Arabidopsis TERMINAL FLOWER1 Is Involved in the Regulation of Flowering Time and Inflorescence Development through Transcriptional Repression

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TERMINAL FLOWER1 (TFL1) is a key regulator of flowering time and the development of the inflorescence meristem in Arabidopsis thaliana. TFL1 and FLOWERING LOCUS T (FT) have highly conserved amino acid sequences but opposite functions. For example, FT promotes flowering and TFL1 represses it; FT-overexpressing plants and TFL1 loss-of-function mutants have a similar phenotype production of terminal flowers in the shoot apex. FT is believed to function in a transcriptional activator complex by interacting with FD. Here, we demonstrate that TFL1 is involved in the transcriptional repression of genes that are activated by FT. We analyzed transgenic plants overexpressing TFL1 fused to a transcriptional repressor domain (TFL1-SRDX) or an activator domain (TFL1-VP16). Plants carrying 35S:TFL1-SRDX showed delayed flowering similar to 35S:TFL1 plants, and plants carrying 35S:TFL1-VP16 showed an early flowering phenotype and produced terminal flowers. Furthermore, the tfl1 and 35S:TFL1-VP16 plant phenotypes were strongly suppressed by the fd mutation, and TFL1 interacted with FD in the cell nucleus, as shown by bimolecular fluorescence complementation experiments. We conclude that TFL1 negatively modulates the FD-dependent transcription of target genes to fine-tune flowering time and the development of the inflorescence meristem.

INTRODUCTION

In higher plants, a phase transition from vegetative to reproductive development (flowering) is tightly coordinated through a diverse array of signaling networks that integrate external stimuli, such as light and temperature (Koornneef et al., 1991; Thomas and Vince-Prue, 1997; Davis, 2009). In Arabidopsis thaliana, flowering is promoted under long-day (LD) conditions and delayed under short-day (SD) conditions and at low temperature (Lempe et al., 2005; Samach and Wigge, 2005; Strasser et al., 2009). These environmental signals are integrated in the shoot apical meristems (SAMs); vegetative SAMs produce leaves, and reproductive SAMs produce flowers. A reproductive SAM is called inflorescence meristem (IM) and establishes inflorescence architecture (Wigge et al., 2005; Conti and Bradley, 2007). An IM may maintain itself while it produces floral meristems (FMs), giving rise to an indeterminate inflorescence. Determinate inflorescences are formed when the IM develops into an FM, forming a terminal flower. Wild-type Arabidopsis has indeterminate inflorescence architecture (Shannon and Meeks-Wagner, 1991; Alvarez et al., 1992; Bradley et al., 1997). Inflorescence architecture and the timing of flowering are closely related in Arabidopsis; overexpression of flowering genes, such as FT, or of meristem identity genes, such as APETALA1 (AP1) and LEAFY (LFY), induce the development of terminal flowers and early flowering phenotypes (Mandel and Yanofsky, 1995; Weigel and Nilsson, 1995; Kardailsky et al., 1999; Kobayashi et al., 1999).

TERMINAL FLOWER1 (TFL1) controls flowering time and inflorescence architecture (Shannon and Meeks-Wagner, 1991; Alvarez et al., 1992). As mutations in TFL1 accelerate flowering time, TFL1 is believed to be a negative regulator of flowering time (Simon et al., 1996). In addition, the inflorescences of tfl1 mutants are converted from indeterminate into determinate; they produce reduced numbers of flower buds and possess terminal flowers at the shoot apices (Shannon and Meeks-Wagner, 1991; Alvarez et al., 1992; Bradley et al., 1997). In contrast with the loss-of-function phenotypes, overexpression of TFL1 delayed flowering and prevented the IM-to-FM transition, resulting in the extension of the IM stage, the production of bract-like leaves, and the proliferation of secondary flowers (Ratcliffe et al., 1998; Hanzawa et al., 2005). Thus, TFL1 is a negative regulator of the phase changes of the SAM from vegetative to reproductive and from IM to FM.

TFL1 shares 71% similar amino acid residues (including 55% identical residues) with FT. Both TFL1 and FT belong to the CETs (CENTRORADIALIS, TFL1, and SELF PRUNING) gene family and exhibit homology with a phosphatidylethanolamine binding protein (PEBP) (Pnueli et al., 2001). Despite these significant sequence similarities, FT and TFL1 are functionally antagonistic: overexpression of FT leads to an earlier commencement of flowering, reduces the number of flower buds, and leads to the production of terminal flowers. Single amino acid substitutions, H88Y in TFL1 and the corresponding Y85H in FT, convert TFL1 into an agent that induces earlier flowering and FT into one that...
delays flowering, respectively (Hanzawa et al., 2005). These amino acid substitutions affect an external loop in the PEBP structure (Ahn et al., 2006), but the molecular basis of the functional switch induced by the mutation remains obscure. Previously, TFL1 and FT were reported to interact with the bZIP transcription factors FD and FD PARALOG (FDP), which regulate the expression of several FM identity genes (Abe et al., 2005; Wigge et al., 2005).

To explain these facts, one may assume that TFL1 is involved in an FD/FDP-dependent transcriptional complex, as FT is; the complex acts as a transcriptional repressor when TFL1 participates, but FT turns it into a transcriptional activator. Instead, TFL1 may promote the transcription of floral inhibitors by participating in a different complex: TFL1 may interact with unknown FD-like transcription factor(s) to induce floral inhibiting genes, whereas the FT-FD complex may activate floral promoting genes. Other hypotheses of TFL1 function are based on its intracellular localization. TFL1 was detected in nuclei and cytoplasm, but FT colocalized with FD in nuclei (Abe et al., 2005; Conti and Bradley, 2007). TFL1 and FT are also known to be signal molecules in cell-to-cell and leaf-to-SAM communication, respectively (Conti and Bradley, 2007; Corbesier et al., 2007; Jaeger and Wigge, 2007; Mathieu et al., 2007; Tamaki et al., 2007). The TFL1 gene is transcribed in cell layer 3 in the SAM, and the protein is shuttled between the cell layers in the IM. By contrast, FT is generated in leaves that sense environmental signals and then is moved into SAMs. Recently, TFL1 was reported to play a role in protein trafficking to protein storage vacuoles (PSVs) (Sohn et al., 2007). Thus, TFL1 may shuttle FD from nuclei to PSVs to inhibit FD-dependent transcription, whereas FT recruits FD to nuclei where transcription occurs.

To elucidate the molecular mechanisms underlying the control of flowering time and inflorescence architecture by TFL1, we examined the functions of TFL1 proteins fused with the transcriptional activator domain VP16 or the repressor domain SRDX in planta (Parcy et al., 1998; Ng and Yanofsky, 2001; Ohta et al., 2001; Mitsuda et al., 2006). Here, we show that plants over-expressing TFL1-VP16 exhibit dominant-negative phenotypes. In turn, TFL1-SRDX acts as a native TFL1, suggesting that the native TFL1 functions as a transcriptional repressor or a carrier of a repressor. We also demonstrate genetic and molecular interactions between FD and TFL1. We propose that TFL1 plays a crucial role in the transcriptional repression of FD-dependent events.

RESULTS

**tfl1** Mutants Exhibit a Reduced Response of Flowering Time to Temperature

The tfl1 mutant produces fewer rosette leaves per plant than the wild type and a reduced number of flower buds in the primary inflorescence (Shannon and Meeks-Wagner, 1991; Alvarez et al., 1992). To quantify the tfl1 mutant phenotype, we counted the number of rosette leaves at bolting as a measure of flowering time and the number of flower buds between the last lateral shoot and the terminal flower in the primary shoot as an indicator of inflorescence phenotype, for the tfl1 mutant alleles in the Columbia (Col) background (see Supplemental Figure 1 online).

First, we characterized the influence of tfl1 alleles on flowering time, examining tfl1-1, a severe allele, and tfl1-17, a null allele, along with several weak alleles, including tfl1-11, tfl1-13, and tfl1-14. Arabidopsis is a LD plant; the flowering time of the wild type is delayed under SD conditions. SD conditions induced delayed flowering also in plants carrying tfl1 mutant alleles (Shannon and Meeks-Wagner, 1991). At 22°C and our standard LD conditions, tfl1 mutants clearly flowered earlier than the wild type, but the difference in rosette leaf number at flowering between the wild type and tfl1 mutants was small (Figure 1A). Under SD conditions, the rosette leaf numbers of tfl1 mutants and the wild type were increased but not generally lower in all the mutants than in the wild type (Figure 1A). The flowering time of the wild type was retarded at 16°C under LD conditions, but this retardation was smaller in tfl1 mutants (Figure 1B). These results suggested that tfl1 mutants are responsive to the photoperiod but are less sensitive to the temperature (Strasser et al., 2009). This finding proved useful in further analyses of the control of flowering time in the tfl1 background; plants carrying the null allele tfl1-17 flowered only slightly earlier than the wild type at 22°C, but this difference was greatly enhanced at 16°C (Figures 1A and 1B).

The numbers of flower buds above the last lateral shoot excluding the terminal flower are shown in Figure 1C. The wild type always produced more than 20 lateral flower buds, while five or fewer lateral buds were generated in the tfl1 mutants. Thus, we confirmed the phenotypic traits of the tfl1 alleles. tfl1-1, a severe allele, and tfl1-17, a null allele, were analyzed further.

**TFL1-SRDX Simulated the Original TFL1 Function**

The TFL1 homolog FT is involved in LD-dependent transcriptional regulation (Abe et al., 2005; Wigge et al., 2005). Considering its structural similarity to FT, TFL1 can be hypothesized to be a component of transcriptional complexes. Intriguingly, neither TFL1 nor FT possess any sequence domains characteristic of previously characterized transcription factors. Also, tfl1 mutants are defective in protein trafficking to PSVs (Sohn et al., 2007). To elucidate the molecular function of TFL1 in a possible transcriptional complex, we fused a transcriptional activator or a repressor domain with TFL1. If TFL1 does not participate in transcriptional regulation, plants carrying any of these modified TFL1 constructs would be expected to show the same phenotypes as the plants carrying wild-type TFL1. However, if TFL1 plays an important role in transcription, one of the fusion proteins should rescue the loss-of-function mutant tfl1, while the other one should disrupt the normal function of TFL1 in wild-type plants.

We used VP16 as a transcriptional activator domain and SRDX as a repressor domain (Parcy et al., 1998; Ohta et al., 2001). TFL1 was fused with VP16 (TFL1-VP16) or SRDX (TFL1-SRDX) and with the cauliflower mosaic virus 35S promoter to overexpress the constructs in Arabidopsis. Overexpression lines in Col, tfl1-1, and tfl1-17 null mutants were obtained and analyzed. To test the effects of TFL1-SRDX, we determined the numbers of rosette leaves and flower buds in plants overexpressing TFL1-SRDX in the wild-type (35S:TFL1-SRDX/Col) and tfl1 mutant background (35S:TFL1-SRDX/tfl1). The phenotypes of 35S:TFL1-SRDX
plants in the T1 generation varied similarly as observed in 35S:TFL1 plants (frequency distributions of flowering time of T1 generations are shown in Supplemental Figure 2 online). Although the number of rosette leaves varied, 17 out of 20 35S:TFL1-SRDX/Col lines and 10 out of 14 35S:TFL1-SRDX/TFL1-VP16 lines displayed late flowering phenotypes in the T1 generation. Similar results were observed in 35S:TFL1-SRDX/tfl1-1.

We selected several representative lines to produce T2 generations and examined the inheritance of the phenotype (Figures 2B and 2C, Table 1). As we had observed in T1 generations, 35S:TFL1-SRDX in the Col as well as the tfl1 background caused T2 plants to delay flowering time at 22°C (Table 1; see Supplemental Figure 3A online). 35S:TFL1-SRDX in both backgrounds increased the number of flower buds. In plants overexpressing TFL1-SRDX in either the tfl1 or wild-type background, sepals were converted into bract-like organs, flowers sometimes lacked petals, and secondary flowers proliferated as seen in typical TFL1-overexpressing lines (Figure 2D; the TFL1-overexpressing phenotype is shown in Ratcliffe et al., 1998). Some of these secondary flowers developed long peduncles (Figure 2E). When such an elongated peduncle developed close to the apex of the inflorescence, we regarded it as the last lateral shoot for flower bud scoring. Therefore, flower bud numbers of 35S:TFL1-SRDX/Col (#5) and 35S:TFL1-SRDX/tfl1-1 (#2 and 4) were below 10. Since these lines did not produce terminal flowers, we assumed the terminal flower phenotype to be rescued. In summary, TFL1-SRDX rescued early flowering and the terminal flower phenotype in the tfl1 mutant, and this effect was dominant over the effects of the native protein. These results showed that TFL1-SRDX retained the native TFL1 function and that the transcriptional repressor form of TFL1 did not disrupt the function of TFL1.

**TFL1-VP16 Overexpression Caused a Dominant-Negative Phenotype**

Since TFL1-SRDX behaved like the original TFL1, we wondered whether the TFL1-activator fusion perturbed TFL1 function. We made Col and tfl1-17 plants overexpressing TFL1-VP16 fusion genes. Interestingly, overexpression of TFL1-VP16 in Col (35S:TFL1-VP16/Col) induced a terminal flower phenotype that resembled that of tfl1 mutants (Figure 2A). We found fewer than 10 flower buds in the inflorescences of 13 out of 14 independent 35S:TFL1-VP16/Col T1 lines, whereas vector control plants generated more than 20 buds. In the tfl1-17 mutant backgrounds, none of the 35S:TFL1-VP16 lines rescued the tfl1 phenotype.

To confirm the dominant-negative phenotype caused by TFL1-VP16 overexpression, we examined phenotypes of several representative T2 lines. 35S:TFL1-VP16 lines in the Col and tfl1-17 backgrounds had reduced numbers of flower buds in their inflorescences (Table 1). The shoot apices carried terminal flowers in these lines. Although 35S:TFL1-VP16/Col line #3 produced numerous flower buds, the inflorescence carried a terminal flower. In secondary shoots, the effects of TFL1-VP16 were subtle. The tfl1 plants usually had secondary stems possessing only a terminal flower, but secondary stems of 35S:TFL1-VP16/Col plants were elongated and inflorescences with a few flower buds were generated (Figure 2A). Thus, 35S:TFL1-VP16 caused dominant-negative effects in Col, although the inflorescence phenotypes of the 35S:TFL1-VP16/Col lines were weaker than those in tfl1 mutants (Table 1).

We also determined flowering time in the above lines. Col lines carrying TFL1-VP16 showed early flowering compared with the wild type at 16°C, but the effect was small at 22°C. Our observation showed that the effects of 35S:TFL1-VP16 were subtle in tfl1 mutants. Flowering times of 35S:TFL1-VP16/tfl1-17 lines...
were similar to that of 
tfl1-17
, indicating that TFL1-VP16 induced its effects only when the native TFL1 was functional. These inflorescence and flowering time phenotypes suggested that in contrast with TFL1-SRDX, TFL1-VP16 acts in a dominant-negative manner to disrupt normal TFL1 function.

To exclude the possibility that the effects of TFL1 fusion proteins were due to altered protein structure or changes of the molecular mass of the proteins, we examined the phenotypes caused by overexpression of TFL1-GFP (green fluorescent protein) and FT-VP16. As we had observed with TFL1 and TFL1-SRDX-overexpressing plants, the tfl1 phenotype was complemented in several individual TFL1-GFP-overexpressing lines (see Supplemental Figure 2 online). Although phenotypic variations similar to those in TFL1-overexpressing plants were observed, plants overexpressing TFL1-GFP exhibited a late flowering phenotype with aberrant flowers in both the Col and the tfl1 background. Moreover, FT-VP16-overexpressing lines displayed early flowering phenotypes in Col and ft-101 as also observed in FT-overexpressing plants (Figure 2F), suggesting that the VP16 fusion did not perturb FT function, which is in line with a previous report (Wigge et al., 2005). Thus, we concluded that the function of TFL1 was not disturbed by the changes in molecular mass and conformation caused by the extra peptides in our constructs. The dominant-negative phenotypes of TFL1-VP16-overexpressing plants were not due to the disturbance of the original protein function but to the fusion of the protein with a transcriptional activator domain.

Genes Activated by FT Are Repressed by TFL1-SRDX

Next, we attempted to identify genes that are regulated by TFL1-mediated mechanisms. According to previous reports, TFL1 represses the expression of AP1 and LFY (Gustafson-Brown et al., 1994; Liljegren et al., 1999) and interacts with FD and FDP (Abe et al., 2005). Considering the structural similarity between TFL1 and FT and the early-flowering phenotype of 35S:TFL1-VP16, it appears plausible that both proteins are involved in the same regulatory pathway. FRUITFULL, SEPALLATA1 (SEP1), SEP3, and AP1 are downstream elements of FT-dependent regulation in the SAM (Teper-Bamnolker and Samach, 2005; Wigge et al., 2005). Therefore, we investigated the expression of these meristem identity genes and LFY in plants overexpressing TFL1-SRDX or TFL1-VP16. To normalize mRNA amounts in the SAM, expression of the SAM-specific gene CLAVATA3 (CLV3) was measured as a standard of SAM-specific total mRNA (Fletcher et al., 1999).

As described above, the early flowering phenotype of 35S:TFL1-VP16 lines became evident at 16°C, while 35S:TFL1-SRDX lines showed delayed flowering at 22°C. Therefore, we determined mRNA abundance in these lines grown at the appropriate temperatures. 35S:TFL1-SRDX plants grown for 3 weeks under SD conditions were transferred into LD at 22°C. RNA was isolated from shoot apical regions harvested 7 d before and 0, 2, 6, 10, and 14 d after transfer to LD. On the other hand, the apical regions of 35S:TFL1-VP16 plants that had been grown under LD at 16°C for 3, 4, 5, 6, and 7 weeks after germination were harvested for RNA isolation.

We found that overexpression of TFL1-SRDX repressed all genes examined in the Col and tfl1 background (Figure 3, left panels). In the tfl1-17 mutant, transcription of these genes was initiated within 2 d of the transfer to LD at 22°C, when the plants started bolting. Expression of these genes was induced at 10 d after transfer in Col, which corresponds to the bolting time. TFL1-SRDX overexpression repressed the transcription of these genes, which probably caused the delay in flowering time.
To determine whether TFL1-SRDX represses the target genes more strongly than TFL1 itself, we compared the expressions of meristem identity genes in 35S:TFL1-SRDX and 35S:TFL1 plants. We selected lines of 35S:TFL1-SRDX and 35S:TFL1 showing similar flowering time and isolated RNA from shoot apical regions at the initiation of bolting. The expression level of the meristem identity genes in both 35S:TFL1-SRDX and 35S:TFL1 were significantly lower than those in Col and tfl1-17 (see Supplemental Figure 4 online). However, no significant differences between 35S:TFL1-SRDX and 35S:TFL1 were observed.

Plants overexpressing TFL1-VP16 in the Col background showed early flowering, and the amounts of AP1, SEP1, SEP3, and LFY transcripts were higher than in the wild type at 6 weeks after germination (Figure 3, right panels). This timing coincided with the initiation of bolting in 35S:TFL1-VP16/Col. By contrast, in tfl1-17, the expression levels of AP1, SEP1, and SEP3 at 6 weeks did not differ significantly from those in 35S:TFL1-VP16 and nontransgenic lines. This observation was consistent with the fact that overexpression of TFL1-VP16 had small effects on flowering time in tfl1-17. Though it remained unclear whether the genes examined were directly or indirectly regulated by TFL1, these genes clearly were controlled by TFL1.

The genes examined were repressed by TFL1-SRDX overexpression, which induced the same phenotype as overexpression of TFL1 did. On the other hand, overexpression of TFL1-VP16 had similar effects as the tfl1 mutation on these genes. Therefore, we suggest that TFL1 is involved in the transcriptional repression of these genes.

The bZIP Transcription Factor FD Is Associated with TFL1-Dependent Transcriptional Repression

To address how TFL1 participates in transcriptional repression, we focused on the bZIP transcription factors FD and FDP. Previously, FT and TFL1 were reported to interact with FD and its paralog FDP, although the interactions between TFL1 and FD or FDP were weaker than those between FT and the transcription factors (Abe et al., 2005; Wigge et al., 2005). We performed yeast two-hybrid assays and confirmed that TFL1 weakly interacts with FD and FDP (see Supplemental Figure 5 online). Thus, both FD and FDP appear to be involved in the TFL1-dependent transcriptional repression complex.

fd mutants are known to develop late flowering phenotypes, but the flowering time of fdp mutants was unclear. Therefore, we attempted to find mutants with altered flowering time among the TILLING lines carrying fdp mutations. In contrast with the delayed flowering observed in fd-1 mutants (Table 2; see Supplemental Figures 3B and 3C online), we could not find any line in the fdp TILLING populations showing obvious effects on flowering time (see Supplemental Figure 6 online). Because these lines are substitution mutants, they may be leaky and retain original protein function. Therefore, the absence of flowering phenotypes in these lines does not necessarily imply that FDP does not affect flowering. In any case, further studies of flowering time in these fdp mutants seemed futile, and we focused on fd mutants in our subsequent genetic analyses.

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### Table 1. Phenotypes of Col and tfl1 Plants Overexpressing TFL1-SRDX and TFL1-VP16

<table>
<thead>
<tr>
<th>Plant Lines</th>
<th>Inflorstone Phenotype</th>
<th>Flowering at 22°C</th>
<th>Flowering at 16°C</th>
</tr>
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<tr>
<td></td>
<td>n</td>
<td>Leaf No. ± SE</td>
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<tr>
<td>Col</td>
<td>6</td>
<td>&gt;20</td>
<td>15</td>
</tr>
<tr>
<td>tfl1-1</td>
<td>6</td>
<td>1.17 ± 0.31</td>
<td>14</td>
</tr>
<tr>
<td>tfl1-17</td>
<td>6</td>
<td>2.00 ± 0.37</td>
<td>18</td>
</tr>
<tr>
<td>TFL1-SRDX/Col</td>
<td></td>
<td></td>
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<tr>
<td>#2</td>
<td>6</td>
<td>3.67 ± 2.03**</td>
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<tr>
<td>#15</td>
<td>6</td>
<td>&gt;20</td>
<td>5</td>
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<td>#3</td>
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<td>7.67 ± 3.29†</td>
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<td>1.33 ± 0.99</td>
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<td>#15</td>
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<td>&gt;20†</td>
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<td>#13</td>
<td>6</td>
<td>&gt;20‡</td>
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<td>#14</td>
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<td>10.17 ± 1.17**</td>
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<td>&gt;20</td>
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<tr>
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<td>6.50 ± 0.89**</td>
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<td>TFL1-VP16/tfl1-17</td>
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The statistical significance of differences between samples in each background was evaluated by the two-tailed multiple t test with Bonferroni correction following ANOVA. Symbols *, †, and ‡ indicate significant difference in comparison with Col, tfl1, and tfl1-17, respectively. Single and double symbols represent P < 0.05 and P < 0.01, respectively. Bar graphs of flowering time based on this table are shown in Supplemental Figure 3A online.
To dissect the genetic interactions between TFL1 and FD, we introduced the *tfl1* mutation into the *fd* mutant by crossing and introduced the 35S:TFL1-SRDX construct into *fd* by transformation (Figure 4). Since *fd-1* is a mutation in the Landsberg erecta (*Ler*) background, we used the *tfl1* mutant in *Ler*, *tfl1-2*. The flowering times of *Ler*, *tfl1-2*, *fd-1*, and the *fd-1 tfl1-2* double mutants were determined (Table 2; see Supplemental Figure 3B online). We found no significant difference between *fd-1* and *fd-1 tfl1-2* at 22 and 16°C. Though the *tfl1* mutant exhibited a pronounced early flowering phenotype at 16°C, this was not seen in plants carrying the *tfl1* mutation in the *fd* background. Thus, while the *tfl1-2* mutation caused early flowering in *Ler*, the *tfl1* mutation had little effect on flowering in the *fd-1* background.

**Figure 3.** Expression of Meristem Identity Genes in 35S:TFL1-SRDX and 35S:TFL1-VP16 Plants.

Left panels: Gene expression in 35S:TFL1-SRDX plants at 22°C. Plants were transferred from SD to LD at day 0. For RNA isolation, the shoot apical region of 10 to 15 plants were collected at 7 d before and at 0, 2, 6, 10, and 14 d after the transfer. Right panels: Gene expression in 35S:TFL1-VP16 plants under LD at 16°C. RNAs were isolated from the shoot apical regions of 10 to 15 plants that were 4, 5, 6, and 7 weeks old. Expression levels were determined by real-time quantitative PCR; the ratio of each gene expression level relative to *CLV3* expression is shown. Results from three biological replicates are shown as means ± SE. Since harvested plants were not completely synchronized regarding phase transition, both between replicates and within each series, some data sets show high variances (large error bars).
We also determined the inflorescence phenotype. Similar to fd-1, the fd-1 tfl1-2 double mutant generated more than 20 flower buds in the primary shoot, while tfl1-2 produced less than two buds (Figures 4A to 4C, Table 2). Although the reduction of flower buds was suppressed in the fd-1 tfl1-2 plants, a terminal flower was still present at the shoot apex (Figures 4E and 4F). Because the fd mutation suppressed early flowering and inflorescence phenotype of tfl1 but still allowed for the development of a terminal flower, we concluded that fd is partially epistatic to tfl1.

We produced transgenic lines overexpressing TFL1-VP16 in the fd-1 and Ler background (3SS:TFL1-VP16/fd-1 and 3SS:TFL1-VP16/Ler, respectively). 3SS:TFL1-VP16 slightly accelerated flowering of Ler, but there was no effect in fd-1 at 22°C (Table 2; see Supplemental Figure 3C online). At 16°C, overexpression of TFL1-VP16 showed similar effects as the tfl1-2 mutation in Ler, but the early flowering effect appeared reduced in fd, as the difference in leaf number at flowering time between 3SS:TFL1-VP16/fd-1 and fd-1 was small (Table 2; see Supplemental Figure 3C online). Thus, TFL1-VP16 overexpression accelerated flowering in wild-type Ler but had no significant effect in fd-1. We observed similar effects with respect to inflorescence phenotype. 3SS:TFL1-VP16/Ler lines but not 3SS:TFL1-VP16/fd-1 lines showed reduced numbers of flower buds in the primary shoot (Figure 4D, Table 2). The fd mutation strongly suppressed the 3SS:TFL1-VP16 phenotypes, similarly to its suppression of the tfl1 phenotype. In both cases, however, terminal flowers still developed at the shoot apices after the production of numerous flower buds (Figures 4F and 4G).

Flowering time and development of the inflorescence shoot are correlated; for example, early flowering plants overexpressing FT produced terminal flowers (Kardailsky et al., 1999; Kobayashi et al., 1999). It appeared possible that the late flowering of fd-1 masked the tfl1 phenotype. Therefore, we studied the flowering time and inflorescence phenotypes in ft-101 tfl1-17. The ft tfl1 double mutant had been reported to be phenotypically intermediate between ft1 and ft (Hanzawa et al., 2005; Ahn et al., 2006). In our tests, ft-101 tfl1-17 plants had an intermediate flowering phenotype; they flowered later than tfl1-17 but earlier than ft-101 plants. The ft-101 and ft-101 tfl1-17 lines flowered with 55.4 ± 2.42 (mean ± se) and 35.4 ± 1.12 rosette leaves, respectively (ft-101 versus ft-101 tfl1-17, P < 0.01). Both ft-101 and ft-101 tfl1-17 produced more than 20 flower buds on the primary stem, but only the shoots of ft-101 tfl1-17 ended with terminal flowers.

In comparison with the late flowering phenotype of ft tfl1 and ft, neither the tfl1 mutation nor overexpression of TFL1-VP16 significantly accelerated flowering in fd plants. The early-flowering effects of the tfl1 mutation and of TFL1-VP16 overexpression apparently were cancelled out by the fd mutation. Taken together, our results suggested that FD is required for TFL1 function in the context of the transcriptional regulation of target genes and the regulation of the timing of flowering. The inflorescence architecture in tfl1 mutants and plants overexpressing TFL1-VP16 was rescued by the fd-1 mutation; terminal flowers were produced in all cases, although sometimes they were weakened. As TFL1 represses AP1 and overexpression of AP1 produces terminal flowers (Gustafson-Brown et al., 1994; Mandel and Yanofsky, 1995), it seemed possible that the phenotypes we observed were due to the ectopic expressions of AP1. We made ap1-7 tfl1-17 double mutants (Figures 4H and 4I) and found that the phenotypes of ap1 and tfl1 were additive with
a terminal flower still present. Thus, the terminal flower phenotype of tfl1 is not due to the ectopic expression of AP1. We conclude that terminal flowers are produced in fd tfl1 double mutants independently of AP1 function.

**TFL1 Interacts with FD in the Nucleus**

Since genetic analyses supported the idea of an interaction between TFL1 and FD, we investigated possible interactions on the molecular level. As the most convenient and reliable method to examine molecular interactions and localizations in living plant cells, we employed a bimolecular fluorescence complementation (BiFC) technique (Hu and Kerppola, 2003). To make BiFC constructs, the C-terminal half of yellow fluorescent protein (cYFP) was fused to the C terminus of TFL1 via a linker (TFL1- cYFP), and the N-terminal half of YFP (nYFP) was fused to the N terminus of FD (nYFP-FD). Each construct was under the control of 35S promoter and was transformed into Agrobacterium...
tumefaciens. Transformed agrobacteria were mixed in desired combinations and infiltrated into epidermal cells of Nicotiana benthamiana leaves (Voinnet et al., 2003). The fluorescent signal of YFP, which indicates molecular interactions of the fusion proteins, was observed in the nuclei of the cells when 3SS:TFL1-cYFP and 3SS:nYFP-FD were coinfiltrated (Figure 5). No fluorescent signal was observed in the combination of 3SS:TFL1-cYFP/3SS:nYFP or 3SS:nYFP-FD/3SS:cYFP (see Supplemental Figure 7 online). This result supports our hypothesis that TFL1 interacts with FD in the nucleus and functions in transcriptional complexes. Similar experiments using FT instead of TFL1 (coinfiltration of 3SS:FT-cYFP and 3SS:nYFP-FD) also showed the YFP fluorescent signal specifically in the nucleus (Figure 5; see Supplemental Figure 7 online; Abe et al., 2005).

Since the cellular localization of TFL1 when it does not interact with FD is unclear, we studied the intracellular localization of TFL1 protein using GFP fusion and Agrobacterium infiltration. N. benthamiana epidermal cells expressing 3SS:TFL1-GFP showed fluorescent signal in the cytoplasm and nucleus (Figure 5). Since the TFL1-GFP fusion gene complemented tfl1 mutants when expressed in the TFL1 genomic context (K. Goto, unpublished data), TFL1-GFP retains the original function of TFL1. Although the 3SS promoter produced an excess of TFL1 protein in the cells, these results suggested that the intracellular localization of TFL1 protein is not restricted. TFL1 localized to both cytoplasm and nucleus, whereas interactions of TFL1 and FD were observed only in the nucleus. Thus, TFL1 may function in cytoplasm as well as the nucleus, which is consistent with the postulated function in transcriptional regulation.

**DISCUSSION**

In this study, we described the negative role of TFL1 in FD-dependent transcription. TFL1 is known as a floral repressor and a regulator of IM development. TFL1 and FT share high sequence similarity, but they have opposite functions. FT acts in FD-dependent transcription (Abe et al., 2005; Wigge et al., 2005). To shed light on the molecular function of TFL1, we characterized plants overexpressing TFL1 fused with either a transcriptional activator domain (VP16) or a repressor domain (SRDX). TFL1-VP16 overexpression caused early flowering and the development of terminal flowers, and TFL1-SRDX overexpression delayed flowering and rescued the tfl1 mutant similarly to

![Figure 5. BiFC Analysis of Protein Interactions between TFL1 or FT and FD, and TFL1 Localization in N. benthamiana Leaf Epidermis Cells.](image-url)

G, green channel image showing fluorescence of YFP (BiFC) or GFP; BF, bright-field image; Merged, merged image of G and BF. TFL1 and FD, coexpression of 3SS:TFL1-cYFP and 3SS:nYFP-FD; TFL1-GFP, expression of 3SS:TFL1-GFP; FT and FD, coexpression of 3SS:FT-cYFP and 3SS:nYFP-FD. Bars = 50 μm.

[See online article for color version of this figure.]
overexpression of TFL1 (Figure 2). Plants overexpressing TFL1-VP16 displayed a dominant-negative phenotype, but 3SS:FT-VP16 and 3SS:TFL1-GFP gave the same phenotypes as 3SS:FT and 3SS:TFL1, respectively. The VP16 domain itself did not interfere with the function of FT (Wigge et al., 2005). Moreover, although the molecular mass of GFP is larger than that of VP16, GFP did not disrupt TFL1 function. We concluded from these observations that the dominant-negative phenotype induced by overexpression of TFL1-VP16 was not caused by a structural modification of the TFL1 molecule but by the functional change conferred by the fusion with VP16. The fusion with the transcriptional activator domain turned TFL1 into an activator component in the transcriptional regulator complex, which conflicted with the function of native TFL1 as a repressor component. In this scenario, it is plausible to suggest that TFL1-SRDX rescued the tf1 mutant phenotype by acting as a transcriptional repressor. We conclude that TFL1 is involved in the transcriptional repression mechanisms that regulate FM identity genes that are also FT targets (see Supplemental Figure 8 online).

The fact that TFL1 and FT have antagonistic roles in transcriptional regulation is intriguing. It is noteworthy that these proteins possess none of the canonical transcriptional activator or repressor domains (Ahn et al., 2006). As a possible explanation, we propose that TFL1 and FT act as mediators in the transcriptional regulatory complex. In other words, TFL1 and FT interact with FD and also interact with a corepressor and a coactivator, respectively (see Supplemental Figure 8 online). The identity of these postulated transcriptional regulators targeted by TFL1 and FT remains to be revealed. We attempted to isolate candidate proteins by yeast two-hybrid screening and found that several isoforms of 14-3-3 proteins interacted with TFL1 (K. Goto, unpublished data). Some 14-3-3 proteins interact with SP and Hd3a, a tomato (Solanum lycopersicum) TFL1 homolog and a rice (Oryza sativa) FT homolog, respectively, and are thought to be adhesive proteins but not transcriptional regulators (Pnueli et al., 2001; Purwesrati et al., 2009).

In this work, we identified nontranscription factors that act as transcriptional regulators by fusing them with an activator or repressor domain (Mitsuda et al., 2006). Numerous studies of transcription factors have used this approach (Groszmann et al., 2008; Ikeda and Ohme-Takagi, 2009; Mitsuda and Ohme-Takagi, 2009; Eklund et al., 2010). Here, we applied the method to TFL1, which does not contain any of the functional domains typically found in transcription factors or regulators nor does it contain any recognizable nuclear localization signal, DNA binding domain, or activation or repression domain (Ohshima et al., 1997; Ahn et al., 2006). Our study showed that TFL1-VP16 overexpression induced dominant-negative phenotypes, whereas TFL1-SRDX and FT-VP16 acted like the native TFL1 and FT, respectively. These observations provide additional evidence that TFL1 and FT function as transcriptional mediator molecules.

While TFL1-VP16 significantly reduced the number of flower buds even in tf1 mutants, it had a significant effect on flowering time in Col wild-type plants but not in tf1 mutants (Table 1). Considering that 3SS:TFL1-VP16 did not evoke a similarly strong phenotype as overexpression of FT did, it seems possible that TFL1 function was lost in the TFL1-VP16 fusion protein and that this protein interfered nonspecifically with the function of the transcriptional complex. However, in the presence of FT, the function of TFL1-VP16 may be somewhat masked, since FT interacts with FD more strongly than TFL1 does (see Supplemental Figure 5 online). When flowering is induced, FT moves into the SAM and forms a transcriptional complex with FD. Although TFL1 is expressed at this stage and the TFL1 protein may be involved in the same transcriptional complex, FT may exclude TFL1 and switch the complex from a repressing to an activating mode. This possible scenario explains why 3SS:TFL1-VP16 showed a weaker phenotype than 3SS:FT, though further corroboration is required.

We demonstrated interaction between TFL1 and FD in the nucleus by a BIFC assay and showed that the TFL1 protein is present in nucleus and cytoplasm (Figure 5). These findings suggest that TFL1 is a nucleocytoplasmic shuttling protein that may play a role in FD-dependent transcription. The nuclear and cytoplasmic localization of TFL1 was also observed in Arabidopsis using GFP-TFL1 with a native promoter and enhancer (A. Nakayama, M. Takahashi, and K. Goto, unpublished data). Intriguingly, previous work suggested that TFL1 is located exclusively in the cytoplasm (Conti and Bradley, 2007; Sohn et al., 2007) and that it may be involved in protein trafficking to PSVs (Sohn et al., 2007). It is possible that TFL1 has pleiotropic functions, including that of a carrier of proteins from the nucleus to PSVs. Since TFL1 and FT also act as mobile signals between cells (Conti and Bradley, 2007; Corbesier et al., 2007; Jaeger and Wigge, 2007; Mathieu et al., 2007; Tamaki et al., 2007), intra- and intercellular translocation may be essential characters of these proteins. A clarification of possible functions of TFL1 in the cytoplasm is required.

One of the most direct experiments to demonstrate that TFL1 functions in a transcriptional complex is the chromatin immunoprecipitation (ChIP) assay directed against the target gene. Wigge et al. (2005) showed that the putative target region of FD in the AP1 promoter is concentrated by anti-FT antibodies in 3SS:FD plants. We performed ChIP assays using wild-type plants, but the AP1 target region was concentrated by neither TFL1 nor FT-specific immunoprecipitation. Our observation, however, is in agreement with a recent report (Benlloch et al., 2011) that suggested that AP1 is not a direct target of FT. Thus, both TFL1 and FT may interact with DNA indirectly, whereas FD binds to DNA directly. Unfortunately, ChIP techniques for molecules that interact with DNA indirectly are not yet established. Therefore, it would appear necessary to identify FD targets through ChIP assays based on FD-specific immunoprecipitation first and then search for TFL1-specific (and FT-specific) target regions in known FD targets.

The fact that fd tf1 and 3SS:TFL1-VP16/fd plants showed a weak terminal flower phenotype (Figure 4, Table 2) suggested that FD is not the only molecule that interacts with TFL1. FDP may be one of the candidates because it is closely related to FD and interacts with TFL1 and FT (see Supplemental Figure 5 online). We found no late or early flowering phenotypes and aberrant inflorescences in any fdp TILLING line population. However, the mutations in these mutants are amino acid substitutions. Therefore, no null or defective mutants might be included in the sample, even though some mutant proteins differed from
the amino acid substitutions found in natural variants (see Supplemental Figure 6 online). Attempts to genetically analyze the control of flowering time using these mutants appear futile. Although FDP function is still unclear, we found that TFL1 interacted with FD in the nucleus similarly as FT did and that the TFL1-FD complex regulates flowering time in Arabidopsis. Further studies on FDP, including functional and expression analyses, are now underway.

In Arabidopsis, SD photoperiod and low temperature delay flowering time (Blázquez et al., 2003). Although tfl1 mutants showed early flowering under SD conditions and at 16°C, the phenotype was more obvious at low temperature (Figures 1A and 1B). SD conditions more effectively delayed flowering time than did low temperature in ftfl1 mutants, suggesting that the temperature regulation of flowering is defective in ftfl1 mutants. Consistent with a previous report (Strasser et al., 2009), our observations suggested that TFL1 is involved in the thermen-
sory pathway rather than the photoperiod pathway of flowering time regulation. Recently, it was shown that a TFL1 homolog is involved in the vernalization pathway of Arabis alpina, a perennial close relative of Arabidopsis (Wang et al., 2011). Further inves-
tigations are required to find out how the TFL1 molecular func-
tions presented here fit into the temperature-related regulation of flowering time.

TFL1 belongs to the CETS family of genes that encode PEBPs (Pnueli et al., 2001). PEBPs are evolutionally conserved among eukaryotes and play important roles in diverse organisms. For example, they are involved in Alzheimer’s disease in humans and the control of grain yield in rice (Jones et al., 2008; Huang et al., 2009). However, the molecular functions of PEBPs are largely unknown. PEBPs have been reported to function as Raf-1 kinase inhibitor proteins in mammals and as Ser protease inhibitors in yeast (Yeung et al., 1999; Mima et al., 2005). Here, we demon-
strated that one PEBP, TFL1, is involved in transcriptional repression. It is interesting to see how PEBPs have been re-
cruited to serve a wide variety of functions in the evolution of eukaryotes. The biochemical functions of plant PEBPs are not known. The flowering plants examined so far possess genes that belong to the TFL1 and FT clades (Ahn et al., 2006). It is interesting that the two types of PEBP have antagonistic effects on flowering time and inflorescence development. Since flowering time and in-
florescence architecture determine the number of seeds set, they should be regulated coordinately and appropriately. Regulated transcriptional repressors add flexibility for the precise control of flowering time beyond the regulatory mechanisms that target the promoters of flowering genes. It appears that plants employ the divergent functions of the members of the same protein family to fine-tune the transition from the vegetative to the reproductive phase.

METHODS

Plant Materials and Growth Conditions

We used Arabidopsis thaliana Col and Ler accessions for wild-type controls. The background ecotypes of ftfl1-1, ftfl1-11, ftfl1-13, ftfl1-14, ftfl1-
17, and ft-101 are Col, ftfl1-2, and ft-1 and originally came from Ler. The ft-
101 ftfl1-17 and ftfl1-2 double mutants were made by artificial crossing. The constructs on binary vectors were introduced into Arabi-
dopsis using Agrobacterium tumefaciens C58 (Clough and Bent, 1998), and T1 transformants were selected on kanamycin plates. Transgenic lines with a single locus T-DNA insertion were subsequently identified based on the 3:1 segregation ratio between kanamycin resistance and sensitivity.

For flowering time determination, seeds were placed on 0.9% agar with half-strength Murashige and Skoog salt, 1% sucrose, and 25 μg mL−1 kanamycin. Seeds were kept for 2 to 3 d at 4°C and then moved to growth chambers under LD (16 h light/8 h dark) or SD (10 h light/14 h dark) with an illumination rate of 50 to 60 μmol m−2 s−1 of white fluorescence light at 22 or 16°C. This time was defined as day 0. Germinated plants were selected for their resistance to kanamycin. Plants were grown on plates for 1 week in LD or 2 weeks in SDs and then transferred to soil (Metromix 350: vermiculite = 1:1), except for flowering measurements at 16°C for which plants were grown on plates until scoring. To determine flowering time, the number of rosette leaves was counted when the flower stalks were around 3 to 5 cm, at the time the first flowers bloomed.

Plasmid Constructions

To produce transgenic plants overexpressing the modified FT and TFL1, we prepared 35S:TFL1-VP16, 35S:TFL1-SRDX, 35S:FT-VP16, and 35S:
TFL1-GFP on the binary vector pCGN1547 (McBride and Sommerfelt, 1990).

To prepare VP16- and SRDX-fusion constructs, TFL1 and FT coding regions were amplified by PCR using primers with linker sequences at the 3’ termini instead of stop codons. The linker sequence 5′-GGAGAGGG-
TACCCCCGGGCTC-3′ was translated to a GGTPGGL peptide. For instance, the TFL1-VP16 sequence encodes a TFL1-GGTPGGL-VP16 protein. The amplified fragments were cloned into pENTRY VP16 or pENTRY SRDXG version 2 (Mitsuda et al., 2008). pENTRY VP16 has the VP16 sequence from pVP16 C1 (Novagen) instead of SRDX in pENTRY SRDXG version 2. The resultant plasmids were subjected to Gateway cloning into 35S:NOS-GW/pCGN, which has the cauliflower mosaic virus 35S promoter (35S), Gateway rta cassette (Invitrogen), and the napoline synthase (NOS) terminator on pCGN1547. TFL1-GFP was constructed by fusing the TFL1 cDNA and GFP (SGST) coding regions via the same linker as used for the VP16 fusion. TFL1-GFP was then subcloned by BamHI and PstI digestion and ligation into 35S:NOS/pCGN version 2, which has 35S, a multicloning site, and NOS on pCGN1547. The amplified se-
quences were confirmed by DNA sequencing.

For the BiFC assay, PCR fragments amplified with the specific primers for TFL1, FD, and FT were subcloned into entry vectors pDONR221 or pENTRY 1a (Invitrogen). In TFL1 and FT, stop codons were replaced by the same linker as used for TFL1-VP16. The resultant plasmids were transferred by Gateway Cloning (LR reaction) into the split YFP vectors pB4NY0 and pB4CY2 (gifts from Shoji Mano).

PCR primers used for plasmid construction are shown in Supplemental Table 1 online.

RNA Isolation and Quantitative RT-PCR

Plants grown at 22°C for 3 weeks under SD (10-h-light/14-h-dark cycle) conditions were moved into a growth camber with LD conditions and then harvested for RNA isolation at various times (the transfer date was defined as day 0). For sampling at 16°C, plants were grown under LD conditions. Total RNA was isolated from the shoot apical region of 10 to 15 plants using the RNeasy plant mini kit (Qiagen). Elimination of genomic DNA and reverse transcription were performed on 1.0 μg of total RNA with the QuantiTect reverse transcription kit (Qiagen). Real-time quantitative PCR was performed with the LightCycler 1.5 system (Roche Applied Science)
and the QuantiTect Probe PCR kit (Qiagen). Gene-specific primers and labeled probes were designed using ProbeFinder for Arabidopsis (version 2.41; Roche) or Primer Express (version 1.5; Applied Biosystems). Details of primers and probes are described in Supplemental Table 2 online. Each expression value was quantified in comparison with standard molecular amounts and was divided by the CLV3 expression value.

BiFC Assay

Agrobacterium tumefaciens–based transient transformation was performed according to a previous description (Voinnet et al., 2003). Agrobacterium cultures carrying plasmids for BiFC and p19 were grown overnight at 28°C in 10 mL YEB plus selective antibiotics, collected by centrifugation, and resuspended in infiltration medium (10 mM MgCl₂, 150 μM mL⁻¹ acetosyringone, and 10 mM MES-NaOH, pH 5.6). Cells were kept at 28°C in this infiltration medium for 3 h and then infiltrated into the abaxial air spaces of 3- to 4-week-old Nicotiana benthamiana plants. YFP fluorescence was observed 2 to 3 d after infiltration. Fluorescent signals and bright-field pictures were taken using an LSM 5 PASCAL confocal laser scanning microscope (Carl Zeiss) controlled by the LSM 5 software.

Photography

Higher magnification pictures of the inflorescence shoot were taken with a DP70 digital camera (Olympus) attached to an MZ-APO binocular (Leica). Extended focus images were created from Z-stacks with DynamicEye software (Mitani).

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative (AGI) or GenBank/EMBL databases under the following accession numbers: TFL1 (AT5G03840), FT (AT1G65480), AP1 (AT1G69120), SEP1 (AT5G15800), SEP3 (AT1G24260), FRUITFULL (AT5G60910), LFY (AT5G61850), CLV3 (AT2G27250), FD (AT4G35900), and FDP (AT2G17770). Current information regarding the FDP coding sequences is inconsistent (AT5G61850), CLV3 (AT2G27250), FD (AT4G35900), and FDP (AT2G17770).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Diagram of the Location of the Mutations in the tfl1 Alleles Used in This Study.

Supplemental Figure 2. Frequency Distributions of the Flowering Times of T1 Transgenic Populations.

Supplemental Figure 3. Bar Graphs of Flowering Times Shown in Tables 1 and 2.

Supplemental Figure 4. Expression of Meristem Identity Genes in 35S:TFL1-SRDX and 35S:TFL1 Plants.

Supplemental Figure 5. Protein Interactions between TFL1, FT, FD, and FDP in the Yeast Two-Hybrid Assay.

Supplemental Figure 6. tdp TILLING Alleles and Their Flowering Time.

Supplemental Figure 7. Negative Controls for BiFC Analysis.

Supplemental Figure 8. A Model for TFL1 Molecular Function in Transcription.

Supplemental Table 1. List of PCR Primers Used for Plasmid Construction.

Supplemental Table 2. List of PCR Primers and Probes Used in Real-Time Quantitative PCR.

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AUTHOR CONTRIBUTIONS

K.G. conceived and designed the research, S.H. performed most experiments, and S.H. and K.G. wrote the article.

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Arabidopsis TERMINAL FLOWER1 Is Involved in the Regulation of Flowering Time and Inflorescence Development through Transcriptional Repression

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