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Circadian clock components in *Arabidopsis* III. LHY/CCA1/GI in regulating the floral integrator genes *LFY*/*SOC1*/*FT* to control flowering time and shoot architecture

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Abstract LHY and CCA1 are clock components and *TFL1* encodes a shoot-identity gene in *Arabidopsis*. When combined with *tfl1*, *lhy cca1* results in precocious and ectopic expression of a floral integrator gene, *LFY*. We have shown that *lhy cca1* and *GI-ox* cause early flowering and increase the expression of two floral integrator genes, *FT* and *SOC1*. *FT* and *SOC1* are required for the early flowering of *GI-ox* under SD. Here we demonstrate that *tfl1* dramatically reduced the generation time of *GI-ox* as well as *lhy cca1* plants. *tfl1* enhanced the precocious expression of the meristem-identity gene *AP1* in *GI-ox* in a similar way to that in *lhy cca1*. However, *tfl1* did not affect the mRNA levels of *FT* and *SOC1* in *lhy cca1* and *GI-ox*, suggesting that the additive phenotypes in *lhy cca1 tfl1* (and *GI-ox tfl1*) are attributable to the concurrent up-regulation of three genes, *FT/SOC1* and *LFY*. The terminal flower phenotype of *tfl1* was enhanced by *lhy cca1* and *GI-ox* under SD, suggesting that a proper balance between FT and TFL1 with antagonistic roles is important for the photoperiodic control of architecture in *Arabidopsis*. Our results indicate that GI mediates between the circadian clock and three floral integrator genes, *FT*, *SOC1* and *LFY*, to control the photoperiodic flowering.

Key words: CCA1, floral integrators LFY/FT/SOC1, photoperiodic flowering, LHY, TFL1.

The circadian clock acts as the time-keeping mechanism in photoperiodism. Two myb-ralated proteins, LATE ELONGATED HYPOCOTYL (LHY) and CIRCADIAN CLOCK ASSOCIATED 1 (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 (SD) (Mizoguchi et al. 2002). A circadian-clock controlled flowering pathway comprising the genes *GIGANTEA* (*GI*), *CONSTANS* (*CO*) and *FLOWERING LOCUS T* (*FT*) promotes flowering specifically under long days (LD) in *Arabidopsis*. Within this pathway, *GI* regulates circadian rhythms and flowering and acts earlier in the hierarchy than *CO* and *FT* (Suarez-Lopez et al. 2001), suggesting that *GI* might regulate flowering indirectly by affecting the control of circadian rhythms. We investigated the relationship between the roles of *GI* in flowering and the circadian clock using *lhy cca1* double mutants, that are impaired in circadian-clock function (Mizoguchi et al. 2002), plants overexpressing *GI* (*GI-ox* or *35S*:*GI*) and *gi* mutants (Mizoguchi et al. 2005). *GI* acts between the circadian oscillator and *CO* to promote flowering by increasing *CO* and *FT* mRNA abundance (Mizoguchi et al. 2005). In addition, circadian rhythms in expression of genes that do not control flowering are altered in *GI-ox* and *gi* mutant plants under continuous light (LL) and continuous darkness (DD), and the phase of expression of these genes is changed under diurnal cycles. Therefore, *GI* has a general role in controlling circadian rhythms, and this is different from its effect on the amplitude of expression of *CO* and *FT*. The effect of GI on flowering is not an indirect effect of its role in circadian-clock regulation, but rather that GI also acts in the nucleus to more directly promote the expression of flowering-time genes (Mizoguchi et al. 2005).

The effect of GI on flowering probably involves FT-independent pathways, because *ft* only partially suppresses the early flowering caused by *lhy cca1* or *GI-ox* (Mizoguchi et al. 2005). LFY, FT, and SUPPRESSOR OF OVEREXPRESSION OF CO 1 (SOC1) integrate four flowering pathways: the photoperiod, GA, vernalization, and autonomous pathways (Boss et al. 2004; Calvino et al. 2005; Hayama and Coupland 2004). We have recently shown that *SOC1* functions redundantly with *FT* to promote flowering via the LHY/CCA1/GI pathway (Fujiwara et al. 2005b). Messenger RNA levels of the *SOC1* expression are increased in *GI-ox* and *lhy cca1* plants and *gi* partially suppresses the up-regulation in *lhy cca1* under SD. The

Abbreviations: AP1, APETALA 1; CCA1, CIRCADIAN CLOCK ASSOCIATED 1; DD, continuous dark; FT, FLOWERING LOCUS T; GA, gibberellic acid; LFY, LEAFY; LHY, LATE ELONGATED HYPOCOTYL; LD, long day; LL, continuous light; SD, short day; SOC1, SUPPRESSOR OF OVEREXPRESSION OF CO 1; TFL1, TERMINAL FLOWER 1.

phase of *SOC1* expression is shifted in plants overexpressing *LHY* (*lhy-1*), and the *gi* mutation does not affect the phase shift, suggesting that LHY regulates *SOC1* expression both in *GI*-dependent and independent manners (Fujiwara et al. 2005b).

To understand the molecular mechanisms underlying the early flowering of *lhy cca1* mutants, we screened for mutations that enhanced the phenotype of *lhy cca1* under SD (Fujiwara et al. 2005a). We have shown that one of the enhancer mutations is a novel allele of a shoot-identity gene, *terminal flower 1* (*tfl1*) (Shannon and Meeks-Wagner 1991). Triple loss-of-function of *lhy cca1 tfl1* causes precocious and ectopic expression of the floral integrator gene *LFY* and a meristem-identity gene *APETALA1* (*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.

Although the terminal flower phenotype of *tfl1* has been shown to be largely suppressed by SD (Shannon and Meeks-Wagner 1991), molecular mechanisms underlying the photoperiod-dependent control of the shoot architecture has not been elucidated. To further investigate the roles of the circadian clock components LHY/CCA1/GI in regulating the floral integrator genes *LFY/SOC1/FT* to control flowering time and shoot architecture in *Arabidopsis*, we studied phenotypes of *GI-ox tfl1* and compared them to those of *lhy cca1 tfl1*. In this paper, we demonstrate that GI mediates between the circadian clock and three floral integrator genes, *FT*, *SOC1* and *LFY*, to control the photoperiodic flowering. The terminal flower phenotype of *tfl1* is enhanced by *lhy cca1* and *GI-ox* even under SD, supporting the idea that a proper balance between FT and TFL1 with antagonistic roles is important for the photoperiodic control of architecture in *Arabidopsis* (Fujiwara et al. 2005a; Karadailsky et al. 1999; Kobayashi et al. 1999).

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), *GI-ox* (*35S*:*GI-B*; Mizoguchi et al. 2005), *lhy-11 cca1-1 tfl1-2* (Fujiwara et al. 2005a), *tfl1-2* (Alvarez et al. 1992), *gi-3* and *gi-6* (Fowler et al. 1999) mutants were described previously. The double mutant lines, *GI-ox tfl1-2, gi-3 tfl1-2* and *gi-6 tfl1-2*, were made by crossing. 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, in situ hybridization and histological analysis

RT-PCR was performed with *AP1* (Nakagawa and Komeda, 2004), *FT* (Blazquez and Weigel 1999), *SOC1* (Blazquez et al. 2002) and *TUB* (Kobayashi et al. 1999) primers and the products were analyzed as described (Fujiwara et al. 2005a; Fujiwara et al. 2005b) with some modifications. The RT-PCR analysis was performed twice with independent RNA samples. Procedure for histological analysis was same as that reported for the *in situ* hybridization (Fujiwara et al. 2005a). Staining was used an aqueous 0.1% toluidine blue solution.

Results

Mutation in TFL1 *significantly enhanced the early flowering phenotype of* GI-ox *in SD*

A double mutant of *GI-ox tfl1-2* was constructed to test the effects of the *tfl1* mutation on *GI-ox*. We found that this double mutant line displayed an extremely early flowering phenotype similar to that in *lhy-11 cca1-1 tfl1-2* under SD (Figure 1A; Fujiwara et al. 2005a). In LD, flowering time of *GI-ox tfl1-2* was almost the same as that of *tfl1-2* (Figure 1B). This result supports our idea that GI plays key roles in the downstream of LHY and CCA1 to control flowering (Calvino et al. 2005; Fujiwara et al. 2005b; Mizoguchi et al. 2002; Mizoguchi et al. 2005).

Photoperiod-insensitive mutations of lhy cca1 *and* GI-ox *result in* tfl1 *in the terminal flower phenotype, even under SD*

The terminal flower phenotype of *tfl1* depends on the photoperiod, and this phenotype is largely suppressed under SD (Shannon and Meeks-Wagner 1991). Both *lhy cca1* and *GI-ox* plants are photoperiod-insensitive and flower early under both LD and SD (Mizoguchi et al. 2002; Mizoguchi et al. 2005). We tested whether *lhy cca1* mutations and *GI-ox* caused loss-of-sensitivity to the photoperiod *in tfl1* in terms of the terminal flower phenotype. A triple *lhy cca1 tfl1* mutant and a double *GI-ox tfl1* mutant displayed characteristic *tfl1* phenotypes, not only under LD but also under SD (Figure 2A; Fujiwara et al. 2005a). By contrast, *gi* plants flower later than wild-type plants under LD and as late as wild-type plants under SD, indicating that *gi* mutants are also photoperiod-insensitive (Boss et al. 2004; Hayama and Coupland 2004). The terminal flower phenotype of *tfl1* was largely suppressed by *gi* under LD (Figures 1E, 2A). The *gi tfl1* plants flowered slightly earlier than *gi* under LD (Figures 1C, D, 2A).

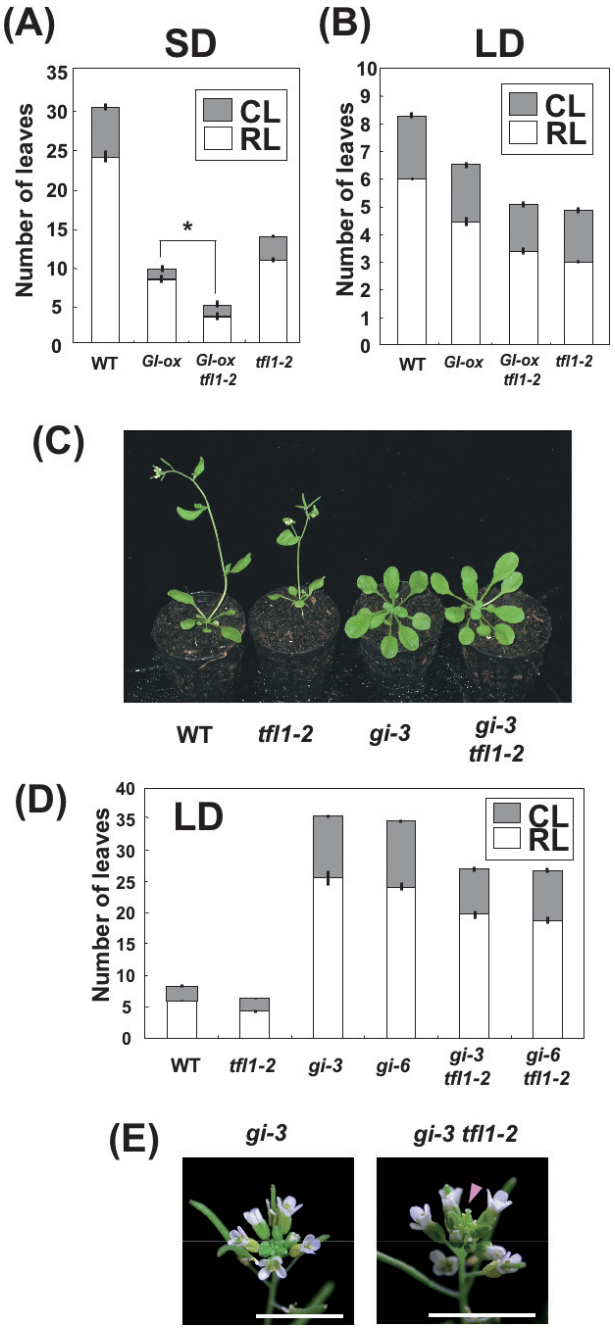

Figure 1. Flowering phenotype of wild-type, *GI-ox*, *GI-ox tfl1*, *tfl1-2*, *gi-3*, *gi-6*, *gi-3 tfl1-2*, and *gi-6 tfl1-2* plants. (A, B). The flowering times of wild-type (WT), *GI-ox*, *GI-ox tfl1-2*, and *tfl1-2* plants under SD (A) and LD (B). (C, D) The flowering times of wild-type, *tfl1-2*, *gi-3*, and *gi-3 tfl1-2* plants under LD. (**C**) Plants grown for 38 days under LD. The flowering times of *gi-6* and *gi-6 tfl1-2* are included in (D). The numbers of rosette (open boxes) and cauline (gray boxes) leaves at flowering were scored, and the data are presented as the mean±SE. Difference in flowering times indicated by an asterisk (A) was statistically significant using Student's *t*-test ($P<0.0005$). (E) Terminal flower phenotype of *tfl1* was largely suppressed by *gi* under LD. Pictures of the top of the main stem of *gi-3* and *gi-3 tfl1-2* 10 days after bolting. Plants were grown under LD. Bars represent 1 cm. A pink arrowhead in the picture for *gi-3 tfl1-2* indicates a terminal flower.

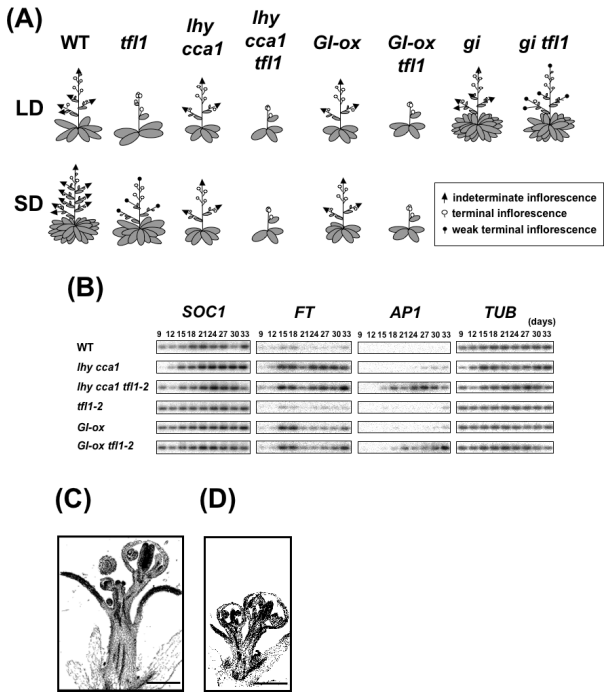

Figure 2. Shoot architecture of wild-type, *tfl1*, *GI-ox tfl1*, *GI-ox*, *lhy cca1 tfl1*, *lhy cca1*, *gi tfl1* and *gi* plants under LD and SD and the mRNA abundance of *SOC1*, *FT* and *AP1* in these mutants. (A) Model illustrations of shoot architectures of wild-type (WT) and the mutants are shown. Arrowheads, open circles and filled circles indicate indeterminate inflorescence, terminal inflorescence and weak terminal inflorescence, respectively. (B) RT-PCR analysis of the expressions of the floral integrator genes *FT/SOC1*, the meristem identity gene *AP1*, and *TUB* in WT, *lhy-11 cca1-1* (*lhy cca1*), *lhy-11 cca1-1 tfl1-2* (*lhy cca1 tfl1*), *tfl1-2* (*tfl1*), *GI-ox*, *GI-ox tfl1-2* plants. (C, D) Anatomy of inflorescences of *lhy cca1* and *lhy cca1 tfl1* under SD. Longitudinal sections of *lhy cca1* (C) and *lhy cca1 tfl1* (D) inflorescences including the first flowers. *lhy cca1* and *lhy cca1 tfl1-2* plants were grown for 42 and 33 days, respectively. The bars represent 500 μm.

Precocious expression of AP1 *in* Gl-ox tfl1 *under SD*

A meristem identity gene, *AP1*, is expressed in the floral meristem after the transition from the vegetative to the reproductive phase, 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 analyzed the level of *AP1* expression in *GI-ox tfl1* and control plants under SD. Consistent with the flowering time (Figure 1A), the induction of *AP1* expression was markedly advanced in *GI-ox tfl1* in a similar way to that in *lhy cca1 tfl1* (Figure 2B; Fujiwara et al. 2005a).

Mutation in TFL1 *does not affect* FT *and* SOC1 *expression in* Gl-ox *and* lhy cca1 *under SD*

To test whether the early flowering of either *GI-ox* or *lhy cca1* enhanced by *tfl1* was owing to greater accumulation of *FT* and *SOC1* mRNA, the levels of *FT* and *SOC1* expression in *GI-ox tfl1*, *lhy cca1 tfl1*,

and control plants were examined (Figure 2B). The expression level of *FT* and *SOC1* was higher in *GI-ox* and *lhy cca1* than in the wild type and *tfl1*, as reported (Figure 2B; Fujiwara et al. 2005b; Mizoguchi et al. 2005). The expression patterns of *FT* and *SOC1* were quite similar in *lhy cca1* and *lhy cca1 tfl1* and in *GI-ox* and *GI-ox tfl1*, suggesting that *tfl1* did not affect *FT* and *SOC1* expression in *lhy cca1* or *GI-ox* under SD (Figure 2B).

Discussion

Clock mutations such as *lhy-11 cca1-1*, *GI-ox*, *gi-3* and *gi-6*, with or without *tfl1* mutations, were used to study the roles of LHY, CCA1, and GI in regulating three floral integrator genes (*FT*, *SOC1* and *LFY*) to control flowering time and shoot architecture in *Arabidopsis*. These are discussed in more detail in the following sections.

Interpretation of the genetic relationship between LHY/CCA1/GI *and* TFL1 *in regulating three floral integrator genes that control flowering:* LFY, FT, *and* SOC1

Figure 3 presents a schematic model of the hypothetical interactions between the circadian clock genes (*LHY*, *CCA1*, and *GI*), the floral activator gene *CO*, floral integrator genes (*LFY*, *FT*, and *SOC1*), the shoot identity gene *TFL1*, and meristem identity genes in controlling flowering time and shoot architecture. We have shown that *GI-ox* or *lhy cca1* increased *FT* expression under SD (Figure 2B; Fujiwara et al. 2005b; Mizoguchi et al. 2005). The *tfl1* mutations enhanced the early flowering of *GI-ox* (Figure 1A) or *lhy cca1* (Fujiwara et al. 2005a) plants under SD, and the acceleration was correlated with precocious *AP1* expression (Figure 2B; Fujiwara et al. 2005a). The *tfl1* mutations did not affect *FT* or *SOC1* expression in *GI-ox* or *lhy cca1* plants under SD (Figure 2B). By contrast, the *tfl1* mutations enhanced the precocious and ectopic expression of a meristem identity gene, *LFY*, in *lhy cca1* under SD (Fujiwara et al. 2005a). FT and LFY have parallel functions downstream from the photoperiod-dependent and -independent pathways of floral induction (Boss et al. 2004; Kardailsky et al. 1999; Kobayashi et al. 1999). In *35S*:*FT 35S*:*LFY* plants, the vegetative phase was bypassed, and a terminal flower was produced immediately upon germination (Kardailsky et al. 1999; Kobayashi et al. 1999). *35S*:*FT tfl1* plants flowered even earlier than *35S*:*FT* plants and often formed only a single, terminal flower on the main shoot (Kardailsky et al. 1999). The *lhy cca1 tfl1* under SD showed an intermediate phenotype in the shoot architecture between those of *35S*:*LFY 35S*:*FT* and *35S*:*FT tfl1* (Figure 2C, D; Kardailsky et al. 1999; Kobayashi et al. 1999). Therefore, the additive effect on flowering time in *lhy cca1 tfl1* may be attributable to the concurrent up-regulation of two genes, *FT* and *LFY*. 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 (Figures 2A, 3).

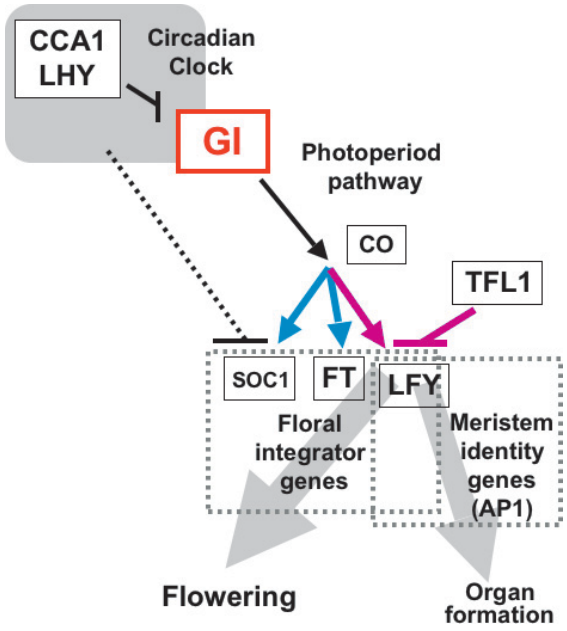

Figure 3. Schematic representation of the roles of the circadian clock components *LHY/CCA1/GI* in regulating the floral integrator genes *LFY/SOC1/FT* to control flowering time and shoot architecture in *Arabidopsis*.

LFY, FT and SOC1 are floral integrator genes. The activation of the photoperiod pathway by *lhy cca1* and loss-of-function of *tfl1* causes precocious and ectopic expression of *LFY* shown in pink. The *tfl1* mutation enhances the precocious expression of AP1 in *lhy cca1* or *GI-ox* under SD. Although *lhy cca1* or *GI-ox* increases the mRNA abundance of *FT* and *SOC1*, *tfl1* does not affect the expression of these genes in the *lhy cca1* or *GI-ox* background (blue arrows). The additive phenotypes in *lhy cca1lhy cca1 tfl1* (and *GI-ox tfl1*) may be attributable to the concurrent up-regulation of three genes, *FT/SOC1* and *LFY*. A proper balance between *FT* and *TFL1* with antagonistic roles is important for the photoperiodic control of architecture in *Arabidopsis* (Fujiwara et al. 2005a; Karadailsky et al. 1999; Kobayashi et al. 1999). GI mediates between the circadian clock and three floral integrator genes, *FT*, *SOC1* and *LFY*, to control the photoperiodic flowering.

How do the clock components LHY/CCA1/GI affect shoot architecture in Arabidopsis?

Arabidopsis is a facultative LD plant and flowers much earlier under LD than SD. Under SD, *Arabidopsis* produces more rosette/cauline leaves and flower buds, indicating that the photoperiod affects many aspects of plant architecture (Figure 2A). The terminal flower phenotype of the *tfl1* mutant is largely suppressed under SD (Shannon and Meeks-Wagner 1991). This phenotype was even seen under SD when *tfl1* was combined with *GI-ox* or *lhy cca1* (Figure 2A; Fujiwara et al. 2005a). Transgenic plants that overexpress *CO*, *FT*, or *AP1* show

a terminal flower phenotype similar to that of *tfl1* (Kardailsky et al. 1999; Kobayashi et al. 1999; Mandel and Yanofsky 1995; Simon et al. 1996). These results suggest that the activation of the floral activators in the photoperiod pathway is required for the terminal flower phenotype of *tfl1*. The early flowering phenotype of *GI-ox* or *lhy cca1* plants is day-length-independent, and the *FT* expression is even increased under SD (Fujiwara et al. 2005a; Mizoguchi et al. 2002; Mizoguchi et al. 2005). A proper balance between FT and TFL1, which have antagonistic roles, may be important for the photoperiodic control of architecture in *Arabidopsis*.

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