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Photoperiod pathway regulates expression of *MAF5* and *FLC* that encode MADS-box transcription factors of the FLC family in *Arabidopsis*

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Abstract The control of flowering by day length was the first photoperiodic response to be described and is also one of the most characterized in many plants. The circadian clock plays a pivotal role in the photoperiodic flowering pathway. In *Arabidopsis*, flowering time is controlled by the photoperiod as well as by the gibberellic acid and vernalization/autonomous pathways. FLOWERING LOCUS C (FLC), a MADS box protein, has been shown to be a key floral repressor in the vernalization/autonomous pathways. Although the roles and regulation of floral activators GIGANTEA, CONSTANS, and FLOWERING LOCUS T in the photoperiodic flowering pathway have been well characterized, those of the floral repressors are not well understood. Here, we demonstrate that the *MADS AFFECTING FLOWERING 5* (*MAF5*) gene, one of the *FLC* family members, shows a diurnal expression pattern in light/dark cycles and that both gain- and loss-of-function mutations in the photoperiod pathway affect the gene expression of *MAF5* and *FLC*. These results highlight the possible roles of MAF5 and FLC in crosstalk between the photoperiod and vernalization/autonomous pathways in *Arabidopsis*.

Key words: Circadian clock, CO, FLC, GI, MAF5, photoperiod.

Temporal control of gene expression is quite important for organ development, metabolism, reproduction, and many other physiological events. These physiological events are thought to be well coordinated by endogenous biological rhythms called circadian rhythms (Fekih et al. 2009b; Niinuma et al. 2007; Salome and McClung 2004; Searle and Coupland 2004). In higher plants, many biological events are under the control of circadian rhythms, including the regulation of flowering time (Boss et al. 2004; Calvino et al. 2005; Mizoguchi et al. 2006; Mizoguchi and Yoshida 2009; Salome and McClung 2004). Molecular genetics using *Arabidopsis* have identified four major pathways that affect the regulation of flowering: the photoperiod, gibberellic acid (GA), vernalization, and autonomous pathways (Boss et al. 2004; Fekih et al. 2009b).

Recently, crosstalk among the different genetic pathways has been demonstrated. For example, gene expression of the floral activator, *SUPPRESSOR OF OVEREXPRESSION OF CO1* (*SOC1*), is influenced by all of the flowering pathways, and *FLOWERING LOCUS T* (*FT*) is regulated by both the photoperiod and the vernalization/autonomous pathways (Boss et al. 2004; Fekih et al. 2009b; Mizoguchi et al. 2006,). *FLOWERING LOCUS C* (*FLC*) encodes a MADS box protein and is a major floral repressor in the autonomous and vernalization pathways (Boss et al. 2004; Searle and Coupland 2004). FLC negatively regulates *FHA/ cryptochrome 2* (*CRY2*) gene expression (El-Din El-Assal et al. 2003). FHA/ CRY2, a blue light receptor, also plays a role in the photoperiod pathway (Boss et al. 2004; Searle and Coupland 2004). Double mutations between the autonomous pathway (*fca* or *fpa*) and the photoperiod pathway (*fha*, *ft*, or *fe*) cause synergistic increases in mRNA and protein expression of FLC (Rouse et al. 2002).

Flowering time is fine-tuned through a balance of both positive and negative activities (Calvino et al. 2005; Fujiwara et al. 2008; Mizoguchi and Yoshida 2009; Yoshida et al. 2009). In *Arabidopsis*, several molecules act as floral activators, including GIGANTEA (GI), CONSTANS (CO), FT, SOC1, and LEAFY (LFY) (Boss et al. 2004; Fekih et al. 2009b; Searle and Coupland 2004). At least four classes of proteins, the FLC family (FLC, MAF1 [MADS AFFECTING FLOWERING1] to MAF5), TERMINAL FLOWER 1 (TFL1), SHORT VEGETATIVE PHASE (SVP) and SCHLAFMUTZE (SMZ)/SCHNARCHZAPFEN (SNZ), play roles in the

Abbreviations: CO, CONSTANS; CCA1, CIRCADIAN CLOCK ASSOCIATED 1; FLC , FLOWERING LOCUS C; GI , GIGANTEA; LHY, LATE ELONGATED HYPOCOTYL; MAF5, MADS AFFECTING FLOWERING 5; SOC1, SUPPRESSOR OF OVEREXPRESSION OF CO1
This article can be found at http://www.jspcmb.jp/

repression of flowering (Fekih et al. 2009a, 2009b; Fujiwara et al. 2008; Hartmann et al. 2000; Ratcliffe et al. 2003; Schmid et al. 2003). *MAF2-5* genes are tandemly arranged in the bottom of the Chromosome 5 (Ratcliffe et al. 2003). The *FLC* family members and *SVP* encode the MADS box protein transcription factor (Boss et al. 2004; Hartmann et al. 2000; Ratcliffe et al. 2003; Searle and Coupland 2004). TFL1 is highly similar to the floral activator FT, although they have opposite effects on flowering (Kardailsky et al. 1999; Kobayashi et al. 1999). Two closely related genes, *SMZ* and *SNZ*, encode AP2 proteins (Schmid et al. 2003).

Gene expression of the floral activators, *GI*, *CO*, and *FT*, are regulated by a circadian clock and temporal expression of the genes is quite important for plants to determine when to flower under a variety of photoperiods (Mizoguchi et al. 2005; Mizoguchi et al. 2006). The circadian clock that generates an about 24 h rhythm, is composed of several components (Mizoguchi et al. 2006; Niinuma et al. 2007; Salome and McClung 2004), including two homologous genes, *LATE ELONGATED HYPOCOTYL* (*LHY*) and *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*), which encode single myb transcription factors (Mizoguchi et al. 2006; Niinuma et al. 2007; Salome and McClung 2004). LHY and GI/CO have been shown to have opposite effects on *FT* expression; over-expression of *LHY* (*lhy-1*) lowered the expression level of *FT* in long-days (LDs), whereas over-expression of *GI* (*35S:GI*) or *CO* (*35S:CO*) resulted in increased expression (Fowler et al. 1999; Fujiwara et al. 2005a, 2005b, 2005c; Mizoguchi et al. 2005; Samach et al. 2000; Suarez-Lopez et al. 2001). Suppression of *FT* expression in *lhy-1* was overcome by either *35S:GI* or *35S:CO* (Mizoguchi et al. 2005; Suarez-Lopez et al. 2001). Up-regulation of *FT* expression in either *lhy-11 cca1-1* or *GI*-ox was suppressed by *co-2* under light/dark cycles such as long-days (LD) and short-days (SD; Mizoguchi et al. 2005). These data suggest that the transcriptional cascade "*LHY*/*CCA1*–*GI*–*CO*–*FT*" plays an important role in the photoperiodic flowering pathway (Mizoguchi et al. 2005; Suarez-Lopez et al. 2001). Although *flc* loss-of-function affects leaf movements that are under the control of circadian rhythms (Swarup et al. 1999), information on the transcriptional control of the floral repressor genes by a circadian clock has been quite limited compared to that on the floral activators. To elucidate the connection between the photoperiodic and the vernalization/autonomous pathways in the control of flowering, we investigated the transcript levels of the floral repressor *FLC* and its paralogs *MAF1* to *MAF5* in *Arabidopsis* mutants that exhibit altered sensitivity to the photoperiods.

Here, we demonstrate that the transcript level of *MAF5*, one of the members of the FLC family, shows a diurnal oscillation and that the expression level is affected by mutations in the photoperiod pathway in *Arabidopsis*. FLC, one of the major floral repressors does not show oscillations in its gene expression but the level of expression is altered by mutations in the photoperiod pathway. These results highlight the transcriptional regulation of a floral repressor *FLC* and its paralog *MAF5* by the circadian clock components in *Arabidopsis*. A hypothetical model on the potential crosstalk between the photoperiod and the vernalization/autonomous pathways involved in the control of flowering in *Arabidopsis* is discussed.

Materials and methods

Plant materials and growth conditions

The L*er* ecotype of *Arabidopsis thaliana* was used unless otherwise indicated. The *gi-3*, *gi-6*, *co-2*, *fca-1*, *35S:GI* (line A), *lhy-1*, and *35S:GI lhy-1* have been described previously (Mizoguchi et al. 2005). Double mutants were constructed by crossing lines homozygous for each mutation. Plants used for the RT-PCR were grown on soil or agar plates in controlled-environment rooms under LD (16 h light/8 h dark) or SD (10 h light/14 h dark) conditions for 10 days. For continuous light (LL) experiments, the LD-grown plants were transferred to LL conditions. For the measurement of flowering times, plants were grown on soil under LD (16 h light/8 h dark) and SD (10 h light/14 h dark) conditions. Flowering time was measured by scoring the number of rosette and cauline leaves on the main stem. Data are presented as means±SEM. Measurement of flowering time was performed at least twice with similar results.

RT-PCR analysis

RT-PCR was performed with 1 μg of total RNA using a SuperScript^{TM} First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) as previously reported (Mizoguchi et al. 2005; Oda et al. 2004). The *MAF1*, *MAF2*, *MAF3*, *MAF4*, *MAF5*, *FLC* (Ratcliffe et al. 2003), *SOC1* (Blazquez et al. 2002), *GI*, *CCA1*, *TIMING OF CAB EXPRESSION1* (*TOC1*; Nakagawa et al. 2004), and *TUB2* (Kobayashi et al. 1999) primers have been previously described.

PCR products were separated on 1.5% agarose gels and transferred to Biodyne B Membranes (Nippon Genetics, Tokyo, Japan). RT-PCR products were cloned by pGEM-T Easy Vector System I (Promega, Madison, WI), and plasmids were extracted for PCR templates to amplify DNA fragments. The fragments were radiolabeled to be probes. 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, Mannheim, Germany), 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, 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 wild type samples after standardization to the *TUB* control. Highest values of the wild type samples in each experiment are shown as 1.00.

All the RT-PCR analyses were performed at least twice and usually with independent RNA samples.

T-DNA insertional mutants of maf5

Two mutant lines carrying a T-DNA insertion within At5g65080 were obtained from the SALK collection (SALK_048316 and SALK_085852, renamed *maf5-1* and *maf5-2*, respectively). The plants that were homozygous for the T-DNA insertion were genotyped by PCR using the primers, salk048316sense (5′-TTCAGGATCTCCGACCAGTTTA-3′), salk048316anti (5′-TACCCTCACAAAGTATTGAAGC-3′), salk085852sense (5′-TGCTGCTACTAAGTGATTGCTT-3′), salk085852anti (5′-CCGTTGATGATTGGTGGTT-ACT-3′) and pROK2A1 (TGGTTCACGTAGTGGG-CCATCG). T-DNA insertion sites in the *maf5-1* and *maf5-2* alleles were confirmed by sequencing the PCR fragment.

Results

Diurnal oscillation of MAF5 *gene expression*

MAF5 gene expression showed a diurnal pattern under SDs and peaked at Zeitgeber time (ZT)16 and decreased to trough level at around ZT0 (Figure 1A). The floral activator *SOC1* also showed a diurnal expression as previously reported (Blazquez et al. 2002). In contrast, the transcript levels of other members of the *FLC* family were almost constant.

Monogenic loss-of-function of maf5 *affects neither flowering time nor rhythmic expression of CCGs*

Two *maf5* mutant lines were obtained from the SALK collection (Figure 1B, C). The *maf5* plants did not show any difference from the wild type plants in terms of total leaf number under LDs (Figure 1D, E) and SDs (Figure 1F, G). Therefore, even though MAF5 might act as a floral repressor (or activator), it appeared to play a relatively subtle role in determining flowering time under the conditions tested.

We next examined whether the expression of the clock-controlled genes (CCGs) was altered in the *maf5* lines (Figure 1H–K). In wild-type plants, *CCA1* expression peaked around subjective dawn at ZT 0, ZT 24, and ZT 48, as reported previously (Mizoguchi et al. 2002). The *maf5* did not significantly affect the free-running rhythms (FRRs) or the amplitude of the *CCA1* expression (Figure 1H). Similar results were obtained for the other CCGs, *GI* (Figure 1J) and *TOC1* (data not shown), which normally reach peak expression in the evening (Mizoguchi et al. 2002; Salome et al. 2004; Searle and Coupland 2004). As a control, *lhy* loss-of-function shortened FRRs of *CCA1* and *GI* as previously reported (Figure 1I, K; Mizoguchi et al. 2002, 2005). No statistical difference was observed in the rhythmicity of the expression of CCGs between *maf5* and wild-type plants (data not shown). These results suggest that MAF5 may play a role in the output pathways controlled by photoperiods. Alternatively, a gene may exist that has a redundant function with MAF5 in controlling the CCG expressions.

Regulation of the MAF5 *gene expression by the photoperiod pathway*

LHY, CCA1, and GI are closely associated with circadian clock functions in *Arabidopsis* (Fekih et al. 2009b; Mizoguchi et al. 2002; Mizoguchi et al. 2005; Niinuma et al. 2007; Salome and McClung 2004), and mutations of these genes alter the expression patterns of the CCGs. Genotypes carrying the mutations in the autonomous pathway, such as *fca-1*, showed high expression of *FLC*, whereas mutations in the photoperiod pathway, such as *gi*, *co*, and *fha*, did not affect the transcript level of *FLC* based on Northern blot analysis (Rouse et al. 2002). We examined whether mutations of the photoperiod pathway affected the expression level of *FLC* together with *MAF5* under SDs using RT-PCR because the expression level of *FLC* is not sufficiently high to be detected by Northern blot analysis in the L*er* ecotype. *GI* gain- and loss-of-function increased and lowered the overall expressions of the *MAF5* and *FLC*, respectively (Figure 2A, B). The transcript level of *FLC* did not show a diurnal oscillation (Figure 2B). Loss-of-function mutation of *co* lowered *MAF5* and *FLC* gene expressions in a similar way to that of *gi* (Figure 2A, B). Consistent with a finding that *co* is epistatic to *gi* (Mizoguchi et al. 2005; Suarez-Lopez et al. 2001), *co* mutation largely suppressed the up-regulation of the *MAF5* and *FLC* genes by *35S:GI* (Figure 2A, B). In contrast, the increased expression of the *MAF5* and *FLC* genes by *35S:GI* was not significantly affected by *lhy-1*, and only slight decrease of the *MAF5* and *FLC* mRNA level was observed in *35S:GI lhy-1*. The *fca* loss-of-function mutations in the autonomous pathway increased *MAF5* and *FLC* gene expression as reported previously (Ratcliffe et al. 2003).

To test whether FLC functioned as a negative regulator of flowering in *35S:GI* plants like it does in wild-type,

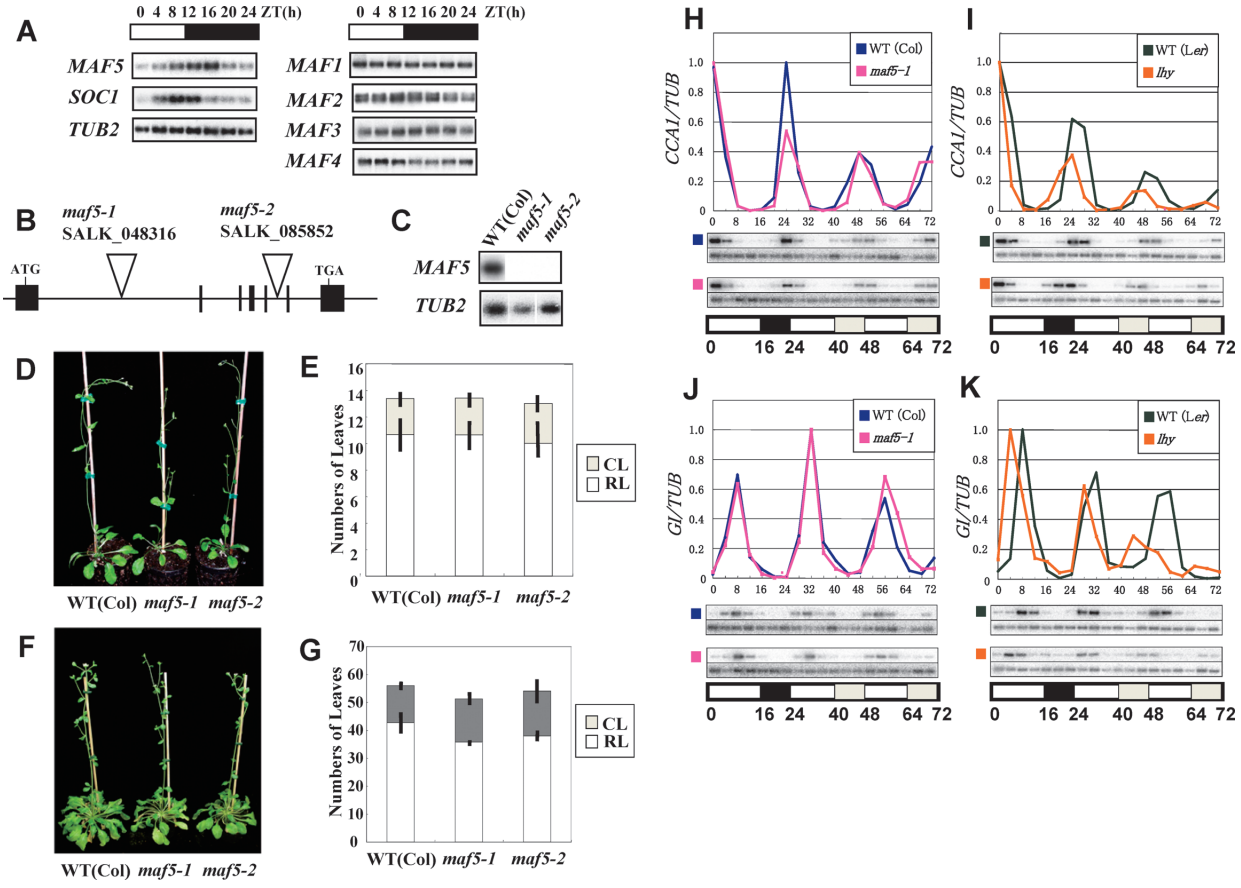

Figure 1 *MAF5* transcript level shows that diurnal oscillation and T-DNA insertions in the *MAF5* gene do not significantly affect the flowering time and circadian rhythms. (A) The RT-PCR analysis of *MAF5*, *SOC1*, *MAF1*, *MAF2*, *MAF3*, *MAF4*, and *TUB2* expression in L*er* wild-type (WT) plants under SDs. (B) T-DNAs were inserted in the 1st and 5th intron in *maf5-1* and *maf5-2*, respectively. (C) The *MAF5* transcript was not detected in the *maf5* seedlings but detected strongly in the WT controls by RT-PCR. Plants were grown under SDs for 10 days and harvested on ZT16. WT (Col, left), *maf5-1* (middle), and *maf5-2* (right) were grown under LDs (D, E) and SDs (F, G). Open and filled boxes represent the numbers of rosette leaves (RL) and cauline leaves (CL), respectively. Each experiment was performed at least twice with similar results. (H-K) The circadian oscillations of transcript levels of CCGs did not change in *maf5*. The RT-PCR analysis of *CCA1* (H) and *GI* (J) in the WT (Col) and *maf5-1* (Col). As controls, the RT-PCR analysis of *CCA1* (I) and *GI* (K) in WT (L*er*) and *lhy-12* (L*er*) is also shown. Plants were entrained under LD conditions for 10 days and then placed under LL conditions. Open, filled, and hatched boxes indicate light, dark, and subjective dark periods, respectively. Each experiment was performed at least twice with similar results. Essentially similar results were obtained with *maf5-2* (data not shown).

flowering time of *35S:GI fca* was compared with those of control plants under SDs and LDs (Figure 2D–G). The *fca* mutation largely delayed the flowering time of the *35S:GI* plants; *35S:GI* flowered earlier than the wild type under SDs and increased expression levels of two floral integrator genes *FT* and *SOC1* (Fujiwara et al. 2005b; Mizoguchi et al. 2005). Mutation of *fca* increased the expression levels of the floral repressor gene *FLC* and its paralog *MAF5* (Figure 2A; Ratcliffe et al. 2003). The late flowering phenotype of the *fca* plants was associated with lowered expression of *FT* and *SOC1* (Samach et al. 2000). Therefore, the delay of flowering time in the *35S:GI fca* was also likely to be associated with decreased expression of *FT* and *SOC1*. *lhy cca1* promoted flowering and increased expression of *FT* and *SOC1* in a similar way to those of *35S:GI* under SDs (Fujiwara et al. 2005a, 2005b, 2005c; Mizoguchi et al. 2005). The *fca* mutation lowered the expression of *FT* and *SOC1* (Figure 2C) and delayed flowering of *lhy cca1* under SDs (Fujiwara et al. 2008). These results suggest that highly accumulated FLC proteins by *fca* in the *35S:GI* plants probably decrease the expression levels of *FT* and *SOC1* and cause late flowering.

Discussion

MAF5 gene expression showed a diurnal rhythm in light/dark cycles (Figure 1A). Furthermore, mutations in the photoperiod pathway affected not only the *MAF5* but also *FLC* gene expression under SDs (Figure 2A, B). *35S:GI* and *gi* increased and decreased the *MAF5* and *FLC* expression, respectively (Figure 2A, B). Loss-of-function mutation in *CO*, a downstream factor of GI, suppressed the up-regulation of the *MAF5* and *FLC* expression by *35S:GI* (Figure 2A, B). To test whether the regulation of the *MAF5* and *FLC* expression by CO was

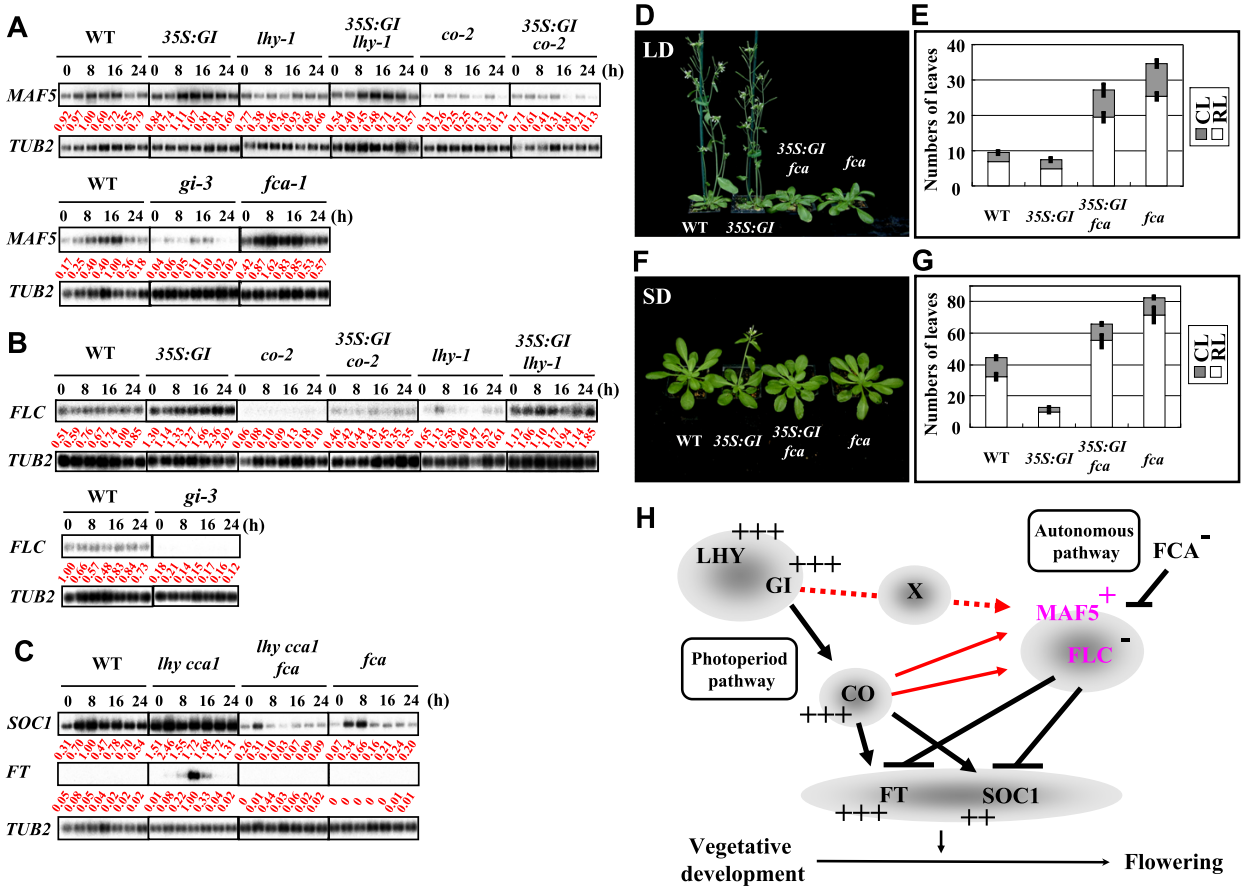

Figure 2 Mutations of the photoperiod pathway affect *MAF5* and *FLC* gene expressions. The RT-PCR analysis of *MAF5* (A), *FLC* (B), *SOC1* and *FT* (C), and *TUB2* (A–C) expression in the wild-type (WT), *35S:GI*, *lhy-1*, *35S:GI lhy-1*, *co-2*, *35S:GI co-2*, *gi-3*, *fca-1*, and *35S:GI fca-1* in the L*er* ecotype under SDs. ZT 0 is the time point just before lights on. 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 wild type samples after standardization to the *TUB* control. Highest values of the wild type samples in each experiment are shown as 1.00. Each experiment was performed at least twice with similar results. Flowering times of the L*er* WT, *35S:GI*, *35S:GI fca-1*, and *fca-1* plants under LDs (D, E) and SDs (F, G). Open and filled boxes represent the numbers of rosette leaves and cauline leaves, respectively. Each experiment was performed at least twice with similar results. (H) A hypothetical model on regulations and functions of the *MAF5* gene. In the photoperiod pathway, CO mediated two floral activators (FT and SOC1) and components of the circadian clock (LHY and GI). The expressions of *CO*, *FT*, and *SOC1* showed diurnal oscillation with relatively higher amplitude (+++ or ++). *MAF5* gene expression was affected by the photoperiod pathway and showed a diurnal oscillation with moderate amplitude (+). *FLC* expression was constant under light/dark cycles (−), although both of the *MAF5* and *FLC* expressions were affected by *co* mutation. These may reflect different regulations of *MAF5* and *FLC* by CO. Although CO is required for the up-regulation of the *MAF5* and *FLC* expressions by *35S:GI*, over-expression of *CO* was not sufficient to increase the gene expressions. This indicates that an unidentified factor (X) may also be required for controlling *MAF5* and *FLC* expressions. FLC is a major floral repressor in the vernalization/autonomous pathway and down-regulated the expressions of *FT* and *SOC1*. FCA negatively regulated *FLC* expression in the autonomous pathway, and *fca* also influenced *MAF5* gene expression.

direct, we examined the transcript level of the genes in *35S:CO:GR* (Simon et al. 1996) with or without dexamethazone (DEX). The mRNA level of *FT* started to increase within 1 hour after the CO-activation by DEX as reported previously (data not shown, Yamaguchi et al. 2005). Expression levels of *MAF5* and *FLC*, however, were not significantly affected by the CO-activation (data not shown). These results suggest that CO may be required for the up-regulation of the *MAF5* and *FLC* expression by *35S:GI*, but over-expression of CO is not sufficient to increase *MAF5* and *FLC* expression. An unidentified factor shown as X in Figure 2H may also be required for the up-regulation of the *MAF5* and *FLC* expressions. We found that expression of some of the *MAF* genes was suppressed in *lhy-21 cca1-11* (Ws) but not in *lhy-12 cca1-101* (L*er*) (Fujiwara and Mizoguchi, unpublished data). The suppression did not occur in wild type Ws and L*er*, suggesting that the natural variation between Ws and L*er* together with *lhy cca1* mutation were involved in the suppression of *MAF* genes. A gene for the natural variation may be one of candidates for the X shown in Figure 2H.

Over-expression of *MAF5* altered flowering time, suggesting that MAF5 might be involved in the regulation of flowering together with other members of the FLC family (Table 1; Ratcliffe et al. 2003). The *maf5*

Table 1. A summarized view of the feature of FLC family genes and SOC1

Gene Name	AGI/ GenBank acc.no.	Function on flowering	Effect on response to vernalization		Transcript levels	Flowering time after vernalization		Diurnal oscilla-tion	References
			KO	OX		KO	OX		
FLC (FLF, AGL25)	At5g10140	repressor	weak	weak	down	early	late[a,b]	No	Koornneef et al. (1994), Lee et al. (1994), Michaels et al. (1999, 2001), Parenicova et al. (2003)
MAF1 (FLM, AGL27)	At1g77080	repressor	N.D.	insensitive	down	early	late[a,b]	No	Alvarez-Buylla et al. (2000), Ratcliffe et al. (2001), Scortecci et al. (2001)
MAF2 (AGL31)[g]	At5g65050	repressor	similar to wt[c]	insensitive (Col)	no change[d]	slightly early[e]	late[a,b]	No	Alvarez-Buylla et al. (2000), Ratcliffe et al. (2003)
MAF3 (AGL70)[g]	At5g65060	N.D.	N.D.	N.D.	down	N.D.	late[a,b]	No	Ratcliffe et al. (2003), Parenicova et al. (2003)
MAF4 (AGL69)[g]	At5g65070	N.D.	N.D.	N.D.	slightly down	N.D.	late[a,b]	No	Ratcliffe et al. (2003), Parenicova et al. (2003)
MAF5 (AGL68)[g]	At5g65080	N.D.	N.D.	N.D.	up	wild type	late[a,f]	Yes	Ratcliffe et al. (2003), Parenicova et al. (2003)
SOC1 (AGL20)	At2g45660	activator	N.D.	N.D.	up	late	early	Yes	Onouchi et al. (2000), Samach et al. (2000), Lee et al. (2000), Moon et al. (2000), Blazquez et al. (2002)

[a] They show late flowering when oversxpressed in Ler (Michaels et al. 1999; Sheldon et al. 1999; Ratcliffe et al. 2001, 2003).
[b] When overexpressed in Col, they show unsettled flowering time phenotype, and significant number of lines show early flowering (Ratcliffe et al. 2001, 2003).
[c] maf2 shows a similar vernalization response to the wild type, but shows a strong response to brief cold spells (Ratcliffe et al. 2003).
[d] MAF2 transcript levels are reduced after excessively long cold treatments of 10 to 12 weeks (Ratcliffe et al. 2003).
[e] maf2 flowers 2 to 3 days earlier than wt (Ratcliffe et al. 2003).
[f] When overexpressed in Col, they don't show significant late-flowering phenotype (Ratcliffe et al. 2003).
[g] MAF2～5 form a tight cluster at the bottom of chromosome 5 (Ratcliffe et al. 2001).
KO＝knockout
OX＝overexpression
N.D.＝not determined

mutations, however, did not affect flowering time (Figure 1D, E), suggesting that effects of *maf5* on the control of flowering may be subtle compared to those of FLC. There may be a gene with redundant functions with MAF5 in *Arabidopsis*. Some of the FLC family members might have such functions because they show high homology to MAF5. Expression of *FLC*, *MAF1* and *MAF3* were decreased by vernalization (Ratcliffe et al. 2003). MAF5 may also be involved in the vernalization pathway. Flowering time is controlled by multiple pathways such as the photoperiod, GA, autonomous and vernalization pathways. Construction and analysis of double or triple mutants of the FLC family members will be required to better understand the function of MAF5 in such a complex regulation of flowering time by possible crosstalks of the different pathways.

Clock mutations such as *lhy*, *cca1*, *toc1*, and *gi*, affect not only flowering time but also other clock-controlled output pathways such as leaf movement, hypocotyl elongation, and expression of the CCGs (Mizoguchi et al. 2002; Mizoguchi et al. 2005; Mizoguchi et al. 2006; Niinuma et al. 2007; Salome and McClung 2004; Searle and Coupland 2004). In contrast, loss-of-function mutations of floral activator genes such as *co* and *ft* do not affect the general circadian rhythms (Suarez-Lopez et al. 2001). Therefore, CO and FT are components of one of the clock-controlled outputs, i.e., flowering. We tested whether MAF5 played a role in the maintenance of general circadian rhythms using the *maf5* mutants. These mutations, however, did not affect the diurnal and circadian expressions of CCGs (Figure 1H–K), suggesting that MAF5 may not have a role in controlling general circadian rhythms.

The *FLC* gene expression was higher in *35S:GI* and lower in *gi-3* and *co-2* than in L*er* wild-type plants under SDs (Figure 2B). Over-expression of *CO* causes early flowering through up-regulation of *FT* and *SOC1* gene expressions (Samach et al. 2000). The over-expression of *CO* also increases the gene expression of a floral repressor *TFL1* (Simon et al. 1996). Too much activity of the floral activators might use the floral repressor activity of FLC and MAF5 as a break to limit early flowering (Figure 2H). Alternatively, MAF5 (and FLC) might function both as a floral repressor and an activator with different partners. In this case, MAF5 might act as one component of the floral activator complex in early flowering plants promoted by *35S:GI*. In contrast, in the late-flowering plants caused by *fca-1*, MAF5 might play a role in a different complex as a floral repressor. Recent findings that MADS box proteins can form a ternary

complex with different combinations support this idea (de Folter et al. 2005).

In this study, we found that *MAF5* and *FLC* gene expressions are affected by the photoperiod pathway and the clock mutation, *lhy-1*. Temporal and synergistic control of the gene expression for a set of floral activators (e.g., *GI*, *CO*, and *FT*) and repressors (e.g., *FLC* and *MAF5*) by a clock both in leaves and shoot apex might be important for the fine-tuning of flowering.

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