

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 PRR9, 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 *prp9 prp7 prp5* 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 phosphoryl group from the sensor kinase. The PR domain is involved in protein–protein interactions (24, 25) and *TOC1* PR is crucial for transcriptional repression (26). In contrast, *PRR9*, *PRR7*, and *PRR5* have a repression motif in an intervening region (IR) between the PR and CCT, and act as transcription 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 the 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 *PRR5* 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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PRR5 fused to *FLAG* under the control of the cauliflower mosaic virus 35S promoter (*35Spro:PRR5-FLAG*), or with a plasmid containing a series of truncated *PRR5s* (*35Spro:PRR5#1-FLAG* to *35Spro:PRR5#4-FLAG*) (Fig. 1A). Bombardment with *35Spro:PRR5-FLAG* (*PRR5-FLAG*) resulted in a significant reduction of *CCA1pro:LUC* activity compared with that with the control vector (*FLAG*), indicating that *PRR5* represses the *CCA1* promoter in transient assays. Introduction of an effector plasmid harboring IR and CCT of *PRR5* (*PRR5#3-FLAG*) resulted in a significant reduction of *CCA1pro:LUC* activity, whereas any expression of PR (*PRR5#1-FLAG*), PR and IR (*PRR5#2-FLAG*), or CCT (*PRR5#4-FLAG*) did not, suggesting that both IR and CCT of *PRR5* are required for repressing *CCA1* promoter activity. The IR and CCT in *PRR9* and *PRR7* are also sufficient for the repression of *CCA1* promoter (Fig. 1B).

To examine whether a CCT is required for *PRR5* association with its target genes, we performed a transient expression assay in which the protein of interest was fused to a tandem construct of the stringent transcriptional activation domain VP16 (VP), such that if the protein of interest associates with the promoter, the VP-fused protein activates promoter activity (29). Expression of *PRR5-VP* or *PRR5#4-VP* resulted in significant activation of the *CCA1* promoter (Fig. 1C). The TOC1 CCT is sufficient for DNA binding in vitro, and both the *toc1-1* and *PRR3*-type mutations attenuate activity (26). We thus generated two independent mutations [*toc1-1* type (mtA: Ala538Val) or *PRR3* type (mtB: Arg543His)] within the CCT of *PRR5* (Fig. 1D) and assayed for their effect on the *CCA1* promoter. Expression of *PRR5mtA* or *PRR5mtB* did not result in any significant reduction of *CCA1* promoter (Fig. 1E). These results suggest that *PRR5* associates with the *CCA1* promoter through its CCT.

To further investigate whether a CCT is crucial to the DNA-binding activity of *PRR5* in vivo, a ChIP assay was performed for plants overexpressing *PRR5-FLAG*, *PRR5mtA-FLAG*, or *PRR5mtB-FLAG* (Fig. 1F). Plants were grown under 12-h light/12-h dark conditions (LD), and harvested at Zeitgeber time 10 (ZT10 indicates 10 h after lights are turned on), when native *PRR5*

protein associates with target promoters in vivo. Amplicons located in the upstream region of *CCA1* and *ASCORBATE PEROXIDASE 3* (*APX3*) were quantified by quantitative PCR (qPCR). The amplicons located at the *CCA1* promoter, but not upstream of *APX3*, was enriched in ChIP DNA from *PRR5-FLAG*-expressing plants, indicating that *PRR5-FLAG* associates with the *CCA1* promoter in vivo (Fig. 1F). In contrast, *PRR5mtA-FLAG* and *PRR5mtB-FLAG* associated with *CCA1* less often. These ChIP-qPCR analyses indicated that a functional CCT is crucial for interactions between *PRR5* and the *CCA1* promoter in vivo.

To determine the biological importance of the *PRR5* CCT, we measured hypocotyl lengths of plants overexpressing *PRR5-FLAG*, *PRR5mtA-FLAG*, or *PRR5mtB-FLAG* (Fig. 1G). The *PRR5-FLAG* plants grew short hypocotyls, a well-known phenotype of lines that overexpress *PRR5* (30). Hypocotyls of *PRR5mtA-FLAG* plants were significantly shorter than the wild-type ($P < 0.05$), but the hypocotyls of *PRR5mtA-FLAG* and *PRR5mtB-FLAG* were longer than those of *PRR5-FLAG*, even though exogenous proteins were expressed at levels similar to those of *PRR5-FLAG*-expressing plants (Fig. 1H).

Identification of Direct-Target Genes of *PRR5*. To determine the genes bound by *PRR5* on a genomic scale, we conducted ChIP-seq for *FLAG-PRR5-GFP* protein expressed under the control of the *PRR5* promoter in a *prr5* mutant background (*PRR5pro:FLAG-PRR5-GFP/prr5*) (Fig. S1) (14). DNA libraries for deep sequencing were generated from the immunoprecipitated fraction (ChIP DNA) and input DNA fraction (input DNA), and analyzed with 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) $q < 10^{-50}$]. 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).

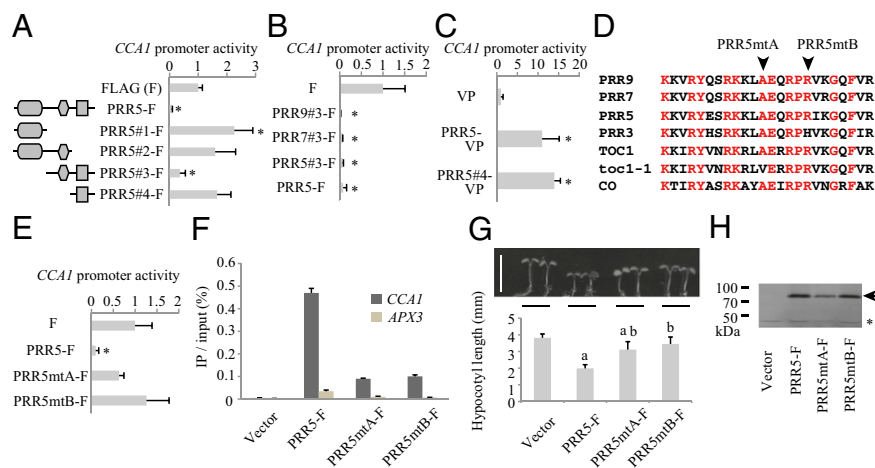


Fig. 1. *PRR5* associates with *CCA1* through its CCT in vivo. (A) Effect of truncated *PRR5* constructs #1 to #4 on *CCA1* promoter activity in *Arabidopsis* seedlings (Right), and a schematic of each construct (Left). Ellipses indicate PR, diamonds indicate a repression motif, and squares indicate 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-VP* (full-length CCT)-VP on *CCA1* promoter activity. (D) Amino acid sequence alignment of the C-terminal portion of CCT. Red indicates conserved residues among *PRR9*, *PRR7*, *PRR5*, *TOC1*, and *CONSTANS* (CO). Arrows indicate amino acid substitutions in *toc1-1* (*PRR5mtA*) or *PRR3* (*PRR5mtB*). (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 (5 mm). (H) Expression of *PRR5mtA-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–C, E, 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.

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 *prp9 prp7 prp5* (Fig. S4), significant overlaps ($P < 10^{-16}$) were found between genes similarly regulated both in PRR5-VP and *prp9 prp7 prp5*, 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 *prp9 prp7 prp5* (28). PRR5-VP plants flowered significantly later than the wild-type (Fig. S4), and thus showed a phenotype similar to *prp5*. 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 ($P < 10^{-9}$). “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 ($P < 10^{-5}$). We were especially interested in TFs because three categories related to TFs were enriched. The direct-target TF group includes six MYB TFs [*CCA1*, *LHY*, *EARLY PHYTOCHROME RESPONSIVE 1* (*EPR1*)/also called as *REV-EILLE7* (*RVE7*), *RVE1*, *RVE3*, and *RVE8*], three DOF TFs [*CYCLING DOF FACTOR 2* (*CDF2*), *CDF3*, and *CDF5*], four C2C-CO-like TFs [*B-BOX DOMAIN PROTEIN 2* (*BBX2*), *BBX6*, *BBX24*, and *BBX29*], three bHLH TFs [*PIF4*, *PIF5*, and *LONG HYPOCOTY IN FAR-RED* (*HFR1*)], three AP2/EREBP TFs [*DREB1A*, *DREB1B*, and *DREB1C*], and three PRRs (*PRR9*, *PRR7*, and *PRR5*). *CCA1*, *LHY*, *RVE8* (32, 33), and PRRs are known to be involved in clock function, *EPR1/RVE7* is in cotyledon opening and flowering-time regulation (34), *RVE1* is in auxin production (35), *RVE3* is in unknown biological process, DOF TFs are in flowering time regulation (36, 37), C2C-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 *SI 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

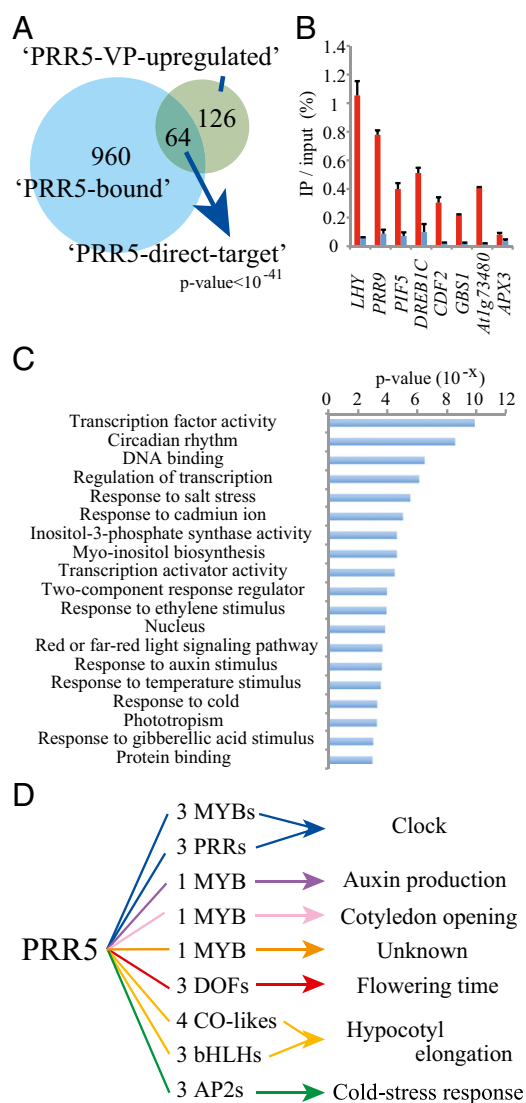


Fig. 2. PRR5 directly controls clock-output pathways by regulating genes encoding transcription factors. (A) Venn diagram of PRR5-bound genes and PRR5-VP up-regulated genes. There is significant overlap between the two gene groups (Fisher’s exact test; $P < 10^{-41}$). (B) ChIP-qPCR assay for representative PRR5-direct-target genes in *PRR5pro:FLAG-PRR5-GFP/prp5*. Red and blue bars indicate percentages of amplicons coimmunoprecipitated with anti-GFP antibody relative to input DNA from the sample harvested at ZT10 and ZT22, respectively. Error bars indicate the SD of three replicates. (C) eGO analysis was performed for PRR5 direct-target genes. (D) Schematic indicating that PRR5 targets key transcription factors in clock-output pathways.

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

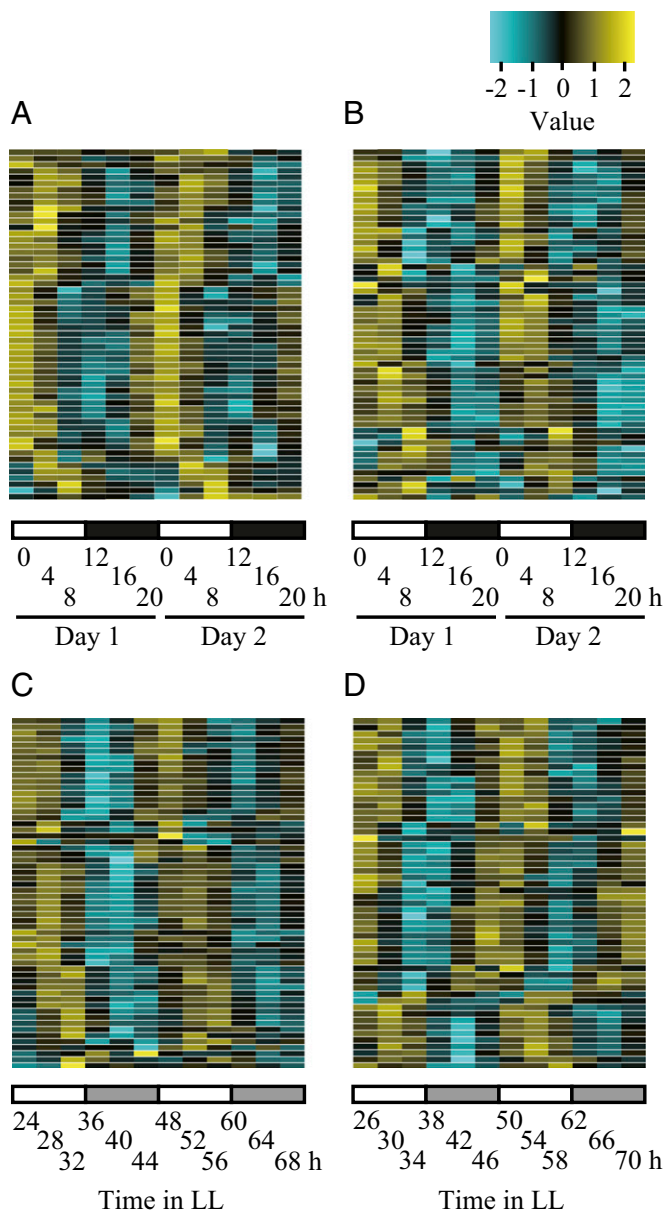


Fig. 3. PRR5 direct-targets are repressed during noon until midnight. Expression of direct-targets under LD (A and B), and constant light conditions (C and D). See also *SI Materials and Methods* for detailed information.

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 LD, 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

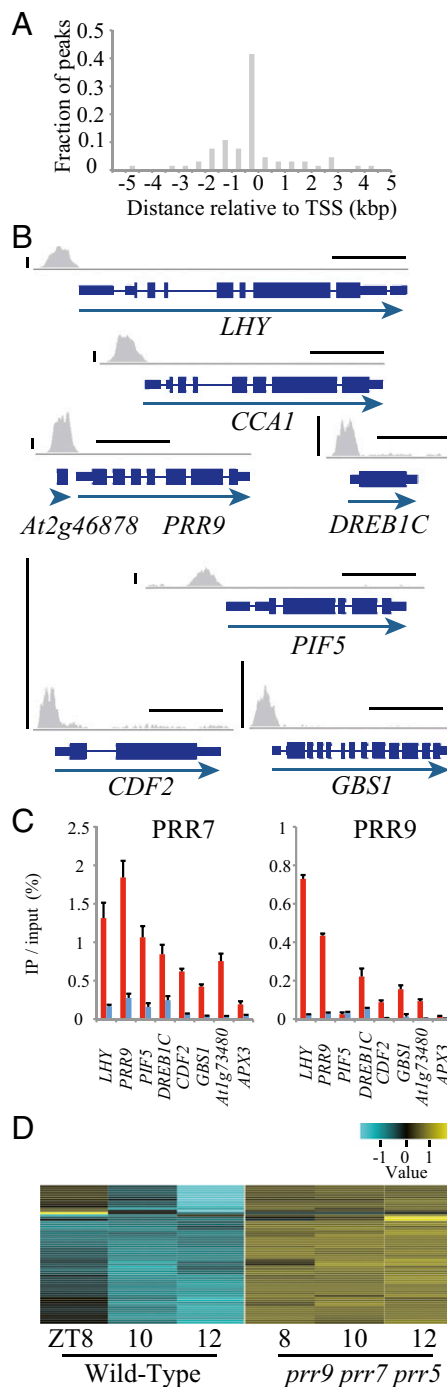


Fig. 4. PRR5, PRR7, and PRR9 repress expression of PRR5-targets. (A) Positions of ChIP peaks relative to transcription start sites (TSS) in the PRR5 direct-targets. (B) Examples of peak distributions around direct-targets. Vertical and horizontal bars indicate 0.5-kb sequence reads and 1.0 kbp, respectively. (C) ChIP-qPCR assay for representative PRR5 direct-target genes in *PRR7pro:FLAG-PRR7-GFP/prr7* and *PRR9pro:FLAG-PRR9-GFP/prr9* plants. Red bars indicate percentages of amplicons coimmunoprecipitated with anti-GFP antibody relative to input DNA from the sample harvested when PRR proteins were expressed, and blue bars are from samples when PRRs were not expressed. (D) Expression of the PRR5 direct-target genes in *prr9 prr7 prr5* at ZT8, ZT10, and ZT12.

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-H1* (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 TGTG motif (26). ChIP-seq analysis for TOC1 revealed that G-box (CACGTG)- or evening element (AAAATATCT)-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 *prp9 prp7 prp5* were compared with PRR5-bound genes. Although the overlap between the down-regulated genes in *prp9 prp7 prp5* 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(mt)-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/gds) under accession no. GSE36361.

Microarrays. Microarray methods and data analyses are described in *SI Materials and Methods*. Microarray data for PRR5-VP-expressin plants were deposited with National Center for Biotechnology Information GEO under accession no. GSE36360.

eGO Analysis. eGO analysis was performed as previously described (46).

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