Short Communication

Possible involvement of FLC in natural variation of activity to enhance the late flowering phenotype of the clock mutant lhy cca1 under continuous light

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Abstract  LHY and CCA1 play key roles in circadian clock functions and photoperiodic flowering in Arabidopsis. Double loss of function of LHY and CCA1 genes (lhy cca1) accelerated flowering under long days or short days, but the lhy cca1 delayed flowering time under constant-light (LL) conditions. FCA encodes an RNA binding protein that plays key roles in the autonomous pathway. Loss of function of FCA increases mRNA level of a major floral repressor gene, FLC. A mutation in FLC gene partially suppressed the late flowering phenotype of the lhy cca1 in LL. Based on this result, we have proposed that FLC may be involved in this process. Increased level of FLC mRNA in fca is responsible for the delay of flowering and the late flowering phenotype of the fca is suppressed by vernalization. In this paper, we isolated an enhancer of the late-flowering phenotype of the lhy cca1 in LL based on natural variation of two Arabidopsis accessions, Columbia and Landsberg erecata. The enhancer was named ELLCL and mapped near the FLC. This result suggested that the gene responsive to ELLCL might be FLC and was consistent with our previous results. The late-flowering phenotype of lhy cca1 was insensitive to vernalization. By contrast, flowering time of the fca was accelerated by vernalization as reported. These results suggested that posttranslational, but not the transcriptional, regulation of FLC might be involved in this process.

Key words: CCA1, circadian rhythms, FLC, LHY, natural variation.

Fluctuations in the length of the day affect developmental processes and behaviors of many organisms. This phenomenon, called photoperiodism, allows the detection of seasonal changes and anticipation of environmental conditions (Boss et al. 2004; Mizoguchi et al. 2006; Mizoguchi et al. 2007). Photoperiodism was first described in detail by Garner and Allard (1920), who found that many plants flowered in response to changes in day length. The photoperiodic control of flowering time is closely linked to the circadian clock (Mizoguchi et al. 2002; Mizoguchi et al. 2005; Mizoguchi et al. 2006; Niinuma et al. 2007; Súarez-López et al. 2001; Yanovsky and Kay 2002). In Arabidopsis, LATE ELONGATED HYPOCOTYL (LHY) and CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) encode myb proteins essential for clock function (Schaffer et al. 1998; Wang and Tobin 1998). FLOWERING LOCUS T (FT) plays a pivotal role in the photoperiodic flowering affected by the circadian clock (Fujiwara et al. 2005a, 2005b, 2005c; Mizoguchi et al. 2005; Oda et al. 2007) and is an important component of the elusive “florigen” signal (Abe et al. 2005; Huang et al. 2005; Wigge et al. 2005). GIGANTEA (GI) and CONSTANS (CO) are mediators between the circadian clock and the FT in photoperiodic control of flowering in Arabidopsis under conditions of light/dark cycling (Calvino et al. 2005; Mizoguchi et al. 2005; Súarez-López et al. 2001; Yanovsky and Kay 2002).

Under conditions of short day length (SD), the lhy cca1 plants flowered much earlier than did wild-type plants (Fujiwara et al. 2005a, 2005b, 2005c; Mizoguchi et al. 2002). The early flowering of lhy cca1 was associated with and explained in part by phase shifting of GI and CO expression and upregulation of FT expression (Fujiwara et al. 2005a, 2005b, 2005c; Mizoguchi et al. 2005). Furthermore, we demonstrated that impairing the function of circadian clock components LHY and CCA1 delayed flowering under conditions of continuous light (LL) and inverted the photoperiodic response from the LD type to a unique SD type in Arabidopsis (Fekih et al. 2009b; Fujiwara et al. 2008; Mizoguchi and Yoshida 2009; Yoshida et al. 2009). We identified three loss-of-function mutations, short vegetative phase (svp; Fekih et al. 2009a; Fujiwara et al. 2008; Yoshida et al. 2009), flowering locus c (floc; Fujiwara et al. 2008), and early flowering 3 (elf3; Yoshida et al. 2009), which suppressed
the late-flowering phenotype of lhy cca1 under LL by both ethyl methanesulfonate (EMS) mutagenesis and genetic analysis. Based on our results, we proposed novel roles of the oscillator components LHY and CCA1 in the control of flowering time through the ELF3-SVP/FLC pathway in Arabidopsis (Mizoguchi and Yoshida 2009).

Some mutations in key clock genes resulted in severe defects in clock function and differences between phenotypes of plants with these mutations under conditions of light/dark cycling compared with under LL. These circadian clock mutants include lhy cca1 (Fujiwara et al. 2008; Yoshida et al. 2009), elf3 cry2 (Natsui et al. 2010), and prr9 prr7 prr5 (Niinuma et al. 2008). Petiole length of prr9 prr7 prr5 was longer than that of wild-type plants under LD, but shorter under LL (Niinuma et al. 2008). Both lhy cca1 and elf3 cry2 produced fewer and more leaves than wild-type plants under SD and LL, respectively.

Nevertheless, isolation of enhancers and suppressors of these mutants would be important to understand the precise molecular mechanism of inversion in the photoperiodic response type. Therefore, we performed mutagenesis of lhy cca1 seeds with EMS and screening of putative enhancers and suppressors of the clock mutant. Use of natural populations has several advantages for the discovery of genes affecting circadian rhythms of leaf movements, flowering time, and light responses (Koornneef et al. 2004; Maloof et al. 2001; Tajima et al. 2007).

Under LL, lhy cca1 plants produced more leaves before the transition from vegetative to reproductive phase than did wild-type, lhy, and cca1 plants (Figure 1A; Fujiwara et al. 2008). To identify enhancers of the late-flowering phenotype of lhy cca1 by using natural
populations but not by EMS-mutagenesis, lhy-11 cca1-1 (Mizoguchi et al. 2002) was crossed with Col gl1 and F2 plants with more or fewer leaves than the progenitor line lhy-11 cca1-1 were screened under LL (Figure 1B). The gl1 is a mutation in GLABROUS1 (GL1) gene that plays a key role in the trichome development in Arabidopsis (Marks and Feldmann 1989). The gl1 does not affect flowering time. The Col gl1 is often used for genetic analysis of Arabidopsis. In this screen, we isolated five F2 plants (#2, 4, 10, 13, and 22) with significantly more leaves than the lhy-11 cca1-1 plants. The enhancement of the late-flowering phenotype of lhy cca1 was most significant in line #10, and this line was used for further analysis. To test whether how many genes were involved in the late flowering phenotype of the line #10, the line #10 (F3) plants were crossed with lhy-11 cca1-1 plants. In our preliminary experiment, approximately 25% of F2 progeny of crosses between line #10 (F3) and lhy-11 cca1-1 exhibited late flowering phenotype similar to that of the line #10 under LL (data not shown). This preliminary experiment suggested that a natural variation between Ler and Col accessions in the line #10 appeared to be responsible for the enhancement of the late flowering phenotype of lhy-11 cca1-1 under LL. The putative natural variation in the line #10 was named enhancer of the late flowering of lhy cca1 under LL (ELLCL). Other potential enhancer and suppressor lines will be characterized and reported elsewhere.

Line #10 was self-fertilized, and F3 progeny were confirmed to carry both the lhy-11 and cca1-1 mutations by PCR. Therefore, line #10 obtained from the F2 plants that produced significantly more leaves than the lhy cca1 was derived from the lhy cca1. The F3 progeny exhibited a similar late-flowering phenotype to that of line #10 under LL (Figures 1B, 2A, B), indicating that the enhancer phenotype was heritable. Under SD, the lhy-11 cca1-1 flowered much earlier, had shorter hypocotyls and petioles, and produced more round leaves than did Ler and Col wild-type, lhy, and cca1 plants (Figure 2C–G, I). Although ELLCL appeared to delay the flowering time of
*lhyc ca1* under LL (Figures 1B, 2A, B), it did not significantly affect early flowering of *lhyc ca1* under SD (Figure 2I). These observations suggested that the effect of ELLCL on flowering of *lhyc ca1* may be specific to LL condition or masked under SD.

To map ELLCL, line #10 (F3) plants were crossed with *lhyc ca1* plants. All the F1 plants flowered at almost the same time as *lhyc ca1* under LL (data not shown). F2 progeny of crosses between line #10 (F3) and *lhyc ca1* were grown under LL, and their flowering times were scored (Figure 3E, F) and compared with those of Ler (Figure 3A), Col gl1 (Figure 3B), *lhyc ca1* plants. The F2 progeny surrounded by dotted lines in (E, F) were used for mapping of ELLCL.

Figure 3. Frequency of distribution of rosette and cauline leaves number at flowering in *Ler* wild type (A), Col gl1 (B), *lhyc ca1* (C), line #10 (D), and plants from segregating populations derived from two independent crosses between line #10 (F3) and *lhyc ca1* (Ler). (E, F) F2 progeny showed a 3:1 ratio of plants that flowered similarly to *lhyc ca1*, and these flowered significantly later than *lhyc ca1* (*p*<0.05).

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The ratio between the extremely late-flowering plants and others was close to 3:1 \((p/110210.05)\) in two independent crosses (experiment 1, 161:53 and experiment 2, 192:59) (Figure 3E, F). These results indicated that \textit{ELLCL} behaved as a recessive mutation in \textit{lhy-11 cca1-1} (Ler) to enhance the late-flowering phenotype under LL. Alternatively, \textit{ELLCL} in Col may have slightly higher activity to enhance the late flowering of \textit{lhy cca1} in LL compared to that in Ler, but the heterozygotes may not show sufficiently clear enhancement.

The F2 plants with a late-flowering phenotype were used to map the \textit{ELLCL} in the line #10. SSLP markers were used to map the \textit{ELLCL}. Enhancement of late flowering of \textit{lhy-11 cca1-1} was closely linked to the genetic markers ciw15 and nga151 on the top strand of chromosome 5, including the \textit{FLC} locus. There were no significant linkage between the late flowering phenotype and the SSLP markers on chromosome 1, 2, 3 and 4.

We proposed that FLC was likely to contribute to the late flowering of \textit{lhy cca1} under LL based on the following four findings. First, we found that \textit{FLC-Ler} appeared to enhance the early flowering of \textit{svp-31} in Col (Fujiwara et al. 2008). Indeed, \textit{flc-102} mutation (Col) enhanced the early flowering of \textit{svp-31} (Col) (Fujiwara et al. 2008). Second, plants showing overexpression of

\textit{cca1-1} (Ler; Figure 3C), and line #10 (F; Figure 3D). The ratio between the extremely late-flowering plants and others was close to 3:1 \((p/11005.05)\) in two independent crosses (experiment 1, 161:53 and experiment 2, 192:59) (Figure 3E, F). These results indicated that \textit{ELLCL} behaved as a recessive mutation in \textit{lhy-11 cca1-1} (Ler) to enhance the late-flowering phenotype under LL. Alternatively, \textit{ELLCL} in Col may have slightly higher activity to enhance the late flowering of \textit{lhy cca1} in LL compared to that in Ler, but the heterozygotes may not show sufficiently clear enhancement.
FLC (FLC-ox) had similar phenotypes to lhy cca1 in LL, such as late flowering, negative regulation of both FT and SUPPRESSOR OF OVEREXPRESSION OF CO I (SOC1) gene expression, dark green/curled leaves, and short hypocotyls (Fujiwara et al. 2008). Third, suppressor mutants of lhy cca1 that flowered earlier than lhy cca1 were screened in LL (Fujiwara et al. 2008; Yoshida et al. 2009). By RT-PCR, we identified at least two classes of suppressor mutant. These exhibited markedly reduced levels of either FLC or ELF3 expression. Finally, lhy-11 cca1-1 flc-102 (Col) plants flowered earlier than lhy-11 cca1-1 (Col) in LL (Fujiwara et al. 2008).

The ftc did not affect the flowering time of co (Michaelis and Amasino 2001). Therefore, late flowering of lhy cca1 in LL was unlikely to be explained solely by downregulation of CO mRNA levels or destabilization of CO proteins (Valverde et al. 2004). The FLC-ox and lhy cca1 plants had shorter hypocotyls than did wild-type plants under LL (Fujiwara et al. 2008). These findings were consistent with the results of the present study. Our results obtained based on natural variation support the suggestion that FLC may play important roles in the inversion of day-length response by lhy cca1.

These findings raise questions regarding the mechanism by which lhy cca1 delayed flowering in LL. One possibility is that lhy cca1 may increase the levels of FLC and/or SVP mRNA. However, this is unlikely because the level of SVP mRNA was almost the same in Ler wild-type and lhy cca1 plants in LL (Fujiwara et al. 2008). Although FLC mRNA level tended to be higher in lhy cca1, lhy, and cca1 than in Ler wild type, the increase did not explain the delay of flowering time in lhy cca1 (Fujiwara et al. 2008). Neither lhy nor cca1 significantly delayed flowering in LL (Figure 1A). Mutations in the autonomous pathway, such as fca, ld, and fpa, increased FLC mRNA level and delayed flowering (Boss et al. 2004). These mutants were sensitive to vernalization, and this long-term cold treatment suppressed the upregulation of FLC and late flowering in the mutants. To test the sensitivity of lhy cca1 to vernalization, seeds and seedlings of lhy-11 cca1-1 and control plants were exposed to low temperature treatment (4°C) for 1, 2, 3, and 4 weeks, and both flowering times and FLC mRNA levels of these plants were investigated. The fca-1 plants were sensitive to vernalization as reported previously (Figure 4B; Boss et al. 2004). The FLC mRNA level was decreased and the late flowering phenotype of the fca-1 was suppressed by vernalization. In contrast, the late flowering phenotype of lhy cca1 was unaffected by vernalization in LL (Figure 4). The mRNA level of the FLC in lhy cca1 decreased in response to vernalization. These observations indicated that lhy cca1 affected a different pathway from fca-1. One explanation for the late flowering of lhy cca1 in LL is that protein stability or complex formation of floral repressors, such as FLC and SVP, may be affected in lhy cca1 in LL.

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