Conditional Circadian Dysfunction of the *Arabidopsis early-flowering 3* Mutant

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Photoperiodic responses, such as the daylength-dependent control of reproductive development, are associated with a circadian biological clock. The photoperiod-insensitive *early-flowering 3* (*elf3*) mutant of *Arabidopsis thaliana* lacks rhythmicity in two distinct circadian-regulated processes. This defect was apparent only when plants were assayed under constant light conditions. *elf3* mutants retain rhythmicity in constant dark and anticipate light/dark transitions under most light/dark regimes. The conditional arrhythmic phenotype suggests that the circadian pacemaker is intact in darkness in *elf3* mutant plants, but the transduction of light signals to the circadian clock is impaired.

Many plant and animal species exhibit striking developmental adaptations in response to changing daylengths (*1*). This process, photoperiodism, indicates the ability of an organism to sense changes in the duration of days and nights throughout the year. Photoperiodic responses are mediated by the cycling of light and dark periods and have long been thought to be tied to a biological clock. Circadian rhythms, with a periodicity close to 24 hours, continue in the absence of light/dark cycles and can be entrained by environmental stimuli such as light or temperature. A role for circadian rhythms in photoperiodism in plants has been indicated by the cyclic sensitivity of many plant species, including *Arabidopsis thaliana*, to light treatments that markedly alter flowering time (*1*).

Arabidopsis is a facultative long-day plant, flowering earlier under long-day growth conditions than under short-day growth conditions. Of the *Arabidopsis* mutants that display altered flowering time, a few also show a lack of sensitivity to daylength (*2*). The *early-flowering 3* (*elf3*) mutant of *Arabidopsis* flowers earlier than the wild type under a variety of growth conditions and is photoperiod-insensitive with respect to floral initiation (*3*). *elf3* mutants also show the long-hypocotyl phenotype characteristic of plants defective in light reception or the transduction of light signals (*3*). If a biological clock measures the length of light/dark cycles, then daylength-insensitive mutants such as *elf3* may show a defect in the circadian regulation of various plant processes.

Leaf movement rhythms were assayed in plants homozygous for each of four *elf3* mutant alleles isolated in two *Arabidopsis* ecotypes (*3*). Plant leaves normally move in a circadian manner, opening during the day and closing at night (*4*). Leaf movements persist in wild-type *Arabidopsis* seedlings grown in constant light but were undetectable in *elf3* mutants (Fig. 1). Lack of rhythmic leaf movements was observed for all *elf3* alleles tested, whereas both parental ecotypes displayed rhythmic behavior (Table 1) (*5*).

In *Arabidopsis*, as in many plants, transcription of genes encoding the *chlorophyll a/b* binding protein (*cab*) follows a circadian pattern (*6*). This circadian response can be detected in vivo in plants containing a transgenic reporter construct, *cab2-luc*, in which the firefly luciferase (*luc*) gene is under the control of the *cab2* promoter (*6*). The *cab2-luc* transgene was introduced into the *elf3-1* mutant background by genetic crosses (*7*).

As observed for leaf movement rhythms, no circadian rhythm in *cab2-luc* transcription was apparent in *elf3-1* mutant seedlings (Fig. 1). Three independent *elf3-1* F_3 families representing 324 plants were analyzed, and only 11% of the mutant seedlings displayed circadian rhythmicity under constant light conditions (*5*). Of a population of 90 wild-type plants, 100% showed circadian rhythmicity in constant light conditions, with a mean period of 24.5 ± 0.7 hours (*5*). On average, the rhythms in wild-type plants were three- to fourfold stronger than detectable rhythms in *elf3-1* seedlings.

Analysis of hypocotyl elongation suggests that *elf3* mutant plants are defective in both blue- and red-light–dependent inhibition of hypocotyl growth, with some greater defect in response to blue light (*3*). We found that *elf3* mutant seedlings were also defective in responding to either blue- or red-light signals for maintaining circadian regulation of *cab2-luc* expression. Of 126 *elf3-1* mutant seedlings assayed in constant red light, only 6% had detectable circadian rhythmicity, whereas of 131 *elf3-1* mutant seedlings assayed in constant blue light, 23% displayed a circadian rhythm. Of 17 wild-type plants assayed in constant red light, 100% showed circadian rhythmicity, with a mean period of 24.7 ± 0.4 hours, and of 26 wild-type plants assayed in constant blue light, 100% showed circadian rhythmicity, with a mean period of 24.8 ± 0.6 hours (*5*, *8*). Thus, both blue- and red-light signals can cause *elf3* mutants to exhibit arrhythmic *cab2-luc* transcription, although blue light is clearly less effective than red light in causing arrythmicity.

Although arrhythmic mutant alleles identified in other systems (*period* and *timeless* in *Drosophila* and *frequency* in *Neurospora*) result in a lack of circadian rhythms in constant conditions, these mutants can show driven rhythms in light/dark cycles (*9*). However, unlike wild type, arrhythmic mutants show no anticipation of light-to-dark or dark-to-light transitions. Wild-type and *elf3-1* seedlings were assayed for *cab2-*

Table 1. Rhythmicity of leaf movement in wild-type and *elf3* seedlings. Data from at least two similar experiments are presented as the percent of seedlings in each population that display rhythmic leaf movements, followed by percent of total, and mean period ± SD for circadian rhythmic leaf movements (*5*, *14*).

Genotype	Percent of total with rhythmicity (no. tested)	Percent of total with circadian rhythmicity	Mean period ± SD (no. tested)
Columbia	58 (53)	57	24.8 ± 1.2 (30)
elf3-1	5 (44)	5	25.3 ± 0.2 (2)
elf3-2	2 (48)	2	26.6 (1)
Wassilewskija	76 (34)	74	25.5 ± 0.9 (25)
elf3-3	2 (42)	0	–
elf3-4	14 (42)	7	24.5 ± 1.2 (3)

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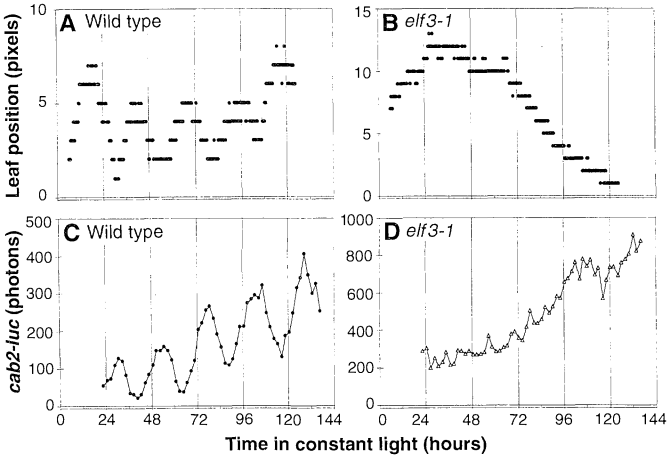

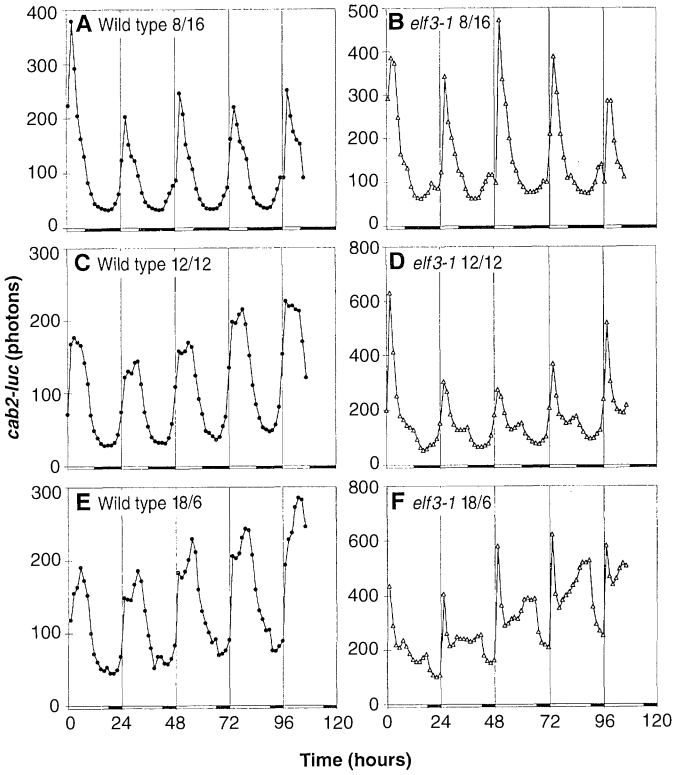

Fig. 1 (left). Circadian responses of Columbia wild type (**A** and **C**) and *elf3-1* mutant (**B** and **D**) in constant light conditions. (A and B) Seedlings were grown for 5 to 7 days before entrainment in 12L/12D for at least 2 days. Leaf position was recorded in constant light in units of screen pixels (*5, 8*). Typical traces from one of three similar experiments are shown for single cotyledons. (C and D) Seedlings were germinated on selective media and grown for 5 days in 12L/12D before transfer to constant light (*6*). Luminescence traces of *cab2-luc* expression are shown for single seedlings from one of three similar experiments. **Fig. 2 (right).** Regulation of *cab2-luc* in wild type (**A**, **C**, and **E**) and *elf3-1* mutant seedlings (**B**, **D**, and **F**) under varying light/dark regimes, indicated with open (light) and filled (dark) boxes on the *x* axis. Seedlings were grown for 5 days in the light/dark cycle indicated before *cab2-luc* expression was recorded in the same conditions (*6*). Arithmetic means were calculated from a population of single-seedling records. Similar results were observed for three independent homozygous *elf3-1* F_3 families. (A) and (B), 8L/16D; (C) and (D), 12L/12D; (E) and (F), 18L/6D.

luc transcription in 12-hour light:12-hour dark (12L/12D) cycles. Rhythmic *cab2-luc* transcription was observed for both wild type and *elf3-1*, although *elf3* seedlings showed an increased response to light at dawn (Fig. 2). Expression of *cab2-luc* in both wild type and *elf3-1* began to rise before the transition from dark to light and began to decrease before the transition from light to dark. Analogous results were observed for seedlings grown in 12L/12D cycles of red or blue light (*10*), suggesting that either wavelength of light is sufficient to drive diurnal *cab2-luc* rhythms in *elf3-1* mutant seedlings. The rise in *cab2-luc* expression in *elf3-1* mutants before subjective dawn in light/dark cycles suggests that *elf3-1* mutant plants retain some functions necessary for the anticipation of light, possibly as a result of a circadian clock.

If circadian responses are intimately connected to the photoperiodic control of flowering, then the wild-type–driven rhythm and wave form of *cab2-luc* expression under different photoperiods may be altered in *elf3* mutants. Driven rhythms of *cab2-luc* in wild-type seedlings grown and analyzed under three different light/dark cycles all maintained a 24-hour period (Fig. 2). In all three photoperiods, changes in expression of *cab2-luc* anticipated both dark-to-light and light-to-dark transitions, although more prominent responses to the light-to-dark and dark-to-light transitions were observed in *elf3-1* mutants than in wild type. The timing of the decrease in *cab2-luc* expression varied with daylength, such that a later decrease in *cab2-luc* expression was observed in longer daylengths. In *elf3-1* mutant seedlings, the wave form of *cab2-luc* expression increasingly diverged from wild type as photoperiod lengthened (Fig. 2). In 8L/16D short-day cycles, rhythmic *cab2-luc* expression was similar between wild type and *elf3-1*, whereas expression of *cab2-luc* in *elf3-1* in 18L/6D long-day cycles

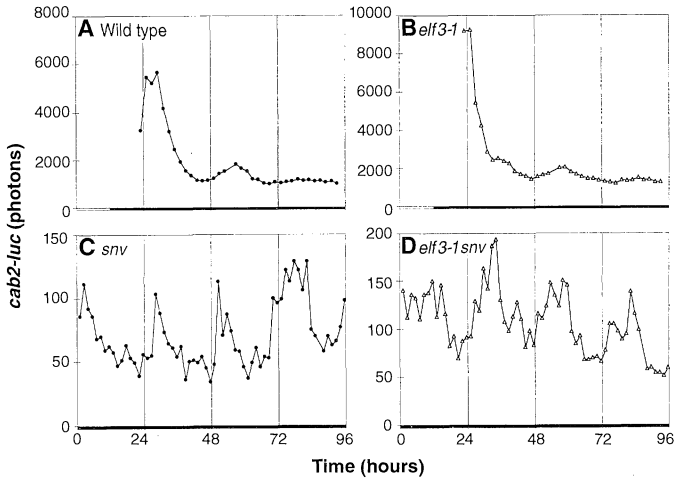

Fig. 3. (**A** and **B**) Seedlings were grown for 5 days in 12L/12D before being transferred to constant dark for measurement of *cab2-luc* expression. Time zero denotes the time of the last dawn before transfer to constant conditions. Because the amount of *CAB2* expression in dark-grown seedlings is less than that in light-grown seedlings, total *cab2-luc* expression was measured in a population of 70 to 100 seedlings. Similar results were observed for three independent *elf3-1* F_3 families in six experiments. (A) Wild type; (B) *elf3-1*. (**C** and **D**) Seedlings were germinated and grown for 5 days in constant darkness before measurement of *cab2-luc* expression under constant dark conditions. Luminescence traces of *cab2-luc* expression are shown for single seedlings. Similar results were obtained in two experiments and in measurements of populations of seedlings. (C) *snv*; (D) *elf3-1 snv*.

resembled that of constant light (Fig. 1) during the day phase. Given that the wave form observed in light/dark cycles results from a combination of circadian regulation and the light induction of *cab2-luc* (*11*), aberrant rhythms in the *elf3* mutant could be due to a defect in one or both of these regulatory pathways.

To further test the hypothesis that a circadian clock remains functional in *elf3-1* mutants, we assayed *cab2-luc* expression under constant dark conditions. Expression in constant dark of *cab2-luc* in populations of wild type or *elf3-1* was similar (Fig. 3, A and B) (*12*). Although the first peak of *cab2-luc* expression observed in constant dark conditions could result from entrainment in light/dark cycles, a second peak in *cab2-luc* bioluminescence was detectable in both wild type and *elf3-1* mutants, indicating the presence of a dampened, long-period rhythm. This observation was confirmed by the use of a mutant background that results in overexpression of *cab2-luc* (*12*). Rhythmic *cab2-luc* transcription was observed in constant dark in both *supernova* (*snv*) and *elf3-1 snv* mutant seedlings (Fig. 3, C and D). *elf3-1* mutants therefore contain a functional circadian clock in darkness but are arrhythmic in continuous white light (LL).

The conditionality of arrhythmic *cab2-luc* expression in the *elf3-1* mutant, with a lack of rhythm observed in constant light but not in constant dark, together with increasingly altered expression of *cab2-luc* in lengthening photoperiods, suggests that a longer daylength—or the lack of a dark period—results in the loss of circadian regulation in the absence of *ELF3* function. An alteration in two distinct circadian responses suggests that the *elf3-1* mutation causes a global defect in circadian regulation. The retention of rhythmicity in *elf3-1* mutant seedlings grown in constant dark suggests that *elf3* is not a simple null mutation of an oscillator component itself. The previously described defect in light inhibition of hypocotyl elongation in *elf3* mutant seedlings is indicative of *ELF3* function being required for light perception or signaling. We favor a model in which the *ELF3* gene product functions on a light-input pathway to the circadian oscillator, and that the aberrant coordination of light and circadian regulatory pathways contributes to the altered flowering time and photoperiodic insensitivity observed in *elf3* mutants.

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5. The KUJATA imaging system recorded cotyledon position every 20 min. Individual traces were analyzed to identify rhythmic behavior and provide a measurement of the period and intensity of rhythmic patterns. Significance levels were taken as 5%. Because the strength of rhythmicity is inversely proportional to relative amplitude, robust rhythmicity was defined as any 19- to 30-hour period with relative amplitude equal to or less than the wild-type mean relative amplitude plus one arithmetic standard deviation. Circadian rhythmicity was defined as robust rhythmicity with a period of 22 to 27 hours. Less than 100% of wild-type seedlings showed robust rhythmicity of leaf movements because of technical difficulties inherent in the assay. Period values are arithmetic means ± SD.
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7. *cab2-luc* transgenic plants of the C24 ecotype were crossed to *elf3-1* plants. F_2 progeny were assayed for kanamycin resistance to identify *cab-luc* transgenic seedlings, and for hypocotyl length to identify *elf3-1* homozygous seedlings. F_3 families were generated from homozygous *elf3-1* F_2 plants containing the *cab2-luc* transgene. Genotype was confirmed by measurements of hypocotyl length and genetic crosses to the *elf3-1* parent. The F_2 population consisting of a cross between the Columbia and C24 ecotypes was used as a wild-type control.
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12. Traces were analyzed as described (*5*). The mean wild-type period (±SD) was 28.6 ± 0.61 hours on a sample size of six, all of which showed rhythmic behavior. The mean *elf3-1* period was 28.8 ± 1.9 hours on a sample size of 18, 16 of which showed rhythmic behavior. The *supernova* (*snv*) mutation was isolated in a screen for alterations in *cab2-luc* expression (*13*). *SNV* maps to a locus distinct from the *cab2-luc* transgene, and the *snv* mutation has no effect on the period, phase, or relative amplitude of circadian regulation of *cab2-luc* expression (*13*). The mean *snv* period was 25.2 ± 2.3 hours on a sample size of 26, 16 of which showed rhythmic behavior. The mean *elf3-1 snv* period was 25.3 ± 1.7 hours on a sample size of 40, 21 of which showed rhythmic behavior.
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Protection from Natural Killer Cell–Mediated Lysis by HLA-G Expression on Target Cells

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The outermost layer of the human placenta is devoid of classical class I human leukocyte antigens (HLA-A, HLA-B, and HLA-C) and class II proteins (HLA-DR, HLA-DQ, and HLA-DP). Although this prevents recognition by maternal T lymphocytes, the lack of class I molecules leaves these cells susceptible to attack by natural killer (NK) cells. However, trophoblast cells directly in contact with the maternal tissues express the class I molecule HLA-G, which may be involved in protecting the trophoblast from recognition by NK cells. Here evidence is provided that expression of HLA-G is sufficient to protect otherwise susceptible target cells from lysis by activated NK1 and NK2 cell lines and clones that are specific for distinct groups of HLA-C alleles. The receptors on NK cells that recognize HLA-G are also identified.

During mammalian pregnancy, hemiallogeneic fetal cells invade the uterine structures and survive without immunological rejection. The outermost extravillous cytotrophoblast cells of the human placenta lack classical major histocompatibility complex (MHC) molecules (*1*). However, the presence of an unusual class I molecule has long been demonstrated (*2, 3*). This molecule was shown to be the product of the HLA-G gene (*4*), originally detected as an intact class I gene not expressed in any cell or tissue examined (*5*). HLA-G is 86% homologous to the HLA-A, -B, -C consensus sequence, but lacks the cytoplasmic tail (*5*). Although reduced expression of this molecule is associated with some abnormalities during pregnancy (*6*), its func-

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