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/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.
repression of flowering (Fekihi 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 hy-1 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 Ler 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™ 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 TUB8 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’-TTCCAGATCTCGACCACTTGA-3’), salk08316anti (5’-TACCCCTTACAAAGTGTAGTCTT-3’), salk085852sense (5’-TGTGCTACTAAGTGATTGCTT-3’), salk085852anti (5’-CCGTTGATGATTGGTGGT-3’) and pROK2A1 (TGTTCCAGCTAGTGGG-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 Ler 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,
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 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
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 (Ler) (Fujiwara and Mizoguchi, unpublished data). The suppression did not occur in wild type Ws and Ler, suggesting that the natural variation between Ws and Ler 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.

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 Ler 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 Ler WT, 35S:GI, 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.
Table 1. A summarized view of the feature of FLC family genes and SOC1

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>AGI/ GenBank acc.no.</th>
<th>Function on flowering</th>
<th>Effect on response to vernalization</th>
<th>Transcript levels</th>
<th>Flowering time after vernalization</th>
<th>Diurnal oscillation</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLC (FLF, AGL25)</td>
<td>At5g10140</td>
<td>repressor</td>
<td>weak</td>
<td>weak</td>
<td>down</td>
<td>early</td>
<td>late&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>MAF1 (FLM, AGL27)</td>
<td>At1g77080</td>
<td>repressor</td>
<td>N.D.</td>
<td>insensitive</td>
<td>down</td>
<td>early</td>
<td>late&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>MAF2 (AGL31)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>At5g65050</td>
<td>repressor</td>
<td>similar to wt&lt;sup&gt;b&lt;/sup&gt;</td>
<td>insensitive (Col)</td>
<td>no change&lt;sup&gt;c&lt;/sup&gt;</td>
<td>slightly early&lt;sup&gt;d&lt;/sup&gt;</td>
<td>late&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>MAF3 (AGL70)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>At5g65060</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>down</td>
<td>N.D.</td>
<td>late&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>MAF4 (AGL69)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>At5g65070</td>
<td>N.D.</td>
<td>N.D.</td>
<td>slightly down</td>
<td>N.D.</td>
<td>late&lt;sup&gt;b&lt;/sup&gt;</td>
<td>No</td>
</tr>
<tr>
<td>MAF5 (AGL68)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>At5g65080</td>
<td>N.D.</td>
<td>N.D.</td>
<td>up</td>
<td>wild type</td>
<td>late&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Yes</td>
</tr>
<tr>
<td>SOC1 (AGL20)</td>
<td>At2g45660</td>
<td>activator</td>
<td>N.D.</td>
<td>N.D.</td>
<td>up</td>
<td>late</td>
<td>early</td>
</tr>
</tbody>
</table>

<sup>a</sup> They show late flowering when overexpressed in Ler (Michaels et al. 1999; Sheldon et al. 1999; Ratcliffe et al. 2001, 2003).

<sup>b</sup> When overexpressed in Col, they show unsettled flowering time phenotype, and significant number of lines show early flowering (Ratcliffe et al. 2001, 2003).

<sup>c</sup> maf2 shows a similar vernalization response to the wild type, but shows a strong response to brief cold spells (Ratcliffe et al. 2003).

<sup>d</sup> MAF2 transcript levels are reduced after excessively long cold treatments of 10 to 12 weeks (Ratcliffe et al. 2003).

<sup>e</sup> maf2 shows flowers 2 to 3 days earlier than wt (Ratcliffe et al. 2003).

<sup>f</sup> When overexpressed in Col, they don’t show significant late-flowering phenotype (Ratcliffe et al. 2003).

<sup>g</sup> 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 Ler 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, *Ihy-1*. Temporal and synergetic 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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References


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