hatched bars indicate subjective warm day. Blue hatched bars indicate subjective cold night. Time is light ZT. Insets, RAE plots (RAE versus period). Each period estimate is an RAE-weighted mean of a group of seedlings. Hours 12 to 84 and 84 to 152 analyzed for free run under LL after LD and WC entrainment, respectively. Error bars represent SEM.
cycle of light and dark, but stops depending on the discontinuation of the environmental rhythm. The closest functional analog to ELF4 may be the FREQUENCY (FRQ) locus of Neurospora crassa. In the absence of FRQ, N. crassa rhythms are of low amplitude, variable length, and not temperature compensated (Merrow et al., 1999, 2006). Previous reports, our own included, have placed ELF4 as part of a light input pathway to the clock. Current data reported allow us to revise this interpretation and state that, because ELF4 is essential for at least two critical clock properties, sustainability and entrainment, it should be considered a core clock component. Assignment of function to FRQ remains a controversial issue (Merrow et al., 1999; Pregueiro et al., 2005; Ruoff et al., 2005; de Paula et al., 2006; Lakin-Thomas, 2006; Schafmeier et al., 2006); whether it becomes so with ELF4 remains to be seen.

MATERIALS AND METHODS

Plant Material and Transgenics

Arabidopsis (Arabidopsis thaliana) ecotype Wassilewskija (Ws-2), elf4-1, and the LUC lines CAB2:LUC1 (6B insertion), CCA1:LUC, and CCR2:LUC are described (McWatters et al., 2000; Doyle et al., 2002). The toc1-1 mutant is in the C24 background and has been described previously (Somers et al., 1998). For the vector to generate lines overexpressing ELF4, first the 35S promoter and Nos terminator were subcloned from pBI121 into pZIP221B as a

Figure 6. ELF4 is expressed in the night and influences the expression level of CCA1 and TOC1. A, ELF4:LUC luminescence activity compared to CCR2:LUC in wild type. B, Luminescence of ELF4:ELF4 LUC and ELF4:LUC in the elf4-1 mutant. C, Long period and high amplitude of CCA1:LUC in ELF4-ox under LL. D, Long period of LHY:LUC in ELF4-ox. Inset, LHY:LUC expression in elf4-1 mutant; note that in elf4-1 the LUC levels are arrhythmic and more than 10-fold lower than the wild type. E, TOC1:LUC expression is high and arrhythmic in elf4-1. F, ELF4-ox displays low TOC1:LUC expression, which is robustly rhythmic. Gray bars indicate subjective day. Time is ZT. Error bars represent SEM. All LUC seedlings were entrained in 16/8 LD cycles. G, ELF4 expression is rhythmic in the toc1-1 mutant. Seedlings were entrained in 12/12 LD cycles. ELF4 level is normalized to β-TUBULIN4 at each time point. Mean values are plotted; error bars represent s.e. The experiment was replicated twice.
HinfIII-EcoRI fragment. The ELF4 coding region was amplified using genomic DNA by PCR with the primers ELF4SS5-L, 5'-AAAAAGACTCC-GGTCCAACTAAAAGAACAT-3' and ELF4SSR-R, 5'-AAAAAGATCTC-GAGCTTGAAGAATAACAAAAG-3'; this fragment was subcloned into the 35S promoter and the terminator as a BamHI fragment. This construct was used to generate multiple homozygous lines that overexpressed ELF4 (ELF4-ex). All tested lines behaved similarly in all assays. Wild-type Ws lines harboring the CAR2:LUC, CCA1:LUC, or CCR2:LUC transgene were crossed into both the elf4-1 mutant and the ELF4-ex line termed ELF4-ox;11; in each case, double homozygous lines were identified in the F2 generation and bulked. We report experiments using the F2 generation of these lines. Gating experiments utilizing the CAB2 clone was a bacterial artificial chromosome clone T28M21 as a translational start site. The C24/11 fragment into pZIPS21B. This fragment is between 1.7 kb and 260 bp upstream of the transcriptional start site. The LUC+ gene from pLUC+ (Promega) was subcloned into this resultant vector as a Nor1-Xhol fragment (pELF4 incomplete: LUC+). A fragment was PCR amplified using an arbitrary upstream primer and the primer 5'-ACCTGCGTCTGGCGTTCTCTATTCA-3'. This PCR product was digested with NorI and the fragment intersected into the Nor1 site of pELF4 incomplete: LUC+, resulting in the completed transcriptional fusion pELF4:LUC+. To generate a vector for TOC1:LUC, PCR against Ws genomic DNA using 5'-TGGCGTCAGACTTCTCTAGACTTCTTCTGAGGAATTTCATCAAAC-3' and 5'-ACT-GATCGATCATGATGAAATAACATCTTGGT-3' was digested with EcoRI and subcloned into a Luciferase vector to generate a PCR that was subcloned as an Nor1 fragment into pELF4 incomplete: LUC+ to generate the transcriptional fusion construct pELF4:LUC+. For experimentation, these constructs were used to transform wild-type Ws or elf4-1, as described in the text, by the floral-dip method (Clough and Bent, 1998). Experiments comparing TOC1:LUC expression in the wild type and elf4-1 represent the averages of 24 lines each from six independent transgenics; all lines behaved similarly. Representative transgenic lines of TOC1:LUC and LHY:LUC were used in crosses to ELF4ox;1; the same LHY:LUC line was similarly introduced to elf4-1 for experimentation.

Growth Conditions

For hypocotyl length measurements, seeds were surface sterilized and plated on 2.2 g/L Murashige and Skoog medium without Suc or vitamins (0.5× Murashige and Skoog) with 2.5 mM 2-(N-morpholino)ethanesulfonic acid (pH 5.7) and 8 g/L agar. Plates were stored in the dark for 3 to 4 d at 4°C, placed in the light in the darkness for 1 d, and irradiated with light for 6 d, as described (Davis et al., 2001). The light sources were as described in Hall et al. (2003). For circadian and real-time PCR experiments, seeds were similarly treated, except that they were plated on 1× Murashige and Skoog medium (4.4 g/L) with 3% Suc and 1% agar before being stratified at 4°C for 48 h. The seeds were then transferred to growth chambers programmed for appropriate light and temperature regimes for 7 d before the start of an experiment. Unless otherwise stated in the text, the fluence rate of white light was 65 μmol m⁻² s⁻¹ and plants were grown at a constant temperature of 22°C.

Rhythm Data Analysis

Luminescence levels were quantified on either a low-light imaging system or an adapted microtiter plate-reading scintillation counter and analyzed essentially as described (McWatters et al., 2000; Thain et al., 2000), using the software package MetaMorph (Universal Imaging Corp.), and the macro suites I&À, TopTemPl, and Biological Rhythms Analysis Software System (Southern and Millar, 2005; available at http://www.amillar.org) and fast Fourier transform-nonlinear least squares (Plautz et al., 1997). Sustainability (precision) of rhythms was derived from measurements of the relative amplitude of error (RAE) as a method that has previously been reported (Alabadi et al., 2006). Where appropriate, data were normalized. Here, normalization was plotted as the quotient of the absolute data point over the mean of the entire dataset, as a method in identical fashion to Hall et al. (2003). This allows the data to be qualitatively compared for each genotype while plotting on the same axes and preserving the waveforms.

Real-Time PCR

Replicated samples of elf4-1, toc1-1, and the wild-type controls Ws-2 and C24 seedlings were collected and immediately frozen in liquid nitrogen, starting at dawn on day 8. toc1-1 and C24 seedlings were collected during a 12/12 LD cycle; elf4-1 and Ws-2 seedlings under LL following the discontinuation of such a cycle. RNA was extracted (Qiagen RNAeasy kit) using an additional DNAse treatment step (Qiagen) as per the manufacturer’s instructions. cDNA was synthesized (ABI TaqMan) and real-time PCR carried out in triplicate in an ABI Prism 3700 using SYBR Green master mix (ABI) and gene-specific primers (ELF4-F, 5'-CGCAGATCACTGAGACAAATG-3'; ELF4-R, 5'-AATGTCTGGAGTCCTGAGATC-3'; TOC1-F, 5'-ATCCTCCGACAA-TCCCTGTGATA-3' TOC1-R, 5'-GCACTCTGCTCACAGGAG-3'; CCA1-F, 5'-TCTGTGTCAGGAGGTCGCAAAT-3'; CCA1-R, 3TGTGTCAGGAGGTCGCAAAT-3'; CCA1-R, ACTTGGCCCGAATTCTGCGG-3'); Levels of circadian gene and the control gene ß-TUBULIN4 in each sample were calculated using the standard curve method (Applied Biosystems, User Bulletin no. 2, 2001 update). Circadian gene expression was then normalized using contemporaneous ß-TUBULIN4 expression from the same sample.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. A, Expression of ELF4 RNA is increased in ELF4-ex plants. B, Representative leaf movement traces of ELF4-ex and Ws images under normal LL.

Supplemental Figure S2. RNA expression of putative central clock genes in elf4-1 and Ws.

Supplemental Figure S3. Period estimates of CCA1:LUC (A) and LHY: LUC (B) in ELF4-ex. Period analysis of TOC1:LUC in elf4-1 (C) and ELF4-ex (D).

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LITERATURE CITED


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ELF4 in the Clock