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Article Addendum

ELF4 as a Central Gene in the Circadian Clock

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Addendum to:

ELF4 is Required for Oscillatory Properties of the Circadian Clock

McWatters HG, Kolmos E, Hall A, Doyle MR, Amasino RM, Gyula P, Nagy F, Millar AJ, Davis SJ

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ABSTRACT

The light-dark cycle of the environment serves as one of the major *Zeitgebers* in entrainment of the circadian clock. The circadian system consists of interconnected feedback loops in which the *CCA1/LHY-TOC1* loop has a central position. Genetic analyses of the *elf4* mutant suggested that it is a positive regulator of *CCA1* and *LHY* expression. Recently, we refined the mode-of-action of *ELF4* in entrainment of the clock, and here hypothesize that *ELF4* expression is interlocked with the *CCA1/LHY-TOC1* loop.

The circadian clock is an internal pacemaker. Primary qualities of a circadian clock include anticipation of daily events at dawn and dusk and a free-running rhythmicity of approximately 24 hours. In addition, circadian organisms can set internal rhythms to match the environmental light-dark cycle. This adjustment occurs daily and is known as entrainment.

Various groups have arranged the molecular components of the *Arabidopsis thaliana* circadian system into a model of transcription/translation feedback loops. Each loop consists of elements specific to certain time points (phases) of the 24 h day. In the most central loop, the expression peak times of the two MYB-like transcription factors *CCA1* and *LHY* are in the morning, and the pseudo response regulator *TOC1* peaks in the evening.[1-3] This loop is required for sustaining rhythms.[4] Several other clock genes—*GI*, *ELF3*, *ELF4*, *PRR7* and *PRR9*—are known to be important for proper periodicity.[5-11] Recently, mathematical modeling has assisted expression analyses and expanded the understanding of the circadian system.[12] Two additional feedback loops containing *GI* and *PRR7-PRR9*, respectively, were proposed to interlock with the *CCA1/LHY-TOC1* feedback loop.[13,14] In these studies, the position of the clock-controlling gene *ELF4* was not fully defined. In previous studies, it was found that *ELF4* genetically acts as a positive regulator of *CCA1*.[5,15] It remained a challenge to further define the mode-of-action of *ELF4* in connection to the performance of the circadian system.

We previously showed that *ELF4* is a clock gene whose expression peaks in the evening.[5] *ELF4* loss-of-function leads to an impaired photoperiod response, circadian dysfunction (imprecision and arrhythmicity) under free-running conditions, and very low amplitude of the light-induced clock gene *CCA1*. Thus, *ELF4* function was suggested to be important for input of light signaling to the clock, which is the process of clock resetting termed entrainment. Two other papers supported this conclusion.[15,16] What was missing from these efforts was a definition of *ELF4*'s placement within the clock network. Accordingly, we sought to expand *ELF4* genetic analyses to address the role of *ELF4* in sensing the photoperiodic signals of the environment and its connection to core clock genes.

In the *Plant Physiology* paper, to which we make this Addendum, we assessed clock performance of genotypes altered in *ELF4* expression and connected this to the expression phenotypes of the core clock genes *CCA1*, *LHY*, and *TOC1* in relation to the light-dark cycle. We found that the *elf4* mutant had elevated levels of *TOC1* expression implicating *ELF4* as a negative regulator of *TOC1*. In contrast, *ELF4* promotes the expression of the morning genes *CCA1* and *LHY*, because the expression of these two genes was markedly attenuated in the *elf4* loss-of-function mutant. A reciprocal effect was seen in *ELF4* overexpresssion lines: *TOC1* levels were low and *CCA1* and *LHY* levels were high. In addition, it was previously reported that *ELF4* expression was increased in the *cca1 lhy* double mutant.[15] Collectively, these expression data leads to a model where *ELF4* has a central position in the interconnected feedback loops (Fig. 1). Our conclusion is thus that *ELF4* is a core-clock gene working in the evening phase of the circadian cycle to drive morning expression of *CCA1* and *LHY*.

The interrelationship between *ELF4* and *TOC1* is still unclear, but as both are evening genes, they appear to act coordinately to control the clock. Under light-dark cycles, we found an early phase of *ELF4* expression in the *toc1* mutant, in agreement with the short-period phenotype of *toc1*. The mean expression level of *ELF4* under a diurnal cycle was unaltered in *toc1*. We hypothesize that *ELF4* expression would eventually dampen in *toc1* lines subjected to an extension of light period, as was seen for *ELF4* expression in the *cca1 lhy* mutant.[15] Thus, it is likely that core-oscillator genes are all codependent. Here, we suggest that *ELF4* is a "full" component of the central-clock machinery. That said, reporter levels driven by the *ELF4* promoter in the *elf4* mutant lead to mean levels of expression. Why *ELF4* is not in an autoregulatory loop to regulate its own overall expression level is an open question. As well, the transcripts of *ELF4* and *TOC1* both dampen in constant darkness and thus how evening genes are required for rhythm persistence in the absence of their transcript accumulation is unknown. We note that the *TOC1* profile has a bimodal peak, but *ELF4* does not.[2,5] Therefore, we wonder if circadian-controlling differences exist in the fine structure of *TOC1* and *ELF4* rhythmic expression.

The importance of *ELF4* activity in the circadian clock was corroborated by expression phenotypes under light-dark cycles (diurnal conditions). In contrast to wild type, the promoter: luciferase profiles in *elf4* were driven by "lights on" and "lights off." Furthermore, *elf4* lost anticipation of the phase of the environmental light-dark cycle and displayed an unusual, fast-resetting behavior after a simulated "jet lag." The light-induced peaks of *CCA1* and *LHY* expression can directly explain these diurnal patterns of gene expression in *elf4* confirming the position of *ELF4* in the central loop of the clock. In addition, the rhythm of gene expression of several clock markers in an *ELF4*-overexpression genotype displayed a long period. This suggests to us that *ELF4* functions in a dose-dependent manner as a repressor of periodicity. This overexpression-phenotype is remarkably different from that seen in other clock-gene overexpression lines (e.g., *CCA1-ox*, *lhy-1* and *TOC1-ox*),[13,17,18] and might indicate differences in post-translational performance among the central clock elements.

Analysis of overexpression lines has been supplemented by loss-of-function studies and it was found that mutations in the clock-oscillator genes differ in phenotype (arrhythmicity vs. short period).[2,19] Notably, when released into continuous light, the clock does not arrest in the *cca1 lhy* double, and in the *elf4* single mutant, there is no arrest until after a half-full circadian cycle.[5,19] This can be interpreted as that other "cogwheels" of the clock continue for a limited period of time when one wheel is blocked. Our idea is that *ELF4* function is analogous to an hourglass, where the oscillator stops after a fixed amount of time (after 12 h, subjective dusk) in absence of *ELF4* activity. Subsequently, the clock enters an arrhythmic state where the amplitude of output rhythms is lost, and this is probably due to the low levels of *CCA1* and *LHY* expression in *elf4*. An unknown factor (*X*) was modeled to function as an inducer of *CCA1* and *LHY* expression.[12,13] Further studies are needed to elucidate whether *ELF4* possess some features of *X* (e.g., the effect of TOC1 on *ELF4*).

Various studies implicate ELF4 in distinct input nodes of light signaling to the oscillator. In an earlier report, increased *ELF4* expression was found in the *elf3* mutant and therefore *ELF3* was defined as a negative regulator of *ELF4* expression.[15] We found that the *elf4* mutant had a minor role in gating the acute induction of *CAB* expression, and this was around subjective dusk. This means that both *ELF3* and *ELF4* have roles in controlling light input to the clock.[20] Similarly, *elf4* arrhythmicity is evident under all wavelengths of light (McWatters H, unpublished), supporting the concept of *ELF4* in both light signaling and in entrainment. Furthermore, *ELF4* acts predominantly in response to the light-dark *Zeitgeber*, as *elf4* mutants can entrain (albeit weakly) to temperature cycles. Temperature as an entrainment cue remains to be tested for many of the clock genes. *PRR7* and *PRR9* have been found to mediate in temperature signaling to the clock,[21] and in another aspect of clock buffering, *CCA1* and *LHY* were found to participate in specific roles of temperature compensation.[22] The poor free-running rhythm of *elf4* after entrainment to temperature cycles indicates that more is yet to be learned about the role of *ELF4* in perception of environmental *Zeitgeber* signals.

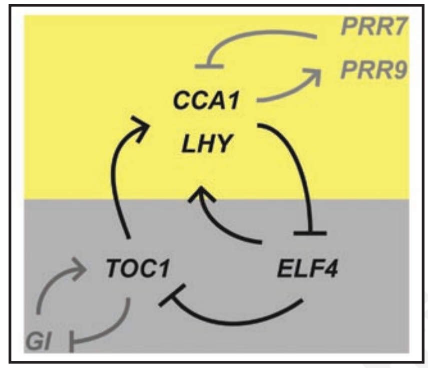

Figure 1. *ELF4* in the three-loop model of the circadian clock. *ELF4* expression is interlocked with the *CCA1/LHY-TOC1* loop in the three-loop model of the central circadian network.[13,14]

The timing of dawn and dusk, which predictably fluctuates in most environments over the year, represents the fixed points for clock entrainment. We provided results supporting the idea that *ELF4* has a central function in the circadian clock and in day-length perception. In future studies, the post-translational relationship among and within the CCA1/LHY-TOC1/ELF4 loops should be explored in order to gain a more detailed view of the feedback mechanism of the circadian clock.

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