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Overlapping and Distinct Roles of *PRR7* and *PRR9* in the *Arabidopsis* Circadian Clock

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Summary

The core mechanism of the circadian oscillators described to date rely on transcriptional negative feedback loops with a delay between the negative and the positive components [1–3]. In plants, the first suggested regulatory loop involves the transcription factors *CIRCADIAN CLOCK-ASSOCIATED 1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*) and the pseudo-response regulator *TIMING OF CAB EXPRESSION* 1 (*TOC1*/*PRR1*)[4]. *TOC1* is a member of the *Arabidopsis* circadian-regulated *PRR* gene family [5,6]. Analysis of single and double mutants in *PRR7* and *PRR9* indicates that these morning-expressed genes play a dual role in the circadian clock, being involved in the transmission of light signals to the clock and in the regulation of the central oscillator. Furthermore, *CCA1* and *LHY* had a positive effect on *PRR7* and *PRR9* expression levels, indicating that they might form part of an additional regulatory feedback loop. We propose that the *Arabidopsis* circadian oscillator is composed of several interlocking positive and negative feedback loops, a feature of clock regulation that appears broadly conserved between plants, fungi, and animals.

Results and Discussion

Loss of *PRR7* and *PRR9* Function Affects Free-Running Circadian Period

The effect of the mutations *prr7-3* ([7]; also known as *prr7-11*, [8]) and *prr9-1* [9] on the circadian clock were analyzed using the circadian reporter *CCR2::LUC* [5]. We confirmed that these mutations lengthen the period 1–2 hr under constant white light (Figure 1A [7–9]). This period defect of *prr7* mutants has been observed for several other T-DNA insertion lines (E.M.F. and S.A.K., unpublished data; [7]). In constant darkness, no change in period length of *CCR2::LUC* rhythms was observed in the *prr7-3* mutant (25.8 ± 0.7 hr [SEM], n = 15), as compared to the wild-type (25.7 ± 0.5 hr, n = 11; data not shown). This is also true of the *prr9-1*mutant [9], indicating that both PRR9 and PRR7 function in the transmission of light signals to the circadian clock.

The inverse relationship between light intensity and the period length of output rhythms has been well established [10]. Mutations in the light input pathway, for example, in the photoreceptors themselves, alter this relationship [11,12]. We measured the period length of the *prr7-3* and *prr9-1* mutants under several different light qualities and quantities. Loss of function of *PRR9* lengthens the free-running period under a wide range of red and blue light fluences (Figures 1B and 1C). In constant blue light, the fluence response curve for *prr9-1* resembles that of the blue light photoreceptor double-mutant *cyrtochrome1 cryptochrome2* (*cry1 cry2* [12]). Unlike *cry* mutants [12], *prr9-1* maintains a long period phenotype under high fluences of red light, suggesting that *PRR9* might act downstream of both blue and red light photoreceptors. The *prr7-3* mutant has a stronger phenotype under red light than under blue light (Figures 1B and 1C). The period length fluence response curve for *prr7-3* in red light resembles that of *phytochromeB* (*phyB*) mutants [11]. However, *prr7-3* but not the *phyB* null mutant retains a period lengthening effect under red light fluences lower than 10 μmol m^{-2} s^{-1}, suggesting that *PRR7* is not exclusively involved in the PHYB signaling pathway. In agreement with these results, *prr7* mutants display a long hypocotyl phenotype under constant red light [8, 13]. Nevertheless, since hypocotyl growth is partly regulated by the circadian clock [14], it remains unclear whether the hypocotyl length phenotypes observed in *prr7* mutants are caused by the circadian defect or by a direct involvement of PRR7 in light-regulated hypocotyl elongation. In summary, these results show that PRR7 and PRR9 play distinct roles in the light input pathway to the circadian clock. The biochemical activity of PRR7 and PRR9 is still unknown. Although no interactions with photoreceptors have been reported for these proteins, PRR9 has been shown to interact with TOC1 in a yeast two-hybrid assay [15]. In turn, TOC1 interacts in vitro with both PHYTOCHROME INTERACTING FACTOR 3 (PIF3), which has been implicated in phytochrome signaling [16–18], and other PIF3-like proteins [19]. However, the in vivo significance of such interactions remains to be investigated in detail.

Loss of both PRR7 and PRR9 Dramatically Affects Circadian Rhythms under Constant Light and Constant Darkness

To investigate the genetic interaction between the *prr7-3* and *prr9-1* mutations, we analyzed the circadian rhythms of the double mutant *prr7-3 prr9-1*. Leaf movement rhythms of *prr7-3 prr9-1* plants had a period length of 36.2 ± 1.7 hr in constant white light (Figure 1D, wild-type plants had a period of 24.3 ± 0.1 hr, *prr7-3* of 25.0 ± 0.2 hr, and *prr9-1* of 24.8 ± 0.2 hr). In agreement with the leaf movement analysis, *CCR2::LUC* rhythms in *prr7-3 prr9-1* displayed a period length of up to 35 hr under

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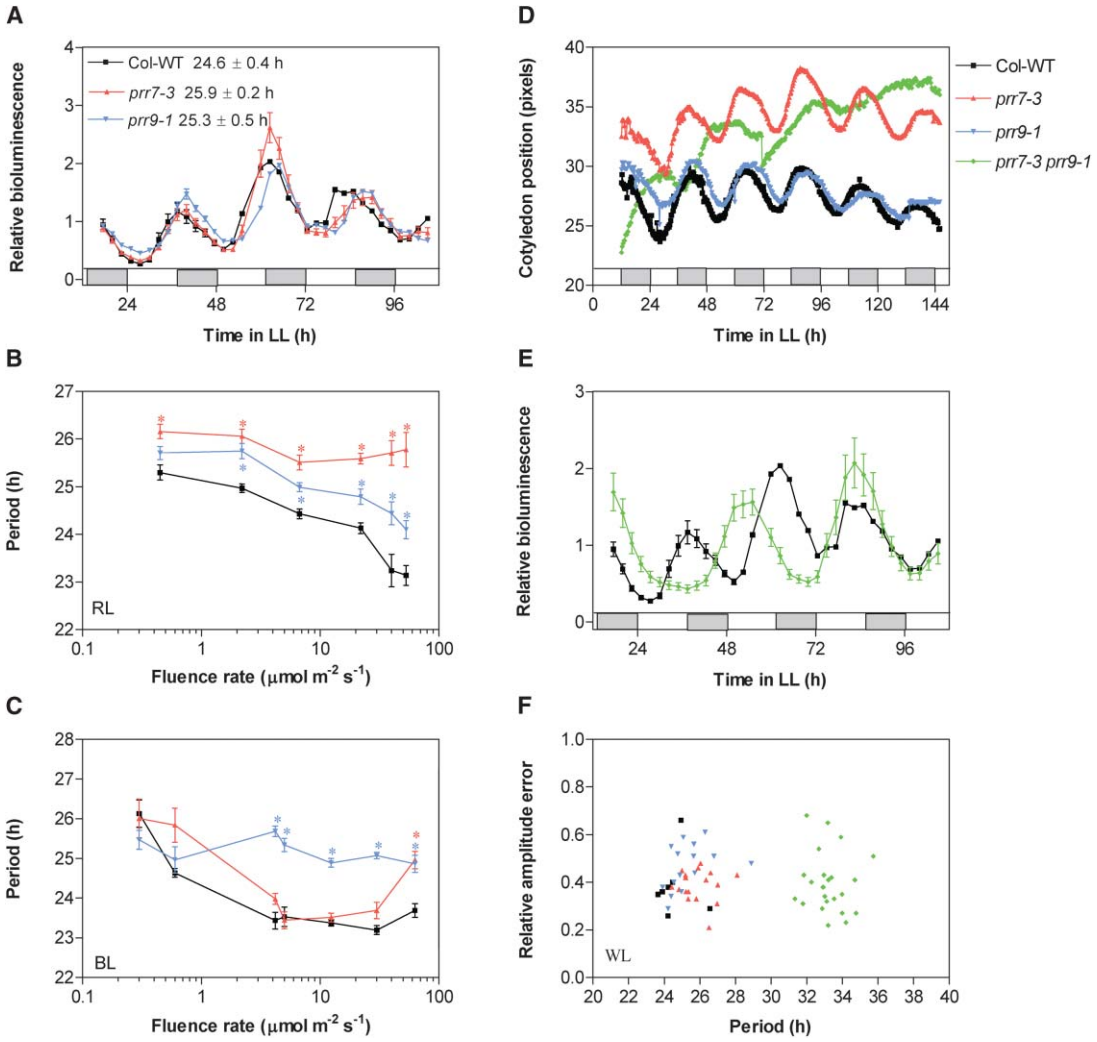

Figure 1. Period Length Effects of the *prr7-3*, *prr9-1*, and *prr7-3 prr9-1*

Seedlings were entrained in white light/dark cycles (12 hr, 70 μmol m^{-2} s^{-1}) for 5–7 days before being transferred to continuous light at CT 0. For bioluminescence assays, single seedlings were imaged every 2.5 hr for 5 days and data (± SEM) were normalized to the mean luminescence value of the respected genotype over the length of the time course. Period length (± SEM) and relative amplitude errors were estimated using fast Fourier transform-nonlinear least-squares analysis (FFT-NLLS) [36, 37].

(A) *CCR2:: LUC* bioluminescence rhythms in wild-type (Col-WT, n = 7), *prr7-3* (n =16), and *prr9-1* (n = 15) under continuous white light (70 μmol m^{-2} s^{-1}, LL). This experiment has been repeated 3 times with similar results.

(B) Period length of *CCR2:: LUC* in Col-WT (n = 5–10), *prr7-3* (n =11–12), and *prr9-1* (n = 10–14) under different fluences of red light. Representative of three independent trials. *, $P < 0.01$ (Student's two-tail t test).

(C) Period length of *CCR2:: LUC* in Col-WT (n = 6–12), *prr7-3* (n =8–12), and *prr9-1* (n = 6–15) under different fluences of blue light. Representative of three independent trials. *, $P < 0.01$ (Student's two-tail t test).

(D) Cotyledon movement rhythms under continuous white light (50 μmol m^{-2} s^{-1}, LL). The data represents the mean position of 19 cotyledons for wild-type (Col-WT), 17 for *prr7-3*, 9 for *prr9-1*, and 5 for *prr7-3 prr9-1*. This experiment has been repeated three times with similar results.

(E) *CCR2::LUC* bioluminescence rhythms in Col-WT (n = 7) and *prr7-3 prr9-1* (n = 25) under continuous white light (70 μmol m^{-2} s^{-1}, LL). This experiment has been repeated three times with similar results.

(F) Period and relative amplitude error estimates of the *CCR2::LUC* bioluminescence rhythms shown in (A) and (E).

constant white light conditions (Figure 1E). Despite this strong period defect, the bioluminescence rhythms remained robust in the double mutant, as observed by the low relative amplitude error values (Figure 1F). These results indicate that *PRR7* and *PRR9* play a partially redundant role in the *Arabidopsis* circadian clock.

The *prr7-3 prr9-1* mutant also displayed a long period phenotype under intermediate fluences of blue and red light (Figures 2A–2D). Under blue light, the relative amplitude errors were similar to wild-type and the single mutants (Figures 2C and 2D); however, under constant red light, *prr7-3 prr9-1* showed higher variability in period length and relative amplitude error (Figures 2A and 2B). Similar results were observed under low fluences of constant red or blue light (Figure S1 in the Supplemental Data available with this article online). Thus, in *prr7-3 prr9-1*, the circadian clock is more compromised under red than under blue light. This finding is in agreement

with the observed light dependencies of the single mutants (Figures 1B and 1C).

In the wild-type, *CCR2::LUC* rhythms remained robust in the dark for at least three days (Figures 2E and 2F). In contrast, in *prr7-3 prr9-1, CCR2* expression dampened after 1 day in constant darkness, although a weak rhythm with a free-running period of about 3 hr longer than in the wild-type could still be detected by FFT-NLLS analysis (Figures 2E and 2F). Rhythmic *CCA1* expression could be detected in the wild-type for the first 2 days after transfer to constant dark conditions (Figures 2G and 2H). However, loss of function of both *PRR7* and *PRR9* caused a rapid dampening of *CCA1::LUC*+ bioluminescence rhythms under constant darkness (Figures 2G and 2H). No damping of *CCA1::LUC*+ luminescence was observed under constant white, red, or blue light conditions in *prr7-3 prr9-1* (Figure S2). These results suggest that PRR7 and PRR9 play a dual role in the circadian clock, being involved in both light input and the central oscillator. The expression of *PRR9* is very low in the dark [20]; therefore, it is difficult to understand a robust role of *PRR9* in this condition. A similar situation has been observed for *CCA1* and *LHY*. Although these genes are expressed at very low levels in constant dark conditions [21], loss of function of both *CCA1* and *LHY* severely disrupts circadian rhythms in constant darkness [22]. *ZEITLUPE* (*ZTL*) and *TOC1* also play a dual role in the circadian clock. In addition to showing circadian defects in constant darkness, *ztl* and *toc1* mutants display light quantity and quality circadian defects, respectively [23–25]. This supports the notion that perception and transmission of ambient light conditions are closely tied with the generation and maintenance of rhythms in *Arabidopsis*.

Expression Level of Clock Regulated Genes in the Double Mutant *prr7-3 prr9-1*

To study how *PRR7* and *PRR9* affect the circadian clock at the molecular level, we analyzed the expression levels of the putative core clock components (*CCA1*, *LHY*, and *TOC1)* and the other two members of the circadian controlled *PRRs* (*PRR3* and *PRR5*) in *prr7-3*, *prr9-1*, and the double mutant *prr7-3 prr9-1*. The expression level of these genes was measured in light/dark cycles and for 3 days after release into constant white light conditions (Figures 3A–3F and Figure S3). After the third day in constant light, the peaks of expression had shifted about 4 hr in *prr7-3* and *prr9-1* and 12–15 hr in *prr7-3 prr9-1*. This corresponds to a period length difference of about 1 hr for the single mutants and 4–5 hr for the double mutant, demonstrating the pervasiveness of the circadian defects caused by the loss of function of both *PRR7* and *PRR9*.

The *prr9-1* mutation caused a slight increase in the maximum level of expression of *TOC1*, in both light/dark and constant light conditions. However, neither loss of function of *PRR7* nor loss of both *PRR7* and *PRR9* caused significant changes in mean *TOC1* expression levels (Figures 3E and 3F). No major change in the expression level of *PRR3* or *PRR5* was observed under constant light conditions (Figure S3). Loss of function of *PRR7* caused an earlier rise in *CCA1* and *LHY* expression in the dark and a slight increase in the amplitude of *CCA1* and *LHY* expression in constant light conditions (Figures 3A and 3C). It has been reported that the *prr7-1* and *prr7-2* mutations display an earlier rise in the second peak of *CCA1* and *LHY* expression in etiolated seedlings after transfer to red light [13]. Taken together, these results suggest that PRR7 plays an inhibitory role on the regulation of *CCA1* and *LHY* expression levels. Although the *prr9-1* mutant showed no significant increase in *CCA1* and *LHY* RNA levels (Figures 3A and 3C), the double mutant *prr7-3 prr9-1* displayed a second peak of expression before dusk and a further enhancement of *CCA1* and *LHY* expression in constant light (Figures 3B and 3D). This increase in *CCA1* and *LHY* RNA levels may be the cause of the long period phenotype in the double mutant. *CCA1* and *LHY* mutants display a short period phenotype of about 3 hr [26, 27]. Strong overexpression of *CCA1* or *LHY* causes arrythmicity in constant conditions and a lagging phase of gene expression under light/dark cycles [21, 28, 29]. However, it is still unclear whether a small increase in CCA1 or LHY could lengthen the period under constant conditions. *PRR7* and *PRR9* RNA levels peak when *CCA1* and *LHY* expression decreases, thus PRR7 and PRR9 might be directly involved in the inhibition of *CCA1* and *LHY* expression.

CCA1 and LHY Act Positively on *PRR7* and *PRR9* Expression

We investigated the role of the transcription factors CCA1 and LHY in the expression of *PRR7* and *PRR9*. Expression levels were measured in the *cca1-1* mutant [26], the line *cca1-1 lhy-R* (50) [22, 25], in which *LHY* expression level is also reduced, and in the lines *cca1-ox* (34) [29], and *lhy-1* [21], which overexpress either *CCA1* or *LHY* under the strong *CaMV 35S* promoter. The peak of *PRR9* expression is dramatically reduced in *cca-1 lhy-R* (Figure 4A), and light induction of *PRR9* expression in dark-adapted plants is also greatly reduced, but still detectable, in this line (Figure S4). This last result suggests the existence of two mechanisms for the light induction of *PRR9* expression, one dependent upon and one independent of CCA1 and LHY. Mutation of both *CCA1* and *LHY* did not influence *PRR7* expression as strongly as it affected *PRR9* expression (Figure 4D). Since the *cca1-1 lhy-R* mutant displayed only a minor reduction in *PRR7* levels, other factors must be involved in the activation of *PRR7*. Presumably these factors also generate the delay in the peak of expression of *PRR7* relative to *PRR9*. Supporting the role of CCA1 and LHY as positive factors, the average expression of *PRR7* and *PRR9* was significantly increased in both the *CCA1* and *LHY* overexpressing lines (Figures 4B–4C and 4E–4F). However, there were differences in the amplitude and phase of *PRR7* and *PRR9* expression between *lhy-1* and *cca1-ox*. Although the phase delay in *lhy-1* has also been observed for other genes [28], there are no significant differences in phase or amplitude of gene expression observed in the single loss of function mutants [27]. Different degrees of overexpression, protein levels, activation states, and/or functions might explain the observations. For example, it has been shown that LHY but not CCA1 protein levels cycle in the overexpressing lines [28, 29].

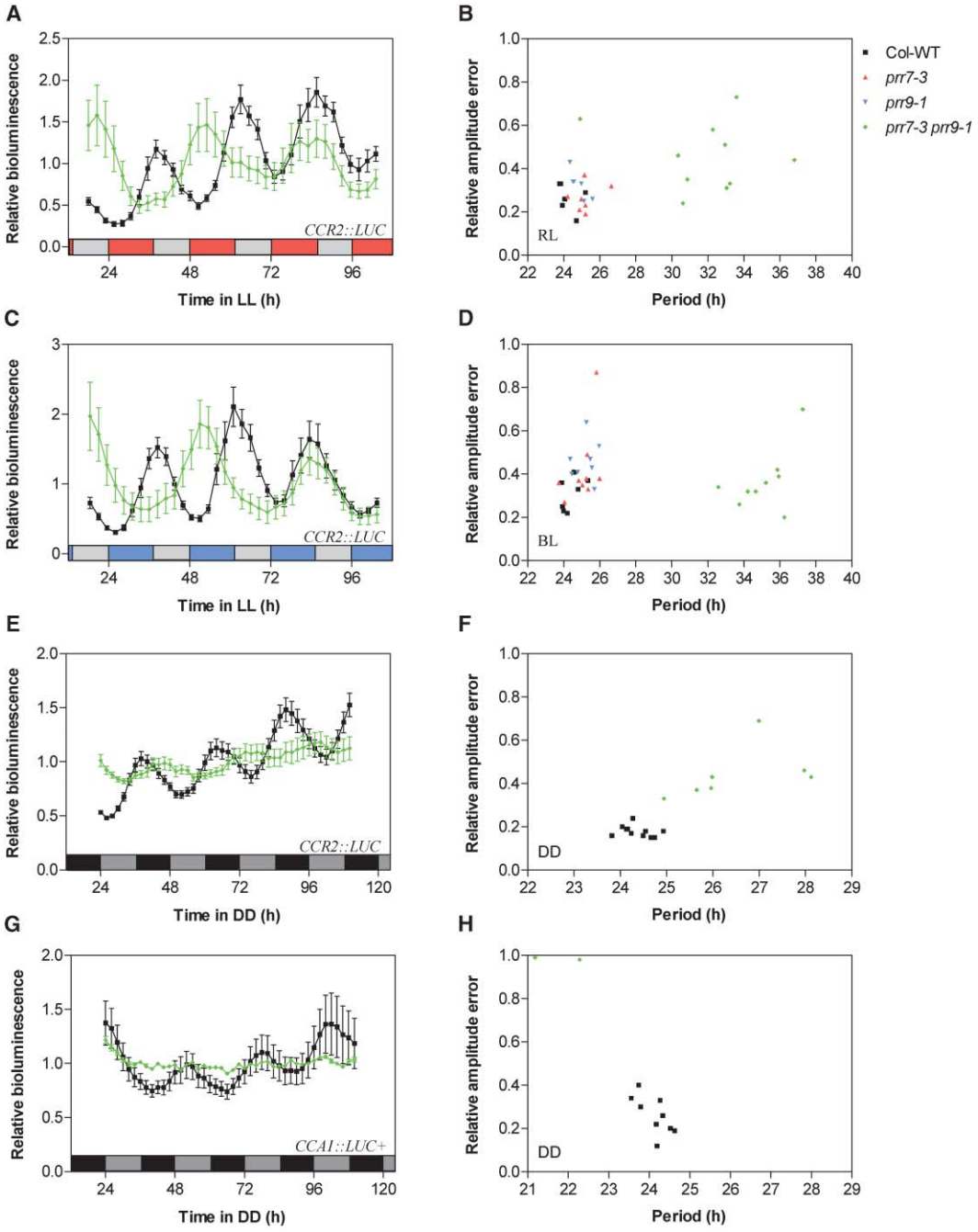

Figure 2. Period Length Effects of *prr7-3 prr9-1* on Bioluminescence Rhythms under Constant Red and Blue Light and Constant Darkness

Seedling entrainment and constant light bioluminescence assays (A–D) were performed as in Figure 1. For the constant darkness experiments (E–H) seedlings were transferred to constant conditions at CT 12 and were imaged every 2 hr in clusters of 6–9 seedlings. Period length and relative amplitude errors were estimated as in Figure 1.

(A) *CCR2::LUC* bioluminescence rhythms in wild-type (Col-WT, n = 6), and *prr7-3 prr9-1* (n = 11) plants in continuous red light (44 μmol m^{-2} s^{-1}, LL).

(B) Period and relative amplitude error estimates of the *CCR2::LUC* bioluminescence rhythms shown in (A) in addition to *prr7-3* (n = 6) and *prr9-1* (n = 6).

(C) *CCR2::LUC* bioluminescence rhythms in Col-WT (n = 7) and *prr7-3 prr9-1* (n = 8) under constant blue light (41 μmol m^{-2} s^{-1}, LL).

(D) Period and relative amplitude error estimates of the *CCR2::LUC* bioluminescence rhythms shown in (C) in addition to *prr7-3* (n = 10), and *prr9-1* (n = 9).

(E) *CCR2::LUC* bioluminescence rhythms under constant darkness (DD). Wild-type (Col-WT, 12 clusters of 6 seedlings) and *prr7-3 prr9-1* seedlings (7 clusters of 9 seedlings).

(F) Period and relative amplitude error estimates of the *CCR2::LUC* bioluminescence rhythms shown in (E).

(G) *CCA1::LUC+* bioluminescence rhythms under constant darkness. Wild-type (Col-WT, 9 clusters of 6 seedlings) and *prr7-3 prr9-1* seedlings (8 clusters of 9 seedlings).

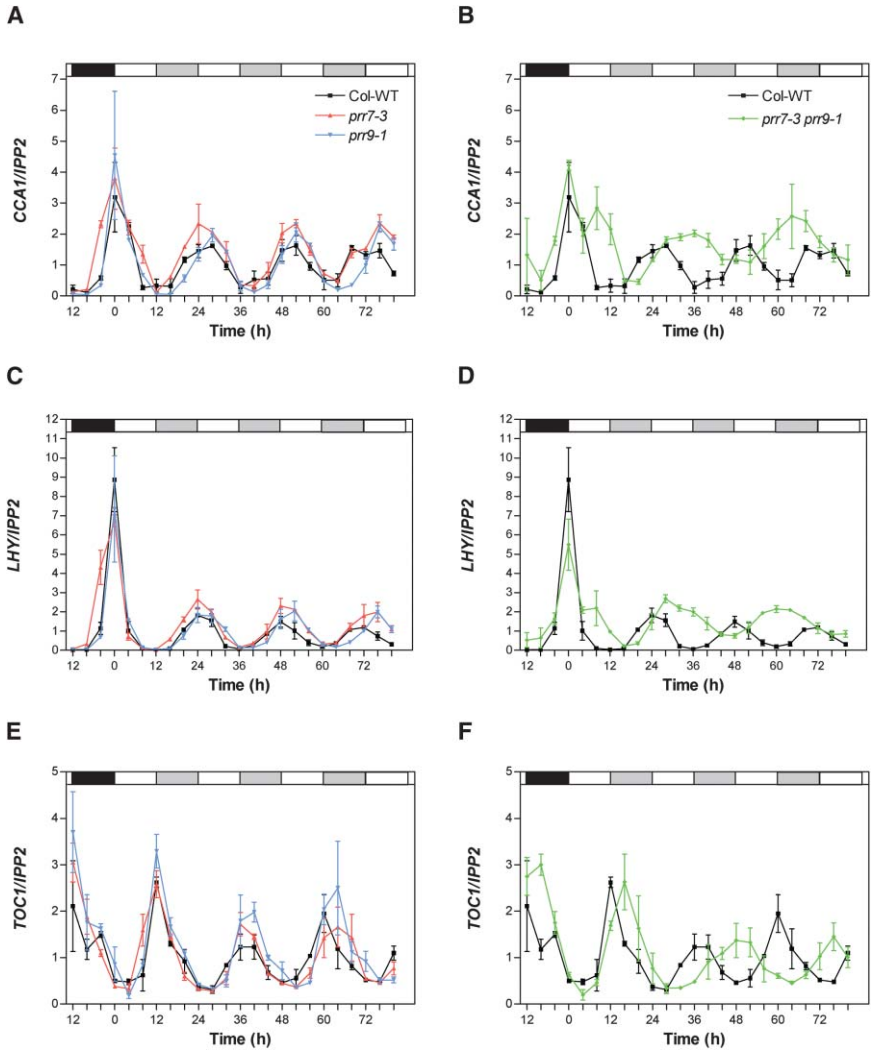

Figure 3. Mutations in *PRR7* and *PRR9* Alter the Cycling of Circadian-Regulated Genes

Seedlings were grown for 15 days in white light/dark cycles (12 hr, 70μmol m^{-2} s^{-1}) and sampled every 4 hr during one light/dark cycle and for 3 days after release into constant white light (70 μmol m^{-2} s^{-1}). The cycling of *CCA1* (A, B), *LHY* (C, D), and *TOC1* (E, F) was analyzed by real-time PCR after reverse transcription as described in the Experimental Procedures section. Values are expressed relative to *IPP2* loading control and normalized to the mean expression level of the wild-type for each gene. Data represents the average ± SEM of two completely independent experiments. Col-WT, *prr7-3*, *prr9-1*, and *prr7-3 prr9-1* were analyzed in parallel. For clarity, the traces were plotted in different graphs: (A), (C), and (E) for Col-WT, *prr7-3*, and *prr9-1*; (B), (D), and (F) for Col-WT and *prr7-3 prr9-1*.

PRR7 and *PRR9* contain promoter elements that may confer direct regulation by CCA1 and LHY. *PRR7* has three CCA1 binding sites (CBS), which are also found in the promoters of the chlorophyll a/b binding protein genes [30], in the region −918/−816 bases from the transcription start site. The *PRR9* promoter contains an evening element (EE) 223 bases before the transcriptional start site. This element has been found in several circadian regulated genes with evening expression, and, in the case of *TOC1*, has been shown to mediate repression by CCA1 and LHY [4]. We investigated whether CCA1 was able to bind these elements in the context of the *PRR7* and *PRR9* promoters by performing electrophoretic mobility shift assays. Addition of extracts from *Escherichia coli* expressing glutathione *S*-transferase (GST)-CCA1 to a probe corresponding to the −918/−816 region of the *PRR7* promoter produced DNA species with retarded mobility (Figure 4G). DNA fragments containing 2 (−871/−816, −918/−841) or 3 (−918/−816) CBS were able to compete for binding. However, a frag-

(H) Period and relative amplitude error estimates of the *CCA1::LUC*+ bioluminescence rhythms shown in (G). The FFT-NLLS program failed to detect a rhythm with a period ranging between 15 and 45 hr for 8 of the 10 seedling clusters of *prr7-3 prr9-1*.

These experiments have been repeated twice with similar results. In (A) and (C), *prr7-3* and *prr9-1* were analyzed in parallel with Col-WT and *prr7-3 prr9-1*, but for clarity the bioluminescence traces of *prr7-3 CCR2::LUC* and *prr9-1 CCR2::LUC* were omitted.

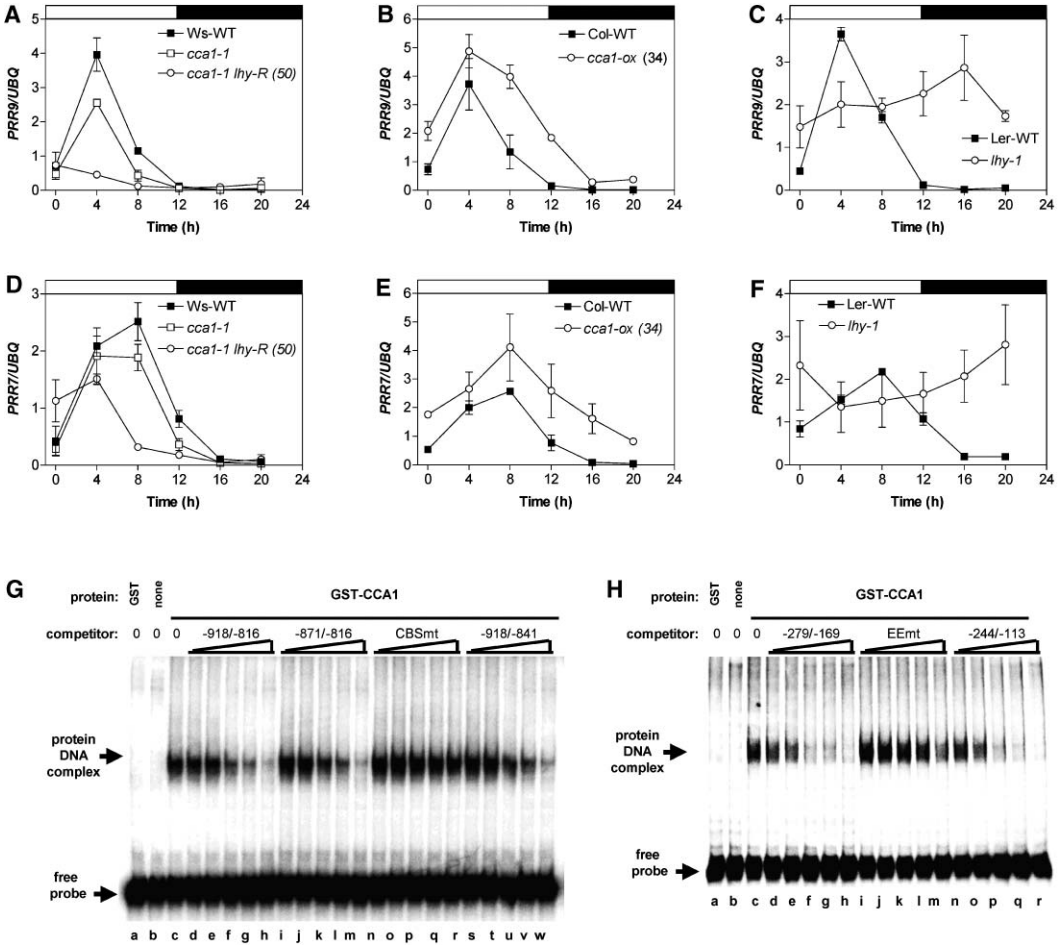

Figure 4. CCA1 and LHY Regulate *PRR7* and *PRR9* Expression

(A–F) Wild-type (Col-WT, Ws-WT, Ler-WT), *cca1-1*, *cca1-1 lhy-R (50)*, *cca1-ox (34)*, and *lhy-1* were grown for 15 days in white light/dark cycles (12 hr, 70 μmol m^{-2} s^{-1}) and sampled every 4 hr during one light/dark cycle. *PRR9* (A–C) and *PRR7* (D–F) RNA levels were analyzed via semiquantitative RT-PCR as described in the Experimental Procedures section. Values are expressed relative to the ubiquitin (*UBQ*) loading control and normalized to the average expression level of the wild-type in each experiment. Data represents the average ± SEM of two completely independent experiments.

(G-H) Cell extracts from bacteria expressing either GST-CCA1 or GST were incubated with a radiolabeled fragment of the *PRR7* promoter (G) or *PRR9* promoter (H). 5×, 10×, 50×, 100×, or 500× M excess of unlabeled competitor DNA was added to each reaction as indicated. Protein/DNA complexes were separated by nondenaturing gel electrophoresis and visualized using a phosphorimager. Specificity of binding is shown by the ability of fragments with wild-type CBS (G) or EE (H) sequences, but not fragments in which these sequences are altered, to compete for binding to GST-CCA1. These experiments have been repeated three times with similar results.

ment corresponding to the −871/−816 region with both CBS mutated (CBSmt) failed to compete, indicating that CCA1 binding to this region was specific for the CBS. Similarly, GST-CCA1, but not GST alone, was able to bind to the −279/−169 region of *PRR9* (Figure 4H). This binding was effectively competed by two different fragments of the PRR9 promoter containing the EE (−279/−169 and −244/−113). Mutation of the EE (EEmt) abrogated the ability of the −279/−169 fragment to compete, demonstrating that CCA1 bound specifically to the EE. This ability of CCA1 to specifically bind *PRR7* and *PRR9* promoter elements, and the fact that CCA1 and LHY protein levels peak a few hours before *PRR7* and *PRR9* RNA levels, suggest that CCA1 and LHY directly regulate *PRR7* and *PRR9* transcription. These transcription factors are able to activate (this study; [26, 30]) or repress transcription of target genes [4]. Although no transcription factor with such dual function has been described in animal circadian systems, it has been shown that members of the nuclear orphan receptor gene family in mammals [31, 32], and basic leucine zipper transcription factors [33] in flies act as either positive or negative elements in circadian interlocking feedback loops.

The results presented here show that PRR7 and PRR9 play overlapping and distinct roles in the circadian clock, being not only involved in the light input pathway but also in the central oscillator. In addition, as is seen for *TOC1*, their expression is regulated by CCA1 and LHY, indicating that *PRR7* and *PRR9* might form additional regulatory feedback loops.

Experimental Procedures

Plant Materials and Growth Conditions

The *Arabidopsis thaliana aprr7-3* and *prr9-1* T-DNA insertion lines in the Colombia-0 (Col) background were isolated from the Salk Collection (http://signal.salk.edu; *aprr7-3* is SALK_030430, *prr9-1* is

SALK_07551). Homozygous T-DNA insertion mutants were identified from segregating F3 by PCR amplification of the T-DNA flanking regions. We did not detect full-length RNA of the corresponding gene in either mutant by Northern blot analysis (data not shown), and therefore, *prr7-3* and *prr9-1* are loss of function mutations. The double mutant *prr7-3 prr9*-1 was generated using the *prr9-1* homozygous mutant as pollen donor. Seedlings were grown on Murashige and Skoog medium [34] with 0.8% agar and 3% sucrose (MS).

Analysis of Circadian Rhythms

Arabidopsis plants homozygous for the *prr7-3*, *prr9-1*, and *prr7-3 prr9-1* mutation were transformed with the *CCR2::LUC* reporter [5] by *Agrobacterium tumefaciens*-mediated DNA transfer [35]. Wild-type (Col) and *prr7-3 prr9-1* were also transformed with the *CCA1::LUC*+ reporter (−319/−848, where +1 denotes the translational start site; see Supplemental Material). T2 seedlings for *prr7-3* and *prr9-1* and T1 seedlings for *prr7-3 prr9-1* were grown for 6 days in light/dark cycles (12 hr light, 12 hr dark; 70 μmol m^{-2} s^{-1}) on gentamycin (75 μg ml^{-1}) MS agar plates. Data on *prr7-3* and *prr9-1* represent the average of seedlings from 2–3 independent transformed lines. Resistant seedlings were transferred to MS plates without selection for analysis. Bioluminescence rhythms of single seedlings under constant light conditions were analyzed as previously described [36]. Expression in constant darkness was imaged and analyzed using the Night Owl imaging system and Win Light software (Perkin-Elmer). For these experiments, groups of seedlings were imaged together. For leaf movement analysis, time-lapse video imaging was carried out in constant white light (50 μmol m^{-2} s^{-1}), and the vertical position of the cotyledons was determined using the Kujata imaging software. Period length and relative amplitude errors were estimated using FFT-NLLS program [36, 37]. The relative amplitude error is a measure of rhythmic strength that varies between 0 (perfect fitted rhythm) and 1 (rhythm not significant).

Expression Analysis by Reverse Transcriptase-Mediated PCR

RNA was extracted using the RNeasy Plant Mini Kit according to the manufacturer's recommendations (Qiagen). For reverse transcriptase-mediated PCR, SuperScript II RNase H^- reverse transcriptase (Invitrogen) was used to synthesize the first-strand cDNA with oligo (dT12-18) primer (Invitrogen) from 2 μg of total RNA at 42°C for 50 min. The cDNA was diluted five times with water and 2 μl were used for PCR amplification. In Figures 4A–4F, RNA was amplified for 20 cycles for *UBQ* and 25 cycles for *PRR9* and *PRR7*. PCR products were detected by Southern blot using standard methodology and quantified using a Phosphorimager (Typhoon 8600, Molecular Dynamics) in the exponential range of amplification. The blots were probed with randomly ^{32}P-labeled DNA probes generated by PCR amplification of cDNA using the same primers used for amplification. Gene expression data were represented relative to average value for the wild-type in each experiment, after normalization to the control *UBQ*. In Figures 3 and S3 and Figure S4, RNA was detected using a real-time PCR detection system (MyiQ single-color real time PCR detection system, Bio-Rad). The gene *IPP2* (isopentenyl pyrophosphate:dimethylallyl pyrophosphate isomerase, AT3G02780) was used as normalization control. The expression of this gene does not cycle in constant light, constant dark, or light/dark cycles. Each PCR reaction was repeated twice. Gene expression data were represented relative to average value for the wild-type in each experiment, after normalization to the control *IPP2*. All experiments were performed twice with completely independent samples. Primers and probes are described in Supplemental Material available on line.

Electrophoretic Mobility Shift Assay

Gel retardation assays were performed using extracts of *E. coli* induced to express either GST or GST-CCA1 as the source of recombinant protein. Saturated cultures of BL21 harboring the pGEX-4T1 and pGEX-CCA1 plasmids were diluted 1:100 in M9 media, grown at 37°C to an OD_{600} of 0.1, and IPTG was added to 0.1 mM. After an additional 4 hr at 37°C, the cultures were harvested and resuspended (resuspension buffer = 20 mM HEPES [pH 7.2], 75 mM KCl, 10% glycerol, 0.1 mM EDTA, 0.1 mM PMSF, 2.5 mM DTT, 1× protease inhibitor cocktail [Roche]) and the cells disrupted using a probe sonicator. After a high-speed spin, the supernatants were collected, aliquoted, frozen in liquid nitrogen, and stored at −80°C. The probes and competitor DNA fragments used in the assay were generated by PCR using cloned promoter fragments as templates. For gel shift assays, cell extract containing approximately 1 fmol of GST-CCA1 (or GST) was incubated with 8 fmol of the appropriate radiolabeled probe in reaction buffer (20 mM HEPES [pH 7.2], 80 mM KCl, 0.1 mM EDTA, 10% glycerol, 2.5 mM DTT, 0.07 μg μl^{-1} BSA, 8 ng μl^{-1} poly dI-dC) and the appropriate unlabeled competitor DNA (competitor DNA was added at 5, 10, 50, 100, and 500 M excess over labeled probe, as indicated). Reactions were incubated for 15 min at room temperature and then resolved by electrophoresis on 5% nondenaturing polyacrylamide gels. After drying, gels were imaged using a PhosphorImager (Storm, Molecular Dynamics). Generation of constructs and probes are described in Supplemental Data.

Supplemental Data

Supplemental data are presented in four figures and an extended Experimental Procedures section available with this article online at http://www.current-biology.com/cgi/content/full/15/1/47/DC1.

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