Two New Clock Proteins, LWD1 and LWD2, Regulate Arabidopsis Photoperiodic Flowering[1][W][OA]

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The "light" signal from the environment sets the circadian clock to regulate multiple physiological processes for optimal rhythmic growth and development. One such process is the control of flowering time by photoperiod perception in plants. In Arabidopsis (*Arabidopsis thaliana*), the flowering time is determined by the correct interconnection of light input and signal output by the circadian clock. The identification of additional clock proteins will help to better dissect the complex nature of the circadian clock in Arabidopsis. Here, we show LIGHT-REGULATED WD1 (LWD1)/LWD2 as new clock proteins involved in photoperiod control. The *lwd1lwd2* double mutant has an early-flowering phenotype, contributed by the significant phase shift of *CONSTANS* (*CO*), and, therefore, an increased expression of *FLOWERING LOCUS T* (*FT*) before dusk. Under entrainment conditions, the expression phase of oscillator (*CIRCADIAN CLOCK ASSOCIATED1* [*CCA1*], *LATE ELONGATED HYPOCOTYL* [*LHY*], *TIMING OF CAB EXPRESSION1* [*TOC1*], and *EARLY FLOWERING4* [*ELF4*]) and output (*GIGANTEA*, *FLAVIN-BINDING, KELCH REPEAT, F-BOX1*, *CYCLING DOF FACTOR1*, *CO*, and *FT*) genes in the photoperiod pathway shifts approximately 3 h forward in the *lwd1lwd2* double mutant. Both the oscillator (*CCA1*, *LHY*, *TOC1*, and *ELF4*) and output (*COLD, CIRCADIAN RHYTHM, AND RNA BINDING2* and *CHLOROPHYLL A/B-BINDING PROTEIN2*) genes have a short period length in the *lwd1lwd2* double mutant. Our data imply that LWD1/LWD2 proteins function in close proximity to or within the circadian clock for photoperiodic flowering control.

Arabidopsis (*Arabidopsis thaliana*) flowers early under long days and is thus categorized as a facultative long-day (LD) plant. In the past decade, both genetic and biochemical studies of Arabidopsis have greatly fueled our understanding of photoperiod control in plants (for review, see Imaizumi and Kay, 2006; Kobayashi and Weigel, 2007). The environmental light signals and the internal circadian clock must function in harmony to achieve proper photoperiod control in plants.

Environmental light signals are perceived by plant photoreceptors, including the red/far-red photoreceptors and blue light photoreceptors. This process inputs the light signal to reset the circadian clock for optimal rhythmic growth and development in plants. Recent studies have also revealed a few regulators that are essential for the proper function of the Arabidopsis circadian rhythm (for review, see McClung, 2006; Yakir et al., 2007). Among those, EARLY FLOWERING3 (ELF3), TIME FOR COFFEE, and LIGHT INSENSITIVE PERIOD1 (LIP1) have been implicated as functioning to input the light signal into the core circadian clock (McWatters et al., 2000; Covington et al., 2001; Hall et al., 2003; Ding et al., 2007; Kevei et al., 2007).

The most well-studied Arabidopsis circadian clock is formed by a negative feedback loop composed of the oscillator proteins CIRCADIAN CLOCK ASSOCIATED1 (CCA1), LATE ELONGATED HYPOCOTYL (LHY), and TIMING OF CAB EXPRESSION1 (TOC1; Schaffer et al., 1998; Wang and Tobin, 1998; Strayer et al., 2000; Alabadi et al., 2001). ELF4, GIGANTEA (GI), LUX ARRHYTHMO/PHYTOCLOCK1 (LUX), TOC1 paralogs, and PSEUDO-RESPONSE REGULATOR5 (PPR5), PRR7, and PRR9 represent members of additional feedback loops of the clock (Yamamoto et al., 2003; Farre et al., 2005; Hazen et al., 2005; Kikis et al., 2005; Nakamichi et al., 2005; Onai and Ishiura, 2005; Martin-Tryon et al., 2007; McWatters et al., 2007). More recently, FIONA1 (FIO1) was found to regulate the clock in close association with the central oscillators (Kim et al., 2008). The proper expression of the clock genes is crucial for the function of plant circadian rhythm. In addition to transcriptional control, the protein stability of TOC1 and the activity of CCA1 can be regulated by ZEITLUPE (ZTL) and casein kinase II, respectively (Mas et al., 2003; Daniel et al., 2004). There exists a complex interlocked network within the Arabidopsis circadian clock (McClung, 2006). More effort is still needed to

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uncover additional clock proteins for a complete understanding of circadian regulation in Arabidopsis.

In the aspect of flowering time control regulated by photoperiod sensing, circadian output from the clock regulates the rhythmic expression of *FLAVIN-BINDING, KELCH REPEAT, F-BOX1* (*FKF1*) and *GI* (Fowler et al., 1999; Park et al., 1999; Nelson et al., 2000; Imaizumi et al., 2003; Mizoguchi et al., 2005). In a blue light-dependent manner, the complex of FKF1 and GI functions as a positive regulator of *CONSTANS* (*CO*) expression via targeted degradation of CYCLING DOF FACTOR1 (CDF1), a repressor of *CO* (Imaizumi et al., 2005; Sawa et al., 2007). In addition, light also regulates CO protein stability (Valverde et al., 2004), a process regulated by SUPPRESSOR OF PHYA-105 (SPA) proteins (Laubinger et al., 2006) and/or COP1 (for CONSTITUTIVE PHOTOMORPHOGENIC1; Jang et al., 2008; Liu et al., 2008). The timing of *CO* gene expression and the regulation of CO protein stability together are crucial for Arabidopsis to sustain correct daylength measurement. The accumulation of CO protein by dusk under LD conditions activates the expression of *FLOWERING LOCUS T* (*FT*; Suarez-Lopez et al., 2001; Yanovsky and Kay, 2002). FT protein was later proven to be one of the "florigen" molecules generated in photoperiod-induced leaves and translocated to the shoot apex for the stimulation of the vegetative-to-floral transition (Corbesier et al., 2007; Jaeger and Wigge, 2007; Mathieu et al., 2007; Tamaki et al., 2007).

Although a good foundation has been laid for studies of photoperiod sensing in Arabidopsis, missing pieces in this big puzzle of fine-tuning and/or modulating photoperiodism still remain to be discovered. Light appears to be the most effective signal in synchronizing the environmental cue and the internal circadian clock in plants. Previous studies indicated that many of the key regulators in circadian and photoperiod control exhibit light-regulated expression characteristics. These observations prompted us to use a reverse genetics approach to find additional light-regulated genes that contribute to photoperiod regulation. We uncovered an early-flowering Arabidopsis mutant defective in both *LIGHT-REGULATED WD1* (*LWD1*) and *LWD2*, both of which encode WD (for Trp and Asp)-containing proteins. Here, we show that the increased expression of *FT* is the likely cause of the early-flowering phenotype in the *lwd1lwd2* double mutant. Our data also indicate that LWD1 and LWD2 are new clock proteins. Their presence is essential for the proper expression phase and period length of both the oscillator and output genes known to participate in Arabidopsis photoperiod sensing.

RESULTS

The *lwd1lwd2* Double Mutant Has an Early-Flowering Phenotype

Our previous transcriptome analyses of Arabidopsis seedling photomorphogenesis and dark-treated leaves revealed many light-responsive genes (Lin and Wu, 2004; Chang et al., 2008). Genes encoding unique protein features were selected for further characterization of their possible contribution in light-regulated processes in Arabidopsis, including photoperiod flowering control. Among the genes examined, LWD1 (At1g12910) shares approximately 90% amino acid sequence identity with LWD2 (At3g26640) in Arabidopsis (Supplemental Fig. S1), so the two genes may have overlapping functions. Arabidopsis mutants carrying T-DNA insertions in *LWD1* or *LWD2* were obtained from the Arabidopsis Biological Resource Center and designated *lwd1* (SALK_006874) or *lwd*2 (SALK_072182; Fig. 1A). *LWD1* or *LWD2* transcripts were undetectable in homozygous *lwd1* or *lwd2* single mutants, respectively, on reverse transcription (RT)-PCR analyses with primers spanning the T-DNA insertion sites (Fig. 1A). Phenotype characterization of the *lwd1* or *lwd*2 single mutants did not uncover obvious phenotypic alterations, which suggested that these two proteins have functional redundancy (data not shown).

The *lwd1lwd2* double mutant was next generated by reciprocal crossing of *lwd1* and *lwd2*. Northern-blot analyses confirmed no full-length *LWD1* or *LWD2* transcripts in the *lwd1lwd2* double mutant (Supplemental Fig. S2). The mutant *lwd1lwd2* showed an early-flowering phenotype under LD conditions, with significantly fewer leaves than in the wild-type ecotype Columbia-0 (Col-0) prior to bolting (7.05 $\pm$ 0.7 versus 8.7 $\pm$ 0.48 leaves; Fig. 1B, left). The early-flowering phenotype of *lwd1lwd2* was more prominent under short-day (SD) conditions (20.65 $\pm$ 3.6 versus 63.3 $\pm$ 2.6 leaves; Fig. 1B, right). Under both LD and SD conditions, the early-flowering phenotype in the *lwd1lwd2* double mutant could be rescued by introducing a genomic fragment of *LWD1* back to the *lwd1lwd2* double mutant (*lwd1lwd2/LWD1* in Fig. 1B). These results indicate that the lost function of *LWD* is indeed responsible for the early-flowering phenotype in *lwd1lwd2* double mutant plants.

Genes in Photoperiodic Pathways Are Differentially Regulated in the *lwd1lwd2* Double Mutant

Previous studies indicated that defects in photoperiodic sensing, vernalization, autonomous, or gibberellin pathways could account for the early-flowering phenotype in Arabidopsis (for review, see Blazquez, 2000; Mouradov et al., 2002; Komeda, 2004). The early-flowering phenotype in *lwd1lwd2* is likely due to the misregulation of genes in one or more of these pathway(s) by the mutation in *LWD1* and *LWD2*. To clarify this, we performed a transcriptomic comparison between wild-type Arabidopsis and the *lwd1lwd2* double mutant with the use of the Affymetrix GeneChip. The expression of genes belonging to the photoperiodic pathways was more severely affected than that of genes in the other three pathways in the *lwd1lwd2* double mutant (Fig. 2). This implies that the defect in photo-

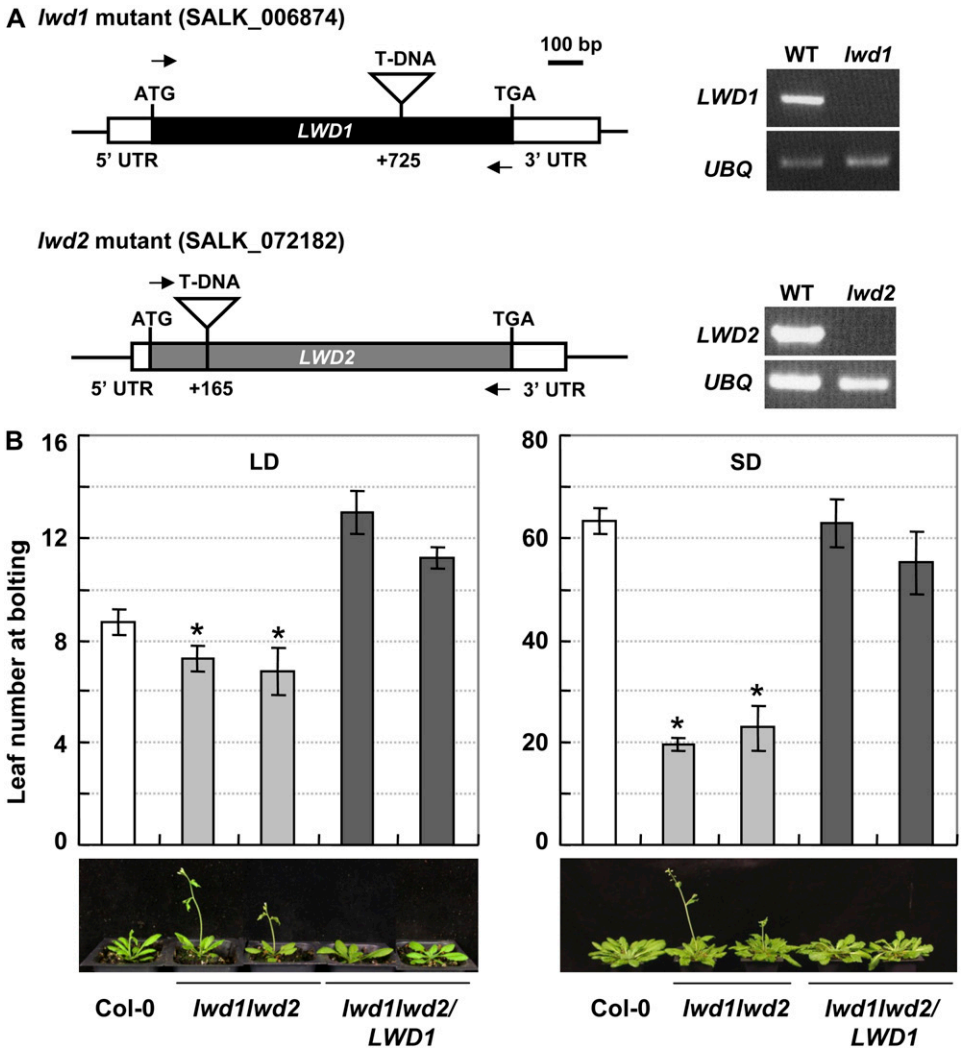

Figure 1. LWD1 and LWD2 regulate flowering time in Arabidopsis under both LD and SD conditions. A, Two lines (SALK_006874 [*lwd1*] and SALK_072182 [*lwd2*]) carried the T-DNA insertion in the intronless *LWD1* and *LWD2* genes. The locations of the T-DNA, 5′/3′ untranslated region (UTR), and ATG/TGA are marked on the *LWD1*/*LWD2* gene models. The level of *LWD1* (*LWD2*) transcript in the wild type and *lwd1* (*lwd2*) was assessed by RT-PCR with the primers listed in Supplemental Primer Table S1 (marked with horizontal arrows). RT-PCR was performed with *UBQ10*-specific primers used as a control for input RNA in the RT reaction. B, Plants of wild-type Arabidopsis (Col-0), *lwd1lwd2* double mutant, and two independent complementation lines (*lwd1lwd2/LWD1*) were grown under LD (16 h of light/8 h of dark) or SD (8 h of light/16 h of dark) conditions. The plants were photographed, and the leaf numbers of each corresponding line were measured at the time of bolting as described in "Materials and Methods." Asterisks indicate that *lwd1lwd2* plants flowered significantly earlier than wild-type plants (Student's *t* test; $P < 0.0005$, $n = 10$).

period sensing is primarily responsible for the early-flowering phenotype in the *lwd1lwd2* double mutant.

FT Is Highly Expressed in *lwd1lwd2* under SD Conditions

In the photoperiodic pathway, the circadian regulation of *CO* and *FT* leads to the correct measurement of daylength information for flowering determination. Previous studies have shown that the higher accumulation of *CO* transcripts/protein before dusk and/or the increased expression of *FT* in the photoperiodic pathway lead to an early-flowering phenotype in Arabidopsis (for review, see Imaizumi and Kay, 2006). To relate these observations to the early-flowering phenotype observed in the *lwd1lwd2* double mutant, we sought to examine the expression of these two genes in *lwd1lwd2*. We used real-time quantitative RT-PCR (qRT-PCR) to measure the transcript abundance of both genes during a 24-h period under both LD and SD conditions. An increased expression of *CO* was observed in *lwd1lwd2* under both LD and SD (Fig. 3). The marginal up-regulation of *CO* resulted in an increased induction of *FT* in *lwd1lwd2* under LD (Fig. 3, left), which was sufficient for *lwd1lwd2* to show an early-flowering phenotype under LD (Fig. 1B). Under SD, likely because of an advanced expression phase (see below), the increased expression of *CO* before dusk led to a higher expression of *FT* in *lwd1lwd2* (Fig. 3, right). The remarkable increase in *FT* transcript abundance in *lwd1lwd2* under SD explains why the *lwd1lwd2* double mutant flowers substantially earlier than wild-type plants under SD.

LWD1/*LWD2* Set the Correct Expression Phase of Circadian Clock-Regulated Genes

Previous reports indicated that CDF1 is a negative regulator of *CO* gene expression. This negative regulation could be derepressed by the targeted degradation of CDF1 by a protein complex composed of FKF1 and GI (Imaizumi et al., 2005; Sawa et al., 2007). Similar to *CO* and *FT*, *GI*, *FKF1*, and *CDF1* are all circadian clock-regulated genes. Since *CO* showed a

Flowering pathway	Systematic	Locus Number	Gene	Repeat 1	Repeat 2
Photoperiodic	249741_at	At5g24470	*PRR5*	5.115	4.051
	247525_at	At5g61380	*TOC1*	4.974	7.311
	247668_at	At5g60100	*PRR3*	4.588	7.554
	252475_s_at	At3g46640	*LUX*	4.027	8.485
	259990_s_at	At1g68050	*FKF1*	3.368	2.142
	267364_at	At2g40080	*ELF4*	2.336	2.045
	264211_at	At1g22770	*GI*	2.222	1.788
	266839_at	At2g25930	*ELF3*	1.821	2.119
	250971_at	At5g02810	*PRR7*	0.708	0.67
	247898_at	At5g57360	*ZTL*	0.626	0.54
	247452_at	At5g62430	*CDF1*	0.502	0.571
	266719_at	At2g46830	*CCA1*	0.193	0.191
	261569_at	At1g01060	*LHY*	0.187	0.178
	266720_s_at	At2g46790	*PRR9*	0.0755	0.0646
Autonomous	250476_at	At5g10140	*FLC*	1.417	0.754
	245848_at	At5g13480	*FY*	1.238	1.53
	255444_at	At4g02560	*LD*	1.044	0.808
	245489_at	At4g16280	*FCA*	0.821	1.241
Vernalizaiton	256944_at	At3g18990	*VRN1*	1.212	1.23
	247695_at	At5g59710	*VIP2*	0.897	0.993
	245280_at	At4g16845	*VRN2*	0.889	0.96
	247565_at	At5g61150	*VIP4*	0.856	0.751
	253645_at	At4g29830	*VIP3*	0.686	0.974
	260813_at	At1g43700	*VIP1*	0.662	0.569
GA	259259_at	At3g11540	*SPY*	1.233	1.359
	266331_at	At2g01570	*RGA1*	1.011	0.671
	262850_at	At1g14920	*GAI*	0.803	0.845

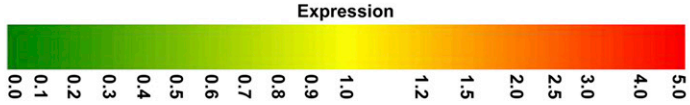

Figure 2. Genes in the photoperiodic pathway are differentially regulated in *lwd1lwd2* plants. Transcriptomic comparison was performed to analyze whether genes in the four pathways regulating flowering time are differentially regulated in 31-d-old wild-type and *lwd1lwd2* plants. Expression changes between wild-type and *lwd1lwd2* plants were color coded with extreme red (for 5-fold up-regulated) and extreme green (for 5-fold down-regulated) in *lwd1lwd2*. The fold change of gene expression in the *lwd1lwd2* double mutant is indicated. *CO* and *FT* were not included in this list because they were classified as "absent" in at least one of the four ATH1 hybridization experiments (see "Materials and Methods"). Expression data shown are listed in Supplemental Table S1.

clear advanced expression phase in the *lwd1lwd2* double mutant grown under SD (Fig. 3), we next sought to examine whether this is caused by the alteration of circadian expression of *GI*, *FKF1*, and *CDF1*. For this purpose, the expression kinetics of these genes was measured in wild-type, *lwd1lwd2* double mutant, and *lwd1lwd2/LWD1* complementation plants. As shown in Figure 4A, the expression level of *CDF1* was higher in *lwd1lwd2* double mutant than in wild-type plants under both LD and SD, whereas *FKF1* showed increased expression in *lwd1lwd2* only under SD. An approximately 3-h advanced expression phase was observed for all three genes known to regulate *CO* expression in the *lwd1lwd2* double mutant under both LD and SD conditions (Fig. 4A). Thus, the mutation in *LWD1* and *LWD2* results in advanced expression of *GI* and advanced and increased expression of *FKF1* and *CDF1*. This change of *GI*, *FKF1*, and *CDF1* expression pattern subsequently leads to an advanced expression phase (approximately 3 h) and, eventually, the accumulation of *CO* and *FT* before dusk in the *lwd1lwd2* double mutant (Fig. 3).

The next question is whether the abnormal function of central oscillators is responsible for the advanced expression phase of these circadian clock-regulated genes in the *lwd1lwd2* double mutant. To answer this, we tested the expression patterns of *CCA1* (morning gene) and *ELF4* (evening gene) under both LD and SD conditions. As shown in Figure 4B, a 3-h advanced expression phase was seen for both genes. Two addi-

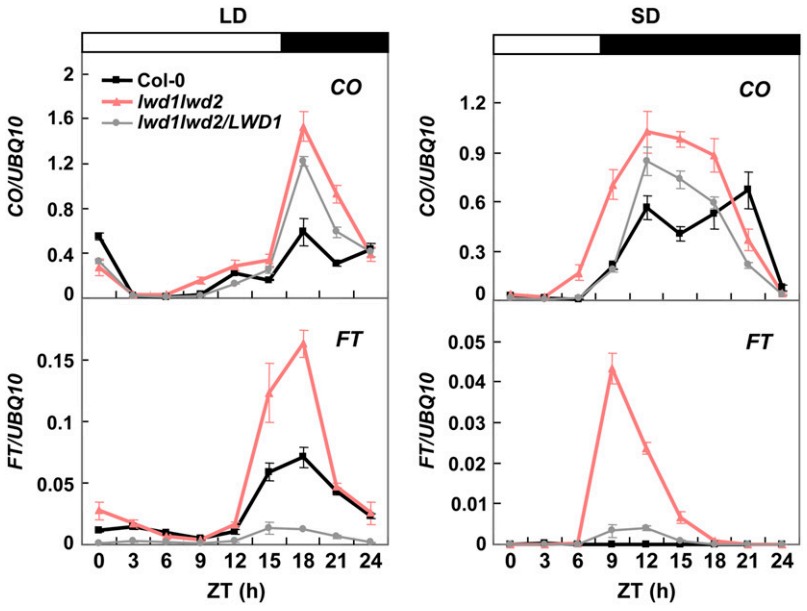

Figure 3. *FT* is highly expressed in *lwd1lwd2* under SD conditions. Eighteen-day-old wild-type, *lwd1lwd2*, and *lwd1lwd2/LWD1* plants grown under LD or SD conditions were harvested at different ZT times for total RNA isolation. White bars denote the light intervals, and black bars denote darkness. qRT-PCR was used to monitor the expression of *CO* and *FT*. Data are means ± SEM from four independent experiments.

tional oscillator genes, *LHY* and *TOC1*, also possessed an advanced expression phase (data not shown).

Functional complementation of *lwd1lwd2* by *LWD1* could restore the expression phase of all genes tested to that seen in wild-type Arabidopsis (Fig. 4, A and B), which indicates that the loss of *LWD1/LWD2* is responsible for maintaining the expression phase of both oscillator and output genes in Arabidopsis.

These expression data suggest that *LWD1/LWD2* are needed to control the proper expression phase of the central oscillator genes. Mutation in *LWD1/LWD2* will advance the expression of the oscillator genes during a 24-h period under both LD and SD conditions. Consequently, the output genes we tested, *FKF1*, *GI*, and *CDF1*, are expressed approximately 3 h earlier under both conditions. Increased transcript levels of *CO* and *FT* in the *lwd1lwd2* double mutant were observed under both LD and SD, whereas the advanced expression phase of these two genes was only seen under SD.

LWD1/LWD2 Regulate the Period Length and Amplitude of Oscillator and Output Genes

Since the advanced expression phase could be coupled with the shortened period length for circadian clock-regulated genes in Arabidopsis (Portoles and Mas, 2007), we next examined the period length of four oscillator genes, *CCA1*, *LHY*, *TOC1*, and *ELF4*, which have an advanced expression phase in the *lwd1lwd2* double mutant (Fig. 4B; data not shown). For this study, wild-type, *lwd1lwd2* double mutant, and *lwd1lwd2/LWD1* complementation plants were entrained under 12 h of light and 12 h of dark for 18 d and then released to continuous light (LL). Samples were collected every 3 h for a total of 72 h for RNA extraction and qRT-PCR analyses. Although these oscillator genes still exhibited a rhythmic expression pattern under LL, the period length of each gene was shorter (approximately 21 h) in the *lwd1lwd2* double mutant than in the wild type under LL (Fig. 5A). On examining the period length of two output genes, *COLD, CIRCADIAN RHYTHM, AND RNA BINDING2* (*CCR2*) and *CHLOROPHYLL A/B-BINDING PROTEIN2* (*CAB2*; Millar and Kay, 1991; Carpenter et al., 1994), we found their period length also shortened (Fig. 5B). Of note, the circadian amplitude of *LHY* and *CCR2* was significantly reduced in the *lwd1lwd2* double mutant under LL.

To test if the short period phenotype of the *lwd1lwd2* double mutant is dependent on the light condition, we further analyzed the expression of *CCA1*, *ELF4*, and *CCR2* in the wild-type, *lwd1lwd2* double mutant, and *lwd1lwd2/LWD1* complementation plants entrained under 12 h of light and 12 h of darkness for 18 d and then released to continuous dark (DD). As shown in Figure 6, *ELF4* and *CCR2* still maintained a rhythmic expression pattern with a shorter (approximately 3 h) period length in the *lwd1lwd2* double mutant than in the wild type under DD. The expression amplitude for *CCA1* was reduced under DD (Fig. 6).

Taken together, our data indicate that functional *LWD1/LWD2* are required for maintaining the period length and amplitude of both oscillator and output genes in Arabidopsis. Because the period length of these genes was shortened to approximately 3 h under both LL and DD in the *lwd1lwd2* double mutant, LWD1/LWD2 are more likely to function close to the central oscillator.

LWD1 Has a Diurnal Expression Pattern

The alteration in both phase and period length of circadian-regulated genes in the *lwd1lwd2* double mutant prompted us to examine whether the expression of *LWD1* and *LWD2* was under the control of the circadian clock in wild-type Arabidopsis. *LWD1* ex-

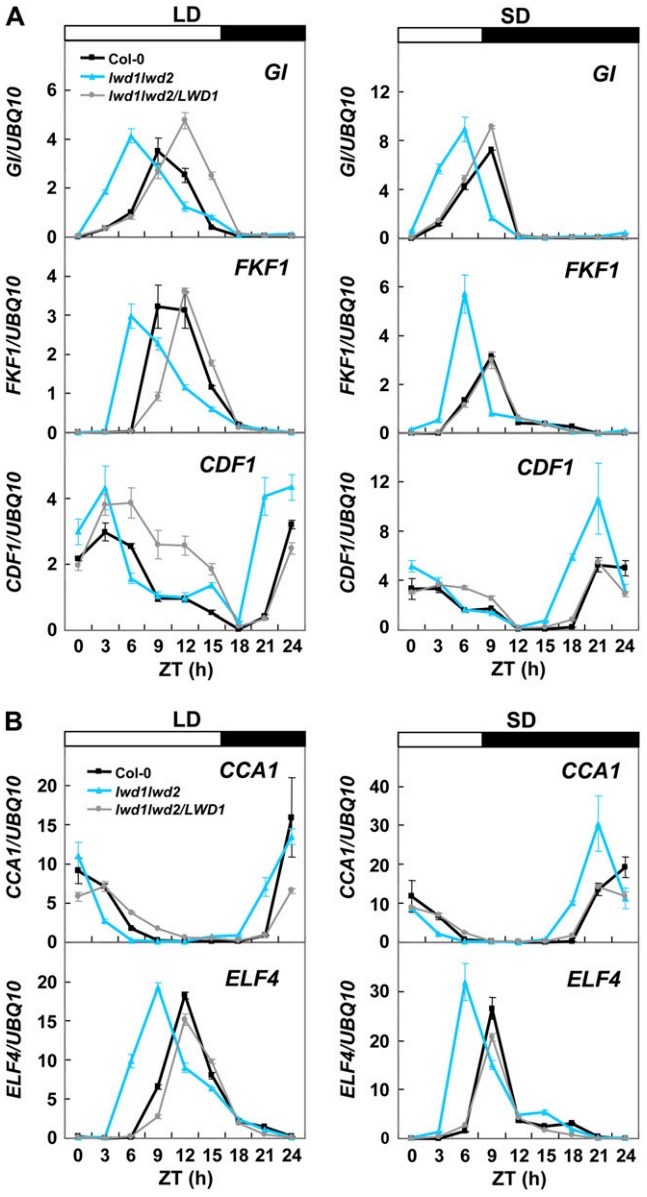

Figure 4. LWDs regulate the expression phase of circadian clock-regulated genes under both LD and SD conditions. Eighteen-day-old wild-type, *lwd1lwd2*, and *lwd1lwd2/LWD1* plants grown under LD or SD conditions were harvested at different ZT times for total RNA isolation. White bars denote the light intervals, and black bars denote darkness. qRT-PCR was used to monitor the expression of *GI*, *FKF1*, and *CDF1* (A) or *CCA1* and *ELF4* (B). Data are means ± SEM from four independent experiments.

hibited a recognizable diurnal expression pattern under LD and SD conditions (Fig. 7). *LWD2*, however, has a constant expression level under both LD and SD conditions. The functional redundancy of LWD1 and LWD2 prompted us to examine the transcript abundance of both genes. In light of the high sequence homology between the *LWD1* and *LWD2* coding regions, the LWD1- and LWD2-specific primers used here were located at the more divergent 3′ untranslated region and thus could be used to unambiguously differentiate the expression patterns of these two genes. qRT-PCR showed that the peak expression of *LWD1* was approximately 2-fold higher in plants grown under SD than LD conditions (Supplemental Fig. S4). The steady-state transcript level of *LWD1* is apparently much higher than that of *LWD2*, especially before dawn, approximately in the range of 40-fold (ZT24 [for zeitgeber time in hours]) and 100-fold (ZT21) higher under LD and SD conditions, respectively.

DISCUSSION

LWD1 and LWD2 Are New Players in Arabidopsis Photoperiod Sensing

We have adopted a reverse genetics approach to characterize two previously uncharacterized light-regulated genes, *LWD1* and *LWD2*. Our data support the notion that LWD1 and LWD2 function in Arabidopsis photoperiod sensing. That the early-flowering phenotype could only be observed in the *lwd1lwd2* double mutant indicates that LWD1 and LWD2 work redundantly in this respect (Fig. 1B). The successful complementation of this phenotype with just *LWD1* further supports this notion. This also explained why these genes were not uncovered in previous genetic screening for Arabidopsis mutants with aberrant flowering time.

Transcriptome analysis revealed the impact of the mutation in *LWD1* and *LWD2* on the photoperiodic pathway (Fig. 2). A detailed comparison of the expression profiles for photoperiodic genes provided an explanation for the early-flowering phenotype in the *lwd1lwd2* double mutant. As summarized in Figure 8, in wild-type Arabidopsis (left), LWD1 expresses in a diurnal pattern and regulates the expression of oscillator genes by a molecular mechanism yet to be identified. Under SD, the circadian clock-regulated *FKF1* and *GI* do not reach their expression peak until dusk approaches. Under this circumstance, CDF1 protein is still present at a sufficient level to repress the expression of *CO* before dusk. Thus, *FT* could only express at a low level that is insufficient to induce flowering. On the contrary, Arabidopsis defective in both *LWD1* and *LWD2* (*lwd1lwd2*; Fig. 8, right) possesses perturbed circadian regulation with an advanced expression phase (left-pointing arrow) for the oscillator and output genes examined. The advanced expression of *CO* results in a higher *CO* transcript level before dusk in *lwd1lwd2* plants (light-blue area). As a result, *FT* is highly expressed in *lwd1lwd2* (up-pointing arrow), which leads to the early-flowering phenotype. Although not as dramatic, a similar scenario could explain the subtle but significant early-flowering phenotype of *lwd1lwd2* under LD (Supplemental Fig. S5). While the aberrant clock function provides the simplest explanation for the increased expression of *FT* in the *lwd1lwd2* double mutant, a slim possibility still

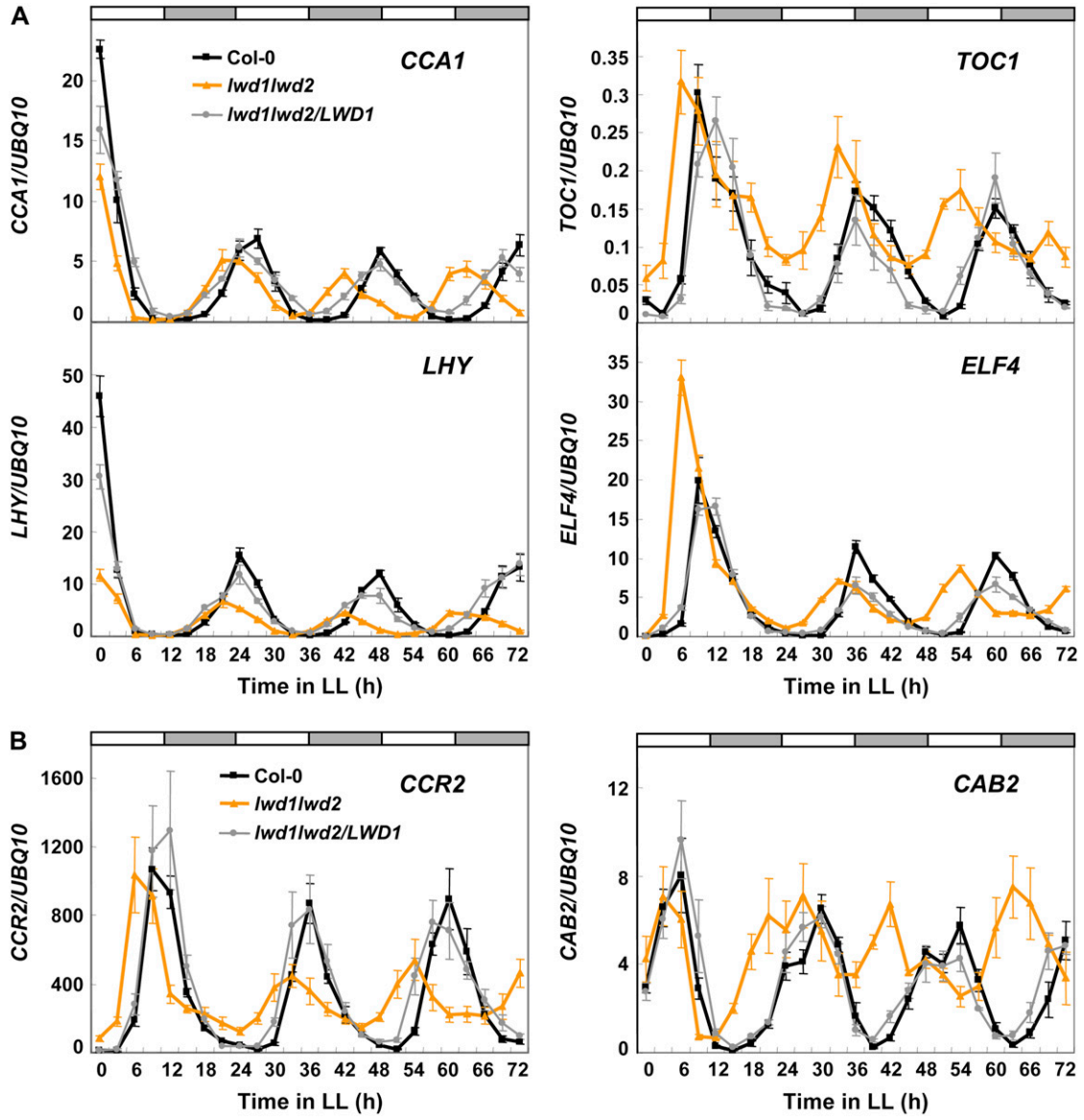

Figure 5. LWDs regulate the period length and amplitude of circadian clock-regulated genes in LL. Eighteen-day-old wild-type, *lwd1lwd2*, and *lwd1lwd2/LWD1* plants grown under 12 h of light and 12 h of dark were transferred to LL (time 0). Samples were harvested at 3-h intervals for a total of 72 h. qRT-PCR was used to monitor the expression of *CCA1*, *LHY*, *TOC1*, and *ELF4* (A) or *CCR2* and *CAB2* (B). Expression is relative to that of *UBQ10*. Data are means ± SEM from four independent experiments.

exists that LWD1/LWD2 may directly regulate the expression of *FT* independent of their impact on circadian oscillators and output genes upstream of *FT*.

LWD1 and LWD2 Are New Clock Components

Previous reports showed that mutation of some Arabidopsis genes results in the alteration of period length for circadian clock-regulated genes in Arabidopsis. Both lengthened and shortened period lengths have been observed in Arabidopsis carrying mutations in the circadian clock-regulated genes. For example, mutation in photoreceptors (*PHYTOCHROME A* [*PHYA*], *PHYB*, *CRYPTOCHROME1*, and *ZTL1*), *PPR7*, and *FIO1* resulted in a longer period length in Arabidopsis (Somers et al., 1998, 2000; Michael et al., 2003; Kim et al., 2008). In contrast, a shorter period length was observed previously in Arabidopsis mutants defective in *CCA1*, *LHY*, *TOC1*, *PRR3*, *PRR5*, *GI*, *LIP1*, and *SENSITIVITY TO RED LIGHT REDUCED1* (Millar et al., 1995; Green and Tobin, 1999; Park et al., 1999; Mizoguchi et al., 2002; Michael et al., 2003; Staiger et al., 2003; Kevei et al., 2007; Martin-Tryon et al., 2007). Our results show that mutation in LWD1/LWD2 affects the period length of the oscillator genes *CCA1*, *LHY*, *TOC1*, and *ELF4* (Fig. 5). Short period length was also observed for the output genes *CCR2* and *CAB2* in the *lwd1lwd2* double mutant (Fig. 5). Since

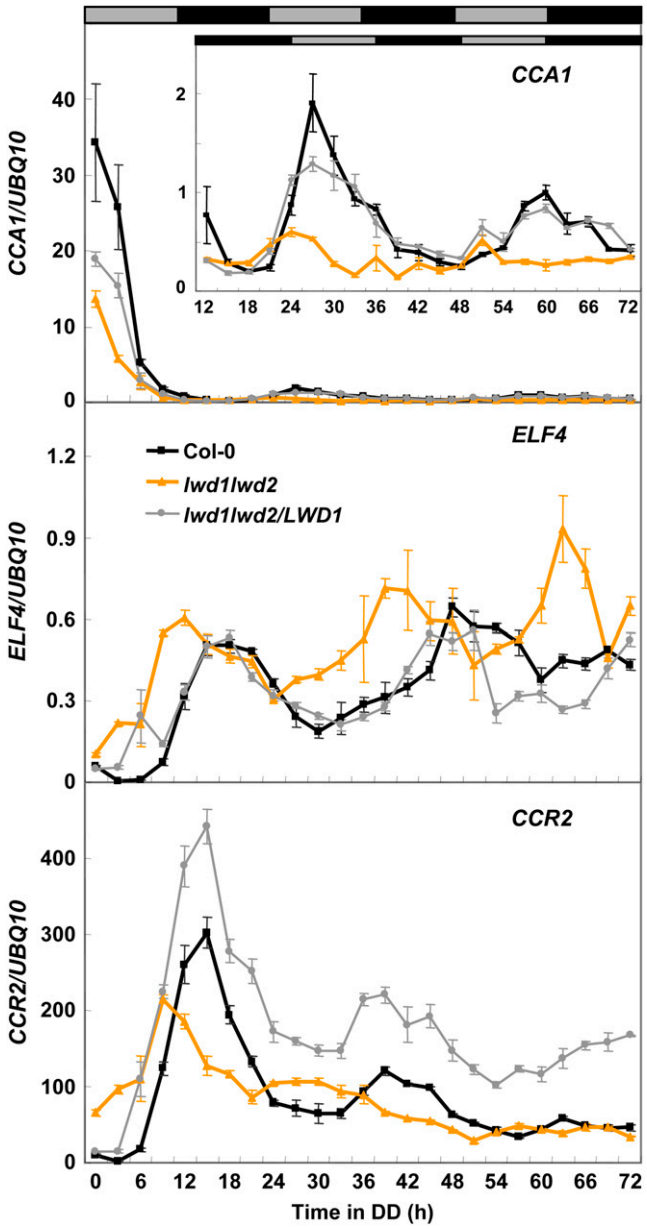

Figure 6. LWDs regulate the period length and amplitude of circadian clock-regulated genes in DD. Eighteen-day-old wild-type, *lwd1lwd2*, and *lwd1lwd2/LWD1* plants grown under 12 h of light and 12 h of dark were transferred to DD (time 0). Samples were harvested at 3-h intervals for a total of 72 h. qRT-PCR was used to monitor the expression of *CCA1*, *ELF4*, and *CCR2*. Expression is relative to that of *UBQ10*. Data are means ± SEM from four independent experiments.

CCR2 and CAB2 are not directly involved in regulation of the flowering process, this indicates that LWD1 and LWD2 have broader influences on clock functions than just regulating flowering time in Arabidopsis. A shorter period length for circadian clock-regulated genes was observed in the *lwd1lwd2* double mutant under both LL and DD (Figs. 5 and 6), indicating that LWD1 and LWD2 are important for maintenance of the period length of circadian clock-regulated genes regardless of light inputs (Figs. 5 and 6). This suggests that LWD1 and LWD2 more likely function in close proximity to or within the clock rather than in the light input pathway. In conclusion, LWD1/LWD2 act as new clock components that play a crucial role in the photoperiodic pathway for flowering time control in Arabidopsis as well as regulate the proper rhythmic expression of genes, *CCR2* and *CAB2*, for the other physiological processes.

We speculate that LWD1 functions in keeping the correct expression phase and amplitude of the morning genes *CCA1* and *LHY* because of the following observations. First, the expression of *LWD1* precedes that of the morning genes and peaks before dawn (Fig. 7). Second, the expression amplitude was significantly diminished only for the morning genes *CCA1* and *LHY* under DD and LL, respectively (Figs. 5 and 6). Third, the expression pattern and transcript level of *LWD1* remained indistinguishable among wild-type, *cca1* mutant, and transgenic plants overexpressing *CCA1* (J.F. Wu and S.H. Wu, unpublished data), which indicates that the expression of *LWD1* does not depend on

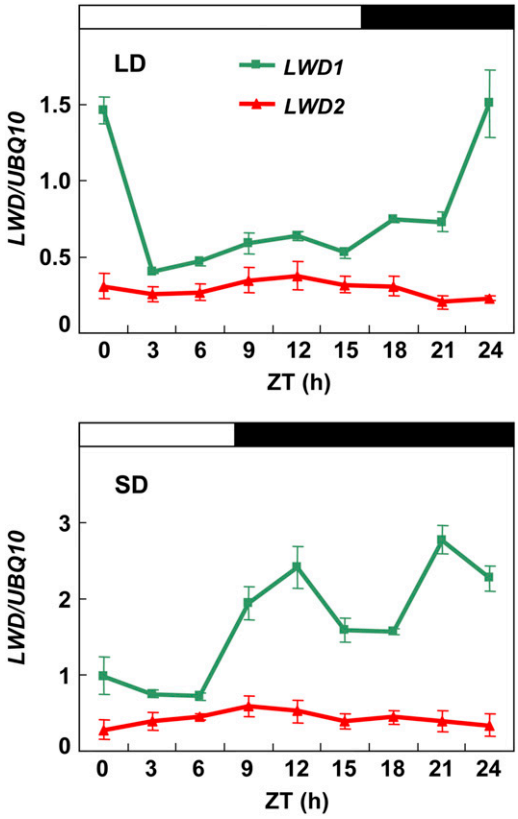

Figure 7. Quantitative expression analyses of *LWD1* and *LWD2* in Arabidopsis. Eighteen-day-old wild-type Arabidopsis plants grown under LD or SD conditions were harvested at different ZT times for total RNA isolation. qRT-PCR was used to monitor the expression of *LWD1* and *LWD2*. Expression is relative to that of *UBQ10*. Data are means ± SEM from four independent experiments.

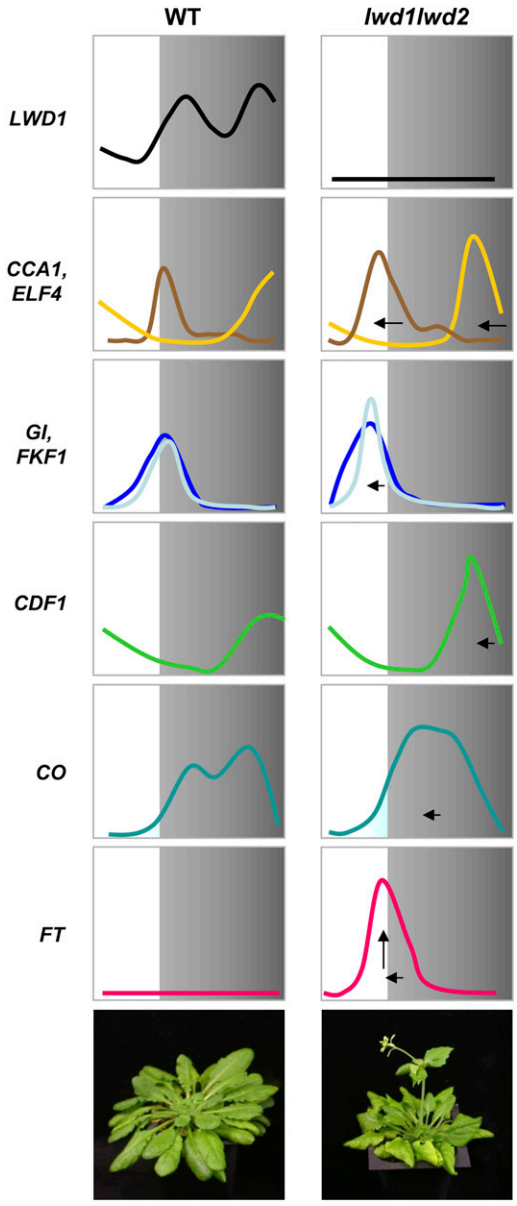

Figure 8. An illustration showing how LWDs regulate the temporal expression pattern of oscillator and output genes in photoperiod sensing under SD conditions. The expression kinetics of *LWD1*, oscillator genes (*CCA1* and *ELF4*), and output genes (*GI*, *FKF1*, *CDF1*, *CO*, and *FT*) under SD conditions show their expression phase and amplitude in wild-type and *lwd1lwd2* plants. Yellow line, *CCA1*; brown line, *ELF4*; blue line, *GI*; light-blue line, *FKF1*. The light-blue area highlights the accumulation of *CO* transcripts. The left-pointing and up-pointing arrows refer to phase shift and higher expression level, respectively.

CCA1. LWD1 might function to delay the expression of the morning genes until dawn approaches. Apparently, the expression of morning genes guarded by LWD1 must occur at the correct phase to reach a desirable amplitude under free-running conditions. That LWD1/LWD2 function to delay the expression phase of the morning genes *CCA1* and *LHY* until dawn in wild-type Arabidopsis is of great interest. Whether LWD1/LWD2 act in the delay mechanism between TOC1 and CCA1/LHY is also worth testing.

Molecular Characteristics of LWDs

A total of 237 WD-containing proteins are annotated in the Arabidopsis genome (van Nocker and Ludwig, 2003). To date, only a few members of the Arabidopsis WD protein superfamily have been characterized. Supplemental Figure S1 shows that LWD1 and LWD2 have five WD repeats. It has been proposed that the WD repeats form a propeller structure and serve as a protein-protein interaction platform (Smith et al., 1999).

Several WD proteins were found to contribute to circadian control. For example, FWD1 (F-box/WD-40 repeat-containing protein 1) was reported to modulate circadian rhythm in *Neurospora* by regulating the degradation of the clock protein FREQUENCY (He et al., 2003; He and Liu, 2005). In Arabidopsis, SPA1 and COP1 modulate the abundance of the circadian protein CO to regulate flowering time (Laubinger et al., 2006; Jang et al., 2008; Liu et al., 2008). Here, we report to our knowledge the first WD proteins, LWD1 and LWD2, that function as clock proteins and regulate Arabidopsis photoperiodic flowering.

One common feature of these WD proteins is that, in addition to WD repeats, extension or additional protein domains are present in these proteins (e.g. a protein kinase domain for SPA1, a RING finger for COP1, and an F-box domain for FWD1). However, no known protein domains in addition to the WD repeats could be recognized in LWD1 and LWD2. The best studied case for WD proteins comprising only WD repeats is TRANSPARENT TESTA GLABRA1 (TTG1), which is the closest homolog of LWD1/LWD2 in Arabidopsis (BLASTP P value of $<10^{-110}$). LWD1, LWD2, and TTG1 form a distinct group of WD-repeat proteins in Arabidopsis, as described previously (van Nocker and Ludwig, 2003). Pair-wise sequence identity comparison and alignment of LWD1, LWD2, and TTG1 are shown in Supplemental Figure S6. TTG1 functions in regulating flavonoid biosynthesis and epidermal cell fate determination through interaction with key bHLH and MYB transcriptional regulators (Broun, 2005; Gonzalez et al., 2008; Zhao et al., 2008). Of interest will be testing whether LWD1/LWD2 directly interact with specific proteins, such as the bHLH and MYB transcriptional regulators in the photoperiodic pathway, to achieve their functions as Arabidopsis clock proteins. Also, one of the *TTG1* mutant alleles, *ttg1-9*, possesses a S282F mutation (Walker et al., 1999). This amino acid is equivalent to Thr-285 of LWD1 and Ser-285 of LWD2, as highlighted in Supplemental Figure S6. Whether this amino acid contributes to the structural integrity of functions of LWD1/LWD2 could be further investigated.

LWD Orthologous Proteins Are Present in Multiple Organisms

At a cutoff of 50% amino acid identity, orthologous proteins of LWD1/LWD2 could be found in a wide spectrum of organisms (HomoloGene:55930 in the National Center for Biotechnology Information database; http://www.ncbi.nlm.nih.gov/sites/entrez?db=homologene). These organisms include *Oryza sativa* (Os02g0524600; NP_001046989.1), *Chlamydomonas reinhardtii* (CHLREDRAFT_130509; XP_001695930.1), *Homo sapiens* (WDR68; NP_005819.3), *Mus musculus* (Wdr68; NP_082222.1), *Xenopus laevis* (MGC82392; NP_001086790), *Danio rerio* (wdr68; NP_956363.1), and *Drosophila melanogaster* (CG14614; NP_608461.1). A multiple sequence alignment of these orthologous proteins is shown in Supplemental Figure S7. The prevalence of LWD1/LWD2 orthologs in a wide spectrum of organisms implies a general involvement of these proteins in growth and/or developmental processes. Reports of biological functions for most of these proteins remain limited. Thus, the further characterization of Arabidopsis LWD1/LWD2 is expected to provide hints for the functional elucidation of these orthologous proteins. Interestingly, a recent report described the high resemblance of the transcriptional feedback loops in circadian clocks of *Chlamydomonas* and Arabidopsis (Matsuo et al., 2008). It will be worthwhile to test whether CHLREDRAFT_130509, the LWD1/LWD2 orthologous protein, also functions in regulating the circadian clock in *Chlamydomonas*.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Two T-DNA insertion lines (Alonso et al., 2003), SALK_006874 (*lwd1*) and SALK_072182 (*lwd2*), were obtained from the Arabidopsis Biological Resource Center. *lwd1* and *lwd2* were crossed to generate the *lwd1lwd2* double mutant used in this study. More than six independent *lwd1lwd2/LWD1* lines were constructed by introducing a 1.4-kb (−263 to +1,175) genomic fragment of *LWD1* into *lwd1lwd2* by floral dipping (Clough and Bent, 1998). The *LWD1* expression in these complementation lines was confirmed by northern-blot analysis (data not shown). Two representative *lwd1lwd2/LWD1* lines, 36-6-3-4 and 32-2-1-2, are shown in Figure 1B. *lwd1lwd2/LWD1* line 36-6-3-4 was used for the qRT-PCR experiments shown in Figures 3 to 6. Seeds of Arabidopsis (*Arabidopsis thaliana*) Col-0, mutant, and transgenic plants were germinated directly in soil and placed at 4°C for 4 d to synchronize the germination. For photoperiod treatment, the plants were grown under LD (16 h of light/8 h of dark) or SD (8 h of light/16 h of dark) at a fluence rate of 80 to 120 μmol m^{-2} s^{-1}.

Determination of Flowering Time

The number of rosette leaves equal to or greater than 2 mm long was recorded for each plant when the primary florescence reached 1 cm above the rosette leaves. This phenotype observation was repeated at least three times. Four to 10 plants for each genotype were planted for scoring for each biological replicate.

Constructs

Sequences for all primers used in this study are listed in Supplemental Primer Table S1. pCAMBIA1390 (CSIRO, Australia) was used to generate *lwd1lwd2/LWD1* complementation lines. A 1.4-kb (−263 to +1,175) genomic fragment of *LWD1* was amplified with the primers pLWD1-*Pst*I-S and LWD1-*Sma*I-2-AS and subcloned into pCAMBIA1390. All constructs used in this study were confirmed by sequencing.

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^{-1} 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 12,000*g*. 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 12,000*g* for 30 min, washed with 75% ethanol, and dissolved in 20 μL of RNase-free water.

Affymetrix ATH1 Genome Array Hybridization and Data Analyses

ATH1 Genome Array hybridization involved use of the Arabidopsis ATH1 Genome Array (Affymetrix). Plants (31-d-old wild-type and *lwd1lwd2* double mutant plants) were grown under 12 h of light and 12 h of dark and harvested at ZT5 to ZT9. Ten micrograms of total RNA was used for cDNA synthesis, labeled by in vitro transcription, followed by fragmentation according to the manufacturer's suggestion (GeneChip Expression Analysis Technical Manual, Rev. 5; Affymetrix). Eleven-microgram labeled samples were hybridized to the ATH1 Genome Array at 45°C for 16.5 h. Washing and staining involved Fluidic Station-450, and the ATH1 Genome Array was scanned with use of the Affymetrix GeneChip Scanner 7G. The results were quantified and analyzed by use of MicroArray Suite 5.0 software (Affymetrix).

Gene expression data for Affymetrix ATH1 were analyzed as described previously (Lin and Wu, 2004). The average intensity of all probe sets of each chip was scaled to 500 so that the hybridization intensity of all chips was equivalent. "Set measurements less than 0.01 to 0.01," "Per Chip: Normalize to 50th percentile," and "Per Gene: Normalize to control mean" were applied for data normalization when Affymetrix data files were imported into GeneSpring 7.2 (Agilent) for further analyses. Genes marked as "present" in all chips analyzed were used for further data analysis shown in Figure 2 and Supplemental Figure S3. Raw data associated with Figure 2 are shown in Supplemental Table S1. The data sets have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE 11762.

qRT-PCR

Total RNA was isolated as described above and quantified by use of a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). cDNA was synthesized from 2 μg of DNase-treated total RNA with the use of SuperScript II reverse transcriptase (Invitrogen) and poly(T) primer. All primers were designed by Primer Express (Applied Biosystems). An amount of 50 μL of real-time PCR contained the following: primers, 5 μL of cDNA (equivalent to approximately 0.25 ng of mRNA), and 25 μL of SYBR Green PCR Master Mix (Applied Biosystems). The names of the primer pairs used for each gene are UBQ10-ABI-1, UBQ10-ABI-2, LWD1-1242-ABI-S, LWD1-1293-ABI-AS, LWD2-1098-ABI-S, LWD2-1231-ABI-AS, CCA1-1695-ABI-S, CCA1-1768-ABI-AS, LHY-1991-ABI-S, LHY-2067-ABI-AS, TOC1-725-ABI-S, TOC1-803-ABI-AS, ELF4-185-ABI-S, ELF4-260-ABI-AS, GI-3513-ABI-S, GI-3563-ABI-AS, FKF1-1583-ABI-S, FKF1-1652-ABI-AS, CDF1-678-ABI-S, CDF1-732-ABI-AS, CO-811-ABI-S, CO-861-ABI-AS, FT-336-ABI-S, FT-388-ABI-AS, CCR2-593-ABI-S, CCR2-679-ABI-AS, CAB2-950-ABI-S, and CAB2-1099-ABI-AS. Sequences and ratios of the primers (5 μM each) were determined experimentally as suggested by the manufacturer and listed in Supplemental Primer Table S1. Real-time PCR involved use of the ABI Prism 7000 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 threshold cycle (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 $2^{-\Delta CT}$ ($\Delta C_T = C_{T,\text{ gene of interest}} - C_{T,\text{ UBQ10}}$) calculated from four independent experiments were multiplied by 100 for the results plotted in Figures 3 to 7.

Supplemental Data

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

Supplemental Figure S1. Amino acid sequence alignment of LWD1 and LWD2.

Supplemental Figure S2. Northern-blot analyses of *LWD1* and *LWD2* in *lwd1lwd2* double mutant plants.

Supplemental Figure S3. Differential gene expression is highly correlated between the two biological replicates of ATH1 hybridization.

Supplemental Figure S4. Absolute quantitation of *LWD1* and *LWD2* transcripts in Arabidopsis.

Supplemental Figure S5. A model showing how LWDs regulate the temporal expression pattern of oscillator and output genes in photoperiod sensing under LD conditions.

Supplemental Figure S6. Sequence comparison of Arabidopsis LWD1, LWD2, and TTG1.

Supplemental Figure S7. Amino acid sequence alignment of LWD1 orthologous proteins.

Supplemental Table S1. ATH1 expression data for genes in four different pathways regulating flowering time.

Supplemental Primer Table S1.

Supplemental Materials and Methods S1.

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