The Plant Cell, Vol. 22: 594–605, March 2010, www.plantcell.org

PSEUDO-RESPONSE REGULATORS 9, 7, and 5 Are Transcriptional Repressors in the *Arabidopsis* Circadian Clock [W][OA]

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An interlocking transcriptional-translational feedback loop of clock-associated genes is thought to be the central oscillator of the circadian clock in plants. TIMING OF CAB EXPRESSION1 (also called PSEUDO-RESPONSE REGULATOR1 [PRR1]) and two MYB transcription factors, CIRCADIAN CLOCK ASSOCIATED1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), play pivotal roles in the loop. Genetic studies have suggested that PRR9, PRR7, and PRR5 also act within or close to the loop; however, their molecular functions remain unknown. Here, we demonstrate that PRR9, PRR7, and PRR5 act as transcriptional repressors of *CCA1* and *LHY*. PRR9, PRR7, and PRR5 each suppress *CCA1* and *LHY* promoter activities and confer transcriptional repressor activity to a heterologous DNA binding protein in a transient reporter assay. Using a glucocorticoid-induced PRR5-GR (glucorticoid receptor) construct, we found that PRR5 directly downregulates *CCA1* and *LHY* expression. Furthermore, PRR9, PRR7, and PRR5 associate with the *CCA1* and *LHY* promoters in vivo, coincident with the timing of decreased *CCA1* and *LHY* expression. These results suggest that the repressor activities of PRR9, PRR7, and PRR5 on the *CCA1* and *LHY* promoter regions constitute the molecular mechanism that accounts for the role of these proteins in the feedback loop of the circadian clock.

INTRODUCTION

The circadian clock controls endogenous biological rhythms that allow a wide range of organisms to adapt to 24-h day-night cycles (Young and Kay, 2001). In eukaryotes, a transcription-translation feedback loop connecting clock-associated genes is thought to form the central oscillator (or core) of the clock (Bell-Pedersen et al., 2005). In *Arabidopsis thaliana*, reciprocal transcriptional regulation between *TIMING OF CAB EXPRESSION1* (*TOC1*; also called *PSEUDO-RESPONSE REGULATOR1* [*PRR1*]) and *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*) has been proposed as the main feedback loop. CCA1 and LHY proteins are MYB transcription factors and repress *TOC1* transcription by binding directly to the *TOC1* promoter around dawn (Alabadi et al., 2001; Mizoguchi et al., 2002; Perales and Mas, 2007). TOC1 protein is expressed in the evening and, in turn, activates *CCA1* expression, partly by antagonizing a transcriptional repressor of *CCA1*, CCA1 HIKING EXPEDITION (CHE) of the TCP (for TEOSINTE BRANCHED1, CYCLOIDEA, and PCF) family (Pruneda-Paz et al., 2009). In addition to *TOC1*, numerous genetic studies have demonstrated the importance of other *PRR* genes in the circadian clock (Eriksson et al., 2003; Kaczorowski and Quail, 2003; Michael et al., 2003; Yamamoto et al., 2003; Farre et al., 2005; Nakamichi et al., 2005; Salome and McClung, 2005; Para et al., 2007; Ito et al., 2009).

The *Arabidopsis PRR* gene family consists of five members (*PRR9*, *PRR7*, *PRR5*, *PRR3*, and *TOC1*), all of which are regulated by the circadian clock, but each of which peaks at different times of the day. *PRR9* mRNA levels are greatest at dawn, *PRR7* peaks in the morning, *PRR5* around noon, and *PRR3* and *TOC1* in the evening (Matsushika et al., 2000). Not surprisingly, the PRR proteins also peak at different times of the day (Mas et al., 2003; Farre and Kay, 2007; Ito et al., 2007; Kiba et al., 2007; Fujiwara et al., 2008). PRR9, PRR7, and PRR5 have a redundant function, but because they are expressed at different times of the day, they are collectively essential to proper timekeeping. This is demonstrated by the loss of rhythmicity in the *prr9 prr7 prr5* triple loss-of-function mutant (Nakamichi et al., 2005). *CCA1* and *LHY* are constitutively expressed at a high level, and *TOC1* is expressed at a low level in the triple mutant, suggesting that PRR9, PRR7, and PRR5 regulate the circadian clock by downregulating *CCA1* and *LHY* expression and by upregulating *TOC1* expression (Nakamichi et al., 2005). Closing the feedback loop, CCA1 and LHY activate transcription of *PRR9* and *PRR7* by directly binding to their promoters (Farre et al., 2005). Although this regulatory framework connecting *PRR9*, *PRR7*, and *PRR5* with *CCA1*, *LHY*, and *TOC1* has been proposed (Farre et al., 2005; Nakamichi et al., 2005; Niwa et al., 2007; Ito et al., 2008, 2009), the molecular mechanism of this interaction remains undetermined.

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www.plantcell.org/cgi/doi/10.1105/tpc.109.072892

PRR proteins feature a pseudoreceiver domain at the N terminus and a CCT (for CONSTANS, CONSTANS-LIKE, and TOC1) motif at the C terminus (Makino et al., 2000; Strayer et al., 2000). The pseudoreceiver domain is similar to the receiver domain of a two-component response regulator, but the key Asp residue that accepts a phosphoryl group from a sensory kinase is not conserved (Makino et al., 2000). The CCT motif is thought to be involved in protein–protein interactions (Wenkel et al., 2006). Although PRR proteins are localized in the nucleus, their exact molecular function is still unknown (Makino et al., 2000; Strayer et al., 2000; Matsushika et al., 2007b; Fujiwara et al., 2008).

In this study, we describe the transcriptional repressor activity of PRR9, PRR7, and PRR5. In addition, PRR9, PRR7, and PRR5 associate with the promoter regions of *CCA1* and *LHY* in vivo, coincident with the timing of decreased *CCA1* and *LHY* expression. These results suggest that PRR9, PRR7, and PRR5 proteins are major transcriptional repressors of *CCA1* and *LHY* and are thus essential for proper clock function.

RESULTS

PRR9, PRR7, and PRR5 Downregulate *CCA1* and *LHY* in *Arabidopsis* Seedlings

To examine whether PRR9, PRR7, and PRR5 regulate *CCA1*, *LHY*, and *TOC1*, at the transcriptional level, we conducted transient assays using reporter plasmids harboring luciferase (*LUC*) under the control of the *CCA1*, *LHY*, or *TOC1* promoters (*CCA1pro:LUC*, *LHYpro:LUC*, or *TOC1pro:LUC*) and effector plasmids harboring *PRR9*, *PRR7*, or *PRR5* genes fused to a gene encoding cyan fluorescent protein (CFP) or a negative control containing CFP only, all under the control of the cauliflower mosaic virus (CaMV) *35S* promoter (*35Spro:PRR9-CFP*, *PRR7-CFP*, *PRR5-CFP*, or *CFP*). We confirmed that the *CCA1*, *LHY*, and *TOC1* promoters generate rhythmic patterns of luciferase activity in the appropriate phases (see Supplemental Figure 1 online). Reporter and effector plasmids were codelivered into cells of *Arabidopsis* seedlings by particle bombardment, and relative bioluminescence of the LUC reporter was measured.

Bombardment with *35Spro:PRR9-CFP*, *35Spro:PRR7-CFP*, or *35Spro:PRR5-CFP* resulted in ~80% reduction in the LUC bioluminescence of *CCA1pro:LUC* and 70% reduction of *LHY-pro:LUC* compared with bombardment of the *35Spro:CFP* control (Figures 1A and 1B). By contrast, *35Spro:PRR9-CFP*, *35Spro:PRR7-CFP*, or *35Spro:PRR5-CFP* did not cause a significant change in the bioluminescence of *TOC1pro:LUC* (*t* test, $P > 0.05$; Figure 1C), suggesting that PRR9, PRR7, and PRR5 each downregulate promoter activity of *CCA1* and *LHY* but act differently toward *TOC1.*

CCA1 and *LHY* Are Immediate Targets of PRR5

To determine whether PRR9, PRR7, and PRR5 directly regulate *CCA1*, *LHY*, and *TOC1* expression, we generated transgenic plants expressing a chimeric fusion protein of PRR5, the hormone binding domain of mouse glucocorticoid receptor (GR), and CFP under the control of the CaMV *35S* promoter (*35Spro:PRR5-GR-CFP*). Such a GR fusion protein becomes biologically functional in the presence of the glucocorticoid steroid hormone dexamethasone (DEX) in plants (Aoyama et al., 1995; Sablowski and Meyerowitz, 1998). This approach is also used to identify the direct target genes of a GR fusion protein (Sablowski and Meyerowitz, 1998).

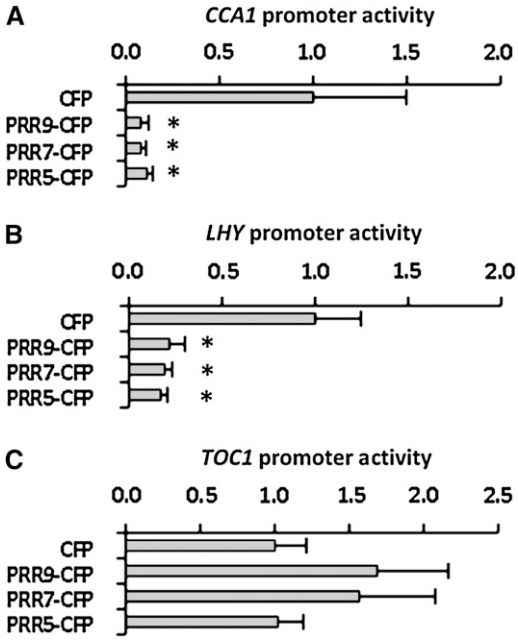

Figure 1. Effect of PRR9, PRR7, or PRR5 on Promoter Activity of *CCA1*, *LHY*, and *TOC1*.

Promoter activities with coexpression of CFP, PRR9-CFP, PRR7-CFP, or PRR5-CFP. Promoter activities are shown relative to values obtained with coexpression of CFP alone. Error bars indicate the SD (biological replicates, $n = 6$). Asterisks indicate values that are statistically different from the CFP control (Student's *t* test; $P < 0.05$). Each experiment was performed twice with similar results.

(A) *CCA1* promoter activity.

(B) *LHY* promoter activity.

(C) *TOC1* promoter activity.

Transgenic *Arabidopsis* seedlings overexpressing *PRR5* produce shorter hypocotyls than the wild type (Sato et al., 2002). Therefore, we first tested whether PRR5-GR-CFP protein becomes biologically active in the presence of DEX by measuring hypocotyl lengths of *PRR5-GR-CFP*–overexpressing plants (*35Spro:5GC*). Two independent *35Spro:5GC* lines had significantly shorter hypocotyls under DEX-treated conditions than under DEX-free conditions (Figure 2A), confirming that the PRR5-GR-CFP protein is biologically functional with DEX treatment.

CCA1, *LHY*, and *TOC1* expression in *35Spro:5GC* plants grown in constant white light conditions for 2 weeks after germination was measured by quantitative PCR (qPCR) with or without a 4-h incubation in DEX. DEX treatment resulted in a 50% decrease in *CCA1* and *LHY* expression but a 20% increase in *TOC1* expression (Figure 2B). To examine whether PRR5 regulates *CCA1*, *LHY*, and *TOC1* through de novo biosynthesis of an intermediary protein, *35Spro:5GC* plants were cotreated with DEX and the translational inhibitor cycloheximide (CHX). DEX

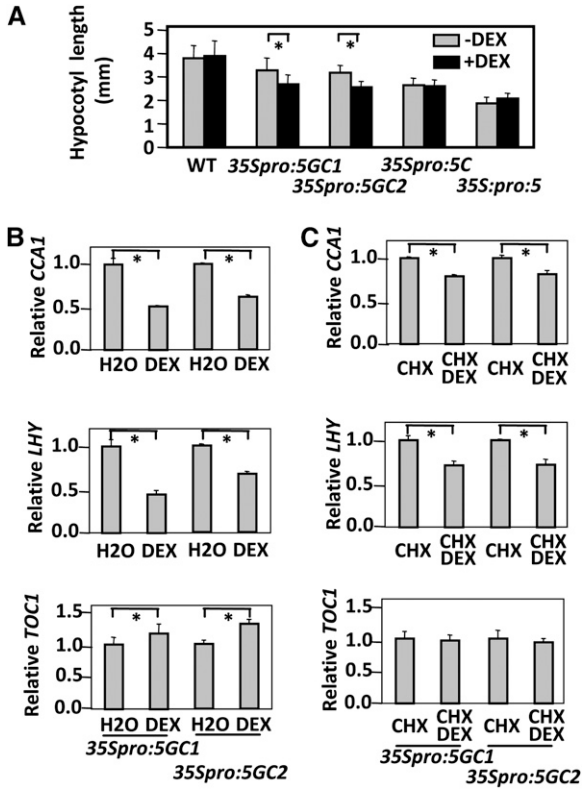

Figure 2. *CCA1*, *LHY*, and *TOC1* Expression in *35Spro:PRR5-GR-CFP*.

(A) Average hypocotyl lengths of 8-d-old *Arabidopsis* seedlings of the wild type, two independent lines of *35Spro::PRR5-GR-CFP* (*35Spro:5GC1* and *35Spro:5GC2*), *35Spro:PRR5-CFP* (*35Spro:5C*), and *35Spro:PRR5* (*35Spro:5*) grown under 10-h-light/14-h-dark conditions with or without DEX (SD; biological replicates, $n = 15$).

(B) *CCA1*, *LHY*, and *TOC1* expression in *35Spro:5GC* seedlings upon DEX treatment. Relative levels of each mRNA to *APX3* mRNA (internal control) under DEX-free conditions were set to 1.0.

(C) *CCA1*, *LHY*, and *TOC1* expression in *35Spro:5GC* seedlings treated with CHX with or without DEX. Relative levels of each mRNA to *APX3* mRNA in 100 μM CHX solution were set to 1.0. Error bars indicate SD (technical replicates, $n = 3$). Each experiment was performed twice with similar results. Asterisks indicate values that are statistically different between –DEX and +DEX (*t* test; $P < 0.05$).

combined with CHX resulted in a 20 to 30% decrease in *CCA1* and *LHY* expression, compared with CHX treatment alone (Figure 2C). However, cotreatment with DEX and CHX did not cause any significant change in *TOC1* expression, compared with CHX treatment alone (Figure 2C, bottom). These results suggest that PRR5 downregulates *CCA1* and *LHY* without de novo biosynthesis of other proteins but upregulates *TOC1* indirectly.

PRR9, PRR7, and PRR5 Have Transcriptional Repressor Activity

To test whether PRR9, PRR7, and PRR5 possess transcriptional repressor activity, transient reporter assays were conducted by codelivering, into *Arabidopsis* seedlings, a *GAL4pro:LUC* reporter plasmid (Fujimoto et al., 2000) and an effector plasmid, each harboring a gene for the GAL4 DNA binding domain (GAL4DB) fused to *PRR9*, *PRR7*, or *PRR5* under the control of the CaMV *35S* promoter (*35Spro:GAL4DB-PRR9*, *35Spro:GAL4DB-PRR7*, or *35Spro:GAL4DB-PRR5*). Expression of GAL4DB-PRR9, GAL4DB-PRR7, and GAL4DB-PRR5 resulted in decreased activity of the GAL4pro:LUC reporter by about half (Figure 3A), indicating that PRR9, PRR7, and PRR5 confer repression activity on GAL4DB.

To clarify which specific region of these PRRs was responsible for the repressor activity, we introduced into *Arabidopsis* seedlings several effector plasmids harboring a series of truncated PRR5 clones fused to GAL4DB (Figure 3B, left). We found that effector plasmids covering the CCT domain (#2) as well as the C-terminal part of the intervening region between pseudoreceiver and CCT (#5) did not cause significant reductions in GAL4pro:LUC reporter activity. However, seedlings expressing effector plasmids that covered a 44–amino acid peptide from the intervening region (#1, 3, 4, and 6) showed reduced reporter activity by about half (Figure 3B), indicating that this region is sufficient to confer repressor activity on GAL4DB. Comparison of this 44amino acid region with those of PRR7, PRR9, and putative orthologs from other plant species lead to the identification of two relatively conserved motifs, $L(^{E}/_{D})(^{L}/_{I})S(^{L}/_{I})(^{R}/_{K})R$ and $SXXSAF(^{S}/_{T})(^{R}/_{Q})(^{Y}/_{F})$. The $SXXSAF(^{S}/_{T})(^{R}/_{Q})(^{Y}/_{F})$ motif was found in PRR3 and TOC1, whereas $L(^{E}/_{D})(^{L}/_{I})S(^{L}/_{I})(^{R}/_{K})R$ was not (data not shown). In addition, $L(^{E}/_{D})(^{L}/_{I})S(^{L}/_{I})(^{R}/_{K})R$ resembles the ETHYLENE RESPONSE FACTOR (ERF)-associated amphiphilic repression (EAR) motif $[(^{L}/_{F})DLN(^{L}/_{F})XP]$ (Ohta et al., 2001).

To determine which motif is responsible for the repressor activity, we generated an effector plasmid expressing the 22–amino acid region harboring LDLSLRR, but not SSASAFTRY (plasmid #7), and another effector plasmid expressing the 25–amino acid region harboring SSASAFTRY, but not LDLSLRR (plasmid #8). Bombardment with these effectors did not result in significant reduction of GAL4pro:LUC reporter activity compared with GAL4DB alone (Figure 3D), indicating that the 44–amino acid region harboring both of the motifs is required for the repressor activity of PRR5. Furthermore, effector plasmids that covered corresponding regions from PRR7 and PRR9 (GAL4DB-PRR7#6 and GAL4-DB-PRR9#6) showed reduced reporter activity by about half (Figure 3E), suggesting that regions harboring both $L(^{E}/_{D})(^{L}/_{I})S(^{L}/_{I})(^{R}/_{K})R$ and $SXXSAF(^{S}/_{T})(^{R}/_{Q})(^{Y}/_{F})$ in PRR9, PRR7, and PRR5 are critical for the repression activity.

To assess whether recruitment to DNA is required for the repression activity of PRR5, GAL4DB-PRR5 or PRR5 was coexpressed with the GAL4pro:LUC reporter. Expression of GAL4DB-PRR5 reduced GAL4pro:LUC activity, whereas PRR5 or CFP did not (Figure 3F). This result suggests that recruitment of PRR5 to a certain DNA region is sufficient to repress transcription from the DNA region.

PRR9, PRR7, and PRR5 Associate with *CCA1* and *LHY* Promoter Regions in Vivo

To investigate whether PRR9, PRR7, and PRR5 associate with promoters of *CCA1* and *LHY* in vivo, we generated transgenic lines expressing FLAG-GUS-GFP (for fusion protein of FLAG, β-glucuronidase, and green fluorescent protein) or

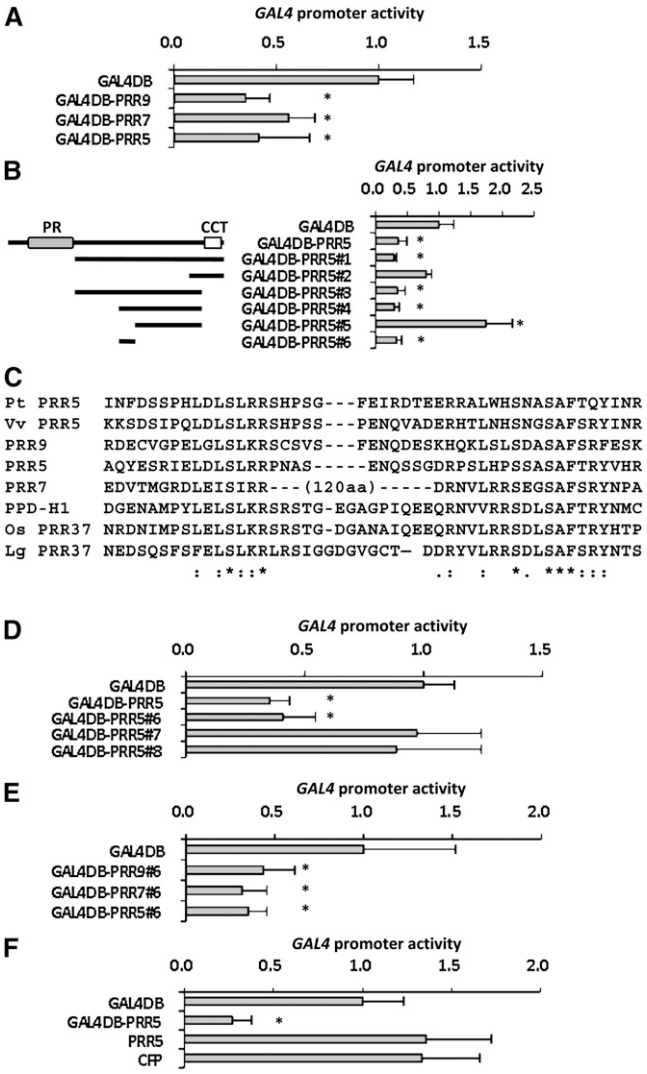

Figure 3. PRR9, PRR7, and PRR5 Act as Transcriptional Repressors.

(A) *GAL4* promoter activity when coexpressed with the GAL4 DNA binding domain (GAL4DB) fused to PRR9, PRR7, or PRR5. *GAL4* promoter activities are shown relative to values obtained with coexpression of GAL4DB alone.

(B) *GAL4* promoter activity when coexpressed with the GAL4DB fused to truncated PRR5 constructs #1 to #6 (right). Schematics of truncated PRR5 constructs fused to GAL4DB (left). PR, pseudoreceiver domain; CCT, CCT motif.

(C) Amino acid sequence alignment of the conserved region in PRR proteins from various plants. The 44–amino acid sequence of PRR5#6 was used for alignment. Asterisks denote amino acids conserved in all the sequences; colons denote similar amino acids. For species information, see the Accession Numbers section in Methods.

(D) *GAL4* promoter activity when coexpressed with the GAL4DB fused to the different motifs in the 44–amino acid region of PRR5.

(E) *GAL4* promoter activity when coexpressed with the GAL4DB fused to corresponding regions in PRR7 and PRR9 described in Figure 3C.

(F) *GAL4* promoter activity determination after coexpression of PRR5 full-length or GAL4DB-PRR5. Error bars indicate SD (biological replicates, $n = 6$). Asterisks indicate values that are statistically different from control (*t* test; $P < 0.05$). Each experiment was performed twice with similar results.

FLAG-PRR-GFP under control of their native *PRR* promoters in a *prr* mutant (*PRR9pro:FLAG-GUS-GFP* in *prr9* [*9pro:FGG*], *PRR9pro:FLAG-PRR9-GFP* in *prr9* [*9pro:F9G*], *PRR7pro:FLAG-GUS-GFP* in *prr7* [*7pro:FGG*], *PRR7pro:FLAG-PRR7-GFP* in *prr7* [*7pro:F7G*], *PRR5pro:FLAG-GUS-GFP* in *prr5* [*5pro:FGG*], and *PRR5pro:FLAG-PRR5-GFP* in *prr5* [*5pro:F5G*]) and performed chromatin immunoprecipitation (ChIP) assays using these lines. To test biological functionality of F9G, F7G, and F5G proteins expressed under a native promoter, we examined the circadian rhythm of *CCA1* and *TOC1* expression in *9pro:F9G*, *7pro:F7G*, and *5pro:F5G*. Each FLAG-PRR-GFP partially (if not fully) complemented the circadian period phenotype of the parental mutant (see Supplemental Figure 2 online), confirming that F9G, F7G, and F5G proteins are biologically functional.

The plants were grown under a 12-h-light/12-h-dark (LD) cycle, and samples were collected when PRR protein levels are at a maximum (i.e., for *9pro:FGG* and *9pro:F9G*, Zeitgeber time 6 [ZT6, 6 h after light on]; for *7pro:FGG* and *7pro:F7G*, ZT6; for *5pro:FGG* and *5pro:F5G*, ZT10). We then analyzed, by qPCR, four different target amplicons from the regions around *CCA1* and *LHY* (#1 to 4 and 5 to 8, respectively), an amplicon located upstream of *TOC1* (# 9), and an amplicon located upstream of *ASCORBATE PEROXIDASE3* (*APX3*; # 10) (Figure 4A). Precise positions of amplicons are shown in Supplemental Figure 3 online. The amplicons 2, 3, 7, and 9 were from the promoter regions used for the transient assays in Figure 1. Amplicons 2 and 3 contain a G-box DNA *cis*-acting element (Schindler et al., 1992) and a TCP binding site (TBS) (Pruneda-Paz et al., 2009), respectively (Figure 4A). Amplicon 7 has three 5A motifs (Spensley et al., 2009) and a G-box. Amplicon 9 has an evening element (Harmer et al., 2000).

Amplicons 2, 3, 6, and 7 were significantly enriched in *9pro:F9G* plants in comparison to the other amplicons (Figure 4B). ChIP analyses for *7pro:F7G* and *5pro:F5G* also indicated a significant enrichment of amplicons 2, 3, 6, and 7 (Figured 4C and 4D). As expected, no significant enrichment was found on any amplicons in the control lines *9pro:FGG*, *7pro:FGG*, and *5pro:FGG*, showing that PRR9, PRR7, and PRR5 specifically associate with *CCA1* and *LHY* promoter regions in vivo. To further test the specificity of the association, protein-DNA complexes from *prr5-11* (Yamamoto et al., 2003) and *35S:PRR5* (Sato et al., 2002) seedlings were used in a ChIP analysis, in which nontagged PRR5 protein was immunoprecipitated with anti-PRR5 antibody (Figure 4E). The same set of amplicons was enriched in *35Spro:PRR5* but not in *prr5-11*. These results show that PRR9, PRR7, and PRR5 are able to associate with the promoter regions of *CCA1* and *LHY* in vivo. Although several known *cis*-acting elements (G-box, TBS, and 5A) exist within these regions, the resolution of our ChIP analyses was too low to specify exactly the binding site of PRR9, PRR7, and PRR5.

PRR9, PRR7, and PRR5 Associate with the Promoter Regions of *CCA1* and *LHY* during the Period from Morning until Midnight

Since PRR9, PRR7, and PRR5 expression is mostly limited to finite but partially overlapping portions of the diurnal cycle, we investigated the levels of association between these proteins and their target promoter regions by analyzing *9pro:F9G*, *7pro:*

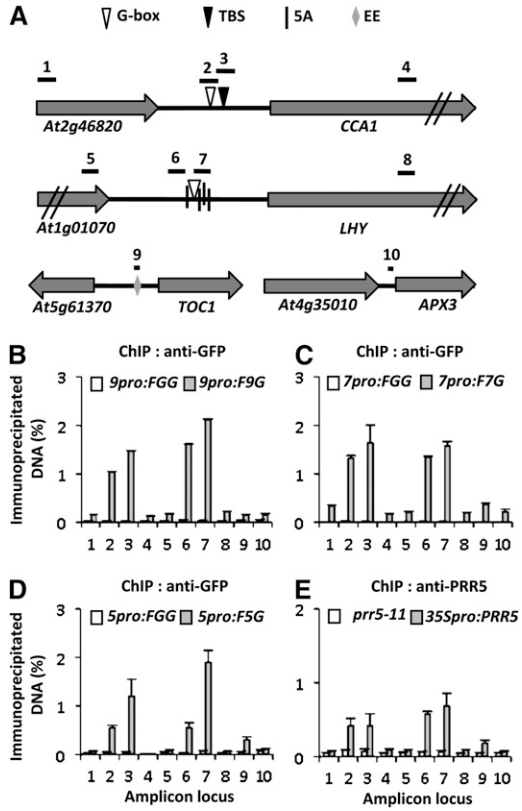

Figure 4. PRR9, PRR7, and PRR5 Associate with *CCA1* and *LHY* Promoters in Vivo.

(A) Schematics of *CCA1*, *LHY*, *TOC1*, and *APX3* loci and the locations of the target DNA fragments (amplicons) used in the ChIP assays. Positions of the 10 amplicons are shown as short horizontal black bars. Arrows indicate full-length coding sequences, with the ATG (translation initiation codon) being located at the tail of the arrow. The open triangle, closed triangle, black line, and diamond are G-box, TBS, 5A, and evening element (EE), respectively.
(B) to **(D)** ChIP assays, with the percentage of DNA fragments coimmunoprecipitated with anti-GFP antibody relative to input DNA presented.
(B) ChIP assays for *PRR9pro:FLAG-GUS-GFP* (*9pro:FGG*) and *PRR9pro:FLAG-PRR9-GFP* (*9pro:F9G*) seedlings
(C) ChIP assays for *PRR7pro:FLAG-GUS-GFP* (*7pro:FGG*) and *PRR7pro:FLAG-PRR7-GFP* (*7pro:F7G*) seedlings
(D) ChIP assays for *PRR5pro:FLAG-GUS-GFP* (*5pro:FGG*) and *PRR5pro:FLAG-PRR5-GFP* (*5pro:F5G*) seedlings
(E) ChIP assays for *prr5-11* and *35Spro:PRR5* etiolated seedlings. Five-day-old etiolated seedlings exposed to white light for 10 h were used. Immunoprecipitation was performed with anti-PRR5 antibody. Error bars indicate SD (technical replicates, $n = 3$). Each experiment was performed twice with similar results.

F7G, and *5pro:F5G* plants grown under LD cycles. The plants were harvested at 2-h intervals from ZT0.

F9G protein levels peaked from ZT2 to ZT6 (Figure 5A, left panel), and the protein became associated with *CCA1* and *LHY* promoters during the same timeframe (Figure 5B, left panels). F7G protein peaked at around ZT8 to ZT12, but its association with *CCA1* and *LHY* promoters was biphasic, with peaks at ZT6 and ZT14 (Figures 5A and 5B, middle panels). F5G protein both peaked and became associated with the *CCA1* and *LHY* promoters between ZT8 to ZT16 (Figures 5A and 5B, right panels). These results suggest a correlation between the timing of PRR9, PRR7, and PRR5 expression and the level of association between each PRR protein with the *CCA1* and *LHY* promoters. Furthermore, our results show that, from ZT2 to ZT16 (morning to midnight), at least one of these proteins is present on the *CCA1* and *LHY* promoters.

We next measured *CCA1* and *LHY* mRNA levels to quantify the effect of PRR9, PRR7, and PRR5 on *CCA1* and *LHY* transcription in vivo. *CCA1* and *LHY* mRNA expression decreased from ZT0 to ZT6, remained at a low level until ZT18, and started to increase at ZT20 (Figure 5C). As shown above, PRR9, PRR7, and PRR5 were expressed and associated with the promoter regions of *CCA1* and *LHY* from ZT2 to ZT16 (Figures 5A and 5B). Therefore, the association patterns of PRR9, PRR7, and PRR5 with the *CCA1* and *LHY* promoters are inversely correlated with the expression of *CCA1* and *LHY*, suggesting that PRR9, PRR7, and PRR5 repress *CCA1* and *LHY* expression in vivo.

Sequential Expression of PRR9, PRR7, and PRR5 Is Required for Repression of *CCA1* and *LHY* from Morning until Midnight

To understand the significance of the sequential expression of PRR9, PRR7, and PRR5 for regulation of *CCA1* and *LHY* transcription, we analyzed the expression patterns of PRR9 and PRR5 proteins and also monitored *CCA1* and *LHY* levels in *prr7 prr5* and *prr9 prr7* double mutants (Farre et al., 2005; Nakamichi et al., 2005; Salome and McClung, 2005). For detection of native PRR9, we developed an anti-PRR9 antibody and confirmed its specificity (see Supplemental Figure 4 online).

In *prr7 prr5* mutants, PRR9 protein accumulated from ZT2 to ZT10, and *CCA1* and *LHY* mRNA expression was minimal from ZT4 to ZT10 (Figure 6B). However, at ZT14, PRR9 protein levels were undetectable, and 2 h later (ZT16), *CCA1* and *LHY* expression began to increase. This inverse correlation between PRR9 levels and *CCA1* and *LHY* transcription suggests that PRR9 represses *CCA1* and *LHY* to a certain level in the *prr7 prr5* mutant but is not sufficient to repress their expression during nighttime.

In the *prr9 prr7* double mutant, PRR5 protein accumulated from ZT10 to ZT18, and *CCA1* and *LHY* expression decreased from ZT12 to ZT18 (Figure 6C). PRR5 protein was not detectable either from ZT0 to ZT8 or from ZT20 to ZT22. *CCA1* and *LHY* expression levels were relatively high from ZT0 to ZT10 and from ZT20 to ZT22 (Figure 6C). These patterns suggest that PRR5 alone can account for the repression of *CCA1* and *LHY* transcription during part of the night, but, without PRR9 and PRR7, this effect is not extended to the daytime. Collectively, the sequential expression of PRR9, PRR7, and PRR5 is important to propagate a proper waveform, long-duration repression state of *CCA1* and *LHY* expression.

DISCUSSION

Transcriptional Repressor Activities of PRR9, PRR7, and PRR5

In this study, we identified PRR9, PRR7, and PRR5 as active transcriptional repressors of *CCA1* and *LHY*. An active

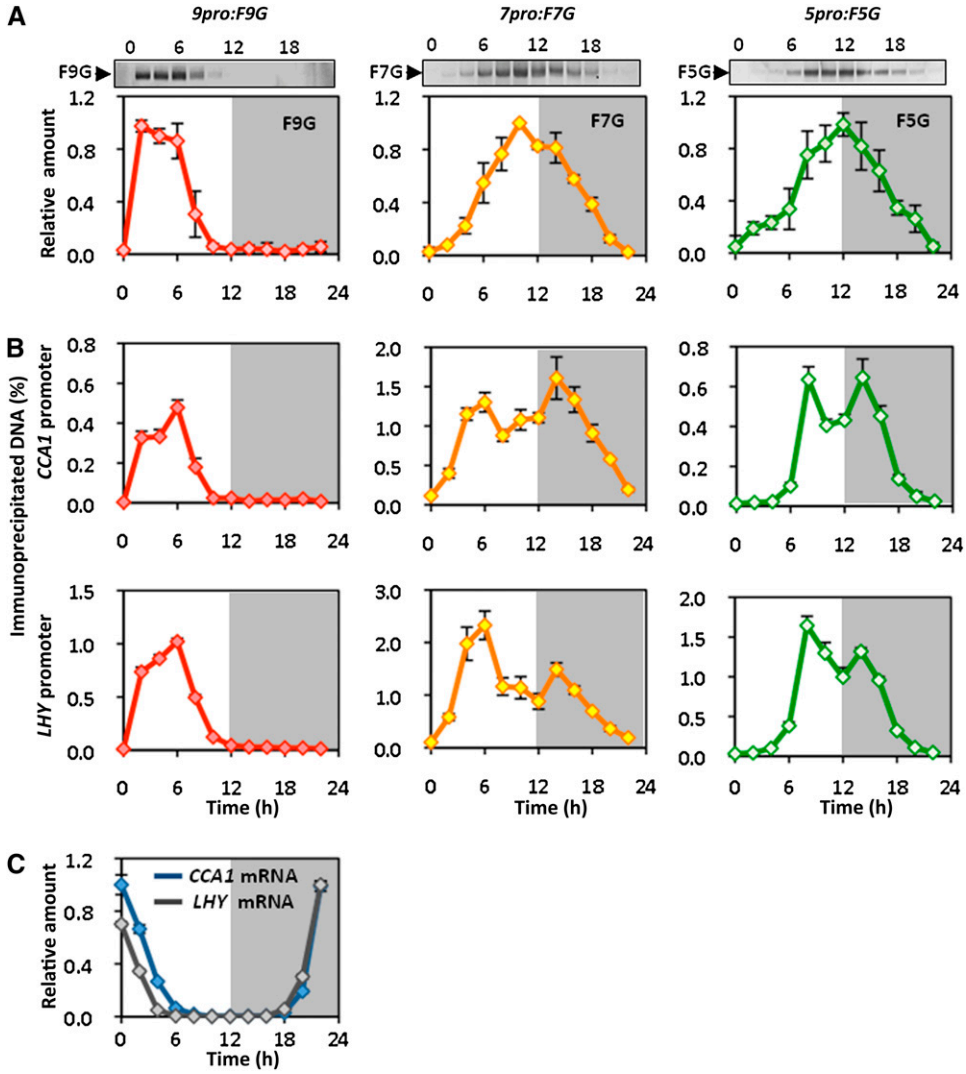

Figure 5. Association Patterns of PRR9, PRR7, and PRR5 with the Promoter Regions of *CCA1* and *LHY* in 12-h-Light/12-h-Dark Conditions.

(A) F9G, F7G, and F5G protein levels in 12-h-light/12-h-dark conditions. *9pro:F9G*, *7pro:F7G*, and *5pro:F5G* plants were grown in 12-h-light/12-h-dark conditions for 2 weeks and cross-linked at 2-h intervals starting at ZT0 (light on). Total protein was immunoblotted by anti-FLAG antibody (top panel). F9G, F7G, and F5G protein amounts normalized with the total protein (bottom). Peak levels were set to 1.0. White and gray areas represent white light and dark conditions, respectively.

(B) Percentages of the amplicon 3 of *CCA1* region (top) and amplicon 7 *LHY* region (bottom) coimmunoprecipitated with anti-GFP antibody relative to input DNA in *9pro:F9G*, *7pro:F7G*, and *5pro:F5G* plants were plotted.

(C) *CCA1* and *LHY* mRNA expression in *5pro:F5G* plants. Expression levels were determined relative to *APX3* mRNA. Peak levels were set to 1.0. Error bars indicate SD (technical replicates, $n = 3$). Each experiment was performed twice with similar results.

transcriptional repressor generally refers to a repressor that contains intrinsic repression domains and has the ability to inhibit transcription via the action of these domains (Ohta et al., 2001). In fact, we found that in PRR9, PRR7, and PRR5, a conserved amino acid sequence between the pseudoreceiver domain and CCT motif (Figure 3C) harboring both $L(^{E}/_{D})(^{L}/_{I})S(^{L}/_{I})(^{R}/_{K})R$ and $SXXSAF(^{S}/_{T})(^{R}/_{Q})(^{Y}/_{F})$ motifs is sufficient for repressor activity (Figure 3E). Interestingly, the $L(^{E}/_{D})(^{L}/_{I})S(^{L}/_{I})(^{R}/_{K})R$ sequence resembles the EAR motif $[(^{L}/_{F})DLN(^{L}/_{F})XP]$ (Ohta et al., 2001), which is conserved in ERF transcriptional repressors. Under our experimental conditions, however, LDLSLRR of PRR5 alone could not confer repression activity (Figure 3D), suggesting that flanking sequences of LDLSLRR are also required.

Although the PRR proteins lack typical DNA binding domains, and no direct interaction between PRR9, PRR7, or PRR5 and upstream regions of *CCA1* or *LHY* was observed in a yeast one-hybrid system (N. Nakamichi, T. Kiba, and H. Sakakibara, unpublished data), our ChIP analysis indicated that PRR9, PRR7, and PRR5 proteins associate with promoter regions of *CCA1* and *LHY* in vivo (Figure 4). We cannot exclude the possibility of direct

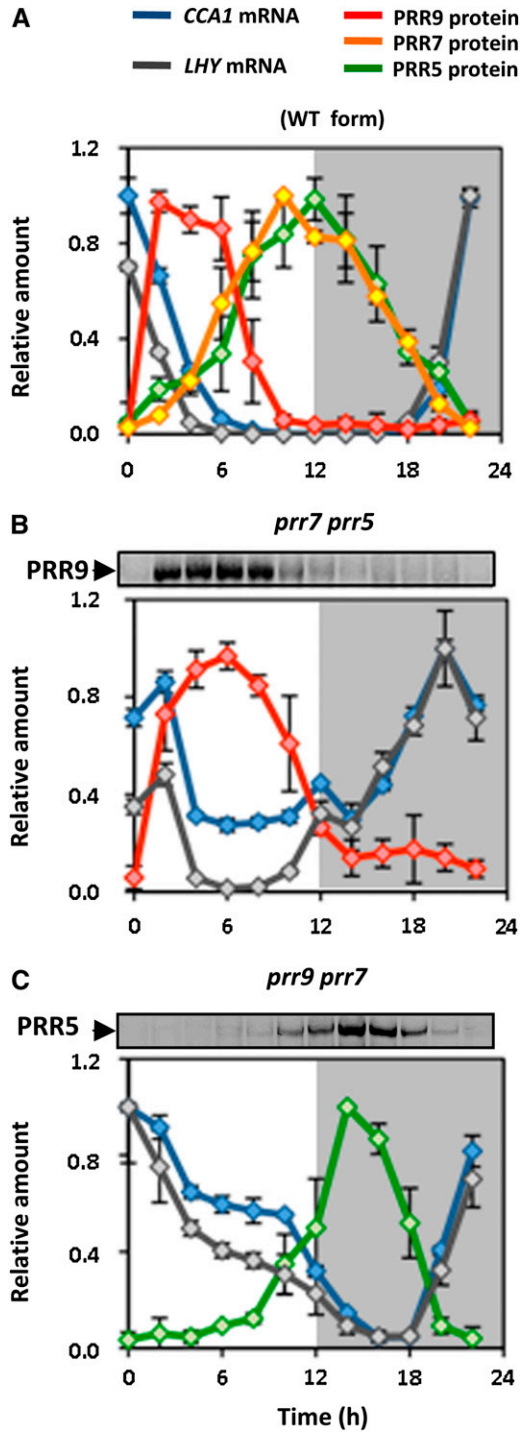

Figure 6. Expression Patterns of PRR9 and PRR5 Proteins, and *CCA1* and *LHY* Expression in *prr7 prr5* and *prr9 prr7* Mutants.

(A) The wild-type waveforms of PRR9, PRR7, and PRR5 protein levels and *CCA1* and *LHY* mRNA expression. These data were also presented in Figures 5A and 5C.

(B) PRR9 protein levels and *CCA1* and *LHY* mRNA expression in the *prr7 prr5* double mutant. The amount of PRR9 protein was normalized with total protein, and the amounts of *CCA1* and *LHY* mRNA were normalized with *APX3* mRNA (SD; technical replicates, $n = 3$). Each experiment was performed twice with similar results.

(C) PRR5 protein levels and *CCA1* and *LHY* mRNA expression in the *prr9 prr7* mutant. The amount of PRR5 protein was normalized with total protein, and the amounts of *CCA1* and *LHY* mRNA were normalized with *APX3* mRNA (SD; technical replicates, $n = 3$). Each experiment was performed twice with similar results. In all panels, peak levels were set to 1.0, and white and gray areas represent white light and dark conditions, respectively.

interaction between PRR9, PRR7, and PRR5 and DNA; however, it would also be possible that an unknown molecule could link PRR9, PRR7, or PRR5 to DNA in vivo. Because the ChIP procedure involves cross-linking, PRR9, PRR7, and PRR5 may associate with these DNA regions through various protein–protein interactions. The CHE protein was proposed as a candidate for bridging TOC1 (PRR1) and the upstream region of *CCA1* (Pruneda-Paz et al., 2009). However, CHE does not bind to the upstream region of *LHY* in yeast (Pruneda-Paz et al., 2009), whereas PRR9, PRR7, and PRR5 associate with the upstream region of *LHY* in planta (Figures 4 and 5). Furthermore, there is no obvious epistatic interaction on *LHY* expression between *PRR9* or *PRR7* and *TOC1* (Ito et al., 2009), implying that PRR9, PRR7, and PRR5 associate with the *LHY* promoter in some way other than in the CHE-TOC1 complex. Although the exact binding mechanism of PRR9, PRR7, and PRR5 to target DNA needs to be determined in future studies, some posttranslational regulation of PRR7 and PRR5 might be involved in the promoter binding mechanism, since protein peaks and promoter-association peaks were not exactly the same for PRR7 and PRR5 (Figure 5).

Matsushika et al. (2007b) showed that overexpression of the C-terminal region (containing both the intervening region and the CCT motif) of PRR5 causes downregulation of *CCA1* but that neither the intervening nor the CCT motif alone could do so, suggesting that cooperation between the two of them is essential (Matsushika et al., 2007b). Since recruitment of PRR5 to the DNA molecule is required for repression (Figure 3E), and the CCT motif is implicated in protein–protein interactions (Wenkel et al., 2006), PRR9, PRR7, and PRR5 may be recruited to the promoter region via such protein–protein interactions and subsequently repress target gene transcription with the repression motif. We cannot exclude the possibility of a molecular function other than transcriptional repression for PRR9, PRR7, and PRR5. However, constant high expression levels of *CCA1* and *LHY* and many day-peaked genes in a *prr9 prr7 prr5* triple mutant (Nakamichi et al., 2005, 2009) suggest that the repressor activity of these proteins is essential to rhythmic expression of their target genes.

PRR9, PRR7, and PRR5 Shape the Waveform of *CCA1* and *LHY* Expression

Sequential expression of *PRR9*, *PRR7*, and *PRR5* under diurnal conditions has attracted the attention of investigators since its discovery (Matsushika et al., 2000). Our time-course ChIP analysis demonstrated that transcriptional repressors PRR9, PRR7, and PRR5 continuously and sequentially associate with the promoters of *CCA1* and *LHY* from morning till midnight (~16 h),

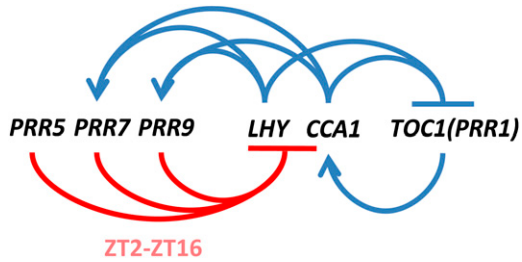

Figure 7. *Arabidopsis* Clock Model Incorporating the Transcriptional Repressors PRR9, PRR7, and PRR5.

CCA1 and LHY repress *TOC1* transcription (blue bar) by binding to the *TOC1* promoter. In turn, TOC1 activates *CCA1* expression (blue arrow) by antagonizing CHE1, the repressor of *CCA1*. As a new addition to this circuit (red), PRR9, PRR7, and PRR5 proteins repress *CCA1* and *LHY* transcription directly from morning until midnight (ZT2 to ZT16). CCA1 and LHY proteins activate *PRR9* and *PRR7* transcription.

exactly the duration when *CCA1* and *LHY* are repressed (Figure 5). Expression of PRR9 and PRR5 coincides with *CCA1* and *LHY* repression in the *prr7 prr5* and *prr9 prr7* mutants, respectively (Figure 6). The duration of *CCA1* and *LHY* repression is shorter (~6 h) in these *prr* double mutants than in the wild type, supporting the notion that sequential expression of PRR9, PRR7, and PRR5 is critical to maintain the repressed state of *CCA1* and *LHY*. In addition, previous studies showed that *CCA1* and *LHY* are expressed at constitutively high levels in the *prr9 prr7 prr5* triple mutant (Nakamichi et al., 2005) but at low levels in *PRR9*-, *PRR7*-, or *PRR5*-overexpressing plants (Sato et al., 2002; Farre and Kay, 2007; Matsushika et al., 2007a). These lines of evidence lead us to propose that the sequential expression pattern of PRR9, PRR7, and PRR5 is required to propagate the proper waveforms of *CCA1* and *LHY*.

PRR9, PRR7, and PRR5 in the *Arabidopsis* Circadian Clock

The central oscillator of the *Arabidopsis* circadian clock is thought to be a multiple gene transcriptional feedback loop (McClung, 2006). In this loop, CCA1 and LHY activate *PRR9* and *PRR7* expression, and PRR9 and PRR7 are negative regulators of *CCA1* and *LHY*. This model is supported by both experimental and mathematical approaches, although the exact mechanisms were previously not clear (Farre et al., 2005; Nakamichi et al., 2005; Locke et al., 2006; Zeilinger et al., 2006). In addition, the position of *PRR5* in the circadian clock has been a matter of conjecture. In this study, we demonstrated a molecular mechanism that closes the loop between PRR9 and PRR7, and *CCA1* and *LHY* (Figure 7). Furthermore, our results show that PRR5 is also involved in the repression of *CCA1* and *LHY*.

Since the *Arabidopsis* circadian clock regulates a large number of genes (output genes) (Harmer et al., 2000; Michael et al., 2008; Hazen et al., 2009), it is not surprising that the expression levels and patterns of these genes are altered in the *prr9 prr7 prr5* triple mutant. This is the case of genes involved in output pathways, such as flowering time regulation, hypocotyl length regulation, cold stress response, and mitochondrial metabolism (the trichloroacetic acid cycle) that are drastically altered in *prr9 prr7 prr5* plants (Ito et al., 2007; Nakamichi et al., 2007, 2009; Fukushima et al., 2009). Our findings that PRR9, PRR7, and PRR5 repress *CCA1* and *LHY* suggest that these proteins could regulate output genes via *CCA1* and *LHY* expression. However, PRR9, PRR7, and PRR5 regulate the flowering pathway and metabolism in mitochondria in a *CCA1*-independent manner (Nakamichi et al., 2007; Fukushima et al., 2009), an indication that the molecular links from PRR9, PRR7, and PRR5 to output genes remain a matter of speculation. Therefore, the identification of direct targets of PRR9, PRR7, and PRR5 other than *CCA1* and *LHY* is an exciting future challenge.

METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana accession Columbia-0 (Col-0) was used as the wild type in this study. Seedlings were grown at 22°C for 14d on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 2% sucrose under white light (80 to 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$) either in constant light or in a 12-h-light/12-h-dark cycle. To obtain etiolated seedlings, seeds were sown on MS plates without sucrose. After 2 d of darkness at 4°C, seeds were exposed to white light (80 to 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for 5 h at 22°C to enhance germination and then incubated for 5d in the dark at 22°C. A T87 *Arabidopsis* cultured cell line was used for real-time bioluminescence assay of *CCA1pro:LUC*, *LHYpro:LUC* and *TOC1pro:LUC* (Nakamichi et al., 2004).

The double mutants *prr7 prr5* (*prr7*, SALK_030430; *prr5*, KG24599; from the KAZUSA DNA Research Institute) and *prr9 prr7* (*prr9*, SALK_007551; *prr7*, SALK_030430) (Nakamichi et al., 2005), *PRR5pro:FLAG-PRR5-GFP* in *prr5* (SALK_006280), *PRR5pro:FLAG-GUS-GFP* in *prr5* (SALK_006280) (Kiba et al., 2007), and *35Spro:PRR5* (Sato et al., 2002) were described previously.

Plasmid Construction

To generate reporter plasmids, the promoter regions of *CCA1*, *LHY*, and *TOC1* were amplified with Prime Star DNA polymerase (Takara) from the *Arabidopsis* Col-0 genome (primer set for the *CCA1* promoter, 5′-CTGAAGCTTCATGCATGGTTAGCTTAGC-3′ and 5′-GGTTCCATG-GTCTCCATCACTAAGCTCCTCTAC-3′ to amplify between positions −854 and 0 relative to the translational start; for the *LHY* promoter, 5′-CACTTAAGCTTCAGCCACTACAATATCACCAC-3′ and 5′- GGAAC-CATGGTAACAGGACCGGTGCAG-3′ to amplify between positions −909 and 0; for the *TOC1* promoter, 5′- GCTAAGCTTCCACTGATGATGAGAT-TAACCATC-3′ and 5′-GTCTTCCATGGACCTCAGCATCTTCATACCC-3′ to amplify between positions −1360 to +570). Each promoter region was fused to the firefly *LUC* gene on modified pSP-*luc*+ (Promega) between the *Hin*dIII and *Nco*I sites as described previously (Nakamichi et al., 2004). These promoters were sufficient to confer rhythmic LUC expression in T87 cells (see Supplemental Figure 1 online). The reporter plasmid in which *LUC* is transcribed under the GAL4 promoter and the reference plasmid were previously described (Fujimoto et al., 2000).

To generate the effector plasmids used in Figure 1, the *35S* CaMV promoter, the coding region of PRR5, PRR7, or PRR9, plus CFP and a NOS terminator were assembled 5′ to 3′ in pBlueScript (Stratagene) as previously described (Yamada et al., 2004) to form the three pBS-PRR-CFP constructs. pBS-PRR5-GR-CFP was generated by a similar method. To make the effector plasmids used in Figure 3, the *35S* promoter, GAL4DB (Fujimoto et al., 2000), GATEWAY reading frame cassette (Invitrogen), 3-FLAG, and NOS terminator were assembled 5′ to 3′ in

pBlueScript to generate pBS-GAL4DB-GW. Full-length coding regions of PRR9, PRR7, PRR5, truncated PRR9, PRR7, and PRR5 (minus stop codon) cloned in pENTR/D-TOPO (Invitrogen) were integrated into pBS-GAL4DB-GW by LR clonase (Invitrogen), generating pBS-GAL4DB-PRR. Truncated PRR5, PRR9, and PRR7 regions were as follows: PRR5#1 (extending from Thr of 173 to Pro of 558), PRR5#2 (from Arg of 468 to Pro of 558), PRR5#3 (from Thr of 173 to Gly of 501), PRR5#4 (from Ara of 286 to Gly of 501), PRR5#5 (from His of 328 to Gly of 501), PRR5#6 (from Ara of 286 to Arg of 329), PRR5#7 (from Ara of 286 to Gln of 307), PRR5#8 (from Glu of 305 to Arg of 329), PRR9#6 (from Arg of 250 to Lys of 296), and PRR7#6 (from Glu of 316 to Ara of 469).

To generate binary vectors harboring the *35Spro:PRR5-CFP* construct, *35Spro:PRR5-GR-CFP*, *PRR5-CFP*, and *PRR5-GR-CFP* were each cloned into binary vector pSK1 (Kojima et al., 1999). To generate a binary vector in which *FLAG-PRR7-GFP* was expressed under its native *PRR7* promoter, the *PRR7* promoter (2223 bp upstream of the inferred initiation codon), 3-FLAG, the GATEWAY reading frame cassette, and a 3′ untranslated region (614 bp) were assembled 5′ to 3′ in the binary vector pBA002a (Kiba et al., 2007), generating pBA-PF7 (*PRR7pro:FLAG-GATEWAY cassette-3′-UTR*). *PRR7* or *GUS* coding regions were amplified and fused to *EGFP* (*PRR7-GFP* and *GUS-GFP*) and integrated into pBA-PF7 by LR clonase, generating *PRR7pro:FLAG-PRR7-GFP* or *PRR7pro:FLAG-GUS-GFP*. The analogous *PRR9* promoter constructs were made in the same way to form *PRR9pro:FLAG-PRR9-GFP* or *PRR9pro:FLAG-GUS-GFP* (*PRR9* promoter; 2045 bp upstream of the inferred initiation codon, PRR9 3′-UTR; 1027 bp).

Transient Transfection Assay in *Arabidopsis* Seedlings

The reporter, effector, and reference plasmids were delivered to 2-week-old *Arabidopsis* Col-0 seedlings by particle bombardment (PDU-1000/He; Bio-Rad) as described previously (Sakakibara et al., 2005). Promoter activity was determined by normalizing luciferase values to *Renilla* luciferase expressed under the control of the CaMV *35S* promoter in reference plasmid pPTRL (Fujimoto et al., 2000). Bioluminescence was detected with a Mithras LB940 (Berthold) as described previously (Yamaguchi et al., 2008).

Real-Time Luciferase Assay

Protoplasts of *Arabidopsis* T87 cultured cells were transfected with a reporter plasmid by a polyethylene glycol–mediated method (Yamada et al., 2004). Cells were entrained to a coordinate circadian rhythm by incubation in constant dark conditions for 12 h and release into constant white light conditions (time 0). Bioluminescence of cells was measured with a real-time monitoring system (Kondo et al., 1993).

Arabidopsis Transformation

Plants were vacuum infiltrated with *Agrobacterium tumifaciens* strain EHA105 harboring binary vectors as described previously (Bechtold et al., 1993). *PRR7pro:FLAG-PRR7-GFP* and *PRR7pro:FLAG-GUS-GFP* were introduced into *prr7* (SALK_030430) (Yamamoto et al., 2003), and *PRR9pro:FLAG-PRR9-GFP* and *PRR9pro:FLAG-GUS-GFP* were introduced into *prr9* (SALK_106072) from the Salk collection (http://signal.salk.edu). *35Spro:PRR5-CFP* and *35Spro:PRR5-GR-CFP* were introduced into the wild type.

Measurement of Hypocotyl Length

Measurement of hypocotyl lengths of *Arabidopsis* seedlings under short-day conditions (10 h light/14 h dark) was described previously (Niwa et al., 2009). Seedlings were grown on MS or MS plus 10 μM DEX (Sigma-Aldrich).

DEX and CHX Treatment

Two independent T3 transgenic seedlings expressing *PRR5-GR-CFP* (two independent lines, *35Spro:5GC1* and *35Spro:5GC2*) were grown on MS (2% sucrose) under constant light conditions for 2 weeks and transferred either to a water control, to 10 μM DEX, to 100 μM CHX (Sigma-Aldrich), or to 100 μM CHX plus 10 μM DEX. After a 4-h incubation, plants were harvested and frozen in liquid nitrogen.

RNA Isolation, Reverse Transcription, and qPCR

For each sample, three to five seedlings were harvested, frozen in liquid nitrogen, and then ground. Total mRNA was extracted with the RNeasy plant mini kit (Qiagen). To synthesize cDNA, 1 μg of each RNA sample was reverse transcribed with SuperScript II (Invitrogen) and oligo (dT20) primer. Real-time qPCR was performed on an ABI PRISM 7000 system (Applied Biosystems) using SYBR Green Extaq II (Takara) and specific primers (see Supplemental Figure 3 online). Reaction conditions were 95°C for 5 min followed by 40 cycles of 95°C for 15 s and 60°C for 31 s. Product sizes were verified by agarose gel electrophoresis and dissociation curve analysis. Amount of a PCR amplicon was quantified by an absolute quantification method using a calibration curve of corresponding DNA.

ChIP Assay

For the ChIP assay, 500 mg of plant sample was cross-linked in 20 mL of 1% formaldehyde solution under vacuum for 40 min. The reaction was stopped by washing twice with ice-cold 0.3 M glycine. Plants were then ground to powder in liquid nitrogen and lysed in 2 mL lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Triton X-100, 50 μM 26S proteasome inhibitor MG132 [Sigma-Aldrich], and complete protease inhibitor cocktail tablets [Roche]). The chromatin complexes were isolated and sonicated, and 50 μL of the chromatin complexes were stored for use as input fractions.

Immunoprecipitation for 1.95 mL of chromatin complexes was performed with anti-GFP antibody (ab290; Abcam) and anti-PRR5-antibody (Kiba et al., 2007), which was bound to Dynabeads Protein G (Invitrogen) for 2 h at 4°C. After washing with lysis buffer and high salt buffer (50 mM Tris-HCl, pH 7.5, 400 mM NaCl, 0.1% Triton X-100, 50 μM MG132, and complete protease inhibitor cocktail tablets), immunocomplexes were eluted from the beads using elution buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 1% SDS).

Cross-linking of immunocomplexes or the input fraction was reversed by incubating at 65°C overnight followed by digestion with 5 μL of Proteinase-K (#9033; Takara) to remove all proteins. DNA was purified by NucleoSpin (Macherey-Nagel) according to the manufacturer's protocol. The amount of each precipitated DNA and input DNA was determined by real-time PCR using specific primers (see Supplemental Figure 3 online).

PRR9 Antibody

Anti-PRR9 antibody was obtained according to the method of Kiba et al. (2007). A cDNA fragment encoding PRR9 amino acid residues 168 to 411 was cloned into the expression vector pQE30 (Qiagen), which adds an N-terminal 6xHis tag. Recombinant protein was expressed in *Escherichia coli* BL21 and purified using a nickel-nitrilotriacetic acid agarose column (Qiagen) and then was used to generate polyclonal anti-PRR9 antiserum of rabbit (Cocalico Biological). The antiserum was immunopurified using its cognate antigen immobilized to a nitrocellulose membrane. Anti-PRR9 antibody was confirmed by detecting native PRR9 protein in the wild type and FLAG-PRR9-GFP protein in *PRR9pro:FLAG-PRR9-GFP*–transformed *prr9* (SALK_106072) (see Supplemental Figure 4 online).

Protein Gel Blot Analysis

To detect FLAG-PRR-GFP proteins (Figure 5), 10 μL of the chromatin complexes was mixed in a 1:1 ratio (v/v) with 2× lithium

dodecyl sulfate sample (SM) buffer (Kiba et al., 2007), boiled at 95°C for 5 min, loaded in a Super Sep Ace 10 to ~20% gradient gel (Wako), and blotted onto an Immobilon-P membrane (Millipore). The membrane was incubated with monoclonal anti-FLAG antibody (F3165; Sigma-Aldrich). Goat anti-mouse IgG conjugated with alkaline phosphatase (170-6520; Bio-Rad) was used as the secondary antibody, and protein signals were detected using the NBT/BCIP system (Roche).

To detect native PRR5 and PRR9, frozen plant materials were ground to a fine powder and suspended in a 1:1 ratio (w/v) with 2× SM buffer and incubated for 5 min at 95°C. Rabbit anti-PRR5 or anti-PRR9 antibodies were used for the primary antibody. Goat anti-rabbit IgG conjugated with alkaline phosphatase (170-6518; Bio-Rad) was used as the secondary antibody. Quantitation of immunodetected proteins was performed using ImageJ software (http://rsb.info.nih.gov/ij/).

Accession Numbers

Sequence data for the genes described in this article can be found in the Arabidopsis Genome Initiative and GenBank/DDBJ/EMBL data libraries under the following accession numbers: *Arabidopsis PRR9* (At2g46790), *PRR7* (At5g02810), *PRR5* (At5g24470), *PRR3* (At5g60100), *TOC1* (At5g61380), *CCA1* (At2g46830), *LHY* (At1g01060), and *APX3* (At4g35000); poplar Pt *PRR5* (B9I296); grape Vv *PRR5* (CAO48570.1); *Lemna* Lg *PRRH37* (AB243684) (Miwa et al., 2006); barley *PPD-H1* (AY970705.1) (Turner et al., 2005); and rice Os *PRR37* (AB189039.1) (Murakami et al., 2005).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. *CCA1pro:LUC*, *LHYpro:LUC*, and *TOC1pro:LUC* Bioluminescence in *Arabidopsis* Cultured Cells.

Supplemental Figure 2. Circadian Rhythm in *9pro:F9G*, *7pro:F7G*, and *5pro:F5G*.

Supplemental Figure 3. The Sequence of Primers Used in mRNA Expression Analysis and ChIP Analysis.

Supplemental Figure 4. Characterization of the PRR9 Antibody.

ACKNOWLEDGMENTS

We thank Masaru Takagi (National Institute of Advanced Industrial Science and Technology, Japan) for providing *GAL4pro:LUC* and pPTRL plasmids, Takashi Aoyama (Kyoto University, Japan) for the mouse GR clone, and Takao Kondo (Nagoya University) and Tokitaka Oyama (Kyoto University) for supporting of real-time bioluminescence monitoring system. We also thank Minami Shimizu (RIKEN Plant Science Center) for DNA sequencing. We thank The Salk Institute Genomic Analysis Laboratory (La Jolla, CA) and Kazusa DNA Research Institute (Chiba, Japan) for T-DNA insertion mutants. We thank Masatoshi Yamaguchi (Nara Institute of Science and Technology, Japan) and our lab members for helpful discussions. This research was supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology (20870040 to N.N.), National Institutes of Health Grant GM-44640 to N.-H.C, a postdoctoral fellowship from the Gulbenkian Foundation, Portugal (ref. 78682) to R.H., and the Special Postdoctoral Researcher's Program from RIKEN to N.N.

Received November 18, 2009; revised February 12, 2010; accepted February 27, 2010; published March 16, 2010.

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PSEUDO-RESPONSE REGULATORS 9, 7, and 5 Are Transcriptional Repressors in the Arabidopsis Circadian Clock

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PLANT CELL 2010;22;594-605;
DOI: 10.1105/tpc.109.072892