

LIGHT-REGULATED WD1 and PSEUDO-RESPONSE REGULATOR9 Form a Positive Feedback Regulatory Loop in the *Arabidopsis* Circadian Clock ^{WJ|OA}

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In *Arabidopsis thaliana*, central circadian clock genes constitute several feedback loops. These interlocking loops generate an ~24-h oscillation that enables plants to anticipate the daily diurnal environment. The identification of additional clock proteins can help dissect the complex nature of the circadian clock. Previously, LIGHT-REGULATED WD1 (LWD1) and LWD2 were identified as two clock proteins regulating circadian period length and photoperiodic flowering. Here, we systematically studied the function of LWD1/2 in the *Arabidopsis* circadian clock. Analysis of the *lwd1 lwd2* double mutant revealed that LWD1/2 plays dual functions in the light input pathway and the regulation of the central oscillator. Promoter:luciferase fusion studies showed that activities of LWD1/2 promoters are rhythmic and depend on functional PSEUDO-RESPONSE REGULATOR9 (PRR9) and PRR7. LWD1/2 is also needed for the expression of PRR9, PRR7, and PRR5. LWD1 is preferentially localized within the nucleus and associates with promoters of PRR9, PRR5, and TOC1 in vivo. Our results support the existence of a positive feedback loop within the *Arabidopsis* circadian clock. Further mechanistic studies of this positive feedback loop and its regulatory effects on the other clock components will further elucidate the complex nature of the *Arabidopsis* circadian clock.

INTRODUCTION

The circadian clock in many organisms generates an ~24-h oscillation in biochemical, physiological, or behavioral processes to anticipate diurnal changes in the environment (Harmer et al., 2001; Young and Kay, 2001).

The circadian system in plants can be separated into three general parts: the input pathway, central oscillator, and output pathway. The input pathway transmits environmental cues to the central oscillator, which generates a rhythm of the output genes for the control of many physiological progresses, such as photoperiodic flowering. Components functioning in input, central oscillator, or output pathways have been identified (Millar, 2004; Más, 2005; Gardner et al., 2006; Hotta et al., 2007; Yakir et al., 2007; de Montaigu et al., 2010).

Recent studies suggested that some components have multiple functions in the *Arabidopsis thaliana* circadian system. For example, PSEUDO-RESPONSE REGULATOR7 (PRR7) and PRR9 function in both the central oscillator and light input

pathway (Kaczorowski and Quail, 2003; Farré et al., 2005). EARLY FLOWERING3 (ELF3) acts as a zeitnehmer (time taker) that represses light input to the clock (McWatters et al., 2000; Covington et al., 2001; Hicks et al., 2001) and is also an integral component of the core oscillator (Thines and Harmon, 2010). Moreover, although GIGANTEA (GI) was originally found as an output component regulating the expression of the flowering time genes *CONSTANS* and *FLOWERING LOCUS T* (Kardailsky et al., 1999; Kobayashi et al., 1999; Samach et al., 2000; Suárez-López et al., 2001), it also contributes to the light input pathway and is a key component of the oscillator (Huq et al., 2000; Mizoguchi et al., 2005; Gould et al., 2006; Martin-Tryon et al., 2007). Other clock components playing dual roles in the light input pathway and within the oscillator include ZEITLUPE (ZTL) (Somers et al., 2000; Kevei et al., 2006) and TIMING OF CAB EXPRESSION1 (TOC1) (Más et al., 2003b; Martin-Tryon and Harmer, 2008). The circadian system is evidently not a unidirectional pathway but rather a complex network (Harmer, 2009). Thus, whether clock components have multiple functions in the circadian system should be carefully evaluated.

A current model indicates that the *Arabidopsis* central oscillator is composed of several negative feedback loops (Harmer, 2009; Imaizumi, 2010; Pruneda-Paz and Kay, 2010). The most well-characterized negative feedback loop consists of CIRCADIAN CLOCK ASSOCIATED1 (CCA1)/LATE ELONGATED HYPOCOTYL (LHY) and TOC1 (Schaffer et al., 1998; Wang and Tobin, 1998; Strayer et al., 2000; Alabadi et al., 2001). The morning genes CCA1 and LHY directly repress the expression of the evening gene TOC1

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during the day, and the accumulation of TOC1 in the evening indirectly activates the expression of morning genes to form a negative feedback loop in a 24-h period. The finding that TOC1 could antagonize in part the function of CCA1 HIKING EXPEDITION (CHE), a transcriptional repressor of *CCA1*, completes the CCA1/LHY-TOC1 negative feedback loop (Pruneda-Paz et al., 2009). In addition, CCA1/LHY could form a morning negative feedback loop with PRR9/PRR7 (Farré et al., 2005; Nakamichi et al., 2010). In this loop, CCA1 and LHY proteins activate PRR9 and PRR7, and PRR9/PRR7 proteins then directly repress the transcription of *CCA1* and *LHY*. Highly complex and interlocked feedback loops within the *Arabidopsis* central oscillator were postulated with the identification of additional central oscillator components, including ELF4, GI, PRR5, ZTL, and LUX ARRHYTHMO (LUX)/PHYTOCLOCK1 (Más et al., 2003a; Yamamoto et al., 2003; Hazen et al., 2005; Kikis et al., 2005; Nakamichi et al., 2005; Onai and Ishiura, 2005; Martin-Tryon et al., 2007).

Components that function within or close to the circadian oscillator have been reported recently. These include FIONA1, TIME FOR COFFEE, LIGHT-REGULATED WD1 (LWD1), and LWD2 (Hall et al., 2003; Ding et al., 2007; Kim et al., 2008; Wu et al., 2008). Whether these clock proteins are part of the pre-existing feedback loops or constitute unidentified regulatory loops within the central oscillator remains to be elucidated.

Here, we report on our systematic study of the function of LWD1/2 in the *Arabidopsis* circadian clock. Our results indicate that LWD1/2 plays dual functions in both the light input pathway and central oscillator of the clock. LWD1/2 functions to attenuate light signals to adjust period length and regulates the expression of multiple oscillator genes indirectly or by targeting their promoters. Interestingly, our data support that LWD1 and PRR9 form a positive feedback loop within the *Arabidopsis* central oscillator. This positive feedback loop endorses the mutual activation of *LWD1* and *PRR9*. PRR9 indirectly activates the expression of *LWD1*, whereas *LWD1*, a nuclear protein, can directly target the *PRR9* promoter. We discuss the implications of a positive feedback loop within the *Arabidopsis* central oscillator.

RESULTS

LWD1/2 Controls the Amplitude and Period Length but Not the Robustness of the *Arabidopsis* Circadian Rhythm under Continuous Light

Our previous study of steady state transcripts of oscillator genes measured by real-time quantitative RT-PCR (qRT-PCR) showed that the *lwd1 lwd2* double mutant has a short period phenotype (Wu et al., 2008). Here, to determine whether *LWD1* and *LWD2* regulate the transcriptional activities of oscillator genes, we generated transgenic *Arabidopsis* harboring promoter:luciferase fusions of central oscillator genes (described in Methods). LUC2 reporter activity was measured in wild-type and *lwd1 lwd2* plants carrying the oscillator promoter:luciferase constructs *CCA1:LUC2*, *LHY:LUC2*, *GI:LUC2*, *TOC1:LUC2*, or *ELF4:LUC2* under continuous light (LL). As shown in Figure 1A, the promoter activity of all oscillator genes was significantly lower, by 3- to 10-fold in

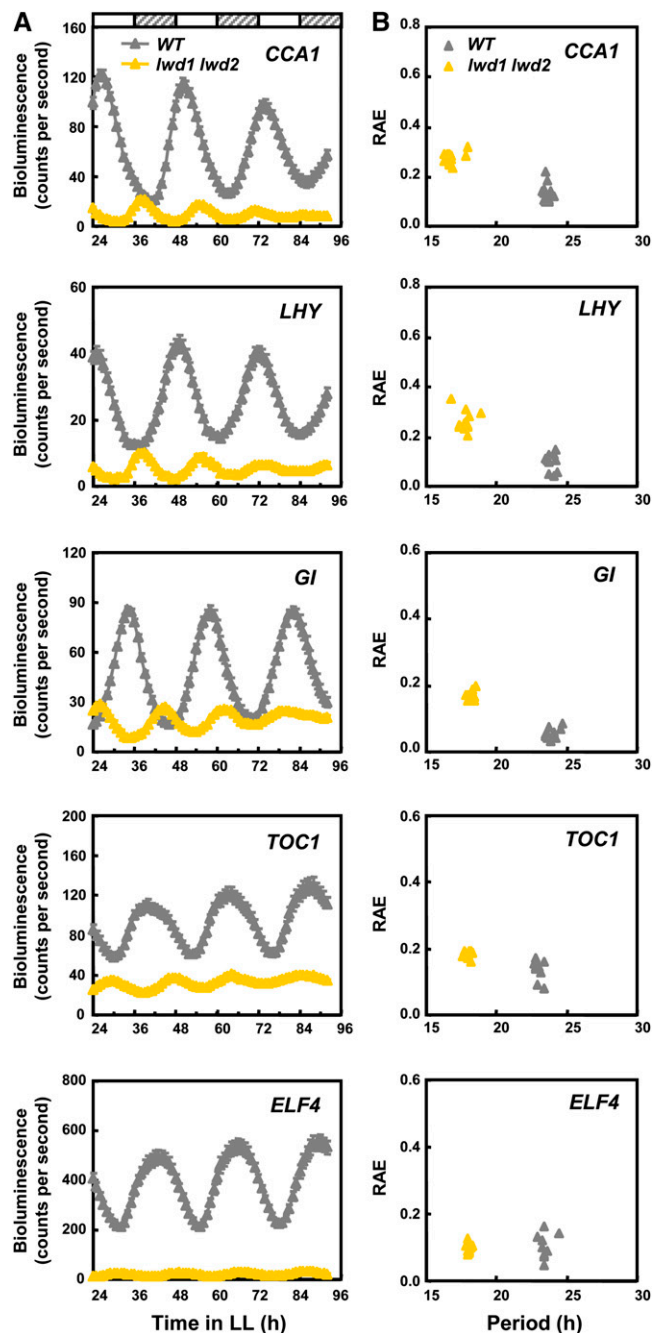


Figure 1. LWD1 and LWD2 Are Required for Maintaining Period Length and Amplitude of Clock Genes in Continuous Light.

(A) Bioluminescence assays were performed to measure the promoter activity of *CCA1*, *LHY*, *GI*, *TOC1*, and *ELF4* in the wild type (WT) and *lwd1 lwd2*. Nine-day-old seedlings grown under 12 h light/12 h dark ($55 \mu\text{mol m}^{-2} \text{s}^{-1}$) were transferred to continuous light ($45 \mu\text{mol m}^{-2} \text{s}^{-1}$) at ZT0. Seedlings were imaged every 1 h for 4 d. Data are means \pm SE of at least nine seedlings. The white and gray regions indicate subjective light and dark periods, respectively.

(B) Period length and RAE for each promoter in the wild-type and *lwd1 lwd2* plants were calculated by the FFT-NLLS analysis according to data from ZT24 to ZT92. Two independent experiments were performed with similar results.

lwd1 lwd2 plants than in wild-type plants under LL. Steady state mRNAs of oscillator genes measured by qRT-PCR showed less striking fold changes (Wu et al., 2008). This finding suggests that the expression of these clock genes is regulated at both transcriptional and posttranscriptional levels when shifted to LL, and *LWD1/2* is primarily required to maintain the transcriptional activities of these clock genes.

Promoter:*LUC2* reporter lines offered better sensitivity to clarify the impact of *LWD1* and *LWD2* on period length, amplitude, and robustness. For all oscillator promoters tested, the period length was 6 h shorter in *lwd1 lwd2* plants than in wild-type plants under LL (Figure 1B). Although the circadian amplitude was lower in *lwd1 lwd2* plants than in wild-type plants (Figure 1A), the oscillator genes still showed rhythmic expression (see Supplemental Figure 1A online for data with normalized bioluminescence). The robustness of the circadian rhythm in *lwd1 lwd2* plants under LL was further examined by fast Fourier transform-nonlinear least squares (FFT-NLLS) analysis. Figure 1B shows that the circadian clock maintained good robustness by the small (<0.4) relative amplitude error (RAE) values. Thus, *LWD1/2* controls the period length and amplitude but not the robustness of the *Arabidopsis* circadian rhythm under LL.

The Amplitude and Robustness of the Circadian Rhythm Is Affected in *lwd1 lwd2* Plants under Continuous Dark

We next investigated whether the *LWD1/2*-dependent regulation of period length depends on light by measuring the promoter activity of oscillator genes in *lwd1 lwd2* plants under continuous dark (DD) after entrainment. Under DD, *CCA1* and *LHY* promoters lost their rhythmicity in *lwd1 lwd2* plants, so the period length of most plants could not be calculated (Figure 2; see Supplemental Figure 1B online). This phenomenon is consistent with a stronger impact of *LWD1/2* on morning genes (Wu et al., 2008). The period length for *lwd1 lwd2 TOC1:LUC2* and *lwd1 lwd2 ELF4:LUC2* seedling populations was more sporadic, and the RAE values were increased. A few *lwd1 lwd2 ELF4:LUC2* plants had a short period length and increased RAE values (>0.6) (Figure 2B), similar to our previous observation by qRT-PCR analyses of *ELF4* mRNA (Wu et al., 2008). Most *lwd1 lwd2 Gl:LUC2* plants maintained their rhythmicity (RAE < 0.6; Figure 2B). Interestingly, the RAE values for *Gl*, *TOC1*, and *ELF4* promoters were higher for *lwd1 lwd2* than wild-type plants (Figure 2B). The quick collapse of the circadian robustness in *lwd1 lwd2* plants grown under DD was likely a result of the much reduced transcriptional activities of oscillator genes in this double mutant.

Unlike the 6-h shortening in period length under LL (Figure 1B), under DD, the period length was comparable between the wild-type and *lwd1 lwd2* plants expressing *Gl:LUC2* (Figure 2B). This light dependency of *LWD1/2* in period length regulation suggests that *LWD1/2* may be involved in transmitting light signals to the central oscillator.

LWD1/2 Also Functions in the Light Input Pathway

The above result implies that *LWD1/2* may be involved in the light input pathway. The period length of the circadian clock under LL

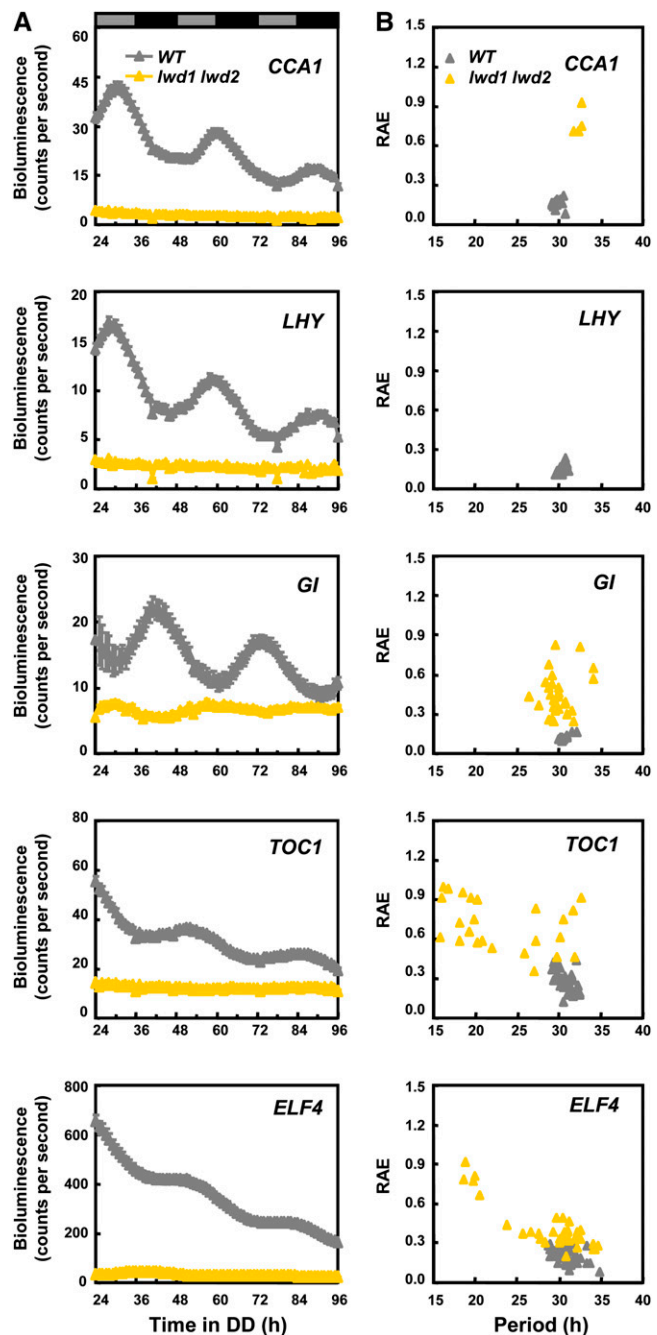


Figure 2. *LWD1* and *LWD2* Are Required for Maintaining Period Amplitude and Robustness of Clock Genes in Continuous Dark.

(A) Bioluminescence assays were performed to measure the promoter activity of *CCA1*, *LHY*, *Gl*, *TOC1*, and *ELF4* in the wild type and *lwd1 lwd2*. Nine-day-old seedlings grown under 12 h light/12 h dark ($55 \mu\text{mol m}^{-2} \text{s}^{-1}$) were transferred to continuous dark at ZT0. Seedlings were imaged every 1 h for 4 d. Data are means \pm SE of at least 10 seedlings. The gray and black regions indicate subjective light and dark periods, respectively.

(B) Period length and RAE for each promoter in the wild-type and *lwd1 lwd2* plants were calculated by FFT-NLLS analysis according to data from ZT24 to ZT96. Three independent experiments were performed with similar results.

is known to decrease with increasing intensity of light input (Aschoff's rule; Aschoff, 1979). When the light input pathway is affected, the slope of the fluence rate response curve (FRC) is altered. To further investigate whether *LWD1/2* contributes to a specific monochromatic light input pathway, we examined the FRC of the *Gl:LUC2* reporter in the wild-type and *lwd1 lwd2* plants under red or blue light conditions. Under red light, the period length was greatly shortened in *lwd1 lwd2* plants compared with wild-type plants especially under high fluence of red light (Figure 3A); a similar shortened period length occurred under blue light with fluence rate between 1.5 and 5 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (Figure 3B). Thus, *LWD1/2* is involved in both red and blue light input pathways but has distinct sensitivities to different light qualities.

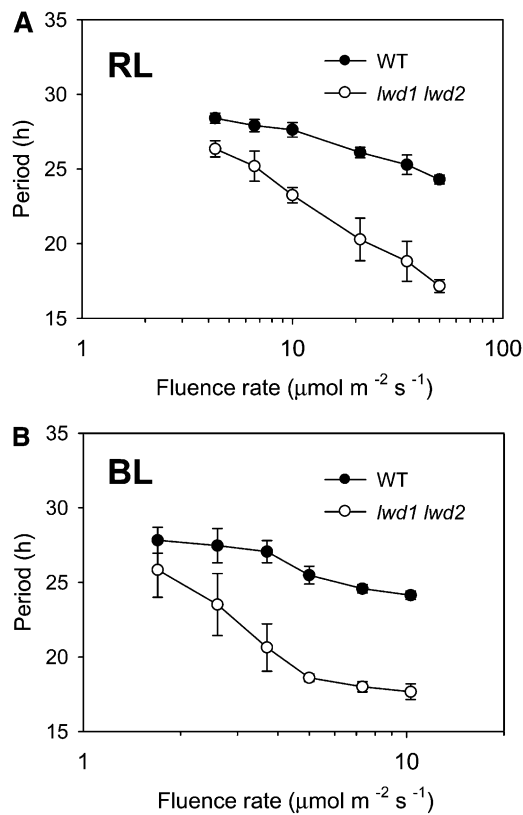


Figure 3. *LWD1* and *LWD2* Function in Red and Blue Light Input Pathways.

(A) Period length of *Gl:LUC2* in the wild type (WT) and *lwd1 lwd2* under different fluence rates of red light (RL).

(B) Period length of *Gl:LUC2* in the wild type and *lwd1 lwd2* under different fluence rates of blue light (BL).

Nine-day-old seedlings grown under 12 h light/12 h dark ($55 \mu\text{mol m}^{-2} \text{s}^{-1}$) were transferred to continuous red or blue light at ZT0. Seedlings were imaged every 1 h for 4 d, and data are means \pm SE of at least 16 seedlings. Period length was calculated by FFT-NLLS analysis according to data from ZT24 to ZT96. Three independent experiments were performed with similar results.

LWD1 and *LWD2* Promoters Are Controlled by the Circadian Clock

According to our previous study (Wu et al., 2008) and the above results, the two clock proteins *LWD1* and *LWD2* function in both the input pathway and the central oscillator. Because the expression of most clock components is regulated by the circadian clock, we next investigated whether the *LWD1* and *LWD2* promoters show rhythmic expression characteristics. For this purpose, we constructed *LWD1:LUC2* and *LWD2:LUC2* reporter lines to monitor the promoters under continuous light. *LWD1/2* showed regular oscillation under LL (Figure 4A). The low RAE (<0.4) indicates that the *LWD1/2* promoter has robust rhythm and is under the control of the circadian clock (Figure 4B). Also, despite the functional redundancy between *LWD1* and *LWD2* (Wu et al., 2008), *LWD1* has a slightly advanced expression phase as compared with *LWD2*.

LWD1/2 Promoter Activity Is Largely Regulated by *PRR9/7*

To reveal which clock oscillator is important for regulating the transcriptional activities of *LWD1* and *LWD2* promoters, we introduced the *LWD1:LUC2* or *LWD2:LUC2* transgene from the wild type into clock mutants by genetic crosses. The clock mutants used in this study were *cca1-1* (Yakir et al., 2009), *lhy-101* (Khanna et al., 2006), *toc1-101* (Kaczorowski, 2004; Kikis et al., 2005), *elf4-101* (Khanna et al., 2003), *gi-2* (Park et al., 1999), *prr5-11*, *prr7-11* (Yamamoto et al., 2003), and *prr9-10* (Ito et al., 2003). The results of the *LWD1* and *LWD2* promoter activity assay with each mutant are shown in Figure 5. For *LWD1*, *PRR9* and *PRR7* represent key positive regulators for the full activity of the *LWD1* promoter because of a marked reduction in *LWD1* and *LWD2* promoter activity in the *prr9* and *prr7* mutant background (Figures 5B and 5F). To a lesser extent, *LHY* also positively regulates the *LWD1* promoter (Figure 5A), whereas the evening clock components *TOC1* and *ELF4* play negative roles on *LWD1* (Figure 5D). In general, *LHY*, *PRR9*, *PRR7*, *TOC1*, and *ELF4* have similar effects on *LWD1* and *LWD2* promoter activities (Figures 5A, 5B, 5D to 5F, and 5H). However, *CCA1* has a negative regulatory role in the activity of the *LWD2* promoter, and *PRR5* has a positive role. Although the changes are minor, the inhibitory role of *CCA1* and the stimulatory role of *PRR5* only on the *LWD2* promoter may explain the slightly delayed expression phase of *LWD2* (Figure 4). *GI* has no effect on *LWD1* or *LWD2* (Figures 5C and 5G). Among all clock genes tested, *PRR9* and *PRR7* represent the key regulators of both *LWD1* and *LWD2* promoters.

We also measured the transcript levels of *LWD1/2* in the *prr9 prr7 prr5* triple mutant but did not observe clear reduction of *LWD1/2* transcripts in the triple mutant than in wild-type plants (see Supplemental Figure 2A online). The *LWD1* transcript level sharply decreased after being released to LL (see Supplemental Figure 2A online). The *LWD1* transcripts also only slightly oscillated as compared with the rhythmic activity of the *LWD1* promoter under LL (Figure 4). These findings indicate that the expression of *LWD1* is likely subjected to posttranscriptional regulation under LL. This posttranscriptional regulation disallows drawing conclusions based on the evaluation of *LWD* mRNAs in the clock mutant (see Supplemental Figure 2A online) or over-expression plants under LL (see Supplemental Figure 2B online).

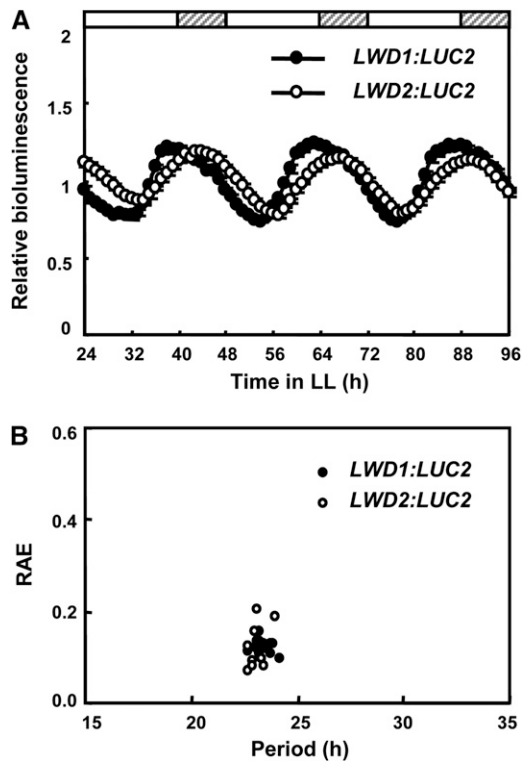


Figure 4. Rhythmic Activity of *LWD1* and *LWD2* Promoters.

(A) *LWD1/2* promoter fusion lines, *LWD1:LUC2* or *LWD2:LUC2*, were grown under 16 h light/8 h dark ($75 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 7 d and then transferred to LL ($45 \mu\text{mol m}^{-2} \text{s}^{-1}$). The white and gray regions indicate subjective light and dark periods, respectively. Data represent means \pm SE of 12 to \sim 15 seedlings.

(B) Period length and RAE were calculated by FFT-NLLS analysis according to data from ZT24 to ZT96. Three independent experiments were performed with similar results.

Nevertheless, results in Figures 5B and 5F clearly demonstrate the positive regulatory roles of *PRR9/7* on the transcriptional activities of *LWD1/2* promoters.

LWD1/2* Positively Regulates *PRR9*, *PRR7*, and *PRR5

The clear impact of *PRR9/7/5* on the promoter activities of *LWD1/2* prompted us to examine whether *LWD1/2* and *PRR9/7/5* constitute a regulatory loop within the circadian clock. For this purpose, we characterized the expression pattern of *PRR9/7/5* in wild-type, *lwd1 lwd2*, and *lwd1 lwd2 LWD1* complementation plants. *lwd1 lwd2 LWD1* is a successful complementation line that can recover the early flowering phenotype of *lwd1 lwd2* (Wu et al., 2008). The plants were entrained under 12 h light/12 h dark for 18 d and then released to LL. Samples were collected every 3 h for 72 h for RNA extraction and qRT-PCR analyses. The period length of *PRR9*, *PRR7*, and *PRR5* was shorter (\sim 3 to 6 h) in *lwd1 lwd2* plants than in wild-type plants (Figures 6A to 6C). Also, the circadian amplitude and maximum expression of *PRR9* and *PRR5* was significantly reduced in *lwd1 lwd2* plants.

We also compared wild-type and *lwd1 lwd2* double mutant plants in terms of promoter activities of *PRR9/7/5* using the reporter gene *LUC2*. The promoter activities of *PRR9* and *PRR5* were significantly impaired in the *lwd1 lwd2* double mutant (see Supplemental Figures 3A and 3C online), similar to other oscillator genes examined in Figure 1. The *PRR7* promoter could not reach the maximal expression activity in the *lwd1 lwd2* double mutant (see Supplemental Figure 3B online), which was faithfully reflected by the compromised *PRR7* transcript level in the double mutant (Figure 6B). Interestingly, the expression of *PRR9* and *PRR7* was increased in *lwd1 lwd2 LWD1* plants (Figures 6A and 6B). This finding is likely due to the higher level of *LWD1* transcripts in *lwd1 lwd2 LWD1* plants than in wild-type plants (see Supplemental Figure 4 online).

The above results indicate that *LWD1* is a positive regulator of *PRR9/7/5*. Combined with the clear impact of *PRR9* and *PRR7* on the *LWD1/2* promoter activities (Figures 5B and 5F), these data suggest that *LWD1/2* and *PRR9/7* form a positive feedback loop in the circadian clock.

***LWD1*-GFP Preferentially Localizes in the Nucleus**

We found that *LWD1* and *LWD2* are required for regulating the proper promoter activities of the oscillators *CCA1*, *LHY*, *GI*, *TOC1*, *ELF4*, *PRR9*, *PRR7*, and *PRR5* (Figure 1; see Supplemental Figure 3 online). Therefore, we next addressed the site(s) of action of *LWD1* protein. To visualize the subcellular localization of *LWD1*, the *LWD1-GFP* (for green fluorescent protein) fusion construct driven by the *LWD1* promoter was introduced into *lwd1 lwd2*. The early flowering phenotype of *lwd1 lwd2* could be successfully rescued in two independent transgenic lines (*lwd1 lwd2 LWD1:LWD1-GFP*), which indicates that *LWD1-GFP* retains the functions of endogenous *LWD1* (see Supplemental Figure 5A online). We further examined the *LWD1-GFP* subcellular localization in one of these transgenic lines. *35S:GFP* seedlings were used as a control to show the ubiquitous subcellular localization of GFP alone (see Supplemental Figure 5B online). *LWD1-GFP* signals observed in *lwd1 lwd2 LWD1:LWD1-GFP* seedlings appeared to localize to the nucleus and to be absent from the nucleoli (Figure 7). To further confirm that the localization is the nucleus and not the perinuclear region, we introduced a nuclear membrane marker, *RanGAP1-RFP* (for red fluorescent protein; Rose and Meier, 2001), into the *lwd1 lwd2 LWD1:LWD1-GFP* complementation lines. The inset of Figure 7 shows that *LWD1-GFP* is surrounded by the nuclear membrane, which indicates that *LWD1-GFP* is distributed in nuclei. A weak *LWD1-GFP* signal was found in the cytoplasm. These data suggest that *LWD1* is preferentially localized in nuclei under the conditions examined.

LWD1* Associates with the Promoters of *PRR9*, *PRR5*, and *TOC1

The nuclear localization of *LWD1* implies that *LWD1* may be involved in the transcriptional control of clock components. Interestingly, *TRANSPARENT TESTA GLABRA1*, the closest homolog of *LWD1/2* in *Arabidopsis*, functions as a transcription coregulator to control trichome cell fate (Zhao et al., 2008). We

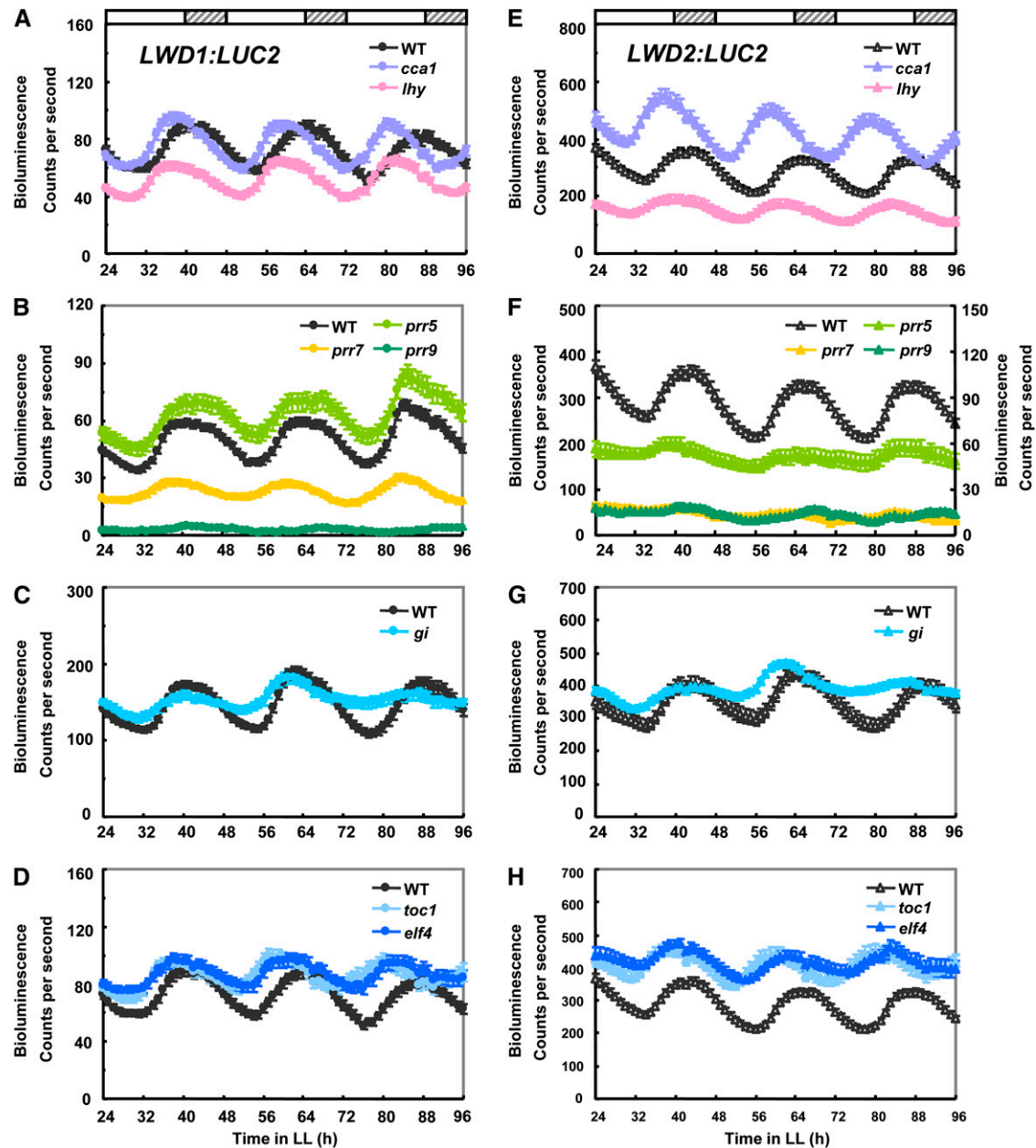


Figure 5. Promoter Activity of *LWD1* and *LWD2* in the Wild Type and Clock Mutants under Continuous Light.

(A) *LWD1:LUC2* bioluminescence assay in *cca1* and *lhy* mutants. WT, wild type.

(B) *LWD1:LUC2* bioluminescence assay in *prr5*, *prr7*, and *prr9* mutants.

(C) *LWD1:LUC2* bioluminescence assay in the *gi* mutant.

(D) *LWD1:LUC2* bioluminescence assay in *toc1* and *elf4* mutants.

(E) *LWD2:LUC2* bioluminescence assay in *cca1* and *lhy* mutants.

(F) *LWD2:LUC2* bioluminescence assay in *prr5*, *prr7*, and *prr9* mutants. The counts per second values for *prr7* and *prr9* were rescaled to increase the plot visibility (y axis to the right of the graph).

(G) *LWD2:LUC2* bioluminescence assay in *gi* mutant.

(H) *LWD2:LUC2* bioluminescence assay in *toc1* and *elf4* mutants.

Seedlings were entrained for 7 d under 16 h light/8 h dark ($75 \mu\text{mol m}^{-2} \text{s}^{-1}$) and then transferred to constant white light ($45 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 4 d. The white and gray regions indicate subjective light and dark periods, respectively. Data represent means \pm SE of 11 to \sim 16 seedlings. The experiments were repeated three times with similar results.

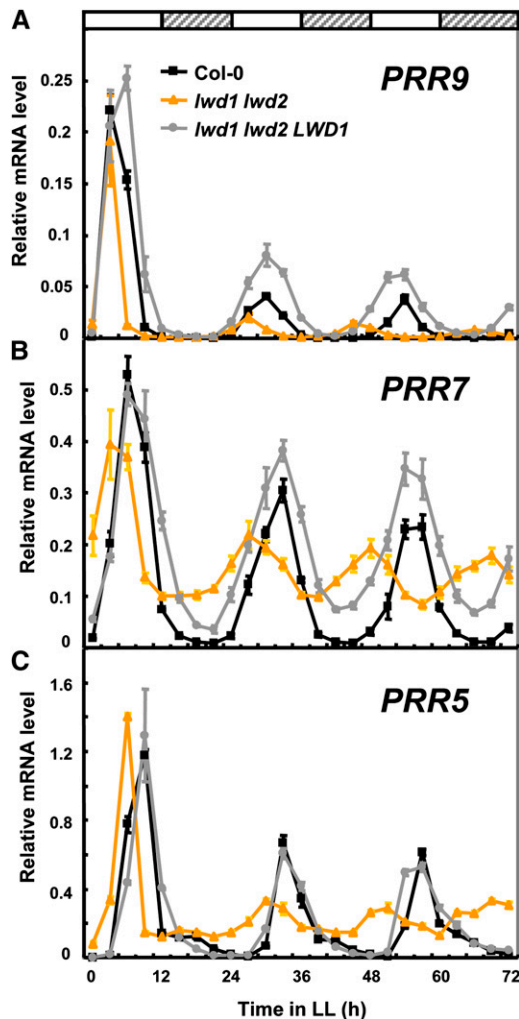


Figure 6. LWD1/2 Regulates the Period Length and Amplitude of *PRR9*, *PRR7*, and *PRR5*.

Real-time qRT-PCR was used to monitor the expression of *PRR9* (A), *PRR7* (B), and *PRR5* (C). Eighteen-day-old wild-type, *lwd1 lwd2*, and *lwd1 lwd2 LWD1* plants grown under 12 h light/12 h dark were transferred to continuous light at ZT0. The white and gray regions indicate subjective light and dark periods, respectively. Samples were harvested at 3-h intervals for 72 h. Expression is relative to that of *UBQ10*. Data are means \pm SE from four independent experiments.

thus hypothesized that LWD1 may function similarly as a coregulator to control target gene expression by interacting with transcription factors and binding to the target genes it regulates.

The reciprocal regulation between LWD1/2 and *PRR9/7* prompted us to first examine whether LWD1 is physically associated with the promoters of *PRR9* and *PRR7*. A chromatin immunoprecipitation quantitative PCR (ChIP-qPCR) assay was performed in *lwd1 lwd2 LWD1:LWD1-GFP* complementation lines, with *lwd1 lwd2 35S:GFP* used as a control. The plants were grown under 12-h-light/12-h-dark cycles, and samples were collected at ZT0, ZT6, and ZT12 to span the expression peaks of

PRR9 and *PRR7*. LWD1-GFP or GFP protein was immunoprecipitated with anti-GFP antibody, and qPCR was used to analyze the precipitated DNA for enrichment of promoters or coding regions of genes examined (Figure 8A). Compared with the 35S:GFP control lines, LWD1-GFP complementation lines showed that the *PRR9* promoter fragment b (–120 to –170 relative to the transcriptional start) was enriched, with no enrichment of the upstream regions of *PRR7* or the intragenic region of *PRR9*, *PRR7*, and *UBQ10* at three time points examined (Figure 8B). Together with the reduced expression of *PRR9* in the *lwd1 lwd2* double mutant (Figure 6A; see Supplemental Figure 3A online), these data imply that LWD1 may function as a coregulator to positively regulate the expression of *PRR9* by associating with its promoter region in planta.

We examined whether *PRR9* positively regulates *LWD1* and *LWD2* by targeting their promoters. The *PRR9* protein did not associate with the *LWD1* or *LWD2* promoters throughout a 24-h period (see Supplemental Figure 6 online). Similar results were obtained for *PRR7* protein (Supplemental Figure 6 online). Thus, within the positive feedback loop formed by LWD1 and *PRR9*, LWD1 could activate *PRR9* by binding with its promoter, whereas the activation of *LWD1* by *PRR9* is indirect. The *PRR9*-dependent activator(s) of *LWD1* remains to be identified.

We also examined the binding of LWD1-GFP to promoters of the other oscillator genes *PRR5*, *CCA1*, *LHY*, *TOC1*, and *ELF4*. As shown in Figure 8B, LWD1-GFP could associate with upstream regions of *PRR5* and *TOC1*. Because the promoter activities of *TOC1* and *PRR5* were greatly changed in the *lwd1 lwd2* double mutant (Figure 1A; see Supplemental Figure 3C online), LWD1 may function as an important coregulator to retain the normal expression of *TOC1* and *PRR5*. However, compared

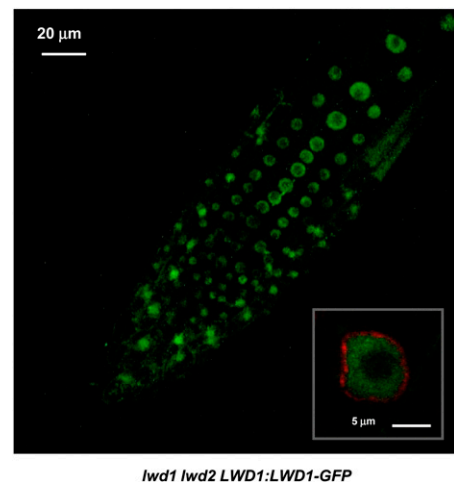


Figure 7. Functional LWD1-GFP Proteins Localize in Nucleus.

Four-day-old *lwd1 lwd2 LWD1:LWD1-GFP* seedlings grown under 12-h-light/12-h-dark conditions were observed at ZT2. GFP signals represent the nuclear localization of LWD1-GFP. Inset shows that LWD1-GFP is surrounded by red fluorescence of RanGAP1-RFP, a nuclear membrane marker.

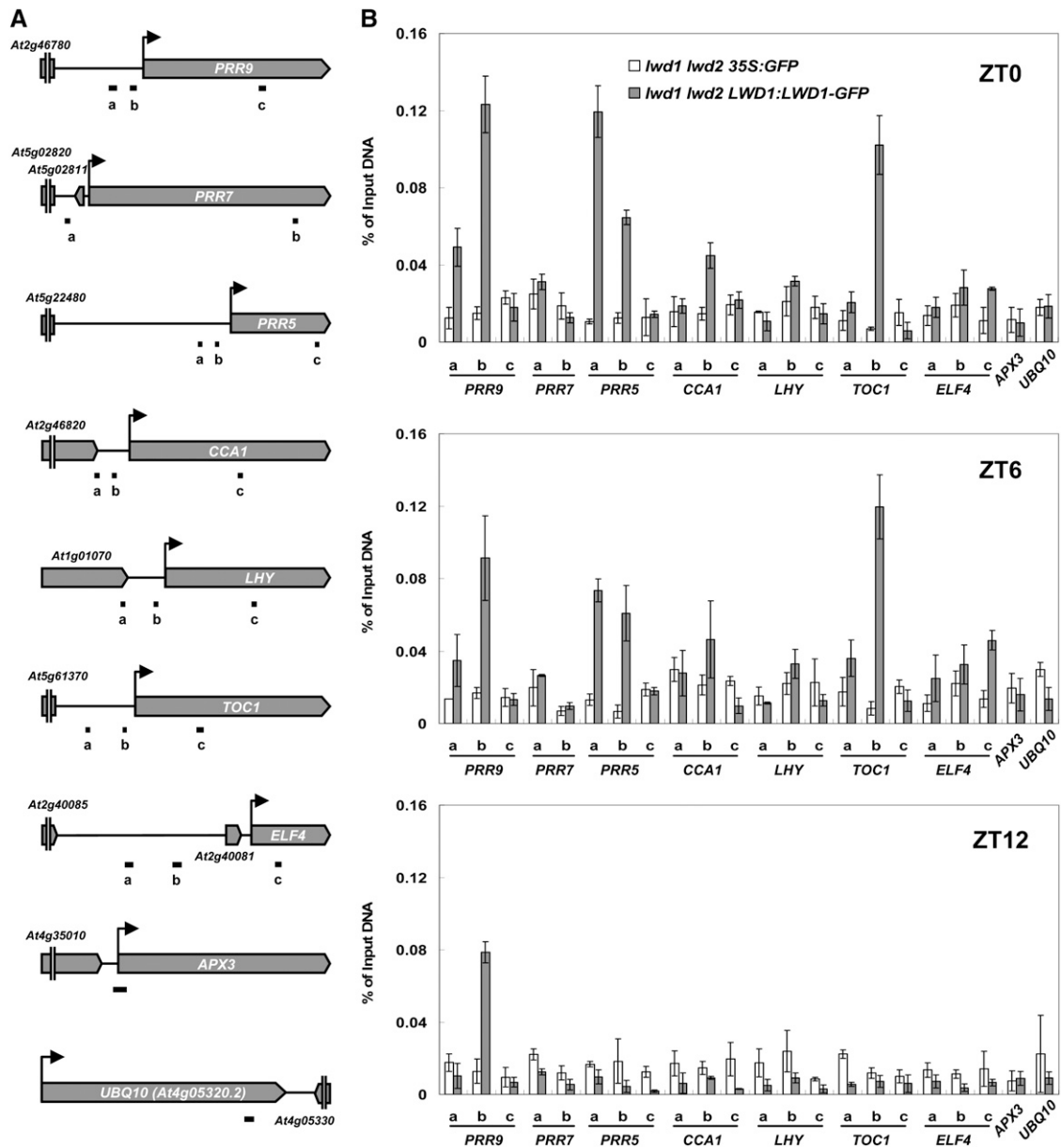


Figure 8. LWD1 Associates with *PRR9*, *PRR5*, and *TOC1* Promoters in Vivo.

(A) Diagram of the gene structures for oscillator genes, *APX3* and *UBQ10*. Transcriptional start and genomic regions are marked with arrow and shaded bars. Target fragments assayed by ChIP-qPCR are marked by horizontal black bars.

(B) ChIP assays were performed with anti-GFP antibody in *lwd1 lwd2 35S:GFP* or *lwd1 lwd2 LWD1:LWD1-GFP* plants. Plants were grown under 12 h light/12 h dark ($55 \mu\text{mol m}^{-2} \text{s}^{-1}$) and harvested at ZT0, ZT6, and ZT12. Immunoprecipitated DNA was quantified by qPCR with specific primer pairs for amplicons a, b, or c corresponding to various regions in genes tested. Amplicons in *APX3* and *UBQ10* were used as an internal control. Results were normalized as percentage of the input DNA. Data are means \pm SD (technical replicates, $n = 3$). Two independent experiments were performed with similar results.

with the significant reduction in *LWD1* promoter activity in the *pr9* mutant, the *pr5* or *toc1* mutant showed less change of *LWD1* promoter activity. This finding indicates a more remote role of *PRR5* and *TOC1* in regulating the *LWD1* promoter (Figure 5). Our results did not reveal clear binding of *LWD1* to the

upstream regions of *CCA1*, *LHY*, *ELF4*, and *PRR7*. Thus, *LWD1* may indirectly regulate their expression. However, we cannot entirely rule out that these genes might possess *LWD1* target site(s) outside of the amplicons tested or that the binding only occurs at time points not examined in this study.

DISCUSSION

LWD1 and PRR9 Constitute a Positive Feedback Loop

Previously, we reported LWD1 and LWD2 as two clock proteins (Wu et al., 2008). In this study, we performed an in-depth characterization of the relationship between LWD1/2 and central oscillator genes. Our study revealed a positive feedback loop consisting of *LWD1* and *PRR9* in the *Arabidopsis* circadian clock (Figure 9). Positive and negative feedback loops exist in the circadian clock of many model organisms (for review, see Doherty and Kay, 2010). Negative feedback loops can contribute to the clock's function in building the 24-h oscillation. However, mathematical modeling suggested that in *Neurospora*, a positive loop could enhance the buffering of the conidiation phase against seasonal photoperiod changes (Akman et al., 2010). Successful integration of positive and negative feedback loops are known to play key roles in maintaining the stability and robustness of the oscillator in *Neurospora* (Lee et al., 2000; Cheng et al., 2001).

A positive feedback loop may not contribute to building the 24-h oscillation like the frequently discussed negative feedback loops. Rather, the positive feedback loop is crucial to guarantee the expression of key regulators such as *PRR9/7* and *LWD1/2* in *Arabidopsis*. Previous studies indicated that the expression of *PRR9/7* is crucial for the direct binding and repression of the morning genes *CCA1* and *LHY* (Nakamichi et al., 2010). Our study also suggested an important regulatory role of *LWD1/2* in the transcription of multiple clock components (Figures 1 and 6; see Supplemental Figure 3 online). The mutual enhancement of *LWD1/2* and *PRR9/7* may ensure a balanced expression of clock components. Of course, a positive feedback loop requires a negative regulator(s) to inhibit an unwanted amplification. Such potential negative regulators for *LWD1/2* are *TOC1* and *ELF4* (Figure 5).

LWD1 Has Multiple Entry Points to the Central Oscillator in *Arabidopsis*

Central oscillator proteins could regulate the expression of multiple oscillator genes. For example, *CCA1* can bind to the promoters of *TOC1*, *PRR9*, *PRR7*, *LUX*, and *CHE* and regulate the expression of these genes (Alabadi et al., 2001; Farré et al., 2005; Hazen et al., 2005; Pruneda-Paz et al., 2009). Our results indicate that *LWD1* directly targets the promoter regions of *PRR9* and *PRR5* (Figure 8). Because both the promoter activities and transcript levels of *PRR9* and *PRR5* are reduced in the *lwd1 lwd2* double mutant (Figure 6; see Supplemental Figures 3A and 3C online), *LWD1* likely serves as a positive regulator of *PRR9* and *PRR5* (Figure 9).

LWD1 also binds to the promoter of *TOC1*, and *TOC1* promoter activity is clearly reduced in the *lwd1 lwd2* double mutant (Figures 1, 8, and 9); however, the transcript level of *TOC1* is slightly elevated in the *lwd1 lwd2* double mutant (Wu et al., 2008). The slightly elevated transcript level of *TOC1* may result from the posttranscriptional regulation of *TOC1* itself or a combination effect of *LWD1/2* and other oscillator genes because of their interconnected nature.

The expression of *CCA1* and *LHY* is repressed by *PRR9* and activated by *TOC1* (Alabadi et al., 2001; Farré et al., 2005;

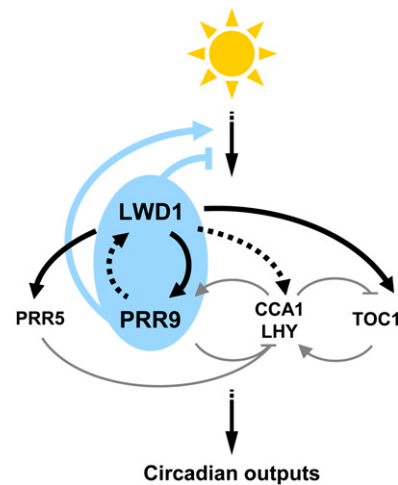


Figure 9. A Model Showing the Contribution of LWD1 in the *Arabidopsis* Circadian Clock.

A positive feedback loop formed by *LWD1* and *PRR9* according to this study is highlighted in blue. The *LWD1/PRR9* loop is also involved in regulating the light input pathway. The reciprocal regulations between *LWD1* and oscillators are marked with black lines according to the promoter activity data collected in this study. Solid and dashed lines represent direct binding and indirect regulation, respectively. Positive and negative regulations are marked with arrows and blunt-ended arrows. Gray lines indicate two known negative feedback loops composed of *CCA1/LHY*, *PRR9*, and *TOC1* reported previously.

Nakamichi et al., 2005, 2010). In the *lwd1 lwd2* double mutant, one would expect an increased expression of *CCA1/LHY* due to the decreased expression of *PRR9* and slightly increased expression of *TOC1* (Figure 6; (Wu et al., 2008). However, the expression of *CCA1* and *LHY* is decreased in the *lwd1 lwd2* mutant (Wu et al., 2008), which suggests that *LWD1/2* could regulate *CCA1* and *LHY* in a *PRR9/7*- and/or *TOC1*-independent manner (Figure 9). These results imply that *LWD1* has multiple entry points to central oscillators. Also, the impact of the decreased transcript levels of *CCA1/LHY* and *PRR5* in the *lwd1 lwd2* double mutant may be dominant to that of the misregulation of *PRR9* and *TOC1*. This suggestion might explain why *lwd1 lwd2* has a short period phenotype, similar to that in *cca1*, *lhy*, or *prp5* mutants. Further studies are needed to decipher the regulatory mechanism of *LWD1/2* on *CCA1/LHY* or *PRR5*.

LWD1/2 Plays a Dual Function in the Circadian System

In addition to functioning as a component of the central oscillator, FRC analysis indicated that *LWD1/2* also acts in the input pathway to modulate the clock sensitivity to light (Figure 3). Thus, *LWD1/2* plays dual functions in both core oscillator loops and the light input pathway (Figure 9). Additional examples of such dual function proteins are *PRR9* and *PRR7*. *prp7* and *prp9* mutants also have a different slope of FRC (Farré et al., 2005). However, in contrast with the short period length in *lwd1 lwd2*, that in *prp7* or *prp9* mutants is longer (Farré et al., 2005).

By functioning in the input pathway, LWD1/2 and PRR9/7 may have an antagonistic function in keeping the proper period length. In this regard, plants use photoreceptors to perceive light stimuli and positive regulators such as PRR9/7 for transmitting the light signals to increase the frequency of the oscillation (Aschoff, 1979). To ensure a proper equilibrium, negative regulators such as LWD1 and LWD2 are required to attenuate the light input and to prevent excessive shortening of the period length. Possibly by antagonizing effects against each other in period length control, the positive feedback regulation between LWD1/2 and PRR9/7 in the circadian system offers plants a safeguard or flexibility to fine-tune the period length under various light intensities in nature.

METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana ecotype Columbia (Col-0) was used as the wild type in this study. The clock mutants used were *lwd1 lwd2* (Wu et al., 2008), *cca1-1* (Yakir et al., 2009), *lhy-101* (Khanna et al., 2006), *toc1-101* (Kaczorowski, 2004; Kikis et al., 2005), *elf4-101* (Khanna et al., 2003), *gi-2* (Park et al., 1999), *prr5-11*, *prr7-11* (Yamamoto et al., 2003), and *prr9-10* (Ito et al., 2003). *cca1-1* used in this study was originally in Wassilewskija ecotype but was introduced into Col-0 background via backcrossing as described (Yakir et al., 2009). Constructs of promoter:luciferase reporter genes were first transformed into Col-0 and then introduced into destination mutants by genetic crossing. For qRT-PCR experiments shown in Figure 6, 18-d-old plants were germinated in soil and placed at 4°C for 3 d to synchronize the germination. The plants were grown under 12 h light/12 h dark at a fluence rate of 80 to 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

Constructs

To generate luciferase reporter plasmids, the synthetic firefly *LUC2* gene derived from pGL4.10 (Promega) was inserted into the *Sall* and *Sacl* sites of pJD301 and fused with the transcriptional terminator sequence of the *Agrobacterium tumefaciens* nopaline synthase gene (*Nos*). The *LUC2:Nos* cassette was subcloned in the binary vector pCambia1390 (CSIRO) at *Sall-EcoRI* sites and fused with a *PstI* to *Sall* fragment of the promoter of interest. Promoter regions of clock genes are *CCA1* (−1418 ~ −1 relative to the translation start) (Kim et al., 2003), *LHY* (−1661 ~ −1) (Kim et al., 2003), *TOC1* (−1558 ~ −1) (Perales and Más, 2007), *ELF4* (−1613 ~ −1) (McWatters et al., 2007), *GI* (−3700 ~ −1) (Onai et al., 2004), *PRR9* (−1368 ~ −1) (Para et al., 2007), *PRR7* (−2000 ~ −1), *PRR5* (−2020 ~ −1), *LWD1* (−1068 ~ −1), and *LWD2* (−949 ~ −1). Primer sequences used for amplification from genomic DNA are in Supplemental Table 1 online. All constructs used in this study were confirmed by sequencing analysis.

Bioluminescence Measurement and Data Analyses

For measurement of luciferase activity, plants grown on half-strength Murashige and Skoog (Murashige and Skoog, 1962) agar plates and entrained under 16-h-light/8-h-dark or 12-h-light/12-h-dark cycles with white light illumination (55 to 75 $\mu\text{mol m}^{-2} \text{s}^{-1}$; GE cool white fluorescent lamps). After 7 or 9 d, each seedling was transferred into black 96-well microplates containing 200 μL solid half-strength Murashige and Skoog medium and 70 or 80 μL of 0.5 mM luciferin (Promega) per well. Bioluminescence activity was measured as described previously (Kim et al., 2008) with minor modification. Light treatment and bioluminescence detection was performed in a temperature- and light-controlled

dark box (Taiwan Hipoint). White, red, and blue lights were produced by LED lamps (B5-430-JD, 435 to ~675 nm; B5-436-30D, 660 \pm 5 nm; and B5-437-KD, 472 \pm 15 nm, respectively; DAINA Electronics). Light intensities were adjusted with neutral density filters (LEE Filters). The fluence rate was measured with use of an LI-250 radiometer (Li-Cor). Each bioluminescence image was recorded by low-light video imaging for 5 to ~10 min using a Peltier-cooled CCD slow scan camera (PIXIS 2048; Roper Scientific). Image processing and quantification involved use of ImagePro Plus software (Media Cybernetics). Data were imported into the Biological Rhythms Analysis Software System (available from <http://www.amillar.org>; Southern et al., 2006) and analyzed using the FFT-NLLS suite. Period lengths were estimated with bioluminescence data obtained from 24 to 96 h under free-running conditions.

RNA Isolation

Total RNA was isolated as described previously (Chang et al., 1993) with minor modifications. Plant tissues were frozen and ground in liquid nitrogen and extracted by vortexing with 8 volumes of extraction buffer (2% hexadecyltrimethylammonium bromide, 2% polyvinylpyrrolidone K 30, 100 mM Tris-HCl, pH 8.0, 25 mM EDTA, 2.0 M NaCl, 0.5 g/L spermidine, and 2% 2-mercaptoethanol) prewarmed at 65°C. The homogenate was then extracted twice with an equal volume of chloroform: isoamyl alcohol (24:1) by vortexing and centrifugation for 15 min at 12,000g. A one-quarter volume of 10 M LiCl was then added to the aqueous phase for selective precipitation of RNA molecules. After overnight incubation at 4°C, the RNA pellet was harvested by centrifugation at 12,000g for 30 min at 4°C, washed with 75% ethanol, and dissolved in 20 μL of RNase-free water.

Real-Time qRT-PCR

qRT-PCR was performed as described previously (Wu et al., 2008). Sequences and ratio of the primers (5 μM each) used for each gene were determined experimentally as suggested by the manufacturer and listed in Supplemental Table 1 online. Real-time qRT-PCR involved use of the ABI Prism 7500 sequence detection system (Applied Biosystems) with programs recommended by the manufacturer (2 min at 50°C, 10 min at 95°C, and 40 cycles of 95°C for 15 s and 60°C for 1 min). The comparative C_T method was used to determine the relative amount of gene expression, with the expression of *UBQ10* used as an internal control. For clarity, mean values of $2^{-\Delta C_T}$ ($\Delta C_T = C_{T, \text{gene of interest}} - C_{T, \text{UBQ10}}$) were calculated from four independent experiments.

Subcellular Localization of LWD1

An *LWD1* genomic fragment was generated by PCR with the primer pair LWD1-Fw and LWD1-Rv (see Supplemental Table 1 online), which resulted in the removal of the termination codon and created an in-frame fusion of GFP to the C terminus of LWD1. The fragment was ligated into the 326GFP vector (Lee et al., 2001) to replace the 35S promoter and create the *LWD1:LWD1-GFP-Nos* fusion cassette. The cassette was subcloned into pCambia1390 and then transformed into the *lwd1 lwd2* double mutant. A RanGAP1 coding sequence was generated by PCR with the primers listed in Supplemental Table 1 online for an in-frame fusion of RFP to its C terminus. The fragment was inserted into the 326RFP vector to create the *35S:RanGAP1-RFP-Nos* fusion cassette, which was subcloned into pCambia2300 and then transformed into *lwd1 lwd2 LWD1:LWD1-GFP* complementation plants. GFP signals were observed in roots by confocal microscopy (Zeiss META 510; Carl Zeiss Micro-Imaging) with the argon laser at 488-nm excitation and band-pass filter at 500- to 530-nm emission. RFP signals were observed with the argon laser at 543-nm excitation and band-pass filter at 585- to 615-nm emission.

ChIP-qPCR Assay

The ChIP assay was performed as previously described (Saleh et al., 2008) with minor modifications. Three-week-old plants grown under 12 h light/12 h dark were harvested at ZT0, ZT6, and ZT12. In total, 1.2 g of plant tissue was cross-linked in 35 mL cross-linking buffer (0.4 M sucrose, 10 mM Tris-HCl, pH 8.0, 1 mM PMSF, 1 mM EDTA, and 1% formaldehyde) under vacuum for 25 min and stopped by adding 2 M glycine (final concentration 125 mM). Plants were then ground to powder in liquid nitrogen and lysed in 12.5 mL nuclei isolation buffer (0.25 M sucrose, 15 mM PIPES, pH 6.8, 5 mM MgCl₂, 60 mM KCl, 15 mM NaCl, 1 mM CaCl₂, 0.9% Triton X-100, 0.1 mM PMSF, and 1× protease inhibitor cocktail [Roche]). The lysate was filtered through two layers of Miracloth (Calbiochem), and the filtrate was centrifuged at 11,000g for 20 min at 4°C. An amount of 0.7 mL cold nuclei lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% SDS, 0.1% sodium deoxycholate, 1% Triton X-100, 0.1 mM PMSF, and 1× protease inhibitor cocktail) was used to resuspend the pellet, which was followed by sonication using the Bioruptor (Diagenode) set at high power and 10-s ON/10-s OFF for 30 min. The chromatin complexes were precleared by adding 30 μL Protein A Sepharose (GE Healthcare) preequilibrated with 1 μg/mL salmon sperm DNA and 1 mg/mL BSA. A one-tenth volume of the chromatin complexes was used as an input control. Immunoprecipitation of 0.7 mL chromatin complexes involved incubation with 1 μL anti-GFP antibody (ab290; Abcam) at 4°C overnight. After incubation, the solution was bound to preequilibrated Protein A Sepharose for 2 h and then washed with nuclei lysis buffer three times, LNDET buffer (0.25 M LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, and 1 mM EDTA) three times, and TE buffer (1 mM EDTA and 10 mM Tris-HCl, pH 8.0) twice. Immunocomplexes were eluted from the beads with use of elution buffer (0.5% SDS and 0.1 M NaHCO₃). Cross-linking of immunocomplexes and the input fraction was reversed by incubation at 65°C overnight and digestion with Proteinase-K (Invitrogen). DNA was extracted by use of a PCR purification kit (Qiagen) according to the manufacturer's protocol. The amount of each precipitated DNA and input DNA was determined by qPCR with the specific primers in Supplemental Table 1 online. The ChIP assay shown in Supplemental Figure 6 online was performed as described (Nakamichi et al., 2010).

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative data library with the following locus identifiers: *LWD1* (At1g12910), *LWD2* (At3g26640), *CCA1* (At2g46830), *LHY* (At1g01060), *TOC1* (At5g61380), *ELF4* (At2g40080), *PRR5* (At5g24470), *PRR7* (At5g02810), *PRR9* (At2g46790), *GI* (At1g22770), *UBQ10* (At4g05320), *APX3* (At4g35000), and *RanGAP1* (At3g63130).

Supplemental Data

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

Supplemental Figure 1. *LWD1* and *LWD2* Are Required for Maintaining Period Length of Clock Genes in Continuous Light and Dark.

Supplemental Figure 2. The Transcript Levels of *LWD1/2* in Wild-Type, *prr9 prr7 prr5* Triple Mutant, and *CCA1* Overexpression Plants.

Supplemental Figure 3. *LWD1* and *LWD2* Are Required for the Full Promoter Activities of *PRR9*, *PRR7*, and *PRR5*.

Supplemental Figure 4. *lwd1 lwd2 LWD1* Complementation Plants Accumulate More *LWD1* Transcripts Than the Wild Type.

Supplemental Figure 5. Transgenic Plants Used to Confirm the Nucleus Localization of *LWD1*-GFP Proteins.

Supplemental Figure 6. *PRR9/PRR7* Do Not Associate with *LWD1/LWD2* Promoters.

Supplemental Table 1. Primers Used in This Study.

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