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Critical Role for CCA1 and LHY in Maintaining Circadian Rhythmicity in *Arabidopsis*

David Alabadí,[2] Marcelo J. Yanovsky, Paloma Más, Stacey L. Harmer, and Steve A. Kay[1]
Department of Cell Biology
and Institute for Childhood and Neglected Diseases
The Scripps Research Institute
10550 North Torrey Pines Road
La Jolla, California 92037

Summary

Circadian clocks are autoregulatory, endogenous mechanisms that allow organisms, from bacteria to humans, to advantageously time a wide range of activities within 24-hr environmental cycles [1]. *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*) are thought to be important components of the circadian clock in the model plant *Arabidopsis* [2–5]. The similar circadian phenotypes of lines overexpressing either *CCA1* or *LHY* have suggested that the functions of these two transcription factors are largely overlapping. *cca1-1* plants, which lack CCA1 protein, show a short-period phenotype for the expression of several genes when assayed under constant light conditions [5]. This suggests that LHY function is able to only partially compensate for the lack of CCA1 protein, resulting in a clock with a faster pace in *cca1-1* plants. We have obtained plants lacking CCA1 and with LHY function strongly reduced, *cca1-1 lhy-R*, and show that these plants are unable to maintain sustained oscillations in both constant light and constant darkness. However, these plants exhibit some circadian function in light/dark cycles, showing that the *Arabidopsis* circadian clock is not entirely dependent on CCA1 and LHY activities.

Results and Discussion

We applied double-stranded RNA interference (RNAi) technology [6] to disrupt LHY function in *Arabidopsis*. RNAi-induced mutation of genes of known function shows reduced activity or complete loss of function phenotypes, indicating that RNAi is a good way to assess gene function [6, 7]. We therefore cloned a 250-bp fragment, encompassing most of the 5′ untranslated region of the *LHY* gene, into a vector designed to produce a self-complementary RNA able to trigger silencing [7]. The selected region shows no homology to any other sequence in the *Arabidopsis* genome and is therefore very unlikely to disrupt expression of any other gene. The *LHY* RNAi construct was used to transform *cca1-1* plants harboring the *ccr2::luc* reporter, a clock-controlled transgene conferring peak luciferase activity near the end of the subjective day [8]. Bioluminescence analysis of many primary *cca1-1 lhy-R* transgenic seedlings indicated that the circadian expression of the reporter was strongly affected in continuous white light (LL) and that this phenotype was fully penetrant (D.A. and S.A.K., unpublished data). We selected T1 lines 48, 50, and 51 for further studies. To determine the extent of gene inhibition, the endogenous *LHY* transcript level was analyzed in T2 seedlings of selected lines by Northern blot. Wild-type, *cca1-1*, and *cca1-1 lhy-R* transgenic seedlings were entrained to 12 hr white light:12 hr dark (LD [12:12]), and tissue samples were collected at different time points during one LD cycle, starting at ZT 0 (ZT, *zeitgeber* time; ZT 0 is lights on). As shown in Figure 1, the amount of *LHY* mRNA at its peak was strongly reduced in lines 48 and 50 compared to wild-type and *cca1-1* peak levels; similar results were obtained for line 51 (M.J.Y, D.A., and S.A.K., unpublished data). This indicates that the *LHY* RNAi transgene efficiently interfered with the accumulation of the endogenous *LHY* RNA. Interestingly, the phase of the peak of the remaining *LHY* RNA level was more advanced in the *cca1-1 lhy-R* seedlings compared to wild-type and *cca1-1* (see below).

To determine the phenotype of plants deficient only in *LHY* expression, we backcrossed T1 lines 48, 50, and 51 to wild-type plants and analyzed F1 seedlings for the expression of the *ccr2::luc* reporter in LL. The F1 population is heterozygous for *cca1-1*, a fully recessive allele (S.L.H, D.A., and S.A.K., unpublished data), and is segregating for the *LHY* RNAi transgene, which has a dominant effect. All F1 plants showed robust cyclic luciferase activity with wild-type amplitude; however, analysis of the bioluminescence data by fast Fourier transform nonlinear least squares (FFT-NLLS; [9]) revealed that one half of this population had a free-running period length (τ) of 23.89 $\pm$ 0.15 hr ($\pm$SEM) (n = 8), while the other group had τ = 20.43 $\pm$ 0.15 hr (n = 8). These data correspond to the cross of line 48. The short-period plants were resistant to the herbicide basta, while the plants with wild-type periods were sensitive to basta, indicating that the *LHY* RNAi transgene had a period-shortening effect on *ccr2::luc* activity rhythms. Data from the cross of line 48 are shown in Figure 2A; similar results were obtained with the other lines (D.A., M.J.Y., P.M., and S.A.K., unpublished data). This period-shortening effect of the *LHY* RNAi transgene demonstrates that loss-of-function alleles of *LHY* have very similar phenotypes to loss-of-function alleles of *CCA1*, supporting the idea that these genes have partially redundant functions in the control of period length.

To determine the phenotype of plants deficient in both CCA1 and LHY functions, wild-type, *cca1-1*, and *cca1-1 lhy-R* T2 seedlings from lines 48, 50, and 51 were grown under an LD (12:12) regime for 7 days and were then transferred to LL for assessment of *ccr2::luc* activity over 5 days. *ccr2::luc* expression free ran with high amplitude and τ = 22.25 $\pm$ 0.10 hr in the *cca1-1* mutant (Figure 2B), compared to the period length of 23.99 $\pm$ 0.15 hr seen in wild-type plants. This shortening of period length in *cca1-1* plants is consistent with previous

[1]Correspondence: stevek@scripps.edu
[2]Present address: Instituto de Biología Molecular y Celular de Plantas (CSIC-UPV), Valencia 46022, Spain.

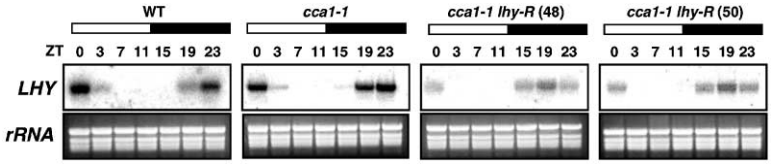

Figure 1. *LHY* RNAi Transgene Reduces *LHY* mRNA

cca1-1 plants homozygous for the *ccr2::luc* reporter were transformed with the *LHY* RNAi construct (see the Supplementary Experimental Procedures) by *Agrobacterium*-mediated DNA transfer [14]. T2 transgenic seedlings were selected by their resistance to the basta herbicide. Total RNA from one-week-old wild-type, *cca1-1*, and selected *cca1-1 lhy-R* T2 seedlings entrained to LD (12:12) was extracted by standard methods. *LHY* transcript levels were analyzed in 10 μg of total RNA by Northern blot as previously described [4]. *rRNA* stained with ethidium bromide serves as a loading control. White and black bars on top of the panels indicate lights on and off, respectively. ZT, *zeitgeber* time.

reports [5]. In contrast, *cca1-1 lhy-R* seedlings were not able to sustain rhythmicity in free-running conditions. A very fast and low-amplitude rhythm dampened after 2–2.5 days in LL (Figure 2C). The level of expression was intermediate or intermediate-high when compared to wild-type. FFT-NLLS analysis showed that 27 of 28 *cca1-1 lhy-R* T2 seedlings assessed in LL were arrhythmic (for these traces, either no rhythm was fitted or the fitted rhythm had a relative amplitude error higher than 0.6, which was used as a cutoff to identify rhythmic traces within the circadian range [10]; data from line 51 are included in the FFT-NLLS analysis, although traces are not shown). We investigated whether this effect seen in white light was also observed with monochromatic, red, and blue lights. FFT-NLLS analysis of bioluminescence from *ccr2::luc* expression showed that 17% of 23 *cca1-1 lhy-R* seedlings from line 48 assessed under constant blue light (fluence rate of 15–18 μmol s^{-1} m^{-2}) had detectable circadian rhythmicity, with $\tau = 16.60 \pm 0.60$ hr, whereas all of the 24 seedlings of this line as-

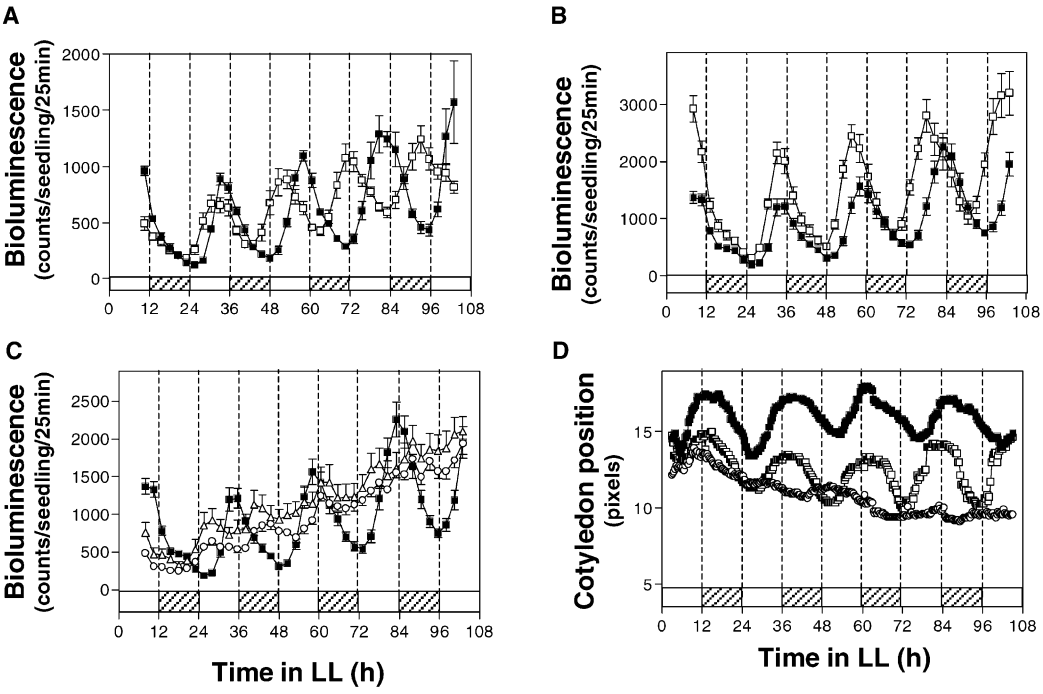

Figure 2. Period Length Depends on Dosage of *CCA1* and *LHY*

(A) Open and closed squares are heterozygous *cca1-1* plants with (n = 8) or without (n = 8) the *LHY* RNAi transgene, respectively. Seedlings of both genotypes were entrained for 6 days to LD (12:12) before being released and were imaged in LL (fluorescent white light, 50–60 μmol s^{-1} m^{-2}). Imaging started the morning of day 7 and continued every 2.5 hr for 4–5 days. Bioluminescence was analyzed as previously described [16].

(B) Wild-type and *cca1-1* plants were entrained and imaged as in (A). Closed squares represent wild-type (n = 16), and open squares represent *cca1-1* (n = 14).

(C) *ccr2::luc* expression damps in *cca1-1 lhy-R* seedlings under LL conditions. *cca1-1 lhy-R* lines 48 and 50 were entrained and imaged as in (A). Wild-type data (B) is replotted along with *cca1-1 lhy-R* T2 lines 48 (open circles, n = 7) and 50 (open triangles, n = 7). Imaging started the morning of day 7 and continued every 2.5 hr for 4–5 days. Bioluminescence was analyzed as previously described [15]. Error bars represent the standard error of the mean.

(D) The *cca1-1 lhy-R* mutation causes arrhythmic leaf movement in LL. Seeds of wild-type (closed squares, n = 10), *cca1-1* (open squares, n = 6), and *cca1-1 lhy-R* line 51 (T2 seeds, open circles, n = 14) were germinated and grown under LD (12:12) cycles for 5 days and were then shifted to LL (fluorescent white light, 20–30 μmol s^{-1} m^{-2}). Recording of cotyledon position (starting on day 6 and continuing every 20 min for 4–5 days) and analysis of the data was performed as previously described [11]. White and dashed bars on the *x* axis indicate subjective day and subjective night, respectively.

sessed under constant red light (fluence rate of 35–40 μmol s^{-1} m^{-2}) were arrhythmic. Similar results were obtained with line 50 (D.A., M.J.Y, P.M., and S.A.K., unpublished data). Of 28 wild-type plants assessed under red light or under blue light, 100% showed circadian rhythmicity. Mean periods of *ccr2::luc* expression for wild-type plants were 24.20 $\pm$ 0.20 and 24.00 $\pm$ 0.20 hr in red light and blue light, respectively. *cca1-1 lhy-R* plants show, therefore, arrhythmic *ccr2::luc* expression under both red and blue light, although the percentage of arrhythmic seedlings is higher under red light than under blue light. See bioluminescence traces of *ccr2::luc* activity for wild-type, *cca1-1*, and *cca1-1 lhy-R* T2 lines 48 and 50 under red and blue light in Figure S1 (see the Supplementary Material available with this article online). The damping of the *ccr2::luc* activity may be due to an arrest of the clock after 2 days in LL, which causes arrhythmicity thereafter. Another possibility, not mutually exclusive, is that a functioning fast-running clock is increasingly masked by deregulated *ccr2::luc* expression in these conditions. Analysis of other outputs would reveal whether there is an underlying fast clock still working in *cca1-1 lhy-R* plants in LL, or if it is actually arrested under these conditions. Both masking of an output and rapid arrest of the clock in LL have been described in *Arabidopsis* [10]. We investigated whether the *cca1-1 lhy-R* mutation affected clock-controlled processes other than *ccr2::luc* expression in LL. We assessed the circadian rhythm in cotyledon movement in wild-type, *cca1-1*, and *cca1-1 lhy-R* plants (Figure 2D). Robust circadian rhythm in cotyledon movement persisted in wild-type and *cca1-1* seedlings. FFT-NLLS analysis revealed mean periods of 24.22 $\pm$ 0.20 and 21.82 $\pm$ 0.67 hr for wild-type and *cca1-1* seedlings, respectively, whereas no consistent circadian rhythms were detected for *cca1-1 lhy-R* seedlings. Biomathematical analysis found 3 of 14 traces within the circadian range for the double mutant plants. On the contrary, all wild-type and *cca1-1* plants showed robust circadian rhythmicity. Together, these results show arrhythmic behavior for two different outputs in *cca1-1 lhy-R* plants under LL, indicating the absence of clock function under these conditions. This clearly indicates an essential role for CCA1 and LHY activities in keeping circadian rhythms in LL.

The endogenous *LHY* gene showed circadian expression in *cca1-1 lhy-R* plants under LD (12:12) cycles, albeit with a phase advance (Figure 1). This suggests the presence of clock function under these conditions in these plants. *elf3* mutants, for instance, have a functional clock under entraining conditions, regardless of their arrhythmic phenotype in LL [10, 11] (M.F. Covington, personal communication). To further characterize the *cca1-1 lhy-R* circadian phenotype under entraining conditions, we analyzed the *ccr2::luc* expression in wild-type and *cca1-1 lhy-R* T2 plants from line 50 growing under different 24-hr LD cycles (Figure 3); similar results were obtained with the other lines (D.A., M.J.Y., P.M., and S.A.K., unpublished data). Both the waveform and phase of the expression of the reporter in wild-type, as in *cca1-1* (D.A., M.J.Y., P.M., and S.A.K., unpublished data), were not significantly affected by the different photoperiods. The peak of *ccr2::luc* expression was

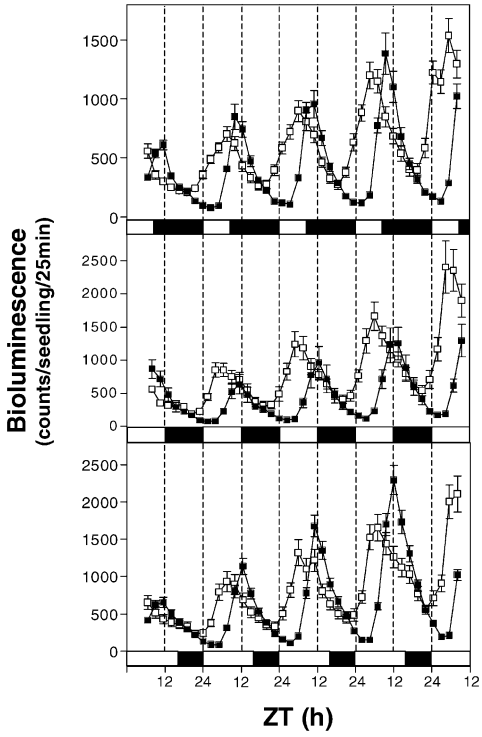

Figure 3. Effect of LD Cycles of Different Photoperiods on *ccr2::luc* Expression in Wild-Type and *cca1-1 lhy-R* Seedlings

LD regimes used: LD (8:16) (upper panel), LD (12:12) (middle panel), and LD (16:8) (bottom panel). *cca1-1 lhy-R* T2 seedlings from line 50 (open squares; n = 12 in both LD [8:16] and LD [16:8], and n = 7 in LD [12:12]) were selected for 5 days under the appropriate LD cycle and were transferred to nonselection media for 2 additional days before being imaged under the same conditions [15]. Wild-type seedlings (closed squares; n = 16 for both LD [8:16] and LD [16:8], and n = 10 for LD [12:12]) were grown for 7 days under the appropriate LD cycle before being imaged under the same conditions. Seedlings were imaged every 2.5 hr, beginning on day 8 and continuing through day 12. Bioluminescence was analyzed as previously described [15]. Error bars represent the standard error of the mean. White and black bars on the *x* axis indicate day and night, respectively.

slightly delayed in LD (12:12) and LD (16:8) photoperiods when compared to LD (8:16). This indicates that photoperiod length is not a major factor regulating the phase of entrainment of *CCR2* in these plants. *cca1-1 lhy-R* plants were also synchronized with the different 24-hr LD cycles, yet the *ccr2::luc* expression showed an earlier phase when compared to wild-type or *cca1-1* plants. *TOC1* expression also showed morning phase in these plants under entraining conditions (see Figure S2). This is consistent with the phase advance detected for the remaining *LHY* expression in these plants (Figure 1) [4]. The phase of *ccr2::luc* peak of activity was not dependent on photoperiod in *cca1-1 lhy-R* plants. Bioluminescence levels in *cca1-1 lhy-R* plants clearly increased in anticipation of dawn in LD (8:16). The anticipation was much less evident in LD (12:12) and was not evident at all in LD (16:8) cycles. This is likely due to a shift of the trough of the rhythm to a later phase in the longer photoperiods. This resulted in bioluminescence levels at dawn, or immediately after dawn, that were closer to

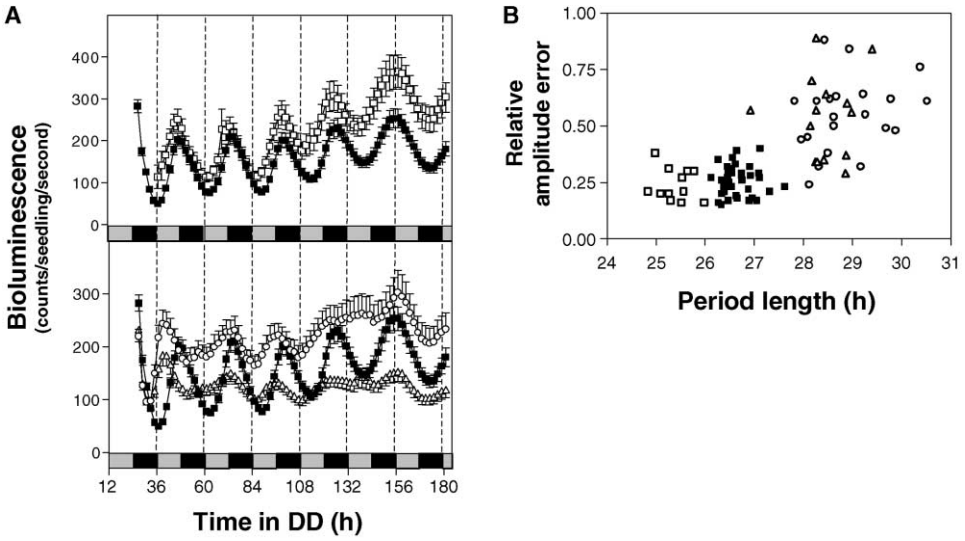

Figure 4. *cca1-1 lhy-R* Mutation Also Affects *ccr2::luc* Rhythms in DD

Seedlings of wild-type, *cca1-1*, and *cca1-1 lhy-R* T2 lines were entrained in LD (12:12) for 6 days before being released and imaged in DD [15]. Transgenic T2 seedlings were grown on selection media for 6 days and were then transferred to nonselection media. Imaging started by the end of the first subjective day and continued every 2 hr for 6 days. Bioluminescence was analyzed as previously described [15].

(A) Closed squares represent wild-type (n = 20); wild-type data have been plotted in the two panels for comparison. Upper panel: *cca1-1* (open squares, n = 12); bottom panel: *cca1-1 lhy-R* T2 lines 48 (open circles, n = 18) and 51 (open triangles, n = 12). Error bars represent the standard error of the mean. Gray and black bars on the *x* axis represent subjective day and night, respectively.

(B) A plot showing the FFT-NLLS analysis of the *ccr2::luc* expression data plotted in (A). The plot shows data for wild-type (closed squares), *cca1-1* (open squares), *cca1-1 lhy-R* lines 48 (open circles) and 51 (open triangles). The strong circadian expression of *ccr2::luc* in wild-type and *cca1-1* plants is reflected by the clustering of data points with low relative amplitude error values, which indicate robust rhythms. Scattered data points with relative amplitude error values closer to 1 in *cca1-1 lhy-R* plants indicate weaker rhythms.

peak values in the shorter than in the longer photoperiods. In all three entraining conditions, the expression levels decreased in anticipation of dusk. Importantly, the anticipation of the light-dark cues that is observed in LD cycles is indicative of the presence of a functional circadian clock in the *cca1-1 lhy-R* plants under these environmental conditions.

To investigate the effects of *cca1-1* and *cca1-1 lhy-R* mutations on clock function in the dark, we assessed *ccr2::luc* rhythms in plants entrained in LD (12:12) cycles, then held in continuous darkness (DD) (Figure 4). The reporter cycled with shorter τ in plants lacking CCA1 function than in wild-type plants (Figure 4A). FFT-NLLS analysis showed that τ was 26.64 $\pm$ 0.15 hr in wild-type and 25.39 $\pm$ 0.24 hr in *cca1-1* plants (Figure 4B). This period-shortening effect is similar to the situation observed in LL in *cca1-1* plants. Inhibition of LHY function in the *cca1-1* background severely affected the expression of *ccr2::luc* in DD (Figure 4A). A wild-type amplitude oscillation during the first 48 hr in DD was followed by a dampened rhythm. *ccr2::luc* expression peaked earlier in *cca1-1 lhy-R* than in wild-type and *cca1-1* plants during the second subjective day in DD, reflecting the phase advance observed in preceding LD (12:12) cycles (Figure 3). FFT-NLLS analysis revealed no consistent circadian rhythms of *ccr2::luc* expression in *cca1-1 lhy-R* plants (Figure 4B; compare the tightly clustered data points and low relative amplitude error values from wild-type and *cca1-1* plants that indicate robust circadian rhythms with the scattered data points and high relative amplitude error values from *cca1-1 lhy-R* plants). This indicates that clock function is highly compromised in these plants. CCA1 and LHY functions are, therefore, required to maintain proper clock function in DD also.

Circadian oscillations of CCA1 or LHY are necessary to sustain circadian rhythmicity, as shown by experiments with plants constitutively expressing these genes [2, 3]. This, together with the damping of rhythms observed in *cca1-1 lhy-R* plants (Figures 2 and 4), supports a critical and partially redundant role for CCA1 and LHY activities in sustaining circadian rhythms under constant conditions. In contrast, the circadian clock appears to be running under LD conditions in *cca1-1 lhy-R* plants. The earlier phase of activity seen in *cca1-1 lhy-R* plants compared to wild-type and *cca1-1* plants in LD cycles may reflect the activity of the very fast clock transiently detected in LL conditions. The phase of entrainment of a circadian clock in a 24-hr LD cycle depends on the period length in constant conditions or τ [12]. The *Drosophila* 16-hr period mutant per^T entrains to an LD (12:12) and shows the peak of activity 6 hr earlier than wild-type flies [13]. If the situation is similar in our double mutant, the *cca1-1 lhy-R* clock must produce a daily circa 6-hr delay to steadily entrain to the 24-hr LD cycle. This delay is, therefore, within the limits of entrainment in *Arabidopsis*.

Our results show that plants null for *CCA1* and with levels of *LHY* expression greatly reduced, most probably below any functional level, have a dramatically compromised ability to maintain circadian rhythmicity in constant light and in constant darkness. Taken together, these results strongly suggest that both *CCA1* and *LHY*

are critical clock components needed to maintain circadian clock function in *Arabidopsis*.

Experimental Procedures

Plant Strains and Growth Conditions

Arabidopsis thaliana ecotype Wassilewskija (WS) was used as wild-type. WS wild-type harboring the *ccr2::luc* transgene was obtained from the F2 of a backcross of *cca1-1* plants (in the WS background) homozygous for the reporter to the WS wild-type. Backcrosses of the *cca1-1 lhy-R* T1 lines 48, 50, and 51 to the WS wild-type were done using the former as pollen donors. After bioluminescence assays, plants were grown in growth chambers at 22°C under long-day conditions (LD [16:8]).

Supplementary Material

Supplementary Material including a description of the *LHY* RNAi cassette cloning and two additional figures showing *ccr2::luc* activity under continuous red and blue light and *TOC1* expression in LD cycles in wild-type, *cca1-1*, and *cca1-1 lhy-R* plants is available at http://images.cellpress.com/supmat/supmatin.htm.

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