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Original Paper

Circadian clock components in Arabidopsis I. The *terminal flower 1* enhances the early flowering phenotype of a mutant, *lhy cca1*

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Abstract Two myb-related proteins, LHY and CCA1 are essential for circadian clock function in *Arabidopsis*. Double loss-of-function of *lhy cca1* shows a photoperiod-insensitive phenotype and a shortened generation time under short-days (SDs). To understand the molecular mechanisms underlying early flowering of *lhy cca1* mutants, we screened for mutations that enhanced the phenotype of *lhy cca1* under SDs. Here we show that one of the enhancer mutations is a novel allele of a shoot-identity gene, *terminal flower 1* (*tfl1*). Triple loss-of-function of *lhy cca1 tfl1* causes precocious and ectopic expression of *LFY* and *AP1* and dramatically reduces the generation time of *Arabidopsis*. The additive phenotype in *lhy cca1 tfl1* may be due to convergence of the autonomous and photoperiod pathways. Reduction of generation time of crops is an important issue for molecular breeding. Our results highlight a possibility that combining loss-of-function of the circadian clock and one of the shoot-identity genes can be applied for the marker-assisted breeding to manipulate flowering time of crops.

Key words: CCA1, circadian clock, photoperiodic flowering, LHY, TFL1.

In *Arabidopsis*, three genetic pathways control flowering time: photoperiod, gibberellin and autonomous pathways (Boss et al. 2004; Hayama and Coupland 2004). In the photoperiod pathway, circadian rhythms play an important role in the control of flowering time. A circadian clock that generates ca. 24 h rhythms, is thought to be composed of several components (Boss et al. 2004; Hayama and Coupland 2004) including two homologous genes, *LATE ELONGATED HYPOCOTYL* (*LHY*) and *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) which encode single myb transcriptional factors (Calvino et al. 2005; Hayama and Coupland 2004; Mizoguchi et al. 2002; Mizoguchi et al. 2005; Schaffer et al. 1998; Wang and Tobin 1998). The *lhy cca1* mutants flower much earlier than *lhy* or *cca1* single mutants and lose free-running rhythms (FRRs) in expression of clock-controlled genes (CCGs) after a few cycles under continuous light (LL) or dark (DD) conditions (Mizoguchi et al. 2002). Over-expression of either *LHY* (*lhy-1*) or *CCA1* (*35S:CCA1*) causes delay of flowering under long-days (LDs) (Schaffer et al. 1998; Wang and Tobin 1998).

In the photoperiod pathway, GIGANTEA (GI), CONSTANS (CO) and FLOWERING LOCUS T (FT) have significant roles in the control of flowering time under regulation by the circadian system (Calvino et al. 2005; Boss et al. 2004; Hayama and Coupland 2004). CO proteins induce flowering under LD conditions partially through the activation of *FT* gene expression (Boss et al. 2004; Hayama and Coupland 2004). The CO protein itself is thought to be activated by light (Hayama and Coupland 2004). Up-regulation of *FT* expression seems to be a major key in switching from vegetative to reproductive phase (Boss et al. 2004; Hayama and Coupland 2004).

During the transition from vegetative to reproductive phase, the primary shoot meristem begins to produce flower meristems instead of leaf primordia on its flanks. Assignment of floral fate is due to the cooperative action of floral meristem identity genes, *APETALA1* (*AP1*), *LEAFY* (*LFY*) and *CAULIFLOWER* (*CAL*) (Boss et al. 2004). TERMINAL FLOWER 1 (TFL1) is a floral repressor that is similar to animal Raf kinase inhibitors (Bradley et al. 1997; Karadailsky et al. 1999; Kobayashi et al. 1999; Ohshima et al. 1997). TFL1 acts to keep the vegetative phase and also keeps the inflorescence meristem indeterminate (Ratcliffe et al. 1998; Shannon and Meeks-Wagner 1991). The *tfl1* mutant shows early

Abbreviations: AP1, apetala 1; CCA1, CIRCADIAN CLOCK ASSOCIATED 1; LHY, LATE ELONGATED HYPOCOTYL; TFL1, TERMINAL FLOWER 1.

flowering and terminal flower phenotypes (Shannon and Meeks-Wagner 1991). The expressions of *LFY*, *CAL* and *AP1* are negatively regulated by TFL1 to keep the meristem indeterminate (Boss et al. 2004; Bradley et al., 1997; Gustafson-Brown et al. 1994; Weigel et al. 1992). Conversely, *LFY*, *CAL* and *AP1* negatively regulate the expression of *TFL1* in the periphery of the inflorescence meristem (Liljegren et al. 1999; Ratcliffe et al. 1999). This balance of expression between *TFL1* and *LFY*/*CAL*/*AP1* maintains the order of floral development (Boss et al. 2004).

Although GI, CO and FT have been shown to mediate the circadian clock and the photoperiodic flowering in *Arabidopsis* (Mizoguchi et al. 2005), molecular mechanisms underlying the early flowering phenotype of the *lhy cca1* under SD has not been fully understood. For example, roles of other floral integrator and meristem identity genes (e.g. *LFY* and *AP1*) in the clock-controlled flowering has not been elucidated.

Here, we investigate molecular mechanisms underlying the control of flowering by a circadian clock, and describe the first isolation of an enhancer mutation of the early flowering phenotype of the *lhy cca1* mutant. The mutation was found to be a novel allele of *tfl1*. Also this is the first demonstration of *LFY* expression by *in situ* hybridization and *AP1* expression by RT-PCR in a clock mutant in plants. Our results suggest that the additive phenotypes in *lhy cca1 tfl1* may be due to precocious and ectopic expression of *LFY* by convergence of activation of the photoperiod pathway and a loss-of-function of a floral repressor TFL1. Production of plants with reduced generation time by combination of mutations of clock and meristem identity genes is discussed.

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* and *tfl1-2* mutants were described previously (Alvarez et al. 1992; Mizoguchi et al. 2002). 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 ($P<0.0005$).

Mutagenesis of lhy cca1 *seeds and phenotypic screening for mutations that accelerate flowering of* lhy cca1

Approximately 5,000 *lhy-11 cca1-1* seeds were mutagenized by imbibition in 0.3% ethyl methanesulfonate (EMS; Sigma Aldrich, St Louis, MO) for 9 h, followed by washing with 0.1 M Na_2SO_3 for 20 min (twice) and distilled water for 30 min (five times). M_2 seeds were collected in pools, with each pool containing ~20 M_1 plants. Approximately 13,000 M_2 seeds representing ~1300 M_1 plants after mutagenesis of *lhy-11 cca1-1* seeds were sown on soil and screened for the early flowering mutants under SD. The mutations in *lhy-11 cca1-1* background were backcrossed to wild-type plants at least once before phenotypic analysis. The *lhy-11 cca1-1 tfl1-2* mutant was made by crossing lines homozygous for *lhy-11 cca1-1* and *tfl1*-2. Detailed information on the construction of lines and genetic segregation ratios is available from the authors.

Semiquantitative RT-PCR and in situ *hybridization analysis*

Plants were grown on soil for 9, 12, 15, 18, 21, 24, 27, 30 and 33 days under SD and aerial parts were used for RNA preparation, as described (Mizoguchi et al. 2002; Oda et al. 2004). RT-PCR was performed with 1 μg of total RNA using a SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen, CA, USA). For PCR amplification by TaKaRa Ex Taq (TaKaRa, Shiga, Japan), cDNAs were diluted to 100 μl with TE buffer and 1 μl of diluted cDNAs were used. For RT-PCR expression studies, the following primers were used: *AP1*, 5′-GTGATGCTGAAGTTGCTCTTG-3′ and 5′-C-CACTGCTCCTGTTGAGC-3′ (Nakagawa and Komeda 2004) and *TUB*, 5′-CTCAAGAGGTTCTCAGCAGTA-3′ and 5′-TCACCTTCTTCATCCGCAGTT-3′ (Kobayashi et al. 1999). Numbers of PCR cycles were as follows; 25 and 18 cycles for *AP1* and *TUB*, respectively. Annealing temperature was 58°C. Primer specificity was verified by sequencing. The 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, WI, USA), and plasmids were used 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% salkosyl, 0.75% Blocking reagent (Boehringer Mannheim, Mannheim, Germany), and 5% dextran sulfate sodium at 65°C for 16 h. Blot was washed with 2× SSC and 0.1% SDS for 20 min and 0.5× SSC and 0.1% SDS for 10 min at 65°C.

For analysis of *LFY* expression, wild-type (L*er*), *lhy-11 cca1-1*, *tfl1-2*, *tfl1-100*, *lhy-11 cca1-1 tfl1-2* and *lhy-11 cca1-1 tfl1-100* plants were grown under SD (10 h light/14 h dark) conditions and harvested at 12, 15, 18, 21, 24, 27, 30, 33, 36, 39 42 and 45 days after sowing. Further time points for wild-type plants were 80 and 93 days. Sections from at least three plants were analyzed

for each time point. *In situ* hybridization was performed as reported (Kouchi and Hata 1993) with some modifications. Tissue was fixed over night at 4°C in FAA solution (Ethanol : acetic acid : formaldehyde : water, 9 : 1 : 1 : 9), dehydrated, embedded and sectioned to 8 mm. After dewaxing and rehydration, sections were incubated for 30 minutes with 3 mg/ml Proteinase K at 37°C. Proteinase action was blocked with 10 minutes postfixation in 10% formaldehyde; 1× PBS. Tissue sections were acetylated 10 minutes in 0.3% acetic anhydride in 50 mM triethanolamine and washed in PBS. The N-terminus region of *LFY* was amplified by PCR and used as a template for making a digoxigenin-labeled RNA probe. Hybridization was performed overnight at 50°C. After the hybridization, sections were washed three times for 10 minutes in 4× SSC at 50°C, incubated for 30 minutes with 20 mg/ml RNase A at 37°C, washed three times for 15 minutes in NTE buffer (500 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5) at 37°C and washed twice for 20 minutes in 0.5× SSC at 50°C. After blocking for 30 minutes with 25% goat serum; 0.5% Tween 20 in 0.1 mM Tris-HCl, pH7.5 containing 150 mM NaCl at 30°C, the antibody incubation and color detection with NBT-BCIP was performed as described previously (Kouchi and Hata 1993).

Results

Screening mutations that enhance an early flowering phenotype of lhy cca1 *under SDs*

To isolate genes that functionally interact with *LHY* and *CCA1* to control flowering, EMS mutagenesis was performed on *lhy cca1* plants and M_2 populations were screened for enhancers of the *lhy cca1* phenotype (Figure 1A, B). Among these lines, one line named *enhancer of lhy cca1 1* (*elc1*) displayed a characteristic morphological phenotype similar to that reported for *tfl1*, in addition to early flowering under SD (Figure 2A, B). We further studied this line and will report detailed characterization of the other mutants elsewhere. F2 progeny of back-crosses between *elc1* and L*er* showed that the dramatic early flowering and the terminal flower phenotypes were tightly linked. F1 progeny of complementation crosses between *tfl1-2* and *elc1* showed the same characteristic *tfl1* phenotype as the parents (data not shown), indicating that the enhancer mutation contained in this line was indeed the *tfl1* mutant allele. This allele was named *tfl1-100*, and *elc1* thus corresponds to the *lhy cca1 tfl1* triple mutant. *tfl1-100* contained a mutation in the open reading frame of the *TFL1* gene by which a cytosine residue 885 bases from the start codon was substituted by thymine, resulting in a proline (P) to leucine (L) amino acid substitution (Figure 1C). This was found to be a novel mutation among reported alleles of the *tfl1* mutations (Bradley et al. 1997; Ohshima et al. 1997; Shannon and Meeks-Wagner 1991). The type and position of the amino acid substitution found in *tfl1-100* was exactly same as that in *ft-6*, a mutation in a homologous gene with an antagonistic function (Figure 1C) (Kobayashi et al. 1999).

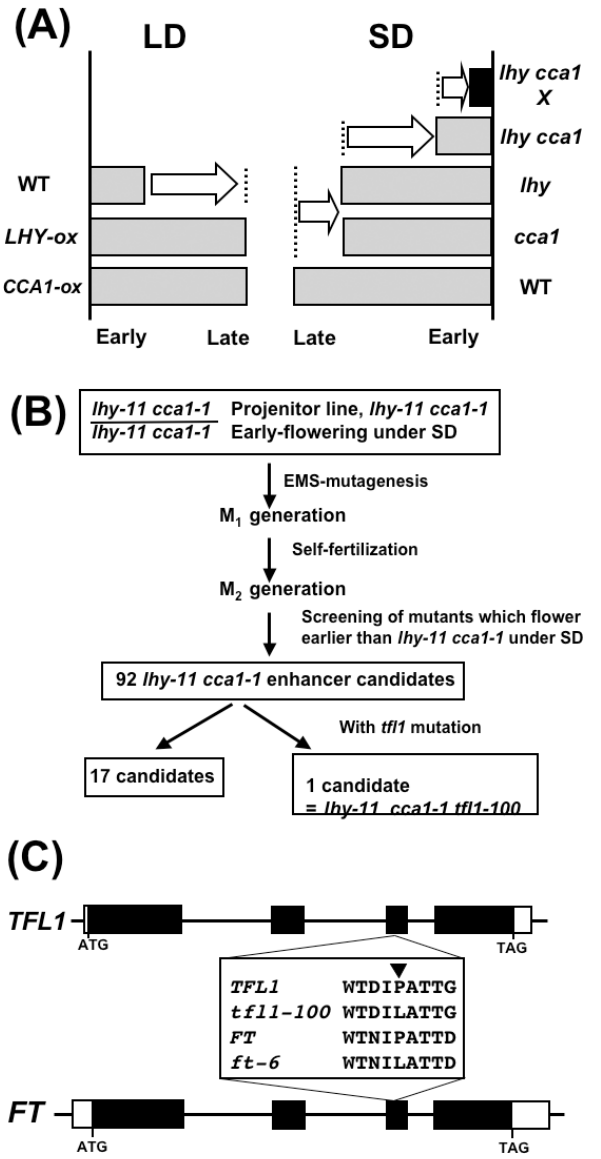

Figure 1. Schematic model for screening mutants that enhance the early flowering phenotype of *lhy cca1* in SD. (A) Overexpression of either *LHY* or *CCA1* delays flowering time in LD, whereas loss-of-function of either *LHY* or *CCA1* accelerates flowering in SD. The *lhy cca1* double mutant lines flower much earlier than either *lhy* or *cca1*. Mutation X can be screened as an enhancer of the early flowering phenotype of the *lhy cca1* in SD. (B) The mutagenesis procedure used to isolate *lhy cca1* enhancer mutations. (C) *tfl1-100* and *ft-6* mutations. Boxes and lines indicate exons and introns, respectively. Open reading frames are indicated by black boxes.

tfl1 *mutations significantly enhance the early flowering phenotype of* lhy cca1 *in SD*

A triple mutant of *lhy-11 cca1-1 tfl1-2* was constructed to confirm the effects of the *tfl1* mutation on *lhy cca1*.

We found that this triple mutant line displayed an extremely early flowering phenotype similar to that in *lhy-11 cca1-1 tfl1-100* under SD (Figure 2A, B). In LD, flowering times of two triple mutant lines were almost the same as those of *tfl1-100* and *tfl1-2* (Figure 2C, D).

Precocious and ectopic expressions of LFY *and* AP1 *in the* lhy cca1 tfl1 *in SD*

It has been shown that GI, CO and FT mediate the circadian clock and the photoperiodic flowering in *Arabidopsis* (Mizoguchi et al. 2005). However, molecular mechanisms underlying the early flowering phenotype of the *lhy cca1* under SD has not been fully understood. For example, roles of other floral integrator and meristem identity genes (e.g. *LFY* and *AP1*) in the clock-controlled flowering has not been elucidated. Therefore, morphological analysis of *lhy cca1* and *lhy cca1 tfl1* was extended by analyzing the expression of a floral marker gene, *LFY* (Weigel et al. 1992), in these mutants. *LFY* is expressed in young leaf primordia at low levels during the wild-type vegetative phase and is up-regulated during reproductive development (Weigel et al. 1992). In the *lhy cca1*, precocious induction of *LFY* mRNA was detected (Figure 3A–D). *tfl1* enhanced the precocious induction and caused the ectopic expression of *LFY* in the *lhy cca1* background (Figure 3E–J). A meristem identity gene, *AP1*, is expressed in the floral meristem after the transition from vegetative to reproductive phase (Hempel et al. 1997), and *AP1* expression is a marker for flower initiation (Hempel et al. 1997). LFY acts redundantly with FT to regulate *AP1* (Ruiz-Garcia et al. 1997). We then analyzed level of *AP1* expression in the *lhy cca1 tfl1* and control plants in SD. Consistent with the flowering time (Figure 2A, B) and the precocious and ectopic expression of *LFY* (Figure 3E–J), induction of the *AP1* expression was largely advanced in *lhy cca1 tfl1* (Figure 3K).

Discussion

Simple interpretation of the genetic relationship between LHY/CCA1 and TFL1 in control of flowering

A schematic model of the hypothetical interactions between circadian-clock genes, a shoot-identity gene *TFL1* and meristem identity genes in controlling flowering time is represented in Figure 4. In the photoperiod pathway, it is proposed that circadian clock components (e.g. LHY and CCA1) are required to measure the photoperiod (Boss et al. 2004; Hayama and Coupland 2004; Mizoguchi et al. 2002; Mizoguchi et al. 2005). By contrast, TFL1 acts as a kind of brake to keep a proper balance in flowering time regulation and in floral meristem formation (Shannon and Meeks-Wagner 1991; Ratcliffe et al. 1998). This is a peculiarity of

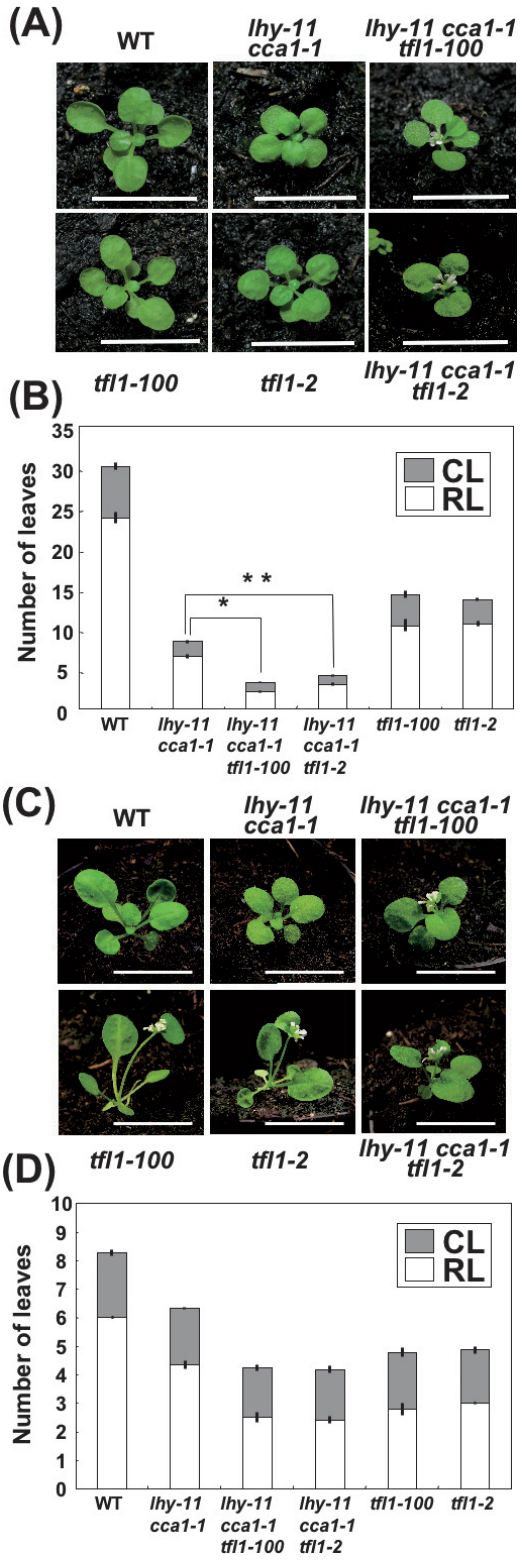

Figure 2. Early flowering phenotype of the *lhy cca1 tfl1*. Flowering time of wild-type, *lhy-11 cca1-1*, *lhy-11 cca1-1 tfl1-100*, *tfl1-100*, *tfl1-2* and *lhy-11 cca1-1 tfl1-2* plants under SD (A, B) and LD (C, D). (A, C) Plants shown 28 days after sowing in SD (A) and 25 days after sowing in LD (C). Bars represent 1 cm. Flowering time in SD (B) and LD (D). Numbers of rosette (open boxes) and cauline leaves (gray boxes) at flowering were scored and the data presented as mean+/−SE. Differences in flowering times indicated by asterisks were confirmed as statistically significant using Student's *t*-test ($P<0.0005$).

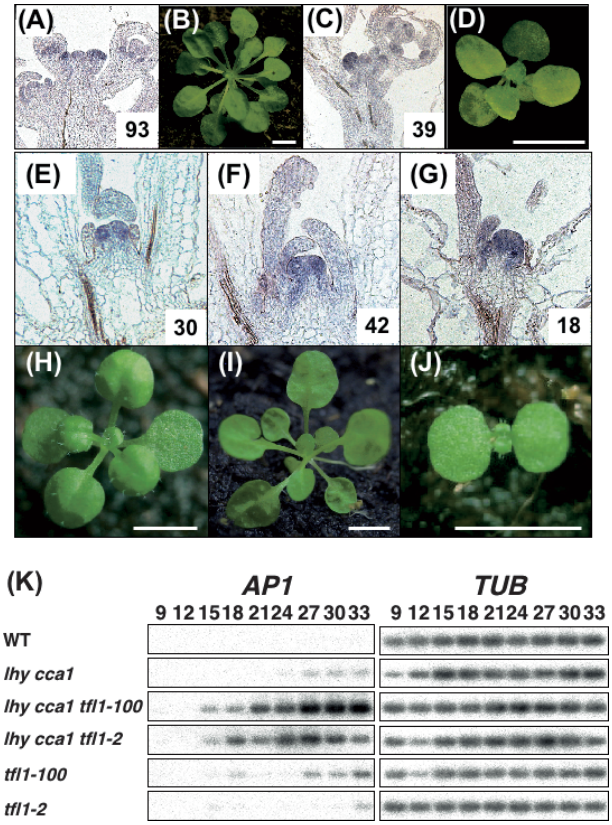

Figure 3. Expression of the *LFY* and *AP1* in the *lhy cca1 tfl1*. (A–J) Localization of *LFY* mRNA in primary meristems of wild-type, *lhy cca1*, *tfl1* and *lhy cca1 tfl1* in SD. Longitudinal sections of primary apices with floral meristems and flowers (A, C) and at early reproductive phase without flowers (E–G) probed with *LFY* antisense RNA. (B, D, H–J) Whole plants. Bars represent 5 mm. Wild-type grown for 93 days (A, B). *lhy cca1* grown for 39 (C, D) and 30 (E, H) days. *tfl1-100* grown for 42 days (F, I). *lhy cca1 tfl1-100* grown for 18 days (G, J). (K) RT-PCR analysis of *AP1* and *TUB* in SD.

Arabidopsis: the *cen* in *Antirrhinum*, a mutation of a homologous gene to *TFL1* does not cause early flowering. Therefore the effect of *tfl1* on flowering time might be different to its effect on meristem identity. In this study, we demonstrated that the *tfl1* enhanced the early flowering phenotype of *lhy cca1*, a double mutant of circadian clock components. This result suggests that LHY/CCA1 and TFL1 may not function in a single linear genetic pathway. Consistent with our idea, TFL1 is proposed to function in the autonomous pathway (Boss et al. 2004; Page et al. 1999; Ruiz-Garcia et al. 1997). If this is the case, one possible explanation is that the additive effect on flowering time in *lhy cca1 tfl1* may be due to convergence of the autonomous and photoperiod pathways. Floral integrator genes and meristem identity genes (e.g. *AP1*) are regulated by both the clock components and TFL1 to promote flowering and to organize floral meristems, respectively.

How do the clock components LHY/CCA1 affect flowering?

Several floral activators, GI, CO, FT and SOC1 have been proposed to be downstream factors of the circadian clock (Boss et al. 2004; Hayama and Coupland 2004; Mizoguchi et al. 2002; Mizoguchi et al. 2005). Among these factors, FT has high homology to TFL1, although these two proteins have opposite functions for flowering (Karadailsky et al. 1999; Kobayashi et al. 1999). CO is proposed to be a downstream factor of GI and an upstream factor of FT/SOC1 in the photoperiod pathway (Hayama and Coupland 2004). The *lhy cca1* causes phase advances of *GI* and *CO* expressions, an up-regulation of *FT* expression and an early flowering phenotype under SD, suggesting that GI plays an important role downstream of LHY/CCA1 in the control of photoperiodic flowering (Mizoguchi et al. 2005). Overexpression of either *CO* or *FT* causes early flowering (Karadailsky et al. 1999; Kobayashi et al. 1999; Simon et al. 1996). This early flowering is enhanced by *tfl1* (Karadailsky et al. 1999; Kobayashi et al. 1999; Onouchi and Coupland, unpublished results). One possible explanation for the dramatic early flowering phenotype of the *lhy cca1 tfl1* may be a coincidence of a gain-of-function of FT and a loss-of-function of TFL1.

The terminal flower phenotype of the *tfl1* mutant is largely suppressed in SD (Shannon and Meeks-Wagner 1991). This phenotype, however, was seen even under SD, if *tfl1* was combined with *lhy cca1* (Fujiwara and Mizoguchi, unpublished). Transgenic plants that overexpress either *CO*, *FT* or *AP1* show a similar terminal flower phenotype to that of *tfl1* (Karadailsky et al. 1999; Kobayashi et al. 1999; Mandel and Yanofsky 1995; Simon et al. 1996). These results suggest that activation of the floral activators in the photoperiod pathway may be required for the terminal flower phenotype of *tfl1*. The early flowering phenotype of the *lhy cca1* is day-length independent suggesting that activity of the floral activators may be increased even under SD. Primary targets of LHY/CCA1 in the control of flowering have not yet been identified. Further analysis of the effects of multiple mutants (combination of both loss- and gain-of-function mutations) on circadian rhythms will provide us much clearer image of the mechanisms by which a circadian clock controls flowering time in *Arabidopsis*.

How can our approach be applied for reduction of generation time in the various crops?

Once we have identified genes whose loss-of-function mutations reduce generation time in model plants such as *Arabidopsis* and rice, this information can be applied for the molecular breeding of crops. For example, we can introduce point mutations into the crops using EMS. After the mutagenesis, it is possible to identify mutations

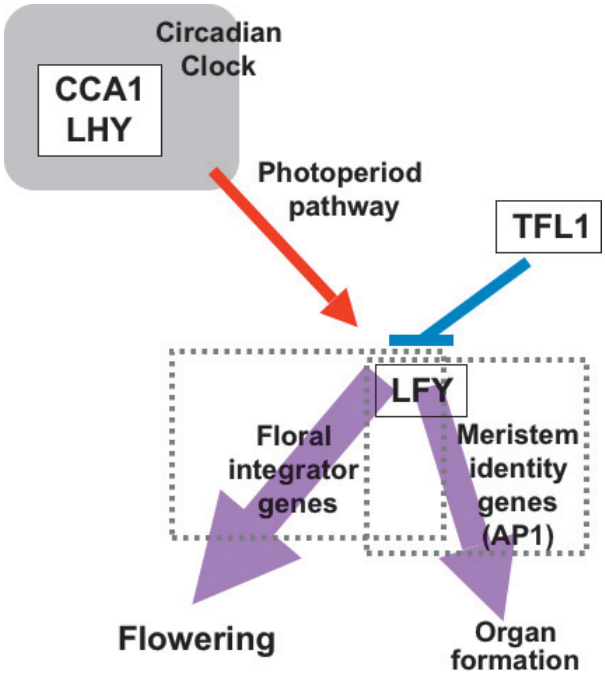

Figure 4. Schematic representation of the hypothetical genetic interactions among circadian clock genes (*LHY* and *CCA1*), a shoot-identity gene *TFL1*, floral integrator and meristem identity genes (*LFY* and *AP1*) for control of flowering in *Arabidopsis*. Activation of flowering by the clock and the photoperiod pathway is shown in red. Inhibitory effect by TFL1 is shown in blue. *LFY* functions both as a floral integrator gene and a meristem identity gene (Boss et al. 2004). *LFY* seems to be a common target of these two pathways to control flowering and organ identity (pink arrows).

of genes of our interests by the TILLING (targeting induced local lesions in genomes) (Slade et al. 2005; Zerr and Henikoff 2005). Phenotypes of single loss-of-function mutants are often quite subtle, if there are genes with redundant function. If some of mutations of such genes are combined, we can see the enhanced phenotypes (Figure 2A, B). Therefore, it is important to seek for not only a phenotype caused by a single mutation but also a strong phenotype caused by a combination of mutations. LHY, CCA1 and TFL1 are common genes widely conserved in plants. Therefore our finding will be applied for other plants including crops if homologous genes for LHY, CCA1 and TFL1 have similar functions to those in *Arabidopsis*. Alternatively, mutations of different sets of genes may be required for other plant species. Comparison of the effects of mutations of homologous genes in different plant species will be next important issue.

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