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

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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 HYPOCOTYL (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; Más, 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 HYPOCOTYL (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; Más, 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 (Más, 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; Más, 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

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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 *LHYL1* 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

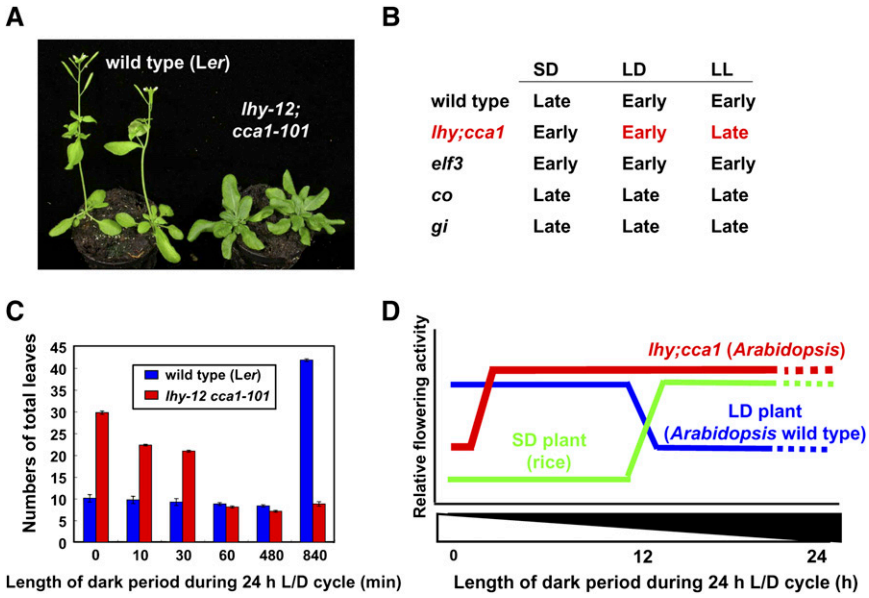

Figure 1. A Change of Daylength Response by *lhy;cca1*.

(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*) (Carré, 2002) or later flowering (*gi* and *co*) than wild-type plants (Figure 1B) (Koornneef et al., 1991; Zagotta et al., 1992; Más, 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

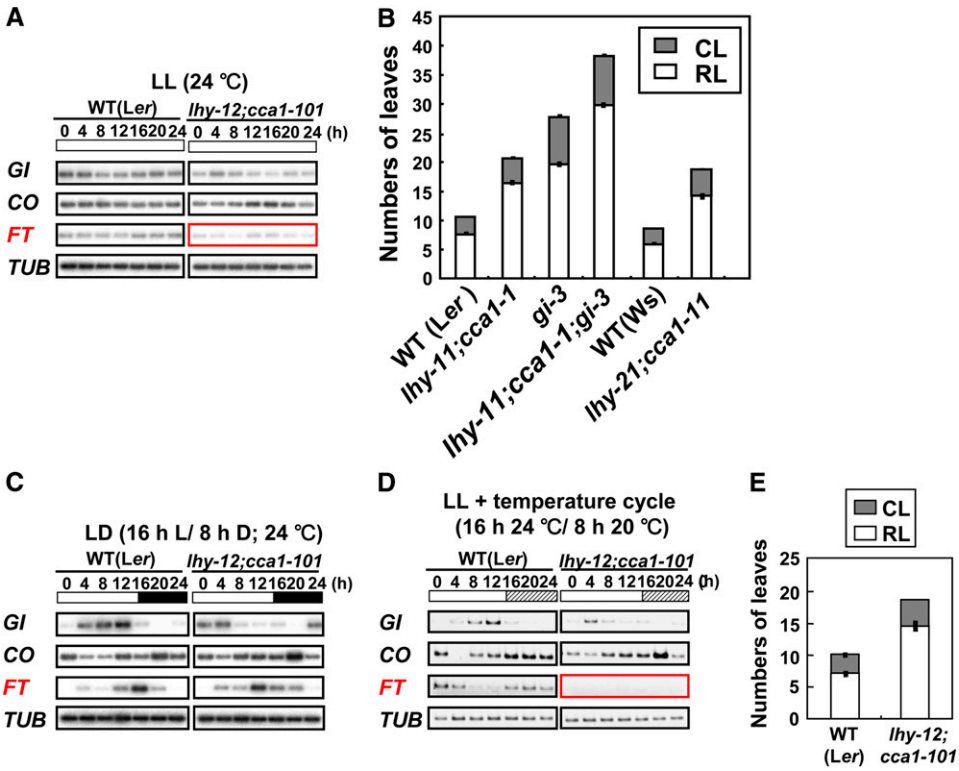

Figure 2. Downregulation of *FT* Expression in *lhy;cca1* under LL.

(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

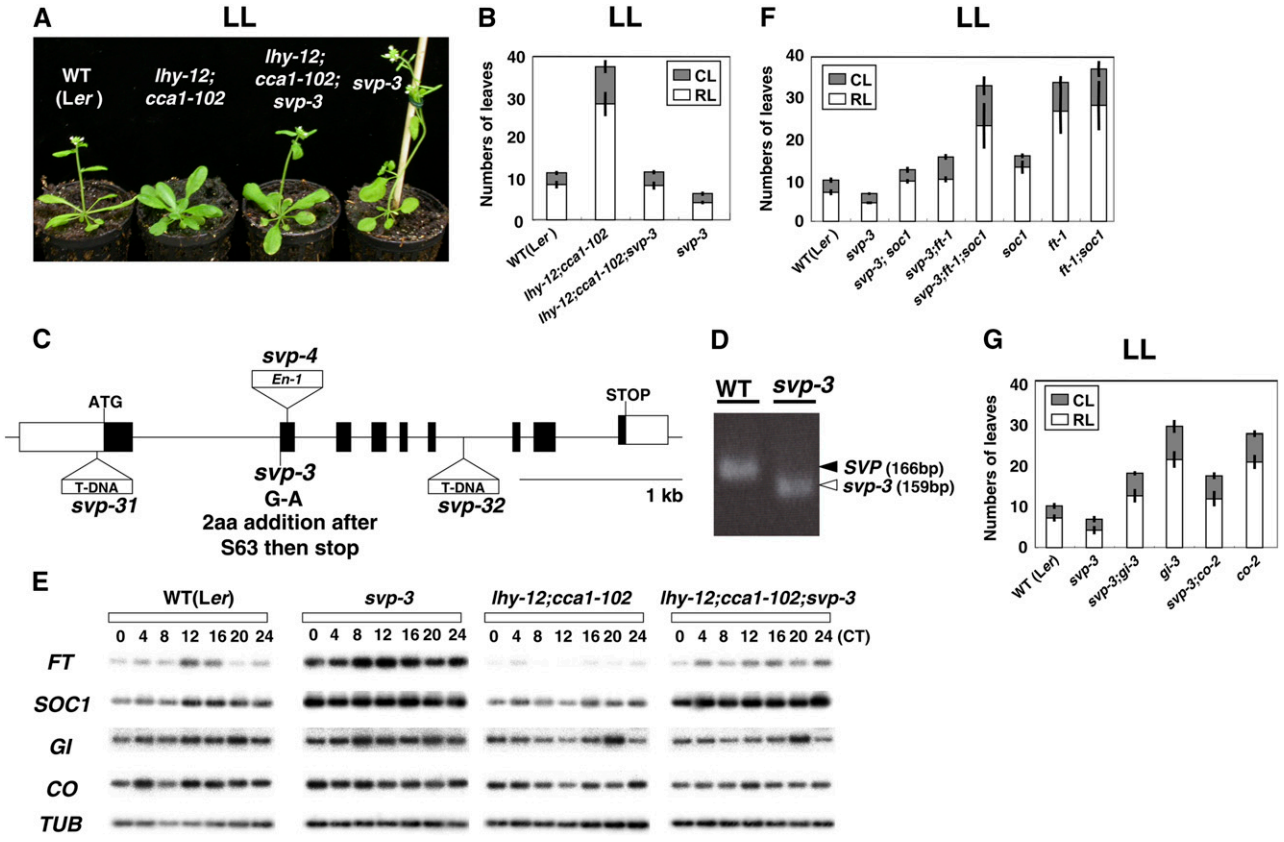

Figure 3. The *svp* Mutation Suppressed the Late-Flowering Phenotype of *lhy;cca1* and Increased *FT* and *SOC1* Expression in LL.

(A) Appearance of L*er* wild type, *lhy-12;cca1-101*, *lhy-12;cca1-101;svp-3*, and *svp-3* plants in LL.
(B) Flowering times of plants shown in **(A)**.
(C) The *svp* alleles in *Arabidopsis*. Black boxes represent exons, white boxes represent noncoding DNA, and black lines represent introns.
(D) Missplicing of *SVP* in *svp-3*. Exons 1 to 3 of *SVP* of the wild type and *svp-3* were amplified by RT-PCR. Different sizes of RT-PCR products are indicated by black and white arrowheads in the wild type (166 bp) and *svp-3* (159 bp), respectively. *svp-3* caused a missplicing and a 7-bp deletion in the *SVP* transcripts.
(E) Expression of *FT*, *SOC1*, *GI*, and *CO* in L*er* wild-type, *svp-3*, *lhy-12;cca1-101*, and *lhy-12;cca1-101;svp-3* plants grown under LL for 12 d. *TUBULIN2* (*TUB*) levels are shown as controls. Open boxes represent LL conditions, and hours from the first sampling are shown.
(F) and **(G)** Flowering times of various mutant plants in LL.
All of these experiments were done at least twice with similar results. Data in **(B)**, **(F)**, and **(G)** are presented as means ± SE.

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* [L*er*] 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;*

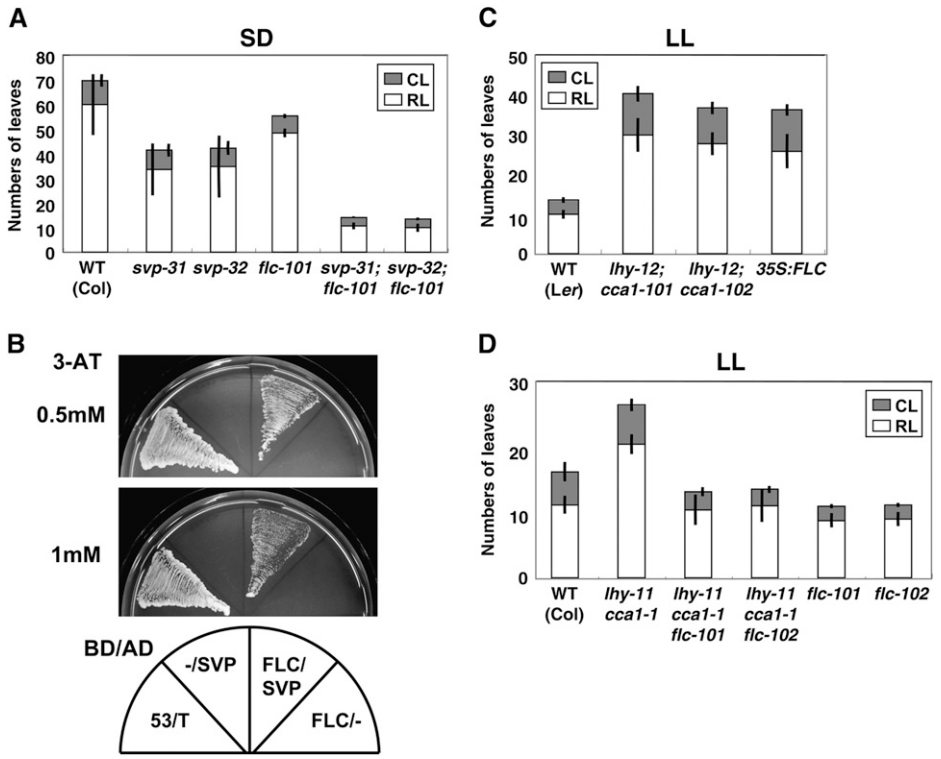

Figure 4. Roles of *FLC* in the Late Flowering of *lhy;cca1* in LL.

(A) Flowering times of *flc-101* (Col), *svp-31;flc-101* (Col), *svp-32;flc-101* (Col), and control plants in SD.

(B) Yeast two-hybrid assay showing direct interaction between SVP and FLC. FLC/SVP indicates that FLC or SVP is fused to the Gal4 activation domain or DNA binding domain, respectively. As a positive control, 53/T was used. Concentrations of the inhibitor of His biosynthesis (3-aminotriazole [3-AT]) used are shown (mM).

(C) Flowering times of L*er* wild-type, *lhy-12;cca1-101*, *lhy-12;cca1-102*, and *35S:FLC* plants in LL.

(D) Flowering times of Col wild-type, *lhy-11;cca1-1* (Col), *lhy-11;cca1-1;flc-101*, and *lhy-11;cca1-1;flc-102* plants in LL.

All of these experiments were done at least twice with similar results. Data in **(A)**, **(C)**, and **(D)** are presented as means ± SE.

cca1-102;svp-3 under LL (Figure 3E). 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 *svp-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 *svp-3* in the light period that is important for the determination of flowering (Figure 3E) (Suarez-Lopez et al., 2001; Valverde et al., 2004). In addition, *ft-1;soc1* mutations largely suppressed the early flowering of *svp-3* (Figure 3F), whereas *gi-3;svp-3* and *co-2;svp-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;svp-3* mutants may be responsible for the partial suppression of the late flowering of *lhy-12;cca1-102* by *svp-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 *svp* (Figure 4A) and that SVP 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 downregulation 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 SVP and FLC, as well as the classical GI-CO pathway for floral activation.

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

Although SVP is required for the late flowering of *lhy;cca1* plants in LL (Figures 3A and 3B), *lhy;cca1* did not affect the level of *SVP* 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 SVP activity to delay flowering. In LL, the late-flowering phenotype of *35S:SVP* was much stronger than that in LD

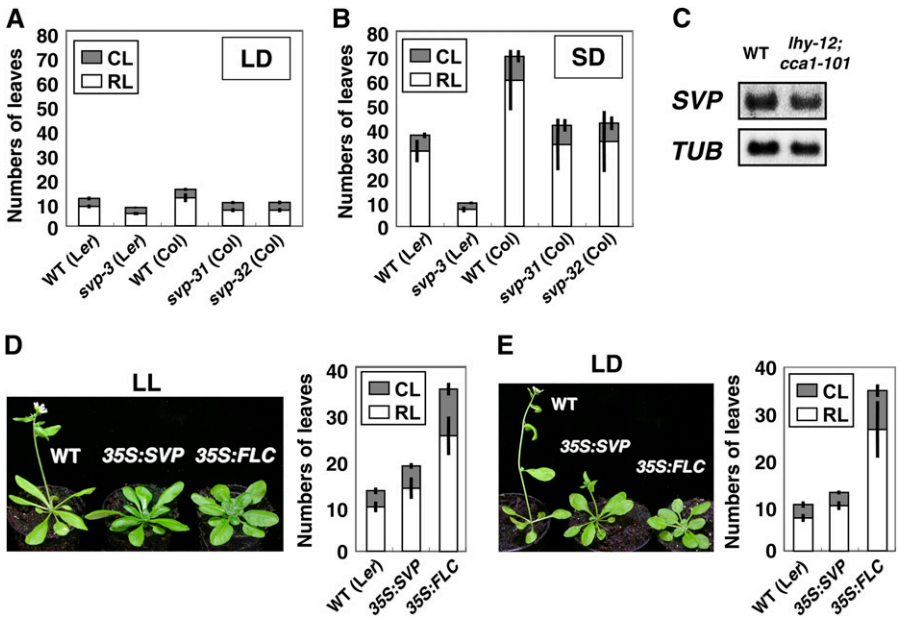

Figure 5. Effects of *SVP* Overexpression on Flowering Time in LL and LD.

(A) and **(B)** Flowering times of wild-type (L*er*), *svp-3* (L*er*), wild-type (Col), *svp-31* (Col), and *svp-32* (Col) plants in LD **(A)** and SD **(B)**.
(C) Expression of *SVP* and *TUB* in L*er* wild-type and *lhy-12;cca1-101* plants grown under LL for 12 d.
(D) and **(E)** *35S:SVP* delayed flowering in LL. Images of L*er* wild-type, *35S:SVP*, 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=proteinandid=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 (L*er*). Higher accumulation of SVP protein was detected at ZT 4, 8, and 12 in the wild type (L*er*). 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 (L*er*). 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

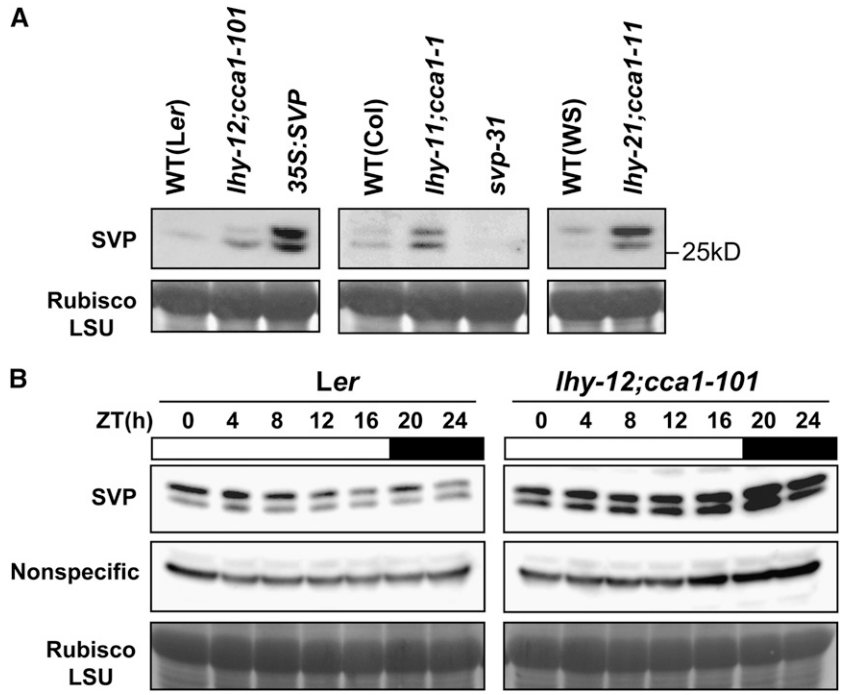

Figure 6. SVP Protein Accumulation.

(A) SVP protein level in the wild type and *lhy;cca1* under LL. Leaf extracts from each plant (30 μg) were subjected to SDS-PAGE and immunoblotted using SVP-specific antibody. Equal protein loading was confirmed by staining ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (Rubisco LSU) with zinc stain (Bio-Rad).

(B) SVP protein level in the wild type (L*er*) and *lhy-12;cca1-101* (L*er*) under LD (16 h of light/8 h of dark).

All of these experiments were done at least twice with similar results.

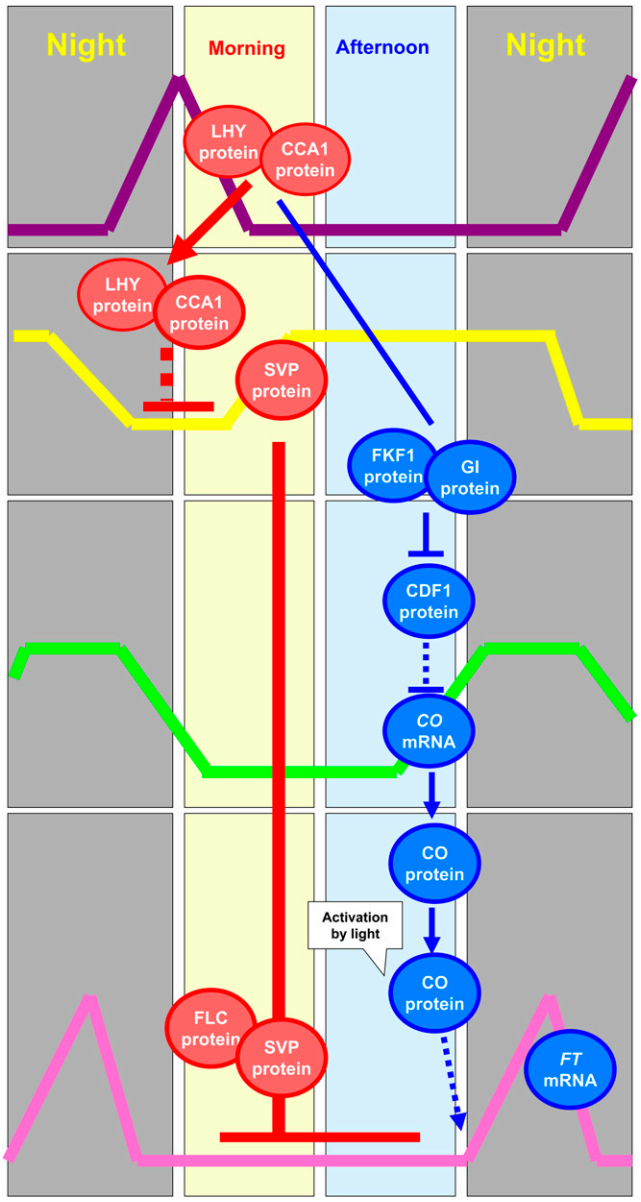

Figure 7. A Schematic Model Showing the Activation and Repression of the *FT* Gene Controlled by LHY and CCA1.

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 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.

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 (Alabadí et al., 2001, 2002; Mizoguchi et al., 2002, 2005; Yanovsky and

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; Sawa 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 *flc* (Figure 4E) but that of *co* was not affected by *flc* (Michaels and Amasino, 1999) and (2) *co* did not show dark-green/curled 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 (Scortecci 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, **h**ow 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 *flc* 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* L*er* 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) (Koornneef et al., 1991), *gi-3* (CS51; Fowler et al., 1999), *lhy-21;cca1-11* (Wassilewskija; CS9380; Hall et al., 2003), and *35S:FLC* (L*er*; Michaels and Amasino, 1999) mutants have been described previously.

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

The *35S:SVP* transgenic plants in L*er* 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* (L*er*) seeds were mutagenized by imbibition in 0.3% EMS (Sigma-Aldrich) for 9 h, followed by washing with 0.1 M Na_2SO_3 (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 L*er* 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 (STGAPVDSESSDTSLR). The synthesized peptides were conjugated with keyhole limpet hemocyanin carrier. Polyclonal antisera 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 (Amersham) 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 Na_2SO_3 (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 *GI* 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 ^{32}P-radiolabeled 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 CEQTM DTCS-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 pGBKT7 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. pGBKT7-53, which encodes a fusion between the GAL4DNA-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 Q6R0H1; GI, At1g22770 and Q9SQI2; CO, At5g15840 and Q39057; FT, At1g65480 and Q9SXZ2; FLC, At5g10140 and Q9S7Q7; SVP, At2g22540 and Q9FVC1; SOC1, At2g45660 and O64645; TUB2, At5g62690 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 2. Quantitative RT-PCR Analysis of *GI*, *CO*, *FT*, and *SOC1* under LL with and without Temperature Cycles.

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, *lhy-12*, *cca1-101*, and *lhy-12;cca1-101* Plants under LL.

Supplemental Figure 6. PCR cycles for RT-PCR.

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