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Temporal Repression of Core Circadian Genes Is Mediated through EARLY FLOWERING 3 in Arabidopsis

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Summary

The circadian clock provides robust, ~24 hr biological rhythms throughout the eukaryotes. The clock gene circuit in plants comprises interlocking transcriptional feedback loops, reviewed in [1], whereby the morning-expressed transcription factors: CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) repress the expression of evening genes, notably TIMING OF CAB EXPRESSION 1 (TOC1). EARLY FLOWERING 3 (ELF3) has been implicated as a repressor of light signaling to the clock [2, 3] and, paradoxically, as an activator of the light-induced genes CCA1 and LHY [4, 5]. We use cca1-11 lhy-21 elf3-4 plants to separate the repressive function of ELF3 from its downstream targets CCA1 and LHY. We further demonstrate that ELF3 associates physically with the promoter of PSEUDO-RESPONSE REGULATOR 9 (PRR9), a repressor of CCA1 and LHY expression, in a time-dependent fashion. The repressive function of ELF3 is thus consistent with indirect activation of LHY and CCA1, in a double-negative connection via a direct ELF3 target, PRR9. This mechanism reconciles the functions of ELF3 in the clock network during the night and points to further effects of ELF3 during the day.

Results

Mutual Regulation of ELF3 and CCA1/LHY Expression

Hypocotyl growth is a circadian output and can be used as an indicator of clock function [6]. elf3-4 seedlings show abnormally elongated hypocotyls as the clock-controlled repression of hypocotyl growth is lost in these plants [7, 8]. To study the interaction between ELF3 and CCA1/LHY, we examined hypocotyl length in loss-of-function mutant backgrounds. Seedlings were grown under short day conditions (6:18 hr light:dark cycles) for 6 days, and hypocotyl length was assessed on day 7. The wild-type ecotype, Wassilewskija (Ws), and cca1-11 lhy-21 seedlings showed hypocotyls of similar length, whereas cca1-11 lhy-21 elf3-4 showed a phenotype very similar to elf3-4 seedlings (Figure 1A). This suggests that the elf3-4 mutant effect on aberrant growth of hypocotyls does not require the LHY and CCA1 transcription factors. Imaging of rhythms in delayed chlorophyll fluorescence (see Figure S1 available online) showed that, like elf3-4 mutants, cca1-11 lhy-21 elf3-4 plants were arrhythmic for this physiological marker in constant light.

CCA1 and LHY RNA expression levels were shown to be very low in elf3 mutant seedlings, suggesting a mechanism for their arrhythmia [5]. We confirmed this through quantitative PCR (qPCR) analysis on 7-day-old seedlings under 12:12 white light:dark (LD) cycles (Figure 1) or transferred from 12:12 red LD to constant light (LL; Figure S2). The high amplitude of CCA1 and LHY expression rhythms in wild-type (100-fold to 1000-fold in LD, 10-fold in LL) collapsed in the elf3-4 plants, which became arrhythmic in LL. Transcript analysis under LD was more informative.

The low-amplitude rhythm in both CCA1 and LHY transcripts (reaching at most 15% of wild-type peak level, Figures 1C and 1D; 40% of wild-type peak, Figures S2A and S2C) showed that the clock’s morning functions were severely impaired in the elf3-4 mutant, though a rhythm could still be driven by the LD cycle. ELF3 RNA levels had a lower-amplitude rhythm in the wild-type (at most 10-fold in LD), whereas in cca1-11 lhy-21 mutants, ELF3 RNA showed little rhythmicity under LD and arrhythmia under LL (Figure 1B and Figure S2F). Circadian control of ELF3 expression [5] requires the morning loop components CCA1 and LHY. ELF3, in turn, regulates these clock genes and gates entrainment signals [2].

ELF3 Is a Key Repressor of Core Circadian Genes

An evening loop, involving at least TOC1 and GIIGANTEA (GI), is proposed to generate the short-period rhythms observed in lhy cca1 double mutants [9]. Through the comparison of clock gene expression in Ws, elf3-4, cca1-11 lhy-21, and cca1-11 lhy-21 elf3-4 plants, we aimed to test the role of ELF3 in the proposed evening loop. Plants were grown under 12:12 LD cycles for 6 days, sampled on day 7, and tested for expression of PRR9, PRR7, GI, and TOC1 (Figure 2; Figure S3). In cca1-11 lhy-21 plants, the evening genes (TOC1 and GI) showed an early-morning peak of high amplitude (Figures 2C and 2D). This is in agreement with previously published data [9–11] and also supports the hypothesis that CCA1 and LHY act to repress evening gene expression in the early morning. In the double mutant, PRR9 showed a lower amplitude rhythm, probably because of the loss of activation of expression by CCA1 and LHY (Figure 2A). The elf3-4 mutant showed a lower amplitude rhythm in gene expression for all measured genes, with notably higher levels (over 10-fold increase compared to wild-type) of PRR9, PRR7, GI, and TOC1 expression in the night, as reported in [12] for GI, as well as slightly higher nighttime expression of TOC1. The aberrant gene expression continued into the early morning, when CCA1 and LHY should be active in the wild-type (Figures 1C and 1D). Such results are consistent with a combination of indirect and direct mechanisms, whereby CCA1 and LHY repress evening gene expression in the morning (Zeitgeber time [ZT] 0–4, where ZT = 0 is defined as the time of lights-on) and ELF3 represses many genes at night (ZT 12–20), before CCA1 and LHY are expressed. From this it could be expected that the cca1-11 lhy-21 elf3-4 triple mutant would show high expression of certain clock genes

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early morning expression characteristic of PRR9 in Ws wild-type plants (filled circles) and elf3-4 mutants (crosses). Data are all normalized against IPP2 expression [25]. Graphs are an average of two to three biologically independent experiments, with normalized data being used to generate SEM error bars. Seedlings were grown in 12:12 white light:dark cycles and sampled every 2 hr from Zeitgeber time (ZT) = 0. ZT = 0 is defined as the time of lights-on. See also Figures S1 and S2.

throughout the LD cycle. This was not observed. Instead, in the triple mutant, all genes were expressed at intermediate levels, without strong responses to the ongoing LD. PRR9 and PRR7 expressions were higher than in cca1-11 lhy-21 but lower than in elf3-4. Evenings genes (TOC1 and GI) lost the early peak observed in cca1-11 lhy-21, but then had the higher nighttime expression characteristic of elf3-4. This suggests that ELF3 influences the circadian network at more than one point and thus affects both morning and evening loops.

**ELF3 Binds In Vivo to the Promoter of PRR9 in the Early Night**

Because ELF3 shows some sequence homology with transcription factors [13], we investigated whether ELF3 physically associates with circadian-controlled promoters. Chromatin immunoprecipitation (ChIP) experiments were conducted using transgenic plants that expressed an ELF3::YFP fusion protein from either the native promoter or the 35S CaMV promoter, being bound at ZT = 14 but not significantly (by Students t test) at ZT = 6 (Figure 3C). ELF3’s apparently rhythmic association with the PRR9 promoter and the increased PRR9 expression observed in the elf3-4 mutant suggest that ELF3 acts as one of the repressors of PRR9 gene expression. Association of ELF3 with the PRR7 promoter was weak, because it was detected only in the 35S::ELF3::YFP plants (Figure S4). Association of ELF4 with PRR7 was comparable to results for PRR9 (Figure S4). Testing 1.3 kbp of sequences upstream of the ATG codon of CCA1 did not reveal any ELF3 or ELF4 association (data not shown), although this promoter fragment is sufficient for rhythmic transcription [15]. However, derepression of the PRR9 promoter is sufficient to explain low levels of CCA1 and LHY expression in the elf3-4 mutant (Figures 1C and 1D), because PRR9 is a known repressor of CCA1 and LHY [16]. The promoter regions required for rhythmic expression of PRR5, TOC1, and GI were also tested, and ELF3 and ELF4 were not found to associate with these (data not shown), suggesting that ELF3 is involved in the regulation of their expression indirectly.

**A Combination of Repressors Is Required for the Control of Circadian-Regulated Light Responses**

In order to investigate the regulation of light signaling via ELF3, a 20 min white-light pulse was applied to seedlings entrained in 12:12 white LD cycles and released into darkness. PRR9 and GI were specifically investigated because they have both...
whether ELF3 protein can associate with DNA. We show that loops with which ELF3 through both the investigation of the transcriptional controls clock genes in darkness, that ELF3 still affects clock gene expression in darkness, that ELF3 still and showed light induction in cca1-11 lhy-21 elf3-4 seedlings, PRR9 type plants showed strong light induction of morning and evening loops of the circadian network. Wild-type plants showed strong light induction of PRR9 (Figure 4A). In cca1-11 lhy-21 double mutants, the expression levels of PRR9 were very low, and a clear acute response to light was observed, which was as large or larger than that in Ws during the predicted night, ZT = 38 (Figure 4A). In elf3-4 and cca1-11 lhy-21 elf3-4 seedlings, PRR9 had a higher level of basal expression in the night, consistent with Figure 2A and with ELF3’s function as a repressor of gene expression in the dark. Little change in expression was observed following a light pulse at either predicted day ZT = 30 or night ZT = 38 (Figure 4A). Notably, the PRR9 expression level was not maximal compared to peak levels (Figure 2A), suggesting that another factor is involved in the gating of light responses in the dark. GI expression was not light responsive at these times in Ws and showed light induction in cca1-11 lhy-21, but not in elf3-4 or the triple mutant (Figure 4B). This again indicates that ELF3 affects clock gene expression in darkness, that ELF3 still controls clock genes in cca1-11 lhy-21 seedlings, and that some repressive functions remain in the triple mutant.

Discussion

This work tests the possibility that ELF3 acts as an activator of CCA1 through both the investigation of the transcriptional loops with which ELF3 is involved and the determination of whether ELF3 protein can associate with DNA. We show that ELF3 has repressive effects on several clock genes. The observed activation of CCA1 in elf3-4 mutants can be explained consistently with ELF3’s repressive function by a double-negative effect via PRR9, the repressor of CCA1 and LHY [18]. ELF3 protein associates with the PRR9 promoter (Figure 3). In elf3-4, the levels of PRR9 are high, so the repression of CCA1 and LHY is greater. However, the high expression of evening genes GI and TOC1 in elf3-4 mutants cannot simply be explained by low levels of CCA1 and LHY, because this high baseline was not observed in cca1-11 lhy-21 mutants.

To investigate the role of ELF3 independently of the influence of CCA1 and LHY, we generated cca1-11 lhy-21 elf3-4 plants. These plants have a growth phenotype similar to the elf3-4 plants (Figure 1A). cca1-11 lhy-21 elf3-4 mutants show high basal levels of clock gene expression in the dark period of 12:12 LD cycles, as in elf3-4, but do not show the characteristic early peaks of PRR7, GI, and TOC1 expression observed in cca1-11 lhy-21 (Figure 2). This high level of gene expression in the dark is also observed in the acute light pulse response data set (Figure 4). Thus, through comparison of the cca1-11 lhy-21 and cca1-11 lhy-21 elf3-4 data, it seems that ELF3 allows rhythmicity in the cca1-11 lhy-21 double mutant. It also suggests that there may be another, normally redundant, factor, which is able to take the role of CCA1/LHY in the early morning (ZT 0–4) and repress the expression of circadian genes. This function is not observed in the cca1-11 lhy-21 double mutant because the component is still being repressed by ELF3.

Association with the PRR9 promoter provides a mechanism for ELF3’s direct (PRR9) and indirect (CCA1/LHY) effects on
The Arabidopsis clock includes repression of gene and assigns this role to ELF3 from the clock network. The fact that ELF3 is essential for the normal operation of the circadian transcriptional feedback loops in light-grown plants, as reported in dark-grown seedlings [21]. The mechanism of ELF3 action presented here links ELF3 directly to the circadian network.

Experimental Procedures

Construction of Multiple Mutant Lines and Transgenic Plants

To create the cca1-11 lhy-21 elf3-4 triple mutant, we crossed the cca1-11 lhy-21 [22] double mutant to elf3-4 [8]. In the F2 progeny, individuals with long hypocotyls were selected and verified by sequencing. These plants were then screened for cca1-11 and lhy-21 mutations. For details on the molecular markers used for genotyping, see Table S1.

The ELF3 promoter and the ELF3 and ELF4 coding sequences (CDS) were amplified by PCR from wild-type Ws genomic DNA by PCR primers with added restriction sites to facilitate cloning. The sequence of primers and the corresponding restriction sites are provided in Table S2. The amplified fragments were cloned in pBluescript SK plasmids and verified by sequencing. The ELF3 promoter fragment contained 2695 nucleotides upstream of the start codon of the ELF3 gene and included the full 5’ untranslated region. The ELF3 and ELF4 CDS fragments included the full coding sequence but not the translational termination codons. The 35S:PHYA-YFP pPCVB812 binary vector has been described [23]. The PHYA cDNA in 35S:PHYA-YFP pPCVB812 was replaced with the ELF3 or ELF4 CDS fragments, resulting in 35S:ELF3-YFP pPCVB812 and 35S:ELF4-YFP pPCVB812. Then the 35S promoter in 35S:ELF3-YFP was replaced by the ELF3 promoter fragment, which yielded ELF3:ELF3-YFP pPCVB812. The binary vectors containing the gene constructs described above were transferred to Agrobacterium tumefaciens GV3101 cells. The constructs were transformed into wild-type Ws (ELF4 construct) and elf3-4-mutant plants (ELF3 construct) by the floral-dip method [24]. Primary transformant plants were isolated based on resistance to Basta herbicide. Ten to 15 independent transgenic lines were produced for each combination of construct and host plant. Lines carrying a single copy of the transgene were selected based on the segregation of Basta resistance and were used for experiments.

Plant Materials and Growth Conditions

All plant lines are in the Ws ecotype. Surface sterilized seeds were stratified for 4 days in the dark at 4 °C before being grown under cool-white fluorescent tubes (70–100 μmol m⁻² s⁻¹) in LD cycles at constant 22 °C. All plants were grown on 1% agar Murashige-Skoog (MS) plates. Photoperiod light conditions were either short day (SD) 6:18 or standard 12:12, as shown in figures. ZT = 0 is defined as the last dark:light transition before measurements start.

Analysis of Gene Expression

For LD time courses, approximately 70 seedlings per sample were harvested for each genotype into 1 ml of RNAlater solution (Ambion). Samples were taken at 2 hr intervals starting at ZT = 0. Total RNA was extracted (Qiagen RNaseasy kit, 74106) according to manufacturer’s instructions. cDNA was synthesized from 1 μg of total RNA, and random hexamer primers were supplied with the Fermentas cDNA synthesis kit. cDNA was diluted 1:5 in RNase-free dH₂O, and qPCR plates (LightCycler 480 multiwell plate 384, Roche) were set up using a Tecan Freedom EVO robot controlled by EVOware standard software with Master Mix containing SYBR Green (Roche), gene-specific primers at 3 μM, and RNase-free dH₂O. The qPCR was conducted in triplicate in a Roche LightCycler 480 controlled by LightCycler 480 SW1.5 software. Transcript levels were normalized to the control transcript IPP2 [25] and were normalized between replicates.

All presented measurements are an average of three independent experiments. Gene-specific primer pairs are listed in Table S2.

Measurement of Hypocotyl Length

Plants were grown under SD (6:18 LD) white-light (70–100 μmol m⁻² s⁻¹) photoperiod conditions on MS and 1% agar plates for 6 days, and hypocotyls with centimeter ruler were imaged using a digital camera. Measurement of hypocotyl length was performed by ImageJ (http://rsb.info.nih.gov/ij/), with hypocotyl length being defined as from V in hypocotyls-cotyledon formation to hypocotyls-root junction.
Chromatin Immunoprecipitation
ChiP was carried out as previously described [26], with the following modifications: seedlings were grown for 3 weeks in 12:12 LD cycles and harvested at either ZT = 6 or ZT = 14; crosslinking with 1% formaldehyde was carried out under a vacuum for a total of 30 min; and samples were resuspended in 4 ml of ChIP dilution buffer and split into four samples. Chromatin was immunoprecipitated using anti-GFP (Clontech). ChIP DNA was analyzed by qPCR on an LC480 (Roche) using SYBR Green Master Mix (Roche). Relative quantities were calculated as a percentage of the input DNA for each sample. Seedlings were grown for 5 days under light:dark 12:12 LD cycles and released into continuous dark from ZT = 12 on day 5. On day 6, samples were either treated with (+) or without (−) a white-light pulse (20 min, 80 μmol m$^{-2}$ s$^{-1}$) 1 hr before sampling on the predicted day at ZT = 30 (white background) and on the predicted night at ZT = 38 (gray background). Error bars indicate the SEM from 4–6 samples. Student’s t test was used to compare treated and untreated samples within a time point and genotype. For clarity, only treated samples that differ significantly from their control are marked with *p < 0.05 or **p < 0.005.

Supplemental Information
Supplemental Information includes four figures and two tables and can be found with this article online at doi:10.1016/j.cub.2010.12.013.

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