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

Norihito Nakamichi, Takatoshi Kiba, Rossana Henriques, Takeshi Mizuno, Nam-Hai Chua, and Hitoshi Sakakibara

Laboratory of Molecular Microbiology, School of Agriculture, Nagoya University, Chikusa, Nagoya, 464-8601 Japan

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 HYOCOTYL (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 HYOCOTYL (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 (Matsushika et al., 2000). 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, CYCOIDEA, 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). Thus, 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 prr9pr7pr5 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.
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, 35Spro:PRR7-CFP, 35Spro: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 LHYpro: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:5GC-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). 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 length 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 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...
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 the GAL4pro:LUC reporter activity. However, seedlings expressing effector plasmids that covered a 44-amino acid peptide from the intervening region by about half (Figure 3B), indicating that this region is sufficient to confer repressor activity on GAL4DB. Comparison of this 44-amino 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 [f(t/7)DLN(f/s)] (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 GAL4DB-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 co-expressed 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 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 [5pro: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 5pro:F5G, 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 binding site (TBS) (Pruneda-Paz et al., 2009), respectively (Figure 4B). As expected, no significant enrichment was found on any amplicons in the control lines 9pro:FGG, 7pro:F7G, 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 3SS: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 3SSpro: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:
and ZT14 (Figures 5A and 5B, middle panels). F5G protein both
with F7G protein peaked at around ZT8 to ZT12, but its association
promoters during the same timeframe (Figure 5B, left panels).
panel), and the protein became associated with
were harvested at 2-h intervals from ZT0.

| A | B | C | D | E |
|---|---|---|---|---|
| G-box | TBS | 5A | EE | 9pro:FGG 9pro:F9G |
| 1 | 2 | 3 | 4 | 5pro:F5G |
| Atg46820 | CCA1 | 6 | 7 | 7pro:F7G |
| 5 | 7 | 9 | 10 | 8pro:F8G 8pro:F7G |
| At1g01070 | LHY | 8 | 11 | 9pro:F5G 9pro:F9G |
| 5 | 7 | 9 | 10 | 8pro:F8G 8pro:F7G |
| At5g61370 | TGC1 | 6 | 7 | 7pro:F7G |
| 5 | 7 | 9 | 10 | 8pro:F8G 8pro:F7G |
| At4g35010 | APX3 | 6 | 7 | 7pro:F7G |
| 5 | 7 | 9 | 10 | 8pro:F8G 8pro:F7G |
| A1 | B1 | C1 | D1 | E1 |
| immunoprecipitated | immunoprecipitated | immunoprecipitated | immunoprecipitated | immunoprecipitated |
| DNA (%) | DNA (%) | DNA (%) | DNA (%) | DNA (%) |
| 1 2 3 4 5 6 7 8 9 10 | 1 2 3 4 5 6 7 8 9 10 | 1 2 3 4 5 6 7 8 9 10 | 1 2 3 4 5 6 7 8 9 10 | 1 2 3 4 5 6 7 8 9 10 |
| 

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 commu-
nonprecipitated with anti-GFP antibody relative to input DNA presented.

(B) ChIP assays for PRR9pro:FLAG-GUS-GFP (9pro:F9G) and PRR9pro:
FLAG-PRR9-GFP (9pro:F9G) seedlings

(C) ChIP assays for PRR7pro:FLAG-GUS-GFP (7pro:F7G) and PRR7pro:
FLAG-PRR7-GFP (7pro:F7G) seedlings

(D) ChIP assays for PRR5pro:FLAG-GUS-GFP (5pro:F5G) 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.

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 pro-
moters 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 be-
 tween 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 6C). 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 trans-
scription, 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 pr7 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 expres-
sion began to increase. This inverse correlation between PRR9
levels and CCA1 and LHY is consistent with the idea that CCA1
and LHY transcription can be repressed by PRR9 at a certain level
in the prr7 prr5 mutant but is not sufficient to repress their expression during nighttime.

In the pr7 prr7 double mutant, PRR5 protein accumulated from
ZT10 to ZT18, and CCA1 and LHY expression decreased from
ZT12 to ZT18 (Figure 6C). PRR5 mRNA was not detectable either
from ZT0 to ZT8 or from ZT20 to ZT22. CCA1 and LHY mRNA 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
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 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.
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), with APX3 mRNA (±SD; technical replicates, n = 3). Each experiment was performed twice with similar results.

**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.
CCA1 and LHY repress TOC1 transcription 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 pr 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 feedback loop is thought to be a multiple gene transcriptional network (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 tricloroacetic 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 14 d on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 2% sucrose under white light (80 to 100 μmol·m−2·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 dark at 4°C, seeds were exposed to white light (80 to 100 μmol·m−2·s−1) for 5 h at 22°C to enhance germination and then incubated for 5 d 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 (pr7, SALK_030430; pr5, KG24599; from the KAZUSA DNA Research Institute) and pr9 prr7 (pr9, SALK_007551; pr7, SALK_030430) (Nakamichi et al., 2005), PRR5pro:FLAG-PRR5-GFP in pr5 (SALK_006280), PRR5pro:FLAG-GUS-GFP in pr5 (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′-TCGAAGGTTCATCATGTTAGTTAGCTAGC-3′ and 5′-GGTTCCATGGTTCATCaCTACTAGTCCTCTAC-3′ to amplify between positions −854 and 0 relative to the translational start; for the LHY promoter, 5′-CAGCTTAAAGCTGCGACCACATATACCAACAC-3′ and 5′-GGAACCATGTTAAACAGGACCGGTCGAC-3′ to amplify between positions −909 and 0; for the TOC1 promoter, 5′-GCTAAAGGTTCCACTAGTAGTACATTAC-3′ and 5′-GCTTTCCATGACCCCTCAGGTTCCTCTAC-3′ to amplify between positions −1360 to +570). Each promoter region was fused to the firefly LUC gene on modified pSP-pro:CATG (Hin II 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 effector plasmids used in Figure 1, the 35S CaMV promoter, the coding region of PRR5, PRR7, or PRR9, plus GFP 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-PRR-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). To generate 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:GFP-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; 1027 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 light 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 pnn7 (SALK_030430) (Yamamoto et al., 2003), and PRR9pro:FLAG-PRR9-GFP and PRR9pro:FLAG-GUS-GFP were introduced into pnn9 (SALK_106072) from the Salk collection (http://signal.salk.edu). 3SSpro:PRR5-CFP and 3SSpro: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, 3SSpro:5GC1 and 3SSpro: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 SDS 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-GR 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-nitriilotriacetic 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 pnn9 (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 2X 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 (At2g48630), LHY (At1g0160), and APX3 (At4g35000); poplar Pt PRRs (B9I296); grape Vv PRR5 (CA048570.1); Lemma Lg PRRH37 (AB243684) (Miwa et al., 2006); barley pPTRL plasmids, Takashi Aoyama (Kyoto University, Japan) for the GAL4pro:LUC and APX3 and APX1expression. Plant Cell 21: 2307–23083.

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

ACKNOWLEDGMENTS

We thank Masaru Takagi (National Institute of Advanced Industrial Science and Technology, Japan) for providing GAL4pro:LUC and pPTYL plasmids, Takashi Aoyama (Kyoto University, Japan) for the mouse GR clone, and Takao Kondo (Nagoya University) and Tokitaka Oyama (Kyoto University) for support 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.
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PSEUDO-RESPONSE REGULATORS 9, 7, and 5 Are Transcriptional Repressors in the 
*Arabidopsis* Circadian Clock
Norihito Nakamichi, Takatoshi Kiba, Rossana Henriques, Takeshi Mizuno, Nam-Hai Chua and Hitoshi 
Sakakibara

*Plant Cell* 2010;22;594-605; originally published online March 16, 2010; 
DOI 10.1105/tpc.109.072892

This information is current as of April 28, 2019

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