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Original Paper

Circadian clock components in *Arabidopsis* II. LHY/CCA1 regulate the floral integrator gene *SOC1* in both GI-dependent and -independent pathways

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Abstract Two myb-related proteins, LHY and CCA1, play key roles in circadian clock function and flowering in *Arabidopsis*. GI mediates between LHY/CCA1 and floral activators (CO and FT) to promote flowering. The effect of GI on flowering probably involves FT-independent pathways, because *ft* only partially suppresses the early flowering caused by *lhy cca1* or overexpression of *GI* (*GI-ox*). LFY, FT, and SOC1 integrate four flowering pathways: the photoperiod, gibberellic acid (GA), vernalization, and autonomous pathways. Roles of *SOC1* and *LFY* in mediating between the clock and control of flowering time have not been elucidated. Here, we demonstrate that SOC1 functioned redundantly with FT to promote flowering via the LHY/CCA1/GI pathway. *GI-ox* and *lhy cca1* increased mRNA levels of the *SOC1* and *gi* partially suppressed the up-regulation in *lhy cca1* under SD. The overexpression of *LHY* (*lhy-1*) shifted the phase of *SOC1* expression, and the *gi* mutation did not affect the phase shift, suggesting that LHY regulates *SOC1* expression both in GI-dependent and independent manners.

Key words: Circadian clock, flowering, GI, LHY/CCA1, photoperiod, SOC1.

Four genetic pathways (the photoperiod, gibberellic acid (GA), vernalization, and autonomous pathways) control flowering time in *Arabidopsis* (Boss et al. 2004; Hayama and Coupland 2004). In the photoperiod pathway, GIGANTEA (GI), CONSTANS (CO), and FLOWERING LOCUS T (FT) have significant roles in the control of flowering time under regulation of the circadian system (Boss et al. 2004; Calvino et al. 2005; Hayama and Coupland 2004; Izawa et al. 2003; Mizoguchi et al. 2005; Suarez-Lopez et al. 2001).

The circadian clock that generates rhythms of ca. 24 h is thought to have several components (Boss et al. 2004; Hayama and Coupland 2004; Mizuno and Nakamichi 2005; Salome and McClung 2004; Southern and Millar 2005), including two homologous genes, *LATE ELONGATED HYPOCOTYL* (*LHY*) and *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*), which encode single myb transcriptional factors (Green and Tobin 1999; Mizoguchi et al. 2002; Schaffer et al. 1998; Wang and Tobin 1998). TIMING OF CAB EXPRESSION 1 (TOC1) and PSEUDO-RESPONSE REGULATOR (PRR) family members also play key roles in the circadian clock function in *Arabidopsis* (Mizuno and Nakamichi 2005). The *lhy cca1* mutants flower much earlier than *lhy* or *cca1* single mutants and lose free-running rhythms in the expression of clock-controlled genes after a few cycles under continuous light or dark (Mizoguchi et al. 2002). The overexpression of either *LHY* (*lhy-1*) or *CCA1* (*35S*:*CCA1*) delays flowering under long days (LD) (Schaffer et al. 1998; Wang and Tobin 1998).

We recently proposed that GI has dual roles within the circadian clock to regulate period length and circadian phase, while also directly promoting expression of a circadian clock output pathway that includes *CO* and *FT* and promotes flowering (Calvino et al. 2005; Mizoguchi et al. 2005). The effect of GI on flowering probably includes the CO- and FT-independent pathways, because *co* or *ft* mutations only partially suppress the early flowering caused by *GI-ox* (*35S*:*GI*) or *lhy cca1* (Calvino et al. 2005; Mizoguchi et al. 2005). However, key factors involved in the CO- or FT-independent pathways have not been identified.

SUPPRESSOR OF OVEREXPRESSION OF CO 1 (*SOC1*) encodes a MADS box protein and functions as a floral integrator gene of the photoperiod, GA,

Abbreviations: CCA1, CIRCADIAN CLOCK ASSOCIATED 1; CO, CONSTANS; GI, GIGANTEA; LHY, LATE ELONGATED HYPOCOTYL; SD, short day; SOC1, SUPPRESSOR OF OVEREXPRESSION OF CO 1; TOC1, TIMING OF CAB EXPRESSION 1.

vernalization, and autonomous pathways (Boss et al. 2004). CO proteins induce flowering under LD conditions through the activation of *FT* and *SOC1* expression (Onouchi et al. 2000; Samach et al. 2000). The up-regulation of *FT* and *SOC1* expression seems to be a major key in switching from the vegetative to the reproductive phase (Boss et al. 2004; Hayama and Coupland 2004). The *SOC1* expression is affected by a clock mutation, *toc1* (Blázquez et al. 2002), but it has not been elucidated whether loss- and gain-of-function of *LHY* and *CCA1* and gain-of-function of *GI* affect the *SOC1* expression or not. Also it has not been tested whether the *SOC1* is really required for the early flowering caused by clock mutations such as *toc1*, *lhy cca1* and *GI-ox*.

Here, we demonstrate that SOC1 functions redundantly with FT in the LHY/CCA1/GI pathway to regulate flowering time in *Arabidopsis*. Also we propose that LHY may regulate *SOC1* expression in both GI-dependent and -independent pathways. The *SOC1* promoter has CCA1/LHY recognition sequences, suggesting that CCA1/LHY may bind the *SOC1* promoter directly to control the *SOC1* expression.

Materials and methods

Plant materials, growth conditions and measurement of flowering time

The Landsberg *erecta* (L*er*) ecotype of *Arabidopsis thaliana* was the wild-type used. The *lhy-11 cca1-1* (Mizoguchi et al. 2002), *lhy-11 cca1-1 gi-3* and *GI-ox* (Mizoguchi et al. 2005), *CO-ox* (Onouchi et al. 2000), *lhy-1* (Schaffer et al. 1998) and *gi-3* (Fowler et al. 1999) mutants were described previously. The *lhy-1 gi-3* mutant was made by crossing lines homozygous for *lhy-1* and *gi-3*. Detailed information on the construction of the double mutant lines and genetic segregation ratios is available from the authors.

Plants were grown on soil in controlled environment rooms under either LD (16 h light/8 h dark) or SD (10 h light/14 h dark) as described (Mizoguchi et al. 2002). Flowering time was measured as described (Mizoguchi et al. 2002). Data are presented as mean+/−SE. Differences in flowering times were confirmed as statistically significant using Student's *t*-test ($P<0.0005$).

Semiquantitative RT-PCR

For SD experiments, plants were grown on soil for 10 days and aerial parts were used for RNA preparation. RT-PCR was performed with 1 μg of total RNA using a SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). cDNAs were diluted to 100 μl with TE buffer, and 1 μl of diluted cDNA was used for PCR amplification by TaKaRa Ex Taq (TaKaRa, Shiga, Japan). For RT-PCR expression studies, the following primers were used: *FT*, 5′-ACAACTGGA-ACAACCTTTGGCAATG-3′ and 5′-ACTATATAG-GCATCATCACCGTTCGTTACTCG-3′ (Blázquez and Weigel 1999); *CCR2*, 5′-CTCTTGAGCTGCCTTCG-3′ and 5′- AGAACATTCATTGGTAATCCC-3′ (Staiger et al. 2003); *SOC1*, 5′-GGATCGAGTCAGCACCAAACC-3′ and 5′-CCCAATGAACAATTGCGTCTC-3′ (Blázquez et al., 2002); *TUB*, 5′- CTCAAGAGGTTCTCAGC-AGTA-3′ and 5′- TCACCTTCTTCATCCGCAGTT-3′ (Kobayashi et al. 1999). Numbers of PCR cycles were as follows; 25, 15, 22 and 18 cycles for *FT*, *CCR2*, *SOC1* and *TUB*. Annealing temperature was 58°C. Primer specificity was verified by sequencing the PCR products. The PCR products were separated on 1.5% agarose gels and transferred to Biodyne B Membranes (Nippon Genetics, Tokyo, Japan). The RT-PCR products were cloned by pGEM-T Easy Vector System I (Promega, Madison, WI), and plasmids were extracted to be templates for PCR to amplify probe DNA. The membranes were hybridized with radioactive probe DNAs in hybridization solution that contained 5×SSC, 0.1% SDS, 0.1% sarkosyl, 0.75% Blocking reagent (Boehringer Mannheim, Mannheim, Germany), and 5% dextran sulfate sodium salt at 65°C for 16 h. The blot was washed with 2× SSC and 0.1% SDS for 20 min, then 0.5×SSC and 0.1% SDS for 10 min at 65°C and then the hybridization signal was visualized using a BioImaging Analyzer (BAS 5000; Fuji Photo Film, Tokyo, Japan); signal intensity was quantified with Science Lab 98 Image Gauge software (version 3.1; Fuji Photo Film, Tokyo, Japan). Values were represented relative to the highest value of the samples after standardization to the *TUB* control. All the RT-PCR analysis was performed at least twice and usually with independent RNA samples.

Results

SOC1 expression in lhy-11 cca1-1 *and* gi *mutants under SD*

The level of *SOC1* expression shows circadian oscillation, suggesting that it is under the regulation of a circadian clock (Blázquez et al. 2002). The expression peak was shifted forward in the *toc1* mutant, which shows an early flowering phenotype under SD (Blázquez et al. 2002; Millar et al. 1995; Somers et al. 1998). The double-loss-of-function line *lhy-11 cca1-1* also shows an early flowering phenotype under SD (Mizoguchi et al. 2002). To determine whether defects of the clock components LHY and CCA1 also affect the pattern of *SOC1* expression, we conducted semi-quantitative RT-PCR analysis. Plants were grown under SD for 10 days and harvested every 4 h for 24 h. The oscillation pattern of the *SOC1* transcript level was changed in *lhy-11 cca1-1* (Figure 1A, B). In the wild-

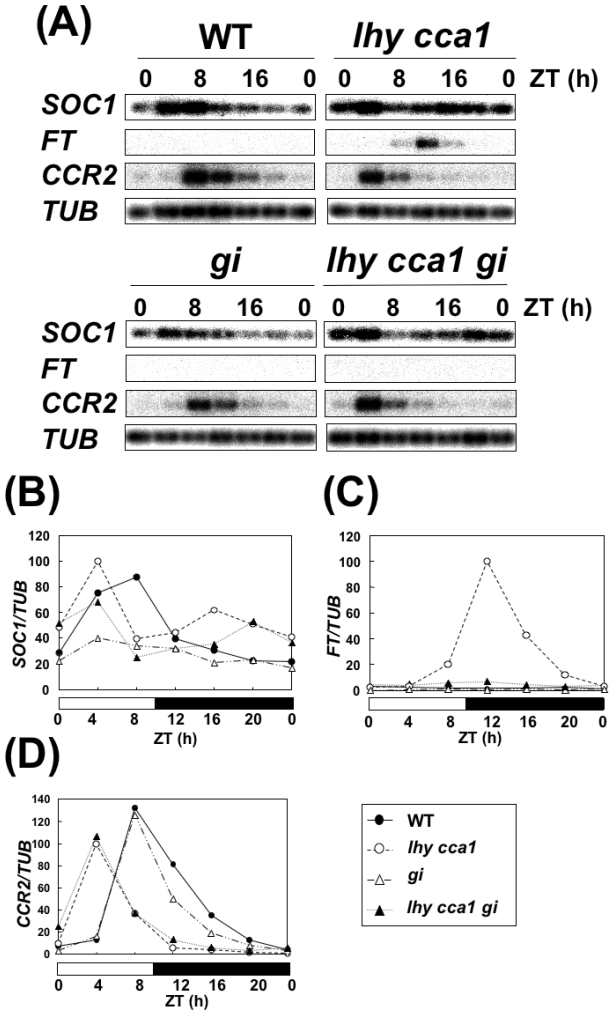

Figure 1. Abundance of *SOC1*, *FT* and *CCR2* mRNA in wild-type, *lhy-11 cca1-1*, *gi-3* and *lhy-11 cca1-1 gi-3* plants grown under SD. (A) The expression of *SOC1*, *FT* and *CCR2* was analyzed using RT-PCR in wild-type (WT), *lhy-11 cca1-1* (*lhy cca1*), *gi-3* (*gi*) and *lhy-11 cca1-1 gi-3* (*lhy cca1 gi*) plants grown under SD. Quantification of *SOC1* (B), *FT* (C), and *CCR2* (D) mRNA abundance from the blots shown in (A). The results are presented as a proportion of the highest value after standardization with respect to *TUBULIN2* levels (*TUB*). Open and solid bars along the horizontal axis represent light and dark periods, respectively. These are measured in hours from dawn (zeitgeber time; ZT). Each experiment was performed at least twice with similar results.

type plant, the transcript level showed diurnal oscillation that peaked around ZT8, as reported (Blázquez et al. 2002), but in *lhy-11 cca1-1*, the peak was shifted to ZT4 and the level was higher than in the wild type during the dark period. The expression patterns of *FT* and *COLD-CIRCADIAN RHYTHM-RNA-BINDING 2* (*CCR2*) were controlled by a circadian clock (Carpenter et al. 1994; Suarez-Lopez et al. 2001) and were analyzed in the same lines as controls. The expression of *FT* was induced in *lhy cca1*, as reported (Figure 1A, C; Mizoguchi et al. 2005). The peak of *CCR2* expression was around ZT8 in the wild type, but was shifted forward to ZT4 in *lhy-11 cca1-1* (Figure 1A, D; Mizoguchi et al. 2005).

The abundance of the *FT* mRNAs was reduced dramatically in the *lhy-11 cca1-1 gi-3* triple mutant compared to *lhy-11 cca1-1* (Figure 1A, C) as reported (Mizoguchi et al. 2005). The phase of expression of *CCR2* was shifted earlier in the *lhy-11 cca1-1* double mutant compared to wild-type plants, but the amplitude of expression was not reduced in the *lhy-11 cca1-1 gi-3* triple mutant (Figure 1A, D) as reported (Mizoguchi et al. 2005). Therefore, in contrast to its effect on *FT* expression the *gi-3* mutation did not alter the amplitude or suppress the phase shift caused by *lhy-11 cca1-1* on *CCR2*. The effect of *gi-3* on the *SOC1* expression in *lhy cca1* background was not so strong, but the up-regulation of the *SOC1* expression in *lhy-11 cca1-1* was lowered by *gi-3* (Figure 1A, B). These results suggest that the GI-dependent pathway controlled by the circadian clock affected the *SOC1* expression.

SOC1 expression in GI-ox *plants under SD*

GI mediates between LHY/CCA1 and CO to promote flowering by increasing *CO* and *FT* mRNA abundance (Mizoguchi et al. 2005). The transcript levels of *SOC1* and *FT* are increased in *CO-ox* plants (Figure 3A, C; Samach et al. 2000). CO is partially required for the early flowering phenotype of *lhy cca1* and *GI-ox* under SD (Mizoguchi et al. 2005). Consistent with this, *SOC1* mRNA expression was increased significantly in *GI-ox* plants compared with that in wild-type plants under SD (Figure 2A, B).

FT and SOC1 function redundantly in the LHY/CCA1/GI pathway to promote flowering

To test whether *SOC1* is required for the early flowering of *GI-ox* plants, the flowering times of double mutants *GI-ox soc1* and *GI-ox ft-1* and a triple mutant *GI-ox ft-1 soc1* were scored under LD and SD (Figure 2C). Under SD, the *GI-ox soc1* plants flowered with 11 leaves more than *GI-ox* plants and 20 leaves fewer than the wild-type control (Figure 2C). As a control, the *GI-ox ft-1* plants also flowered with 21 leaves more than *GI-ox* plants and 10 leaves fewer than the wild-type control as reported (Figure 2C; Mizoguchi et al. 2005). The *GI-ox ft-1 soc1* triple mutant produced >35 leaves more than the *GI-ox* plants under SD. The effect of the *ft-1 soc1* double mutation on the early flowering phenotype of *GI-ox* was also more severe than that of *ft-1* and *soc1* under LD. These results suggest that FT and SOC1 function redundantly in the LHY/CCA1/GI pathway to promote flowering.

SOC1 *expression in* lhy-1 *and* lhy-1 *gi mutants under SD*

We then tested the *SOC1* and *CCR2* expression in *lhy-1* (*LHY-ox*), in which *LHY* mRNA is constitutively overexpressed (Kim et al. 2003; Schaffer et al. 1998). The peak of the *CCR2* expression was shifted to around

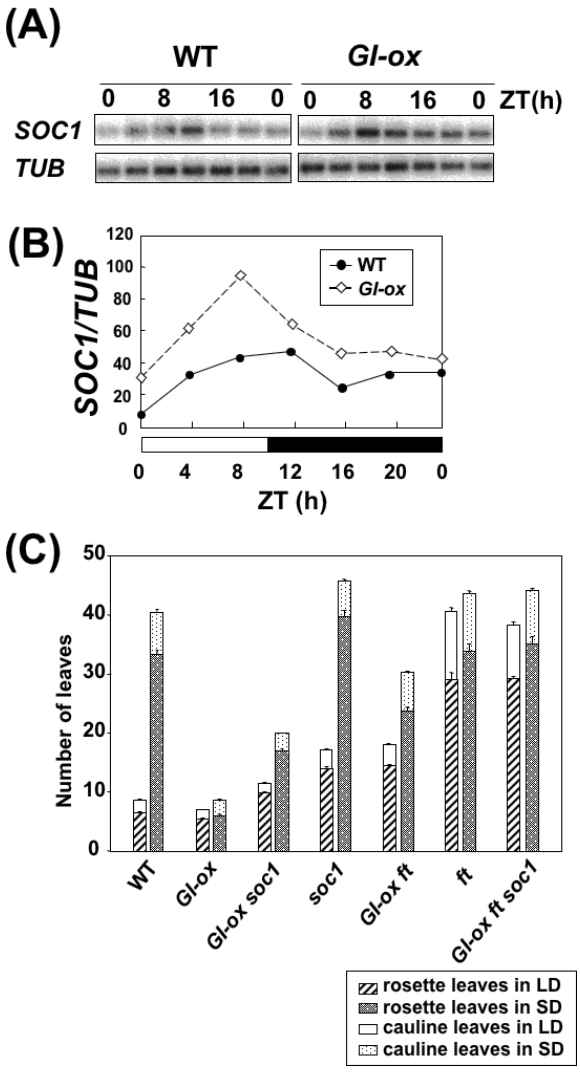

Figure 2. Abundance of *SOC1* mRNA in *GI-ox* plants grown under SD and the flowering times of *GI-ox* plants with and without *soc1* and *ft* mutations. (A) The expression of *SOC1* was analyzed using RT-PCR in wild-type (WT) and *GI-ox* plants grown under SD. (B) Quantification of *SOC1* mRNA abundance from the blots shown in (A). The results are presented as a proportion of the highest value after standardization with respect to *TUBULIN2* levels (*TUB*). Open and solid bars along the horizontal axis represent light and dark periods, respectively. These are measured in hours from dawn (zeitgeber time; ZT). Each experiment was performed at least twice with similar results. (C) The flowering time of *GI-ox* plants with or without *soc1* and *ft-1* was measured under LD and SD. Flowering time was scored by counting the numbers of rosette and cauline leaves on the main stem. The mean leaf number is shown±standard error (SE). Each experiment was performed at least twice with similar results.

ZT20 in *lhy-1* (Figure 3A, D). In *lhy-1 gi-3*, the *CCR2* expression pattern was similar to that of *lhy-1* rather than that of *gi-3* (Figure 3A, D). In *lhy-1*, the phase of *SOC1* expression was changed dramatically, and the peak of expression occurred around ZT16 or ZT20 (Figure 3A, B). The overexpression of CO increased the expression of *SOC1* (Figure 3A, C; Samach et al. 2000), but the loss-of-function of *gi* seemed to have a minor effect on the transcription level of *SOC1*, as reported (Figure 1A, B; Lee et al. 2000; Samach et al. 2000). The pattern of *SOC1* expression in *lhy-1 gi-3* was similar to that of *lhy-1* (Figure 3A, B) but not to that of *gi-3* (Figure 1A, B). These results suggest that *LHY* may regulate the *SOC1* expression both in GI-dependent and -independent manners.

Discussion

A circadian clock affects SOC1 expression, and SOC1 is partially required for the early flowering of GI-ox plants

Disruption of the circadian clock system has been reported to affect flowering time, but how it alters flowering time is less well understood. Recently, we showed that GI mediates between the circadian clock (LHY and CCA1) and floral activators (CO and FT) in the control of flowering (Mizoguchi et al. 2005). A loss-of-function mutation of *gi* almost completely suppressed the early flowering of *lhy cca1* under SD. However, either the *co* or *ft* mutation only partially suppressed the early flowering phenotypes of *lhy cca1* and *GI-ox* plants under SD. These results suggest that the effect of GI on flowering probably includes the CO- and FT-independent pathways.

In this paper, we demonstrated that mutations of clock components (e.g., *lhy-11 cca1-1*, *lhy-1*, and *GI-ox*) affected the level of *SOC1* transcription (Figures 1–3). In addition, the early flowering phenotype of *GI-ox* was partially suppressed by *soc1* (Figure 2C). The double loss-of-function of *ft-1* and *soc1* markedly delayed the flowering time of *GI-ox* plants under both SD and LD (Figure 2C), indicating that SOC1 and FT act redundantly as floral activators in the LHY/CCA1-GI-CO pathway.

The clock components LHY and CCA1 may directly control the expression of SOC1*, a key gene in one of the clock-controlled output pathways*

The peak of *CCR2* expression was dramatically changed in *lhy-1* (ZT8 to ZT20; Figure 3A, D). This phase shift may be caused by the phase shift in the LHY protein level, which shows diurnal oscillation with sharp peaks around dusk in wild-type plants. By contrast, in *lhy-1*, it shows moderate peaks around the end of the light period under light/dark cycles (Kim et al. 2003). The level of *CCR2* transcription is high when the LHY protein is absent, suggesting that LHY negatively regulates *CCR2* expression.

The CCA1-binding site (CBS), AAA/CAATCT, was identified (Wang et al. 1997) and is important for morning-specific transcription of the clock-controlled genes (Michael and McClung 2002). CBS is similar to

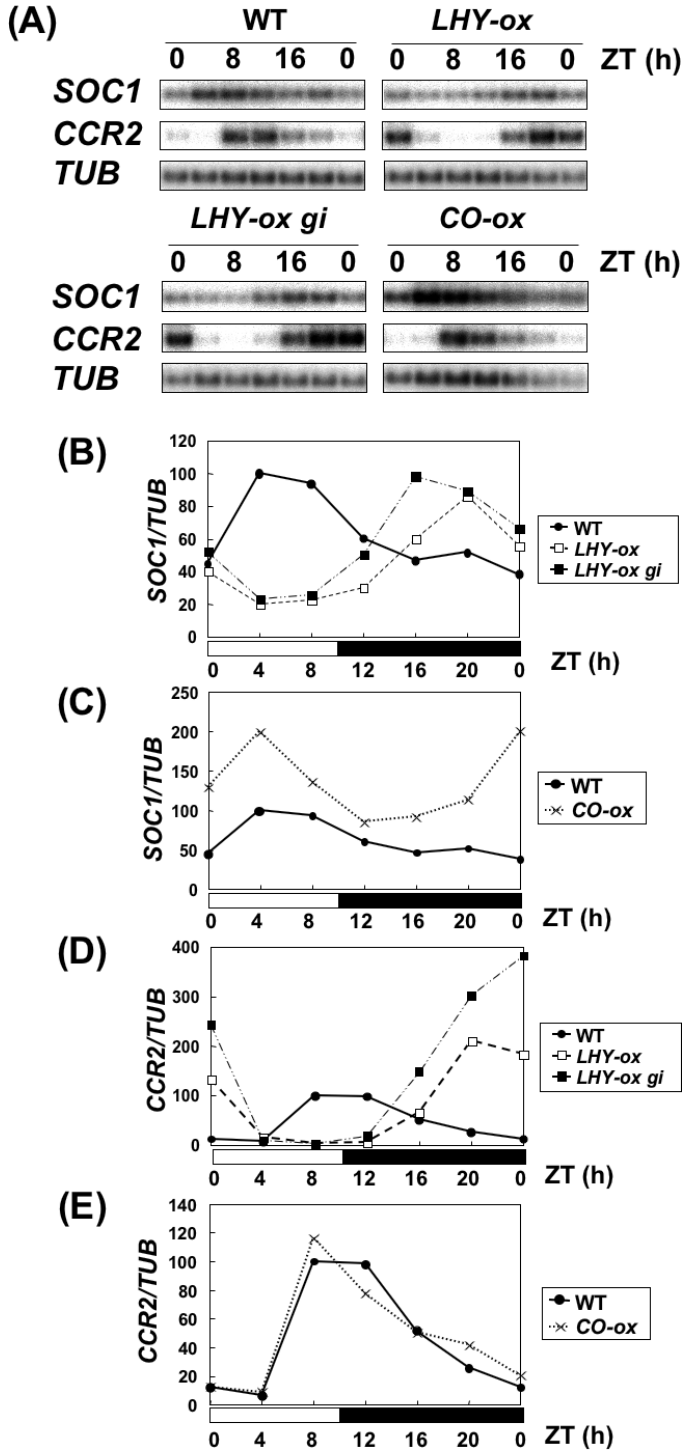

Figure 3. Abundance of *SOC1* and *CCR2* mRNA in *lhy-1*, *lhy-1 gi*, and *CO-ox* plants grown under SD. (A) The expression of *SOC1* and *CCR2* was analyzed by RT-PCR in wild-type (WT), *lhy-1* (*LHY-ox*), *lhy-1 gi-3* (*LHY-ox gi*), and *35S*:*CO* (*CO-ox*) plants grown under SD. Quantification of *SOC1* (B, C) and *CCR2* (D, E) mRNA abundance from the blots shown in (**A**). The results are presented as a proportion of the highest value after standardization with respect to *TUBULIN2* levels (*TUB*). Open and solid bars along the horizontal axis represent light and dark periods, respectively. These are measured in hours from dawn (zeitgeber time; ZT). Each experiment was performed at least twice with similar results.

the eight-nucleotide motif, AAATATCT, found in a computational analysis of *Arabidopsis* cycling genes (Harmer et al. 2000). This motif is called the evening element (EE) and is required for evening-specific transcription of the clock-controlled genes (Harmer et al. 2000; Michael and McClung 2002). LHY and CCA1 recognize the EEs in the *TOC1* promoter (Alabadi et al. 2001). LHY and CCA1 recognize several EEs or CBSs in the *CCR2* promoter (Harmer et al. 2000), suggesting that *LHY* directly binds to the *CCR2* promoter. The

expression of *SOC1* was similar to that of *CCR2* in that there was a phase advance in *lhy-11 cca1-1* and retrogression in *lhy-1* (Figures 1, 3). Therefore, we consulted the *SOC1* promoter sequence and found a CBS (AAAAATCT) and an EE (AAATATCT) in the −267/−260 and −210/−203 regions of the *SOC1* promoter, respectively (Figure 4). Our results suggest that LHY/CCA1 affect the *SOC1* expression both via the GI-dependent and -independent pathways (Figure 4) and that LHY or CCA1 may bind to the *SOC1* promoter directly. There is no report that the clock component regulates the expression of downstream factors in the floral promotion pathway directly. Several pathways regulate *SOC1* expression, including the photoperiod, GA, autonomous, and vernalization pathways (Boss et al. 2004). We propose a possible novel pathway that may regulate *SOC1* expression or direct its regulation by the clock components (Figure 4). Further analysis will be needed to confirm this possibility.

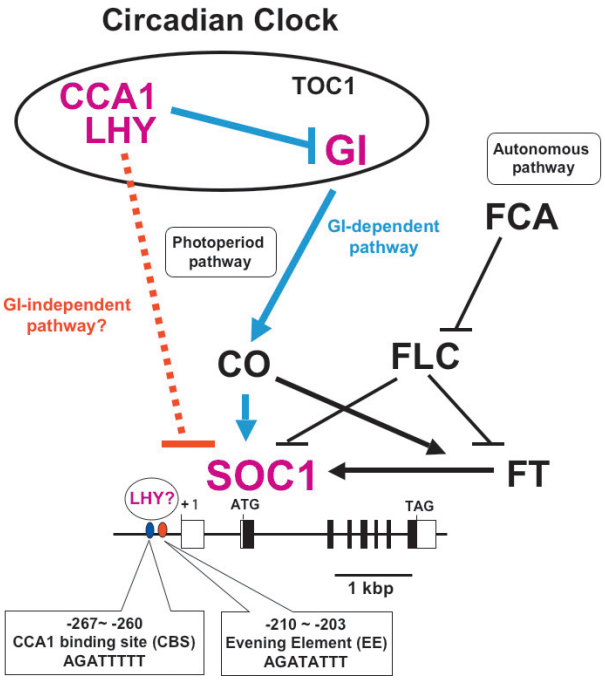

Figure 4. A hypothetical model showing the GI-dependent and -independent regulation of the *SOC1* expression by LHY and CCA1. The circadian clock regulates flowering time through the photoperiod pathway. CO is a key floral activator and mediates between GI and SOC1/FT in the photoperiod pathway. FLC is a floral repressor in the autonomous pathway and shown to negatively regulate the *SOC1* and *FT* expression (Hepworth et al. 2002). Recently, it has been shown that FT regulates the *SOC1* expression (Yoo et al. 2005). SOC1 and FT redundantly act as floral activators in the clock-controlled flowering pathway. GI is required for the up-regulation of the *SOC1* expression in *lhy cca1* and *GI-ox* increases the *SOC1* expression in SD (the GI-dependent pathway shown in blue). The phase of the *SOC1* expression is largely shifted in *lhy-1* under SD. *gi* mutation does not affect the phase shift in the *lhy-1* background. The *SOC1* promoter has a CBS (Wang et al. 1997) and an EE (Harmer et al. 2000) that can be recognized by LHY/CCA1 proteins. These results suggest that LHY/CCA1 may regulate the *SOC1* expression in both the GI-dependent (blue) and -independent (red) pathways. We propose a possibility that LHY/CCA1 proteins may bind the *SOC1* promoter directly to regulate the *SOC1* expression. Further analysis will be needed to confirm this possibility.

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