Circadian Clock Proteins LHY and CCA1 Regulate SVP Protein Accumulation to Control Flowering in *Arabidopsis*  

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The floral regulators GIGANTEA (GI), CONSTANS (CO), and FLOWERING LOCUS T (FT) play key roles in the photoperiodic flowering responses of the long-day plant *Arabidopsis thaliana*. The GI-CO-FT pathway is highly conserved in plants. Here, we demonstrate that the circadian clock proteins LATE ELONGATED HYOCOTYL (LHY) and CIRCADIAN CLOCK-ASSOCIATED1 (CCA1) not only repressed the floral transition under short-day and long-day conditions but also accelerated flowering when the plants were grown under continuous light (LL). LHY and CCA1 accelerated flowering in LL by promoting *FT* expression through a genetic pathway that appears to be independent of the canonical photoperiodic pathway involving GI and CO proteins. A genetic screen revealed that the late-flowering phenotype of the *lhy;cca1* double mutant under LL was suppressed through mutations in SHORT VEGETATIVE PHASE (SVP), a MADS box transcription factor. Yeast two-hybrid analysis demonstrated an interaction between SVP and FLOWERING LOCUS C, and genetic analysis indicated that these two proteins act as partially redundant repressors of flowering time. SVP protein accumulated in *lhy;cca1* plants under LL. We propose a model in which LHY and CCA1 accelerate flowering in part by reducing the abundance of SVP and thereby antagonizing its capacity to repress *FT* expression under LL.

**INTRODUCTION**

Pioneering work by Garner and Allard (1920) classified plants into different daylength response types. They showed that long-day (LD) plants (LDP) take a shorter time to flower when light exposure exceeds a certain critical daylength, while short-day (SD) plants flower earlier when daylength is shorter than a critical length. Subsequent experiments demonstrated that SD plants actually measure the length of the night, which must exceed a critical length to induce flowering, and that these plants do not flower if grown under continuous light (Thomas and Vince-Prue, 1997). Photoperiodic control of flowering time is tightly linked to the circadian clock, which acts as the time-keeping mechanism that measures the duration of the day and night (Suarez-Lopez et al., 2001; Yanovsky and Kay, 2002; Mås, 2005). The circadian clock is an endogenous oscillator with an approximate period of 24 h that can be synchronized, or entrained, to the exact period of daily oscillations in light and temperature (Dunlap, 1999). This process enables an organism to phase its biological activities to the correct time of day.

The LDP are classified into two types, which flower only (absolute LDP) or flower most rapidly (facultative LDP) with more than a certain number of hours of light in each 24-h period (Thomas and Vince-Prue, 1997). *Arabidopsis thaliana* is a facultative LDP and flowers much earlier in a daily regime with a long light period and a short dark period (e.g., 16 h of light/8 h of dark) than in one with a short light period and a long dark period (e.g., 8 h of light/16 h of dark or 10 h of light/14 h of dark). In *Arabidopsis*, two closely related MYB proteins, LATE ELONGATED HYOCOTYL (LHY) and CIRCADIAN CLOCK-ASSOCIATED1 (CCA1), are essential clock components with redundant functions that play important roles in photoperiodic flowering by controlling the rhythmic expression of flowering-time genes (Carre and Kim, 2002; Mizoguchi et al., 2002, 2005). In particular, LHY and CCA1 regulate a flowering pathway comprising the genes GIGANTEA (GI), CONSTANS (CO), and FLOWERING LOCUS T (FT) in light/dark cycles such as LD and SD (Mizoguchi et al., 2002, 2005; Mås, 2005). *FT* gene expression is activated under LDs mainly through a conserved pathway consisting of GI and CO (Mizoguchi et al., 2005). Several other *Arabidopsis* genes, in which mutations also delay or accelerate flowering, have been identified previously (Mås, 2005). The relationship between flowering and daylength in *Arabidopsis* involves rhythmic, circadian clock-controlled expression of CO mRNA. In this model, CO mRNA levels rise and fall over the course of a day and produce an unstable protein. If CO mRNA levels are high when the plant is exposed to light, the CO protein product is stabilized and activates the expression of *FT* (Suarez-Lopez et al., 2001; Valverde et al., 2004; Mås, 2005). Comparative analysis of *Arabidopsis* and rice (*Oryza sativa*), a SD plant, demonstrated that functional differences between the *Arabidopsis* CO and its rice ortholog Heading date1 (Hd1) are the
basis of the reversal in response type (Hayama and Coupland, 2004). In rice, CO represses flowering under LD by repressing expression of the rice ortholog of FT, *Heading date3* (*Hd3a*), whereas in *Arabidopsis*, it activates flowering by activating FT expression (Hayama and Coupland, 2004). FT and Hd3a are candidates for a floral hormone, florigen (Corbesier et al., 2007; Tamaki et al., 2007).

Although GI (Suarez-Lopez et al., 2001; Mizoguchi et al., 2005), FLAVIN BINDING, KELCH REPEAT, F-BOX1 (FKF1), and CYCLING DOF FACTOR1 (CDF1) (Imaizumi et al., 2005) are required for the rhythmic expression of CO mRNA, the molecular mechanism underlying the cooperation between GI and FKF1-CDF1 was largely unknown. Recent characterization of protein–protein interactions between FKF1 and GI (Sawa et al., 2007) and between ZEITLUPE (ZTL) and GI (Kim et al., 2007) has advanced our knowledge of how the circadian clock controls the upregulation of FT transcription just after evening under the inductive LD condition (Rubio and Deng, 2007). The circadian clock controls rhythmic expression of the GI protein by an unidentified mechanism(s) (David et al., 2006). Molecular interaction between GI and ZTL is stabilized by blue light. The ZTL–GI interaction controls the accumulation of the clock component TIMING OF CAB EXPRESSION1 (TOC1), thus allowing robust circadian oscillations in gene expression (Kim et al., 2007). Blue light also induces the formation of an FKF1-GI protein complex, which in turn targets CDF1, a transcriptional repressor of flowering, for degradation (Sawa et al., 2007). CDF1 proteolysis releases transcriptional repression of the CO gene, which allows CO protein expression and LD-dependent accumulation to promote FT expression and flowering.

The effects of loss of function of LHY and CCA1 on flowering time under light/dark cycles such as LD and SD conditions were characterized in detail (Mizoguchi et al., 2002, 2005). Even though abnormal, plants with severe defects in circadian function showed rhythmic expression of clock-controlled genes such as *Cab*, *CCR2*, GI, and *LHY1* under light/dark cycles. This suggests that some defects caused by loss of the internal clock function can be partially rescued by external rhythmic conditions. However, the roles of the circadian clock proteins in the long-term developmental control of animals and plants under continuous conditions without any rhythmic stimuli are not fully understood.

Here, we show that mutations in the circadian clock genes LHY and CCA1 (*lhy*;*cca1*) delay flowering time of *Arabidopsis* under continuous light (LL), although they accelerate flowering under light/dark cycles such as LD and SD. Our genetic studies indicate that two mutations, *short vegetative phase* (*svp*) and *flowering locus C* (*flc*), partially suppress the late-flowering phenotype of *lhy*;*cca1*. Accumulation of a floral repressor protein (SVP) in *lhy*;*cca1* plants under LL and a diurnal pattern of SVP protein accumulation under LD explain a molecular mechanism for the novel activity of LHY and CCA1. Our results demonstrate that

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Figure 1. A Change of Daylength Response by *lhy*;*cca1*.

(A) Wild-type *Arabidopsis* and *lhy*-12;*cca1*-101 at 30 d after sowing in LL.

(B) Summary of flowering phenotypes of the *Arabidopsis* mutant.

(C) Flowering time of the wild type (blue) and *lhy*-12;*cca1*-101 (red) in LL and various light/dark cycles (24 h of light [L], 23 h 50 min of L/10 min of dark [D], 23 h 30 min of L/30 min of D, 23 h of L/1 h of D, 16 h of L/8 h of D, and 10 h of L/14 h of D). Numbers of total leaves when plants flowered were scored, and the data are presented as means ± SE.

(D) A schematic model showing the reversal of daylength response by *lhy*;*cca1*. The daylength responses of wild-type *Arabidopsis* (LD plant) and rice (SD plant) are shown in blue and green, respectively. All of these experiments were done at least twice with similar results.
both an internal biological clock and external rhythms are required for the proper development of Arabidopsis.

RESULTS

Late-Flowering Phenotype of lhy;cca1 under LL

To investigate how the clock genes LHY and CCA1 affect the flowering response to photoperiod, we examined lhy;cca1 double mutants. The lhy-12;cca1-1 and lhy-11;cca1-1 double mutants exhibit early flowering and earlier circadian phase of expression of the flowering-time genes GI and CO under SD (10 h of light/14 h of dark) and LD (16 h of light/8 h of dark) (Mizoguchi et al., 2002, 2005). By contrast, we observed that lhy-12;cca1-101 plants flower later than wild-type plants under LL (Figures 1A to 1C, cca1-101; see Supplemental Figure 1 online). This indicates that LHY and CCA1 are required for the acceleration of flowering in wild-type plants under LL. In addition, the lhy-12;cca1-101 plants flowered earlier as the dark period was extended (Figures 1C and 1D), indicating that the requirement for LHY and CCA1 to delay flowering in light was reduced as the duration of darkness was extended. This effect is in contrast to that observed with other Arabidopsis mutants affecting day-length responses, which exhibit either earlier flowering (elf3) (Carre` , 2002) or later flowering (gi and co) than wild-type plants (Figure 1B) (Koomneef et al., 1991; Zagotta et al., 1992; Mas, 2005) but do not show a reversed response relative to the wild type dependent on the diurnal conditions.

Independent Roles of lhy;cca1 and gi to Delay Flowering under LL

We next analyzed the expression patterns of flowering-time genes in lhy-12;cca1-101 plants under LL. Consistent with the delayed-flowering phenotype, FT mRNA levels were markedly

Figure 2. Downregulation of FT Expression in lhy;cca1 under LL.

(A) Expression of GI, CO, and FT in the wild type and lhy-12;cca1-101 grown under LL (24°C). TUBULIN2 (TUB) levels are shown as controls. At least 20 seedlings for each time point and genotype were used. Open boxes represent continuous light conditions, and hours from the first sampling are shown above the boxes.

(B) Flowering times of the wild type and various mutants under LL. Six to 14 plants of each genotype were used for each trial. CL and RL represent cauline and rosette leaves, respectively.

(C) and (D) Expression of GI, CO, and FT in the wild type and lhy-12;cca1-101 grown under light/dark cycles (16 h of light/8 h of dark, 24°C [C]) and temperature cycles (16 h at 24°C/8 h at 20°C, LL [D]) for 12 d. Open and closed bars along the horizontal axis represent light and dark periods, respectively, in (C). Open and striped bars along the horizontal axis represent warm and cold periods, respectively, in (D). Hours from dawn (ZT) are shown above the bars.

(E) Flowering time of the wild type and lhy-12;cca1-101 grown under temperature cycles (16 h at 24°C/8 h at 20°C, LL). All of these experiments were done at least twice with similar results. Data in (B) and (E) are presented as means ± SE.
lower in lhy-12;cca1-101 mutants than in wild-type plants under LL (Figure 2A; see Supplemental Figure 2D online). However, under these conditions, the mRNA level of GI, a gene that acts upstream of FT in the photoperiod pathway, was only slightly lower in lhy-12;cca1-101 than in wild-type plants (Figure 2A; see Supplemental Figure 2A online). To test whether GI, the most upstream factor of the photoperiodic flowering pathway, is involved in the late flowering of lhy;cca1 plants under LL, we investigated a triple mutant, lhy-11;cca1-1;gi-3. The lhy-11;cca1-1;gi-3 plants flowered significantly later than lhy-11;cca1-1 and gi-3 under LL (Figure 2B). This indicated that LHY/CCA1 and GI encode components of independent genetic pathways that promote the flowering of wild-type plants under LL. These results suggested that the slight decrease of GI expression did not explain the late flowering of lhy-12;cca1-101 plants in LL. Under LL, the CO mRNA level in lhy;cca1 was lower than that in wild-type plants, suggesting that lhy;cca1 might affect CO expression (Figure 2A; see Supplemental Figure 2B online).

**GI-CO–Independent Suppression of FT Expression in lhy;cca1 under LL**

We showed that FT mRNA was detectable in both the wild type and lhy-12;cca1-101 under LD (Figure 2C). Arabidopsis plants show rhythmic expression of clock-controlled genes under temperature cycles (Michael et al., 2003). Under LL, a temperature cycle entrained both wild-type and lhy-12;cca1-101 plants (Figure 2D). Although high CO expression in wild-type and lhy-12;cca1-101 plants under these conditions coincided with exposure to light, FT expression was detected only in wild-type plants and not in lhy-12;cca1-101 plants (Figure 2D; see Supplemental Figure 2G online). The lhy-12;cca1-101 double mutant flowered later than the wild type and lhy-12;cca1-101;gi-3 under LL, indicating that GI-CO–Independent Suppression of FT Expression in lhy;cca1 under LL is a novel mechanism for late flowering in Arabidopsis. These results suggest that GI-CO–Independent Suppression of FT Expression in lhy;cca1 under LL is a novel mechanism for late flowering in Arabidopsis.

![Figure 3](image-url)
later than wild-type plants even under these conditions (Figure 2E). These results indicate that the late-flowering phenotype of lhy-12;cca1-101 under LL is not due simply to the slight decrease of GI or CO mRNA but, rather, to the direct suppression of FT transcription independently of GI or CO or to the negative regulation of CO protein activity (Valverde et al., 2004).

**Suppression of the Late-Flowering Phenotype of lhy;cca1 by svp under LL**

To explore the molecular mechanisms underlying the GI-independent late flowering of lhy;cca1 plants, we screened for ethyl methanesulfonate (EMS)–induced mutations that caused earlier flowering of lhy;cca1 plants under LL. One of these suppressor mutations occurred in SVP, which encodes a MADS box transcription factor and is a previously described repressor of flowering (Figure 3C) (Hartmann et al., 2000).

To map the svp-3 mutation, we crossed lhy-12;cca1-102;svp-3 with the Columbia (Col) wild type. F2 plants with fewer leaves than the wild types (Landsberg erecta [Ler] and Col) in LL were used for mapping. Rough mapping located svp-3 between the genetic markers nga1145 and nga1126 on chromosome 2. Fine mapping of svp-3 showed that the mutation is in a region between BACs F14M13 and T9I22. The interval between these two markers is ~40 kb. To identify the molecular lesion in svp-3, we amplified and sequenced a set of PCR fragments covering the SVP region from lhy-12;cca1-102;svp-3. Genetic mapping and sequencing identified a single mutation (G to A) on the border of the first intron and the second exon of SVP (Figure 3C). This mutation, svp-3, appears to cause a missplicing of SVP mRNA (Figure 3D), possibly leading to a loss of functional SVP. These results indicate that SVP is required for late flowering of lhy-12;cca1-102 under LL; indeed, the lhy;cca1;svp triple mutant line showed an early-flowering phenotype similar to that of wild-type plants under LL (Figures 3A and 3B).

**Suppression of Downregulation of FT in lhy;cca1 by svp under LL**

The impact of the svp-3 mutation on the abundance of the mRNAs of the flowering genes FT and SUPPRESSOR OF OVEREXPRESSION OF CO1 (SOC1) was tested in lhy-12;
Consistent with the flowering times under LL, the level of FT mRNA in lhy-12;cca1-102;svp-3 plants was higher than that in lhy-12;cca1-102 plants but lower than that in vpn-3 LL (Figure 3E). By contrast, the expression of GI and CO, which act earlier in the photoperiod pathway than FT and SOC1, was not affected by vpn-3 in the light period that is important for the determination of flowering (Suarez-Lopez et al., 2001; Valverde et al., 2004). In addition, ft-1;soc1 mutations largely suppressed the early flowering of vpn-3 (Figure 3F), whereas gi-3;vpn-3 and co-2;vpn-3 double mutants flowered earlier than co-2 and gi-3 single mutants (Figure 3G). These results suggest that increased levels of FT and SOC1 mRNA in lhy-12;cca1-102;vpn-3 mutants may be responsible for the partial suppression of the late flowering of lhy-12;cca1-102 by vpn-3 in LL and that repression of FT in lhy;cca1 plants under LL may cause the late-flowering phenotype under these conditions. We did not find a significant difference in SOC1 mRNA level between wild-type and lhy;cca1 plants under LL.

Suppression of the Late-Flowering Phenotype of lhy;cca1 by flc under LL

FLC also encodes a MADS box protein that represses flowering (Michaels and Amasino, 2001). We found that the flc mutation enhanced the early flowering of vpn (Figure 4A) and that VPN interacted with FLC in the yeast two-hybrid assay (Figure 4B). These results suggested that these two proteins might have partially redundant roles in the repression of flowering. Furthermore, 35S:FLC plants exhibited phenotypes similar to those of lhy;cca1 in LL, such as late flowering (Figure 4C), negative regulation of FT expression (see Supplemental Figure 3F online), and dark-green/curled leaves (see Supplemental Figure 4A online). LHY and CCA1 mRNA levels were not affected by 35S:FLC in LL (see Supplemental Figure 4B online). In addition, lhy-11;cca1-1;flc-101 and lhy-11;cca1-1;flc-102 triple mutants were generated, and these triple mutants flowered earlier than lhy-11;cca1-1 (Col) in LL (Figure 4D). The flc mutation did not affect the flowering time of co (Michaels and Amasino, 2001). The lhy-12;cca1-101 plants exhibited dark-green/curled leaves in LL (Figure 1A), but co did not (data not shown). Therefore, late flowering of lhy;cca1 in LL is unlikely to be explained solely by the down-regulation of CO mRNA or protein levels. These results indicate that the delay in flowering caused by LHY and CCA1 under LL requires known floral repressors, such as VPN and FLC, as well as the classical GI-CO pathway for floral activation.

Late Flowering and Downregulation of FT and SOC1 Expression by 35S:VPN

Although VPN is required for the late flowering of lhy;cca1 plants in LL (Figures 3A and 3B), lhy;cca1 did not affect the level of VPN mRNA in LL (Figure 5C). The lhy;cca1 double mutant is sensitive to light (Mizoguchi et al., 2005), and one possible explanation for the late-flowering phenotype of lhy;cca1 in LL is that light may increase VPN activity to delay flowering. In LL, the late-flowering phenotype of 35S:VPN was much stronger than that in LD

Figure 5. Effects of VPN Overexpression on Flowering Time in LL and LD.
(A) and (B) Flowering times of wild-type (Ler), vpn-3 (Ler), wild-type (Col), vpn-31 (Col), and vpn-32 (Col) plants in LD (A) and SD (B).
(C) Expression of VPN and TUB in Ler wild-type and lhy-12;cca1-101 plants grown under LL for 12 d.
(D) and (E) 35S:VPN delayed flowering in LL. Images of Ler wild-type, 35S:VPN, and 35S:FLC plants in LL (D) and LD (E) and flowering times of these plants are shown.
All of these experiments were done at least twice with similar results. Data in (A), (B), (D), and (E) are presented as means ± se.
(Figures 5D and 5E; see Supplemental Figure 3 online). Consistent with the difference of flowering times, expression levels of FT and SOC1 in 35S:SVP plants under LL were lower than those under LD (see Supplemental Figures 3A, 3C, and 3D online). GI and CO expression was not affected by 35S:SVP (see Supplemental Figures 3A, 3B, and 3E online).

**Accumulation of SVP Protein in lhy;cca1 Plants under LL**

To understand the molecular mechanism for the delay of flowering time under LL, we used immunoblots to examine changes of SVP protein levels in lhy;cca1, SVP-ox, svp-31, and control plants under LL. We detected an accumulation of SVP protein in lhy;cca1 and SVP-ox (Figure 6A) plants in LL. As controls, we found an increased level of SVP protein in SVP-ox plants, whereas no detectable level of SVP protein was found in svp-31 plants. This result is consistent with the delayed flowering of lhy;cca1 and can explain why the svp mutation suppressed the late-flowering phenotype of lhy;cca1 under LL. We detected two bands by protein gel blotting using the SVP-specific antibody (Figure 6A). Two types of cDNAs (NP179840 and ABU95407 in the National Center for Biotechnology Information database) that correspond to different lengths of SVP proteins (240 and 235 amino acids) have been identified. Those probably generated by alternative splicing (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=protein&aid=156778051) are likely to be responsible for the two bands.

**Diurnal Change of SVP Protein Level under LD**

The late-flowering phenotype of lhy;cca1 was observed under LL but not under LD and SD. To test whether the accumulation of SVP protein in lhy;cca1 was affected by light/dark cycles, the SVP protein abundance was examined under LD. Zeitgeber time (ZT) is shown as hours from dawn. Seedlings of wild-type plants were harvested at dawn (ZT 0) and then every 4 h for 24 h. SVP protein accumulation showed a diurnal change under LD (Figure 6B). The SVP protein abundance was at trough level at ZT 16, 20, and 24 in the wild type (Ler). Higher accumulation of SVP protein was detected at ZT 4, 8, and 12 in the wild type (Ler). The amplitude of the diurnal rhythm in SVP protein accumulation was reduced in lhy;cca1, and trough levels of SVP protein in lhy;cca1 were higher than those of the wild type (Ler). These results also indicate that both LHY and CCA1 play key roles in the control of SVP protein accumulation.

**DISCUSSION**

In summary, our analysis of Arabidopsis mutants with severe defects in circadian clock function in LL has revealed a role for
Roles of LHY/CCA1 in SVP Accumulation 2967

The plant circadian clock controls the rhythmic expression of the GI gene, which may allow rhythmic accumulation of GI protein, which interacts with ZTL. The ZTL–GI interaction controls the accumulation of the clock component TOC1, thus allowing robust circadian oscillations in gene expression. GI protein also interacts with FKF1, and this FKF1–GI protein complex in turn targets CDF1, a transcriptional repressor of flowering, for degradation. CDF1 proteolysis releases transcriptional repression of the CO gene, which allows CO protein expression and LD-dependent accumulation to activate FT gene expression and promote flowering. This pathway involves three floral activator genes, GI, CO, and FT, and is controlled by the clock proteins LHY and CCA1. All of these events occur in the afternoon and evening. The plant circadian clock also controls rhythmic expression of the LHY and CCA1 genes, which allow rhythmic accumulation of LHY and CCA1 proteins around dawn. By contrast, SVP protein was at trough level around dawn and accumulated LHY and CCA1 in the control of flowering via FT expression. We propose that LHY and CCA1 can regulate flowering independently of their role in regulating the established photoperiodic response pathway through the transcription of GI-CO-FT (shown in blue in Figure 7). We hypothesize that LHY and CCA1 both activate the photoperiodic response pathway that promotes flowering and repress inhibitors of flowering such as SVP and FLC (shown in red in Figure 7). FLC delays flowering by repressing FT expression in the leaf (Searle et al., 2006). In addition, FLC expression in the meristem impairs the response to the FT signal by directly repressing the expression of SOC1 (Searle et al., 2006). FLC and SVP directly repress FT expression (Searle et al., 2006; Lee et al., 2007). One possibility to explain the role of LHY and CCA1 is that they reduce the accumulation of SVP and thereby prevent SVP and FLC from forming a repressive complex that represses FT expression. These data are discussed in more detail in the following sections.

Under SD, lhy-12;cca1-101 plants flowered earlier than LL (Figure 1). Moreover, lhy-12;cca1-101 plants flowered earlier as the dark period was extended, indicating that mutations in circadian clock components appeared to change photoperiodic response type in Arabidopsis (Figures 1C and 1D). When these mutants are grown under LL, the repression of flowering may occur through interactions with two MADS box proteins, SVP and FLC, which cause later flowering. Accumulation of SVP protein in lhy;cca1 plants under LL supports this idea (Figure 6A). However, under light/dark cycles the promotion of flowering in lhy;cca1 mutants through the photoperiodic pathway predominates and early flowering occurs. In wild-type plants, the balance in activity between these pathways differs from that in lhy;cca1 mutants, so that even in LL the promotion of flowering by the photoperiodic pathway overcomes the effect of SVP and FLC. Therefore, by altering the balance between these pathways, lhy;cca1 double mutants exhibit unique characteristics, flowering earlier under SD than under LL.

SVP was required for the late flowering of lhy;cca1 plants in LL (Figures 3A and 3B). However, lhy;cca1 did not affect the level of SVP mRNA in LL (Figure 5C). The hypocotyl length of lhy;cca1 is shorter than that of the wild type under red light, indicating that lhy;cca1 is sensitive to light (Mizoguchi et al., 2005). These findings suggest that light may increase SVP activity to delay flowering. In fact, an increased level of SVP protein was found in lhy;cca1 under LL (Figure 6A). In LL, the late-flowering phenotype of 35S:SVP was much stronger than that in LD (Figures 5D and 5E), and expression levels of FT and SOC1 in 35S:SVP plants under LL were lower than those under LD (see Supplemental Figures 3A, 3C, and 3D online). GI and CO mRNA levels were less affected by 35S:SVP (see Supplemental Figures 3A, 3B, and 3E online). These results are consistent with our ideas that (1) SVP negatively regulates FT and SOC1 expression downstream of GI and CO, and (2) SVP activity may be affected by light. LHY and CCA1 play key roles in the Arabidopsis circadian clock (Alabadi et al., 2001, 2002; Mizoguchi et al., 2002, 2005; Yanovsky and

before and after dawn, thus preventing the unexpected activation of FT expression. These events occur in the first half of the daytime and are required for the precise control of flowering time by the circadian clock.

Figure 7. A Schematic Model Showing the Activation and Repression of the FT Gene Controlled by LHY and CCA1.

The plant circadian clock controls the rhythmic expression of the GI gene, which may allow rhythmic accumulation of GI protein, which interacts with ZTL. The ZTL–GI interaction controls the accumulation of the clock component TOC1, thus allowing robust circadian oscillations in gene expression. GI protein also interacts with FKF1, and this FKF1–GI protein complex in turn targets CDF1, a transcriptional repressor of flowering, for degradation. CDF1 proteolysis releases transcriptional repression of the CO gene, which allows CO protein expression and LD-dependent accumulation to activate FT gene expression and promote flowering. This pathway involves three floral activator genes, GI, CO, and FT, and is controlled by the clock proteins LHY and CCA1. All of these events occur in the afternoon and evening. The plant circadian clock also controls rhythmic expression of the LHY and CCA1 genes, which allow rhythmic accumulation of LHY and CCA1 proteins around dawn. By contrast, SVP protein was at trough level around dawn and accumulated LHY and CCA1 in the control of flowering via FT expression. We propose that LHY and CCA1 can regulate flowering independently of their role in regulating the established photoperiodic response pathway through the transcription of GI-CO-FT (shown in blue in Figure 7). We hypothesize that LHY and CCA1 both activate the photoperiodic response pathway that promotes flowering and repress inhibitors of flowering such as SVP and FLC (shown in red in Figure 7). FLC delays flowering by repressing FT expression in the leaf (Searle et al., 2006). In addition, FLC expression in the meristem impairs the response to the FT signal by directly repressing the expression of SOC1 (Searle et al., 2006). FLC and SVP directly repress FT expression (Searle et al., 2006; Lee et al., 2007). One possibility to explain the role of LHY and CCA1 is that they reduce the accumulation of SVP and thereby prevent SVP and FLC from forming a repressive complex that represses FT expression. These data are discussed in more detail in the following sections.

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before and after dawn, thus preventing the unexpected activation of FT expression. These events occur in the first half of the daytime and are required for the precise control of flowering time by the circadian clock.
Kay, 2002). LHY and CCA1 are shown as negative regulators of GI based on the earlier phase of GI expression detected in a lhy; cca1 double mutant (Mizoguchi et al., 2002, 2005). In the control of flowering time, GI increases the amplitude of CO and FT expression (Mizoguchi et al., 2005).

At least four processes involved in the control of flowering appear to be affected by light. First, the circadian clock can be entrained by light (Yanovsky and Kay, 2002). Second, light plays a key role in the stabilization of CO protein in the process (Valverde et al., 2004). Third, the light-dependent regulation of CO expression by GI-ZTL/FKF1-CDF1 was recently demonstrated (Kim et al., 2007; Sawá et al., 2007). Fourth, we propose a novel role for LHY and CCA1 in the GI-CO-independent process to regulate flowering, based on molecular genetic, biochemical, and yeast two-hybrid analyses. This pathway probably includes the floral repressors encoding the MADS box transcription factors SVP and FLC and regulates the expression of FT and other floral activator genes.

Autonomous pathway proteins, including FCA and FLC, also regulate the expression of FT and SOC1 (Hepworth et al., 2002). Although the lhy-12;cca1-101 double mutant had a tendency to show higher transcript levels of FLC compared with the wild type, we did not get consistent results (see Supplemental Figures 5A to 5C online). Of 21 biologically independent trials, 15 showed a great increase in FLC transcription in lhy-12;cca1-101 compared with the wild type (see Supplemental Figure 5A online). Meanwhile, another three samples showed only moderate increases (see Supplemental Figure 5B online), and the other three showed no differences (Supplemental Figure 5C online). In addition, lhy-12 and cca1-101 single mutants, which flowered much earlier than the lhy-12;cca1-101 double mutant under LL, also showed higher FLC transcription (see Supplemental Figures 5A and 5D online). These data suggest that the FLC transcript level does not show correlation with flowering-time phenotype. For these reasons, we consider that the late-flowering phenotype of lhy;cca1 is not explained by the upregulation of FLC transcription.

Light appears to affect the activities of SVP and FLC to repress the expression of FT and SOC1. Alternatively, the late flowering of lhy;cca1 may be explained by the destabilization of CO protein (Valverde et al., 2004). However, this is not likely because (1) the late flowering of lhy;cca1 in LL was partially suppressed by svp (Figures 3A and 3B) and ftc (Figure 4E) but that of CO was not affected by ftc (Michaels and Amasino, 1999) and (2) co did not show dark-green/curl leaves and short-hypocotyl phenotypes in LL. Loss and gain of function of the MADS box gene FLM/MAF1 caused early- and late-flowering phenotypes, respectively (Scortecchi et al., 2003). The late flowering of 35S:FLM and 35S:SVP is dependent on SVP and FLM activities, respectively. MADS box proteins can interact and constitute heterodimers or homodimers in yeast two-hybrid systems (Folter et al., 2005). In this study, we detected protein–protein interaction between SVP and FLC in the yeast two-hybrid assay (Figure 4B). Complex formation among SVP, FLM/MAF1, and FLC as homodimers and heterodimers (or trimers) (Folter et al., 2005) is likely to play key roles in the LHY/CCA1-dependent flowering pathway.

We show genetically that early flowering of the svp mutant required elevated transcript levels of FT and SOC1, since mutations in these genes suppressed the early-flowering phenotype of svp (Figures 3E and 3F). The addition of the soc1 mutation to the ft-1 mutation, while not so evident in plants with normal SVP, was highly evident in plants with the svp mutation. This suggests that SVP may normally repress SOC1, but not through FT.

Although only a short exposure to darkness appears to be effective to cause the switch from late to early flowering in lhy; cca1, the precise mechanism underlying this effect remains to be elucidated and further analysis will be needed to understand it. Nevertheless, investigations of the multilayered regulation of flowering, through the classical photoperiodic pathway and the MADS box repressors of flowering, play a key role in identifying the relationship between photoperiod and flowering. We have established an unexpected role for LHY and CCA1 in regulating the abundance of the protein encoded by the floral repressor gene SVP. The regulation of this protein is influenced by light, so that it accumulates to high levels in lhy;cca1 plants under LL.

Under LD, the effect of lhy;cca1 on flowering time is reversed, so that the plants flower early. To understand the precise molecular mechanisms relating LHY and CCA1 to SVP stability and to internal and external rhythms will be an important challenge.

Recently, a central role of the interaction between SVP and FLC in the integration of various flowering signals was proposed (Li et al., 2008). The precise mechanisms underlying the negative regulation of flowering in lhy;cca1 under LL are still not clear, because lhy;cca1 mutations did not greatly affect the mRNA levels of SVP (Figure 5C) or FLC (see Supplemental Figures 5A to 5C online) and we did not detect protein–protein interactions between LHY/CCA1 and SVP/FLC (R. Yoshida and T. Mizoguchi, unpublished data). Therefore, how SVP and FLC delayed flowering more strongly in lhy;cca1 mutants than in wild-type plants under LL is unknown. To find the missing link between LHY/CCA1 and SVP/FLC, the characterization of other mutations than svp and ftc that cause lhy;cca1 to flower earlier than wild-type plants under LL will be useful.

METHODS

Plant Materials and Growth Conditions

The wild-type Arabidopsis thaliana Ler ecotype was used unless specified otherwise. The lhy-11;cca1-1 (Mizoguchi et al., 2002), lhy-11;cca1-1:gi-3, lhy-11;cca1-1:co-2, lhy-11;cca1-1:ft-1 (Mizoguchi et al., 2005), co-2 (CS55), ft-1 (CS56), fca-1 (CS52) (Koomen et al., 1991), gi-3 (CS51), Fowler et al., 1999), lhy-21;cca1-11 (Wassilewskija; CS9380; Hall et al., 2003), and 35S:FLC (Ler; Michaels and Amasino, 1999) mutants have been described previously.

T-DNA insertion alleles of SVP (svp-31, 1005231108; svp-32, 4123010) and FLC (ftc-101, 4551274; ftc-102, 1005389859) in the Col background were obtained from the ABRC.

The 35S:SVP transgenic plants in Ler were generated by Agrobacterium tumefaciens–mediated transformation of a construct containing the SVP cDNA linked to the 35S promoter from the pBI121 vector (Clough and Bent, 1998). Plants were grown on soil (Jiffy Mix; Sakata) in controlled-environment rooms or in a plant incubator (CF-305; TOMY) at 24°C under LD (16 h of light /8 h of dark), SD (10 h of light /14 h of dark), or LL unless specified otherwise. For the temperature cycle analysis, plants were grown on soil in a plant incubator (CF-305; TOMY) under LL with the temperature cycle 16 h at 24°C /8 h at 20°C.
EMS Mutagenesis and Phenotypic Screening for Mutations That Accelerate Flowering of *lhy-12;cca1-101* under LL

Approximately 5000 *lhy-12;cca1-101* (Ler) seeds were mutagenized by imbibition in 0.3% EMS (Sigma-Aldrich) for 9 h, followed by washing with 0.1 M Na2SO3 (twice) and distilled water for 30 min (five times). M2 seeds were collected in pools, with each pool containing ~20 M1 plants. Approximately 13,000 M2 seeds representing ~1300 M1 plants after mutagenesis of *lhy-12;cca1-101* seeds were sown on soil and screened for the early-flowering mutants under LL.

Genetic Analysis

The enhancer mutations in the *lhy-12* background were backcrossed to Ler wild-type plants twice before phenotypic analysis.

Measurement of Flowering Time

Flowering time was measured by scoring the number of rosette and cauline leaves on the main stem. Data are presented as means ± SE.

Construction and Analysis of Double and Triple Mutants

Double and triple mutants were made by crossing lines homozygous for each mutation. F2 plants homozygous for one of the mutations were self-fertilized, and F3 families were identified in which phenotypes characteristic of the second mutation were visible.

Immunoblot Analysis

Protein extraction was performed as described previously (Ichimura et al., 2000). SVP-specific antibodies were produced against synthetic peptides corresponding to the amino acid 218 to 233 region of SVP (STGAPVDDSSDTSLLR). The synthesized peptides were conjugated with keyhole limpet hemocyanin carrier. Polyclonal antiserum were raised in rabbits (Invitrogen). For immunoblot analysis, 30 μg of Arabidopsis leaf total protein was separated on a 12% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane by electroblotting. After blocking for 1 h in TBST buffer (50 mM Tris-HCl, pH 8, containing 0.05% Tween 20 and 150 mM NaCl) containing 5% nonfat dried milk at room temperature, the membrane was incubated in the same buffer with the SVP antibody (1:1000 dilution) for 3 h at room temperature. After washing three times in TBST buffer, the blots were incubated with a horseradish peroxidase–conjugated secondary antibody (Amersham) and the complexes were made visible by ECL Plus protein gel blotting detection reagents (Amerham) following the manufacturer’s instructions.

EMS Mutagenesis and Phenotypic Screening for Mutations That Accelerate Flowering of *lhy-12* under SD

Approximately 20,000 *lhy-12* seeds were mutagenized by imbibition in 0.3% EMS (Sigma-Aldrich) for 9 h, followed by washing with 0.1 M Na2SO3 (twice) and distilled water (five times). M2 seeds were collected in pools, with each pool containing ~20 M1 plants. Approximately 50,000 M2 seeds representing ~5000 M1 plants after mutagenesis of *lhy-12* seeds were sown on soil and screened for early-flowering mutants under SD conditions (10 h of light/14 h of dark) in a greenhouse.

RNA Analysis

RNA (20 μg) was separated on 1.2% agarose/formaldehyde denaturing gels and transferred to Biodyne B membranes (Nippon Genetics). Hybridization was done in 0.3 M sodium phosphate buffer (pH 7.0), 7% SDS, 1 mM EDTA, and 1% BSA overnight at 65°C. The blot was washed with 0.2× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate) and 0.1% SDS for 30 min at 65°C. Full-length G′ cDNA was used as a probe (Mizoguchi et al., 2002). Images were visualized using a Bioimaging Analyzer (BAS 5000; Fuji Photo Film); signal intensity was quantified with Science Lab 98 Image Gauge software (version 3.1; Fuji Photo Film).

DNA Gel Blot Analysis

PCR products were separated on 1.5% agarose gels and transferred to Biodyne B membranes (Nippon Genetics). RT-PCR products were cloned by the pGEM-T Easy Vector System I (Promega), and plasmids were extracted for PCR templates to amplify DNA fragments. The fragments were 32P-radio labeled to be probes with specific activity to detect each gene. Membranes were hybridized with the radioactive probe DNAs in hybridization solution that contained 5× SSC, 0.1% SDS, 0.1% sarkosyl, 0.75% blocking reagent (Boehringer Mannheim), and 5% dextran sulfate sodium salt at 65°C for 16 h. The blot was washed first with 2× SSC and 0.1% SDS for 20 min and then with 0.5× SSC and 0.1% SDS for 10 min at 65°C. The hybridization signal was visualized using a Bioimaging Analyzer (BAS 5000; Fuji Photo Film); signal intensity was quantified with Science Lab 98 Image Gauge software (version 3.1; Fuji Photo Film).

Sequence Analysis

Sequence analysis for the results shown in Figure 3 and Supplemental Figure 1 online was performed using the CQM Plus DTC-S-Quick Start kit (Beckman Coulter) following the manufacturer’s instructions.

Yeast Two-Hybrid Assay

For the yeast two-hybrid assay, each gene was amplified by PCR and cloned into the pGBK7T or pGADT7 vector (Clontech Laboratories) (Yoshida et al., 2006). For interaction studies, plasmids containing fusion proteins were cointroduced into Saccharomyces cerevisiae AH109 and grown on medium lacking Leu, Trp, and His in the presence of 0.5 and 1 mM 3-aminotriazole. pGBK7T-S3, which encodes a fusion between the GAL4ADNA-BD and murine p53, and pGADT7-T, which encodes a fusion between the GAL4AD and SV40 large T-antigen, were used as positive controls (53/T).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following Arabidopsis Genome Initiative locus numbers and accession numbers: CCA1, At2g46830 and P92973; LHY, At1g01060 and Q9SQI2; CO, At5g15840 and Q6R0H1; GI, At1g22770 and Q9SQI2; SOC1, At2g45660 and Q9FVC1; TUB2, At1g55650 and Q36R76; CS55; SOC1, 4123010; flc-102, At1g155840 and Q39057; FT, At1g65480 and Q95XZ2; FLC, At1g50140 and Q967Q7; SVP, At2g22540 and Q9FVC1; CS51, At2g45660 and Q64645; TUB2, At1g562690 and P29512. The mutant lines used in this article can be found in The Arabidopsis Information Resource database (http://www.Arabidopsis.org/index.jsp) under the following accession numbers: co-2, CS55; ft-1, CS56; fca-1, CS52; gi-3, CS51; lhy-21; cca1-11, CS9380; svp-31, 1005231108; svp-32, 4123010; flc-101, 4551274; flc-102, 1005389859.

Supplemental Data

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

Supplemental Figure 1. Summary of *lhy* and *cca1* Mutations.
Supplemental Figure 3. Partial Redundant Functions of SVP and FLC in the Control of Flowering.

Supplemental Figure 4. Characterization of 35S:FLC in LL.

Supplemental Figure 5. FLC Expression in Wild-Type, ihy-12, cca1-101, and ihy-12;cca1-101 Plants under LL.

Supplemental Figure 6. PCR cycles for RT-PCR.

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