Transcriptional repressor PRR5 directly regulates clock-output pathways

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The circadian clock is an endogenous time-keeping mechanism that enables organisms to adapt to external daily cycles. The clock coordinates biological activities with these cycles, mainly through genome-wide gene expression. However, the exact mechanism underlying regulation of circadian gene expression is poorly understood. Here we demonstrated that an Arabidopsis PSEUDO-RESPONSE REGULATOR 5 (PRR5), which acts in the clock genetic circuit, directly regulates expression timing of key transcription factors involved in clock-output pathways. A transient expression assay and ChIP-quantitative PCR assay using mutated PRR5 indicated that PRR5 associates with target DNA through binding at the CCT motif in vivo. ChIP followed by deep sequencing coupled with genome-wide expression profiling revealed the direct-target genes of PRR5. PRR5 direct-targets include genes encoding transcription factors involved in flowering-time regulation, hypocotyl elongation, and cold-stress responses. PRR5-target gene expression followed a circadian rhythm pattern with low, basal expression from noon until midnight, when PRR5, PRR7, and PRR5 were expressed. ChIP-quantitative PCR assays indicated that PRR7 and PRR9 bind to the direct-targets of PRR5. Genome-wide expression profiling using a prr7 prr5 prr triple mutant suggests that PRR5, PRR7, and PRR9 repress these targets. Taken together, our results illustrate a genetic network in which PRR5, PRR7, and PRR9 directly regulate expression timing of key transcription factors to coordinate physiological processes with daily cycles.

ChiP-seq | plant

The circadian clock in plants regulates a broad range of biological processes. For example, hypocotyl elongation is observed before dawn and cold-stress responses reach maximal levels in the afternoon in Arabidopsis thaliana (1, 2), all largely because of circadian coordination of these biological processes (clock-output) with daily cycles. The circadian clock mechanism controls the temporal regulation of numerous genes involved in output processes (3–5).

A number of recent studies have described the genetic components of the clock in Arabidopsis. CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) encode morning-expressed MYB transcription factors (TFs) that directly repress TIMING OF CAB EXPRESSION 1 (TOC1, also called PSEUDO-RESPONSE REGULATOR 1 (PRR1)), EARLY FLOWERING 3 (ELF3), ELF4, and LUXARRHYTHMO (LUX) (6–10). ELF3 and LUX associate with upstream region of PRR9, and repress PRR9 expression (11, 12). Expression of PRR9 and PRR7 are activated by CCA1 and LHY (13). CCA1 and LHY are in turn repressed by four PRR proteins, PRR9, PRR7, PRR5, and TOC1 from early daytime through to around midnight (14–16). These TFs form a negative feedback loop for clock function (12, 17, 18). Evidence is accumulating that these TFs directly regulate the expression of genes involved in clock-output pathways. LUX, ELF3, and ELF4 together form the “evening complex” that directly represses expression of PHYTOCHROME INTERACTING FACTOR 4 (PIF4) and PIF5 (19), both of which encode TFs positively regulating hypocotyl elongation (2, 20). CCA1 and LHY bind to the promoter regions of DEHYDRATION-RESPONSIVE ELEMENT BINDING 1 [DREB1, also called C-REPEAT BINDING FACTOR (CBF)] genes encoding TFs involved in cold-stress responses (21). These results suggest that the transcriptional regulators form an interface that allows the clock to regulate output processes.

PRR proteins feature a Pseudo-Receiver (PR) domain at the N terminus and a CONSTANS, CONSTANS-LIKE, and TOC1 (CCT) motif at the C terminus (22, 23). The PR domain resembles the receiver domain of a two-component response regulator, but lacks an aspartate residue that accepts a phosphor yl group from the sensor kinase. The PR domain is involved in protein–protein interactions (24, 25) and TOC1 (CCT) motif at the C terminus (26). In contrast, PRR9, PRR7, and PRR5 have a repression motif in an intervening region (IR) between the PR and CCT, and act as transcriptional repressors (14). Previous studies suggest that these three PRRs redundantly regulate expression of clock-output genes (27, 28). However, it is still not known which genes are the direct targets of these three PRRs and how they are regulated by them. Identifying the direct-target genes is critical for illustrating the entire genetic network of clock-output regulation.

To address this issue, we studied domains within PRR5 and found that PR5 binds to the known target gene CCA1 through the CCT motif in vivo. ChIP followed by deep sequencing (ChiP-seq) coupled with genome-wide expression profiling revealed that a number of genes encoding key TFs for hypocotyl elongation, flowering time, and cold-stress responses were enriched in the population of direct-targets of PRR5. Our results demonstrated that PRR5 functions as a transcriptional repressor that controls various biological processes by directly regulating the timing of expression of its target genes.

Results

PRR5 Associates with CCA1 Through the CCT Motif. To clarify which specific region of PRR5 represses known target genes, such as CCA1, we performed transient expression assays using a luciferase (LUC) reporter plasmid under the control of the CCA1 promoter (CCA1pro:LUC) with an effector plasmid harboring

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession nos. GSE36360 [microarray data] and GSE36361 [ChiP-seq data]).

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**Fig. 1.** PRR5 associates with CCA1 through its CCT in vivo. (A) Effect of truncated PRR5 constructs #1 to #4 on CCA1 promoter activity in Arabidopsis seedlings (Right), and a schematic of each construct (Left). Ellipses indicate PR, diamonds indicate a repression motif, and squares indicate a CCT motif. (B) Effect of truncated (full-length IR and CCT) PRR9, PRR7, and PRR5 on CCA1 promoter activity. (C) Effect of PRR5-VP and PRR5#4 (full-length CCT)-VP on CCA1 promoter activity. (D) Amino acid sequence alignment of the C-terminal portion of CCA1. Red indicates conserved residues among PRR9, PRR7, PRR5, TOC1, and CONSTANS (CO). Arrows indicate amino acid substitutions in tocl-1 (PRR5mA) or PR2 (PRR5mB). (E) Effect of two CCT mutants of PRR5 on CCA1 promoter activity. (F) ChIP-qPCR for CCA1 and APX3 upstream regions in PRR5-FLAG- or mutated PRR5-FLAG-expressing plants. Percentages of the amplicons coimmunoprecipitated with anti-FLAG antibody relative to input DNA are indicated. (G) Hypocotyl length of PRR5-FLAG- or mutated PRR5-FLAG-expressing plants under 10-h light/14-h dark cycles. Typical seedlings are indicated with a scale bar (Left). (H) Expression of PR5mA-FLAG protein in transgenic plants. The arrow and asterisk indicate FLAG-fused protein and nonspecific bands, respectively. Error bars indicate the SD of biological replicates in A–E, F, and G (n = 15 for G, and 3 for others), and the SD of three technical replicates in F. Asterisks indicate a significant change in CCA1 activity compared with coexpression with FLAG (Student t-test; P < 0.05). The "a" and "b" in G indicate one-way ANOVA P < 0.05 compared with Vector and PRR5-F, respectively.

**Fig. 2.** The PRR5-VP in ChIP-Seq reads for FLAG-PRR5-GFP protein expressed under the control of the PRR5 promoter in a pr5 mutant background (PRR5-FLAG-PRR5-GFP/pr5) (Fig. S1) (14). DNA libraries for deep sequencing were generated from the immunoprecipitated fraction (ChIP DNA) and input DNA fraction (input DNA), and analyzed on an Illumina Genome Analyzer II (Fig. S2). Five-hundred forty-two genomic loci (1,024 genes) were significantly enriched in ChIP DNA compared with input DNA [false-discovery rate (FDR) q < 10−5]. These genes make up the in vivo “PRR5-bound” genes (Fig. S3 and Dataset S1), which potentially contain ‘PRR5 direct-target’ genes, but may also contain some false-positive genes because of inherent problems with ChIP and deep-sequencing procedures (31).
To discover PRR5 direct-target genes another way, we performed a DNA microarray experiment with transgenic Arabidopsis overexpressing PRR5-VP in the wild-type background (35Spro:PRR5-VP). When we compared genome-wide gene expression in PRR5-VP plants and prr9 prr7 prr5 (Fig. S4), significant overlaps (P < 10^{-6}) were found between genes similarly regulated both in PRR5-VP and prr9 prr7 prr5, but not between genes oppositely regulated. LHY expression was up-regulated in PRR5-VP during the daytime (Fig. S4), and hypocotyls were longer for PRR5-VP plants, but not for PRR5-ox, thereby resembling the phenotypes of prr9 prr7 prr5 (28). PRR5-VP plants flowered significantly later than the wild-type (Fig. S4), and thus showed a phenotype similar to prr5. These data suggest that PRR5-VP acts in an inverse manner to PRR5. Because PRR5-VP activates a direct-target of PRR5 (Fig. 1C), genes whose expression is significantly increased in 35Spro:PRR5-VP lines compared with wild-type (FDR q < 0.01) potentially contain PRR5 direct-target genes. This strategy may miss potential activated genes by wild-type PRR5, but 190 genes were obtained as PRR5-VP up-regulated genes (Dataset S2).

The comparison between PRR5-bound genes and PRR5-VP up-regulated genes delineated 64 direct-target genes of PRR5 (Fig. 2A and Dataset S3), of which two are the known PRR5 direct-target genes, CCA1 and LHY (14). Overlap between the two gene sets was statistically significant (Fig. 2A), supporting the validity of our strategy. On the other hand, the overlap between PRR5-bound genes and down-regulated genes in PRR5-VP was not significant (Fig. S5). ChiP-qPCR experiments (six genes in Fig. 2B, 43 genes in Fig. S6) confirmed PRR5-binding at most of the PRR5-target loci (45 of 49).

**TFs are Enriched in PRR5 Direct-Targets.** Significantly enriched Gene Ontology (eGO) analysis was performed to explore the biological functions of the direct targets of PRR5 (Fig. 2C). “Transcription factor activity” was the most enriched category in PRR5 direct-targets (P < 10^{-10}). “Circadian rhythm” was the next enriched group (P < 10^{-8}). “DNA binding,” “regulation of transcription,” “response to salt stress,” and “response to cadmium ion” were also enriched (P < 10^{-7}). We were especially interested in TFs because three categories related to TFs were enriched. The direct-target TF group included six MYB TFs [CCA1, LHY, EARLY PHYTOCHROME RESPONSIVE 1 (EPR1)]/also called as REV,EIL,E7 (REV7), REV1, REV3, and REV5, three DOF TFs [CYCLING DOF FACTOR 2 (CDF2), CDF3, and CDF5], four C2C2-CO-like TFs [B-BOX DOMAIN PROTEIN 2 (BBX2), BBX6, BBX24, and BBX29], three bHLH TFs [PIF4, PIF5, and LONG HYPOCOTYL IN FAR-RED (HRF1)], three AP2/EREBP TFs (DREB1A, DREB1B, and DREB1C), and three PRRs (PRR9, PRR7, and PRR5). CCA1, LHY, REV5 (32, 33), and PRRs are known to be involved in clock function, EPR1/REV7 is in cotyledon opening and flowering-time regulation (34), REV1 is in auxin production (35), REV3 is in unknown biological process, DOF TFs are in flowering time regulation (36, 37), C2C2-CO-like (38) and bHLH TFs (2, 20) are in hypocotyl elongation, and AP2/ EREBP TFs are in cold-stress responses (39, 40), suggesting that PRR5 controls diverse biological processes by regulating these TFs (Fig. 2D).

**PRR5 Direct-Targets Are Repressed from Noon Until Midnight.** To examine the expression patterns of the target genes under LD, we tested gene expression in a public microarray database [DIURNAL (4, 41)]. Expression of target genes with valid data (see **Materials and Methods**) showed clear diurnal rhythms (Fig. 3A and B). About 60% of the gene set had an expression peak at ZT0, with the others peaking at ZT4 or ZT8. Even under free-running constant-light conditions, expression of targets with valid data were cyclic, with peaks in subjective dawn to daytime (Fig. 3C and D). Expression troughs of these genes extended from noon to midnight, when the three PRR (PRR9, PRR7, and PRR5) proteins are expressed (14). We also performed RT-qPCR analysis for “invalid genes in DIURNAL” and found similar expression patterns for genes analyzed in DIURNAL (Fig. S7). The majority of direct-targets are expressed in the morning, and thus showed similar expression patterns to CCA1 and LHY (14), suggesting that these genes and CCA1 and LHY are regulated by PRR5 through the same mechanism.

**PRR5 Represses Its Direct-Targets.** To investigate how PRR5 regulates direct-targets, a PRR5-binding profile in ChIP-seq data were visualized. Apparently, PRR5 preferentially binds to upstream regions of direct-targets, supporting the idea that PRR5 regulates gene expression (Fig. 4A and B). ChiP-qPCR analyses using PRR5 CCT mutants suggested that PRR5 regulates its representative
target genes by associating with their upstream regions through the CCT (Fig. S8).

Because PRR5, PRR7, and PRR9 redundantly target CCA1 and LHY (14), we examined whether PRR9 and PRR7 associate with PRR5-target genes using PRR9pro:FLAG-PRR9-GFP/prr9 and PRR7pro:FLAG-PRR7-GFP/prr7 plants (Fig. 4C and Fig. S6). The plants were grown under LL, and samples were collected when PRR proteins are expressed (i.e., ZT4 for PRR9pro:FLAG-PRR9-GFP/prr9, ZT10 for PRR7pro:FLAG-PRR7-GFP/prr7) and not expressed (ZT22 for all plants). Amplicons located in the upstream regions of most of the tested targets were significantly enriched in PRR7pro:FLAG-PRR7-GFP/prr7 when PRR7 is expressed. Similar trends of enrichment were observed for PRR9pro:FLAG-PRR9-GFP/prr9 with some exceptions (e.g., BBX24, CDF3, PIF4, PIF5, and SIGE), indicating that PRR7 and PRR9 share targets with PRR5.

When we surveyed the expression of targets in a microarray dataset for prr9 prr7 prr5 triple mutants (27), most of the targets were up-regulated in prr9 prr7 prr5 compared with the wild-type
from ZT8 to ZT12 (Fig. 4D). Taken together, these results suggest that PRR9, PRR7, and PRR5 coordinately act on the upstream region of the PRR5-targets to repress their expression.

**Discussion**

In this study, a ChIP-qPCR assay using two CCT-motif mutants indicated that PRR5 associates with several target genes through its CCT in vivo (Fig. 1F and Fig. S8). We also found that a CCT is crucial for the biological function of PRR5, because mutations in the CCT resulted in attenuation of PRR5 activity leading to hypocotyl shortening (Fig. 1G). It was reported that Gly-to-Trp change in a CCT of a barley homolog of PRR7 is the most likely cause of *photoperiod-HI* (42). These results suggest that CCT is essential for target gene recognition, through which PRR5-regulated biological processes are controlled.

An in vitro gel-shift assay showed that recombinant CCTs from PRR9, PRR7, PRR5, and TOC1 bind to the TGTT motif (26). ChIP-seq analysis for TOC1 revealed that G-box (CACGTT-) or evening element (AAATAATCTC)-like sequences are enriched in TOC1-bound DNA sequences (16). In our analysis, a G-box motif was found to be enriched around the peak sequences of ChIP DNA (Fig. S9) (43). However, this result should be interpreted with care because ChIP-enriched sequences do not necessarily represent the motif directly bound by PRR5. Because the ChIP procedure involves cross-linking, it is conceivable that bound sequences are a mixture of motifs directly bound and others associated through various protein–protein interactions by PRR5. Previously it was shown that TOC1 occupies the *CCA1* promoter region both by direct binding (16, 26) and through interaction with the CHE transcription factor to regulate *CCA1* (44). Furthermore, we found that PRR7 and PRR5 associate with BBX24, *CDF3, PIF4, PIF5*, and *SIGE* upstream regions, whereas PRR9 associates much more poorly or not at all with these loci (Fig. 4C and Fig. S6). A comparison between TOC1-targets (16) and PRR5-targets reveals that TOC1 binds to 27 genes of 64 PRR5-targets (Dataset S4). Taken together, these results suggest that interactions between PRRs and a certain locus are not solely determined by the CCT binding motif in vivo, and this may cause preferences of target-recognition. Further experiments are required for fully understanding how each PRR is recruited to their target loci in vivo.

Four PRRs directly repress *CCA1* and *LHY* expression from early daytime until midnight (14, 16); however, whether PRR9, PRR7, and PRR5 act as repressors for other target genes was unknown. We proposed that PRR5 represses 64 targets that were found by ChIP-seq coupled with genome-wide expression profiling using PRR5-VP. This strategy might miss genes positively regulated by native PRR5 because native PRR5 may sufficiently activate targets in PRR5-VP plants. To examine the possible PRR5 activation of its targets, 149 down-regulated genes in *prr7 prr5* were compared with PRR5-bound genes. Although the overlap between the down-regulated genes in *prr9 prr7 prr5* and PRR5-bound genes was not statistically significant (*P* > 0.01), 12 genes were found as potential activated targets by PRR5 (Fig. S5). Expression of *UBT71B1* and *AT4G29700* were slightly but significantly up-regulated in the PRR5-ox line, suggesting that PRR5 potentially activates these genes (Fig. S10). Because PRR5 has a repression motif (14), PRR5 may activate *UBT71B1* and *AT4G29700* by an unknown mechanism or with some other transcriptional activators.

PRR9, PRR7, and PRR5 seem to regulate about 60% of the PRR5-target genes by the same mechanism by which they control *CCA1* and *LHY* because circadian expression patterns of these genes were similar to those of *CCA1* and *LHY* (genes whose expression peaked at ZT0 in Fig. 3). The expression peak and trough positions of other genes were slightly different from those of *CCA1* and *LHY*, suggesting that these genes are also regulated by other factors. For example, the *DREB1A* and *DREB1B* promoters are directly regulated by *CCA1* and *LHY* (21), and *PIF4* and *PIF5* promoters are regulated by the evening complex (19). Such differences in TF combinations might be one of the bases for shifting the expression timing of target genes.

The most enriched Gene Ontology category for the targets of PRR5 was TF, suggesting that PRR5 functions as a repressor directly regulating key TFs (PIFs, BBXs, CDFs, DREB1s/CBFs) (Fig. 2D). These TFs control a cascade of gene expression involved in output processes. This kind of hierarchical genetic architecture may be effective in orchestrating the expression of genes involved in certain biological process at the appropriate time of day (3, 4). A similar genetic architecture, in which master clock function TFs directly regulate output TFs, was reported in *Drosophila*, which has a different type of central clock mechanism than plants (45), suggesting that such an architecture is conserved among species.

**Materials and Methods**

**Plant Materials and Growth Conditions.** Transgenic plants and growth conditions are described in *SI Materials and Methods*.

**Transient Expression Assay.** Transient expression assay by particle bombardment was described previously (14). Detailed information is in *SI Materials and Methods*.

**Protein Sequence Alignment.** The alignment for the C-terminal portion of CCT from the proteins was done using ClustalW2 (www.ebi.ac.uk/Tools/msa/clustalw2/).

**Measurement of Hypocotyl Lengths.** Measurement of hypocotyl lengths under 10-h light/14-h dark conditions was described previously (14).

**Protein Gel-Blot Analysis.** Protein gel blotting was performed as previously described (14).

**ChIP-qPCR Assay.** The ChIP-qPCR assay was performed as described previously (14). Anti-FLAG antibody (F3165; Sigma-Aldrich) was used for immunoprecipitation of PRR5(3)-FLAG proteins. Primers used for ChIP-qPCR are listed in Dataset S5.

**ChIP-Seq Analysis.** The methods for ChIP-seq are described in *SI Materials and Methods*. ChIP-seq data were deposited in the National Center for Biotechnology Information GEO (www.ncbi.nlm.nih.gov/geo) under accession no. GSE36361.

**Microarrays.** Microarray methods and data analyses are described in *SI Materials and Methods*. Microarray data for PRR5-VP-expressing plants were deposited with National Center for Biotechnology Information GEO under accession no. GSE36350.

**eG0 Analysis.** eG0 analysis was performed as previously described (46).

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34. Rawat R, et al. (2009) REVVEILLES1, a Myb-like transcription factor, integrates the cir-


36. Fornara F, et al. (2009) Arabidopsis DOF transcription factors act redundantly to re-
duce CONSTANS expression and are essential for a photoperiodic flowering response. Dev Cell 17:75–86.


