Mutation in TERMINAL FLOWER1 Reverses the Photoperiodic Requirement for Flowering in the Wild Strawberry *Fragaria vesca*¹

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Photoperiodic flowering has been extensively studied in the annual short-day and long-day plants rice (*Oryza sativa*) and Arabidopsis (*Arabidopsis thaliana*), whereas less is known about the control of flowering in perennials. In the perennial wild strawberry, *Fragaria vesca* (Rosaceae), short-day and perpetual flowering long-day accessions occur. Genetic analyses showed that differences in their flowering responses are caused by a single gene, *SEASONAL FLOWERING LOCUS* (*FvTFL1*). We show through high-resolution mapping and transgenic approaches that *FvTFL1* is the basis of this change in flowering behavior and demonstrate that *FvTFL1* acts as a photoperiodically regulated repressor. In short-day *F. vesca*, long photoperiods activate *FvTFL1* mRNA expression and short days suppress it, promoting flower induction. These seasonal cycles in *FvTFL1* mRNA level confer seasonal cycling of vegetative and reproductive development. Mutations in *FvTFL1* prevent long-day suppression of flowering, and the early flowering that then occurs under long days is dependent on the *F. vesca* homolog of *FLOWERING LOCUS T*. This photoperiodic response mechanism differs from those described in model annual plants. We suggest that this mechanism controls flowering within the perennial growth cycle in *F. vesca* and demonstrate that a change in a single gene reverses the photoperiodic requirements for flowering.

Plants must time their vegetative and reproductive growth phases accurately in order to ensure their own survival as well as the survival of their progeny. In addition to endogenous cues such as their developmental stage and hormone levels, plants are able to monitor environmental signals, most importantly photoperiod and temperature, for developmental timing (Simpson, 2004; Samach and Wigge, 2005; Turck et al., 2008; Kim et al., 2009; Mutasa-Göttgens and Hedden, 2009). Appropriate developmental timing is especially important for perennial species, which may live for many years, undergoing repeated cycles of vegetative and reproductive (flowering) development, which are regulated by changes in the seasons through the year. Photoperiod is the major environmental cue for developmental timing in several perennial species (Cooper and Calder, 1964; Böhlenius et al., 2006; Gyllenstrand et al., 2007; Heide and Sønsteby, 2007), because it is the only constant environmental signal that undergoes the same cyclical pattern every year, with increasing amplitude toward higher latitudes.

Many perennial plants are economically important crops. Therefore, knowledge of the molecular regulation of flowering and developmental cycling in these species will enhance their cultivation as well as breeding. The woodland strawberry, *Fragaria vesca*, which belongs to the most important family of fruit crops, the Rosaceae, serves as a convenient perennial model (Shulaev et al., 2008, 2011). The presence of different accessions with opposite photoperiodic responses (Heide and Sønsteby, 2007; Sønsteby and Heide, 2008; Mouhu et al., 2009) makes this species a particularly useful model for photoperiodic research among perennials. Short-day (SD) *F. vesca* has a perennial life cycle characteristic of the Rosaceae; flower initiation occurs at the apical meristem under SD and low temperatures during autumn, and flowers emerge the following spring. Under the long days (LDs) of summer, new shoots that have emerged in the uppermost nodes remain vegetative, then as the days become sufficiently short and temperature declines, flower primordia are again produced (Battey et al., 1998; Battey, 2000; Heide and Sønsteby, 2007). Detailed physiological analyses in SD *F. vesca* demonstrated that a short photoperiod is obligatory for flower

¹[This work was supported by the Academy of Finland (grant no. 137439 to T.H.) and the University of Helsinki (grant no. DW–4881545211 to T.H.).

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induction at intermediate temperatures; at temperatures above 20°C, floral development is inhibited; and cool temperatures of about 10°C strongly induce flowering in both SD and LD conditions (Heide and Sonstebey, 2007). Similar to SD F. vesca, the photoperiod sensitivity of several cultivars of octoploid garden strawberry (Fragaria × ananassa) is strongly dependent on temperature (Heide, 1977; Bradford et al., 2010), suggesting that diploid F. vesca may be used as a model for more complex cultivated species.

In contrast to the seasonal flowering SD F. vesca, perpetual flowering accessions of the species (F. vesca f. semperflorens) flower and bear fruits from early summer until late autumn (Brown and Wareing, 1965). In three perpetual flowering accessions, reversed environmental responses compared with SD accessions have been reported. They are LD plants, which flower rapidly under LDs and high temperature, as opposed to SDs and low temperature, which repress flowering (Sonstebey and Heide, 2008; Mouhu et al., 2009). Classical genetic studies have shown that perpetual flowering is caused by recessive alleles of a single repressor gene called SEASONAL FLOWERING LOCUS (SFL; Brown and Wareing, 1965; Albani et al., 2004). Perpetual flowering cultivars (often called remontant or everbearing) are known also in cultivated strawberry. These cultivars have been considered as day-neutral or temperature-dependent LD plants in different studies (Sonstebey and Heide, 2007; Weebadde et al., 2008; Bradford et al., 2010; Stewart and Folta, 2010).

Photoperiodic flowering has been explained by the coincidence of CONSTANS (CO) diurnal expression rhythm and external light signals in both SD and LD model annuals, rice (Oryza sativa) and Arabidopsis (Arabidopsis thaliana), respectively (Suárez-López et al., 2001; Hayama et al., 2003). In both species, CO controls flowering through the CETS (for CEN, TFL1, and FT) family protein FLOWERING LOCUS T (FT), which is thought to be a universal flowering signal (Suárez-López et al., 2001; Hayama et al., 2003; Komiya et al., 2009; Turnbull, 2011). In the LD plant Arabidopsis, CO protein accumulates in the leaf phloem companion cells and activates FT expression only in LDs when the light period coincides with the CO expression peak in the late afternoon (An et al., 2004; Valverde et al., 2004; Corbesier et al., 2007). In contrast, in the SD plant rice, an FT homolog, Heading date3a, is activated when the CO homolog Heading date1 peaks after dusk in SDs (Hayama et al., 2003). After the activation of FT expression, the FT protein moves through the phloem to the shoot apex (Corbesier et al., 2007; Jaeger and Wigge, 2007; Tamaki et al., 2007), where 14-3-3 proteins bridge its interaction with a BZIP transcription factor, FLOWERING LOCUS D (FD; Taoka et al., 2011). The FT/14-3-3/FD complex, in turn, induces flowering by up-regulating the floral meristem identity genes APETALA1 (API) and FRUITFULL (FUL; Abe et al., 2005; Wigge et al., 2005). Another CETS family protein, the floral repressor TERMINAL FLOWERING1 (TFL1), also binds to FD and suppresses the expression of LEAFY, API, and FUL (Ratcliffe et al., 1999; Hanano and Goto, 2011). In Arabidopsis, TFL1 is developmentally regulated and has not been linked to the photoperiodic pathway. At the vegetative stage, TFL1 mRNA is weakly expressed in the lower part of the apical meristem, whereas the protein can move short distances to repress flowering in the main apex. After flower induction, TFL1 is strongly up-regulated to maintain the indeterminate inflorescence meristem (Shannon and Meeks-Wagner, 1991; Bradley et al., 1997; Ratcliffe et al., 1999; Conti and Bradley, 2007).

The functions of key flowering genes seem to be at least partially conserved between annual and perennial species, but their regulation may vary in the perennial context (Albani and Coupland, 2010). FT homologs have been shown to promote flowering in many perennial species (Endo et al., 2005; Böhlenius et al., 2006; Hsu et al., 2006; Kotoda et al., 2010), but at least in Populus trichocarpa, FT is also involved in the control of growth cessation (Böhlenius et al., 2006). Detailed analyses of two Populus FT paralogs have shown that seasonal changes in their expression control the cycling of reproductive and vegetative growth. Winter cold (vernalization) activates FT1, which promotes flowering, whereas LD and high temperature activate FT2, which promotes vegetative growth and prevents bud set (Hsu et al., 2011). In Arabis alpina, the return to vegetative development after flowering is regulated by transient silencing of the FLOWERING LOCUS C (FLC) ortholog PERPETUAL FLOWERING1 (PEP1) by vernalization (Wang et al., 2009). pep1-1 mutants show reduced vegetative growth but also flower perpetually, which is a trait reported in other perennials such as rose (Rosa sp.) and F. vesca (Brown and Wareing, 1965; Iwata et al., 2012). Furthermore, TFL1 homologs have been shown to repress flowering and to control the length of the juvenile phase in apple (Malus domestica), Populus, and A. alpina (Kotoda et al., 2006; Mohamed et al., 2010; Wang et al., 2011).

Recently, Iwata et al. (2012) identified a F. vesca homolog of TFL1 (FvTFL1) as a candidate gene for SFL. They showed that a 2-bp deletion in FoTFL1 is associated with perpetual flowering in F. vesca, but functional validation was lacking (Iwata et al., 2012). Here, we provide functional evidence for FoTFL1 being SFL. Our results further indicate that FoTFL1 is the key component of the perennial photoperiodic pathway in F. vesca; it confers an SD requirement for flowering and controls cycling between vegetative and reproductive phases. We also demonstrate how a 2-bp deletion in FoTFL1 changes the regulation of seasonal life cycles and leads to FoFT-dependent LD flowering.

RESULTS

Mutation in the Floral Repressor FoTFL1 Reverses the Photoperiodic Requirement for Flower Induction

SFL, which causes seasonal flowering in F. vesca (Brown and Wareing, 1965; Albani et al., 2004), was
initially mapped to \textit{F. vesca} linkage group 6 (Sargent et al., 2004). In a recent study, association was found between perpetual flowering habit and a 2-bp deletion in the first exon of \textit{F. vesca} \textit{TFL1} homolog, and the gene was weakly mapped between two markers with a physical distance of approximately 11 Mb in the same linkage group (Iwata et al., 2012). We narrowed down the location of \textit{SFL} within the mapping window of 248 kb, and our extensive linkage analysis in two populations fully supports the association found by Iwata et al. (2012; Supplemental Figs. S1 and S2).

To explore the function of \textit{FoTFL1}, we overexpressed the SD \textit{F. vesca} (PI551792 [National Clonal Germplasm Repository]; abbreviated to VES in the figures) \textit{FoTFL1} and the allele with a 2-bp deletion under the control of the cauliflower mosaic virus \textit{P35S} promoter in the LD accession Hawaii-4 (PI551572; abbreviated to H4 in the figures). Primary transgenic lines overexpressing the wild-type \textit{FoTFL1} allele from SD \textit{F. vesca} (\textit{P35S:FOtFL1}) remained vegetative for at least 10 months under inductive LD conditions, whereas plants overexpressing the mutated \textit{FoTFL1} (\textit{P35S:FOmtFL1}) flowered continuously (Fig. 1, A–C; Supplemental Fig. S3). We further analyzed the flowering time by growing the plants propagated from single-leafed runner cuttings under LD. Both nontransgenic Hawaii-4 and \textit{P35S:FOmtFL1} lines flowered early, whereas \textit{P35S:FOtFL1} lines and SD \textit{F. vesca} were still vegetative after about 10 weeks (Fig. 1D). These data indicate that \textit{FoTFL1} represses flowering under LD and that the 2-bp deletion in \textit{FoTFL1} leads to a nonfunctional \textit{FoTFL1} protein.

In order to further test the hypothesis that \textit{FoTFL1} is \textit{SFL}, we produced transgenic lines in the SD \textit{F. vesca} background and analyzed their flowering phenotypes in LD or after a strong flower induction treatment (4 weeks of SD at 11°C). \textit{FoTFL1} RNA interference (RNAi) silencing lines flowered almost at the same time in both treatments (Fig. 2, A and B). In contrast, nontransgenic control plants remained vegetative in LD and required SD induction treatment for flowering, whereas \textit{P35S:FOtFL1} lines stayed vegetative in both treatments (Fig. 2, A and B; Supplemental Fig. S4). In line with the role of Arabidopsis \textit{TFL1} in the transcriptional repression of floral meristem identity genes (Liljegren et al., 1999; Ratcliffe et al., 1999; Hanano and Goto, 2011), the expression of putative \textit{F. vesca} \textit{API1/FUL} homologs correlated negatively with the expression of functional \textit{FoTFL1} in the transgenic \textit{F. vesca} lines (Figs. 1, E–H, and 2, C and D). In conclusion, our results demonstrate that \textit{FoTFL1} is the major floral repressor \textit{SFL}, which prevents the activation of floral meristem identity genes and flowering under LD conditions in SD \textit{F. vesca}. Moreover, the absence of functional \textit{FoTFL1} reverses the photoperiodic requirements for flower induction.

We also expressed a \textit{P35S:FOtFL1} overexpression construct in the \textit{Arabidopsis} \textit{tfl1-2} mutant, which flowers early especially under SD and produces a determinate inflorescence with a few flowers (Shannon and Meeks-Wagner, 1991; Supplemental Fig. S5B). We analyzed three independent \textit{P35S:FOtFL1} lines in the \textit{tfl1-2} mutant background and showed that \textit{FoTFL1} fully complemented the inflorescence defects and the early-flowering phenotype of the mutant in both SDs and LDs (Supplemental Fig. S5), indicating that \textit{FoTFL1} is an ortholog of \textit{TFL1}.

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure1.png}
\caption{\textit{FoTFL1} is the floral repressor \textit{SFL}. A to C, Phenotypes of SD \textit{F. vesca} (A), LD accession Hawaii-4 (H4; B), and a transgenic line overexpressing functional \textit{FoTFL1} (\textit{P35S:FOtFL1}) in the H4 background (C). Plants were grown under LD for 15 weeks. WT, Wild type. D, Flowering time of SD \textit{F. vesca} (VES), H4, and transgenic lines overexpressing functional and nonfunctional \textit{FoTFL1} (\textit{FoTFL1-ox} and \textit{FromTFL1-ox}), respectively) under the control of the \textit{P35S} promoter. Plants were propagated from runner cuttings. \textit{n} = 3 (VES), \textit{n} = 7 (H4), \textit{n} = 8 (\textit{FOtFL1-ox}2), \textit{n} = 2 (\textit{FOmtFL1-ox}3), and \textit{n} = 6 (\textit{FOtFL1-ox}1 and \textit{FOtFL1-ox}2). E to H, Expression of \textit{FoTFL1} (E), \textit{FvAP1} (F), \textit{FvFUL1} (G), and \textit{FvFUL2} (H) in the primary shoot apices of H4 and transgenic lines grown in LDs. \textit{n} = 3 for H4 and \textit{n} = 1 for each transgenic line. Results for two independent transgenic lines are shown as biological replicates. nd, Not detected.
\end{figure}
FvTFL1 is Not Involved in the Photoperiodic Control of Vegetative Development

Under LDs, strawberry vegetative development typically involves the formation of a single leaf rosette and the differentiation of axillary buds to stolons (also called runners), whereas flower inductive conditions inhibit the emergence of stolons and activate the production of branch crowns (i.e., axillary leaf rosettes; Hytönen et al., 2004, 2009; Heide and Sønsteby, 2007). We analyzed the role of FvTFL1 in the control of vegetative development by using transgenic lines in an SD F. vesca background. Similar to wild-type plants, both FvTFL1 overexpression and RNAi lines continuously produced runners under LD, whereas 3 weeks of SD was enough to stop stolon formation in all lines (Fig. 3, A and B). After the cessation of stolon production in SD, the number of branch crowns started to increase in both wild-type plants and transgenic lines, whereas under LD no branch crowns were formed (Fig. 3, C and D). These results suggest that although the photoperiodic control of vegetative and floral development is tightly connected in strawberries (for review, see Hytönen and Elomaa, 2011), FvTFL1 does not affect vegetative development and is only involved in the photoperiodic control of floral initiation.

Photoperiodic Repression of FvTFL1 Expression Correlates with Floral Initiation

In Arabidopsis, TFL1 is weakly expressed in the lower part of the vegetative apical meristem, but its expression is activated in the inflorescence meristem.
after flower induction (Bradley et al., 1997; Ratcliffe et al., 1999). We analyzed the relative expression of *FvTFL1* normalized against the stable control gene *FvMSH2 MULTICOPY SUPPRESSOR OF IRA1* (Mouhu et al., 2009). *FvTFL1* mRNA was detected in all tissues tested (Supplemental Fig. S6A). Similar to *TFL1*, it was highly expressed in the shoot apex compared with leaves in *F. vesca* grown under LDs (Fig. 4A), but a more detailed analysis revealed a divergent expression pattern. According to in situ hybridization, under LDs *FvTFL1* mRNA was localized in the whole apical meristem, young leaf initials, and vascular tissues (Fig. 4, B and C). Furthermore, strong photoperiodic regulation of *FvTFL1* was detected in the shoot apices but not in the leaves of *SD* *F. vesca* seedlings (Fig. 4D; Supplemental Fig. S6B). Under LDs, *FvTFL1* was highly expressed in the shoot apex, whereas only weak expression was found in plants grown under SDs for 3 weeks (Fig. 4D). However, floral meristem identity genes were not yet activated in SD-grown *F. vesca* seedlings (Fig. 4D; Supplemental Fig. S6A). Similar to *FvTFL1*, strong photoperiodic regulation of *FvTFL1* was detected in the shoot apex compared with leaves in *SD* *F. vesca* seedlings under LDs (Fig. 4A), but a more detailed analysis revealed a divergent expression pattern. According to in situ hybridization, under LDs *FvTFL1* mRNA was localized in the whole apical meristem, young leaf initials, and vascular tissues (Fig. 4, B and C). Furthermore, strong photoperiodic regulation of *FvTFL1* was detected in the shoot apices but not in the leaves of *SD* *F. vesca* seedlings (Fig. 4D; Supplemental Fig. S6B). Under LDs, *FvTFL1* was highly expressed in the shoot apex, whereas only weak expression was found in plants grown under SDs for 3 weeks (Fig. 4D). However, floral meristem identity genes were not yet activated in SD-grown *F. vesca* at this time (Supplemental Fig. S7), indicating that the plants were still vegetative. Because several Norwegian *F. vesca* accessions have been shown to require more than 1 month of SD treatment for flower induction (Heide and Sonsteby, 2007), we grew our *SD* *F. vesca* plants in SDs for 45 d followed by flowering-time analysis under LDs. We observed flower buds about 1 month after the SD treatment in all plants of *SD F. vesca*, whereas all LD-grown plants remained vegetative (Fig. 4E).

In contrast to *SD F. vesca*, *LD-grown Hawaii-4* seedlings flowered rapidly; seedlings raised under SDs for 45 d flowered late, and the plants grown continuously under SDs remained vegetative for several months (Fig. 4, E and F), indicating that *Hawaii-4* is a LD plant. In contrast to seedlings, plants propagated from the stolons of *LD-grown mother plants* were day neutral and flowered continuously under both LDs and SDs (Supplemental Fig. S8). In *Hawaii-4*, *FvTFL1* mRNA expression was low in both photoperiods (Fig. 4D), most likely because of nonsense-mediated mRNA decay, a mechanism that prevents the accumulation of mRNA containing premature stop codons (Conti and Izaurralde, 2005). In association with flowering, the putative floral meristem identity genes *FvAPI* and *FvFUL1* were up-regulated in *Hawaii-4* seedlings grown under LDs (Supplemental Fig. S6). Taken together, our data indicate that the SD requirement for flower induction in *SD F. vesca* is based on the photoperiodic regulation of *FvTFL1*, and the lack of *FvTFL1* function leads to the rapid activation of flowering in *LD-grown Hawaii-4*.

**Regulation of the Perennial Growth Cycle by *FvTFL1***

We further analyzed the expression patterns of *FvTFL1* as well as *FvAP1* and *FvFUL1* in *SD F. vesca* plants grown under SDs followed by LDs and observed the flowering cycle of the plants. Our data showed that *FvTFL1* was gradually down-regulated in the apices of the primary shoots grown under SDs, with a concomitant up-regulation of floral meristem identity genes 4 to 6 weeks after the beginning of the SD treatment (Fig. 5, A–C). When SD-grown plants were returned to LDs after 45 SDs, high *FvTFL1* and low *FvAP1* and *FvFUL1* mRNA levels were again detected in the apices of axillary shoots that emerged after the end of the SD treatment. The observed gene expression patterns were clearly related to flowering, which started about 5 weeks after the end of the SD treatment and continued for about 1 month before declining again (Fig. 5D). In contrast to *SD F. vesca*, *Hawaii-4* continuously produced new inflorescences only under LDs (Fig. 5D). The observed expression pattern of *FvTFL1* in *SD F. vesca* indicates that the photoperiodic regulation of *FvTFL1* mRNA expression is crucial for cycling between vegetative and reproductive phases in *SD F. vesca*.

**FvTFL1 Promotes Flowering under LDs in the *F. vesca* LD Accession**

Functional analysis of FT in several plant species indicates its role as a floregen, a general floral activator (Turck et al., 2008; Turnbull, 2011). To establish the role of *FT* relative to that of *SFL*, we analyzed the
expression patterns of *F. vesca* *FT*-like genes. As demonstrated in Figure 6, A and B, *FvFT1* and *FvFT2* showed contrasting spatial expression patterns in SD *F. vesca*. *FvFT1* had the highest expression level in old leaves, and no expression or weak expression was observed in other tissues, whereas *FvFT2* was expressed almost exclusively in flower buds. The tissue-specific expression pattern of *FvFT1* follows the consensus that *FT* is a mobile signal originating in photosynthetically active leaves (Corbesier et al., 2007; Tamaki et al., 2007). Furthermore, genomic synteny is conserved.

Figure 5. Photoperiod regulates the perennial growth cycle through *FvTFL1*. A to C, Time-course analysis of *FvTFL1* (A), *FvAP1* (B), and *FvFUL1* (C) expression in the primary shoot apices of SD *F. vesca*. LD-grown plants were subjected to SDs for 6 weeks (wk 0–6) and returned to LDs for 5 weeks (+5 LD). Values indicate means ± se (*n*= 3). D, Cumulative number of inflorescences in clonally propagated plants (runner cuttings) of Hawaii-4 (H4) and SD *F. vesca* (VES). Plants were grown under flower-inductive (6 weeks of LD/SD starting from week 0 for H4/VES followed by LD) or noninductive (SD/LD for H4/VES) conditions.

Figure 6. Spatial and temporal expression of *F. vesca* *FT* genes. A and B, *FvFT1* (A) is expressed in old leaves and *FvFT2* (B) in flower buds in SD *F. vesca*. Plants were grown under LD after flower induction. Values indicate means ± sd (*n*= 2). YL, Young unopened leaf; OL, opened leaf; YP, young petiole; OP, petiole of the opened leaf; CR, crown (stem including meristems); RT, runner tip; FB, flower bud; FL, open flower; RO, root. C, Expression of *FvFT1* in leaves of SD- and LD-grown seedlings of *F. vesca* SD accession (VES) and LD accession Hawaii-4 (H4) at the three-leaf stage. Values indicate means ± sd (*n*= 4). D, Diurnal rhythm of *FvFT1* expression is present only under LDs in the SD *F. vesca*. Runner-propagated plants were either grown under LDs or transferred to SDs 8 d before sampling. Values indicate means ± se (*n*= 3). E, Time-course analysis of *FvFT1* in leaves of SD *F. vesca* plants. Plants were moved to flower-inductive SDs at week 0. Values indicate means ± se (*n*= 3).
DISCUSSION

Early physiological studies indicated the existence of photoperiodically controlled flowering activating and inhibitory signals in strawberries (Hartmann, 1947; Guttridge, 1959; Vince-Prue and Guttridge, 1973). Our results confirm that both signals are present in F. vesca and that SFL is a major switch controlling photoperiodic responses. We provide functional evidence that SFL encodes a Fragaria homolog of the floral repressor TFL1 and demonstrate that FoTFL1 is photoperiodically regulated. Our results suggest that in the SD accessions of F. vesca, down-regulation of FoTFL1 under SD allows flower induction to occur only in the autumn, which leads to seasonal flowering the next spring. In contrast, a mutation in FoTFL1 causes rapid FT-dependent LD flowering and continuous initiation of inflorescences in the perpetual flowering LD accession.

**TFL1 Homologs Are Major Floral Repressors in Rosaceae**

A previous study by Iwata et al. (2012) and our data show that perpetual flowering is associated with a 2-bp deletion in F. vesca TFL1 homolog (Supplemental Figs. S1 and S2). We conducted functional analysis of FoTFL1 by overexpression and RNAi approaches. FoTFL1 strongly represses flowering in F. vesca, because the introduction of a P35S:FoTFL1 construct containing functional FoTFL1 into a perpetual flowering LD accession prevented the activation of putative floral meristem identity genes (FoAP1/FUL) and flowering initiation under LD. Furthermore, overexpression of the mutated version of the gene did not change the flowering phenotype, indicating that it does not encode a functional protein. As constitutive overexpression of TFL1 homologs from different species has been shown to cause pleiotropic effects (Bohlenius et al., 2006; Imamura et al., 2011), we used RNAi silencing of FoTFL1 in SD F. vesca to provide further evidence that FoTFL1 is SFL. Silencing of FoTFL1 in SD F. vesca removed the SD requirement for flower induction and changed the plants to LD flowering. As TFL1 homologs prevent LD flowering and cause the seasonal flowering habit in F. vesca (Figs. 1 and 2), control the
length of the juvenile phase in apple (Kotoda et al., 2006), and may also cause seasonal flowering in roses (Iwata et al., 2012), they are possibly major floral repressors and regulators of the perennial growth cycle in the Rosaceae family.

Earlier studies have shown that photoperiod acts in an opposite manner to control flower initiation and vegetative reproduction through stolons (Hytönen et al., 2004; Heide and Sonsteby, 2007). The finding that neither overexpression nor silencing of FoTFL1 affected the photoperiodic control of stolon/branch crown formation from axillary buds showed that FoTFL1 is not directly involved in the regulation of vegetative development of the strawberry shoot. This is consistent with the genetic evidence that two separate single loci, SFL and RUNNERING LOCUS, respectively, control flowering habit and the formation of stolons in F. vesca (Brown and Wareing, 1965). However, FoTFL1 may indirectly affect vegetative growth, because the development of a terminal inflorescence promotes the outgrowth of the uppermost axillary buds as branch crowns by reducing apical dominance (Arney, 1953).

### Regulation of FoTFL1 mRNA Expression Controls Photoperiodic Flowering and Seasonal Growth Cycles

In Arabidopsis, TFL1 is developmentally regulated (Bradley et al., 1997; Ratcliffe et al., 1999), and to our knowledge, no photoperiodic control of TFL1 homologs has been previously reported. Our results suggest that the photoperiodic control of FoTFL1 mRNA level in the shoot apex is a primary mechanism to control photoperiodic flowering in SD F. vesca. In SD-grown plants, FoTFL1 was strongly down-regulated before the activation of floral meristem identity genes and flower initiation. The functionality of transcriptional regulation was supported by the phenotype of FoTFL1 RNAi/ir silenced lines in the SD F. vesca background. In two independent lines, the reduction of FoTFL1 mRNA levels was enough to induce flowering under noninductive LD conditions. In contrast, none of the P35S: FoTFL1 lines flowered under flower-inductive conditions in either SD or LD F. vesca backgrounds, indicating that plants cannot overcome constitutive overexpression of FoTFL1 mRNA. In conclusion, we have identified FoTFL1 as a component of the perennial photoperiodic pathway in F. vesca. We propose that down-regulation of FoTFL1 mRNA expression in the shoot apex of SD F. vesca under SD is required for flower induction and the activation of FoAP1/FUL genes. In contrast, the lack of functional FoTFL1 in LD accessions reverses the photoperiodic requirement for flowering.

The finding that nonfunctional FoTFL1 causes perpetual flowering of LD accessions suggests that in the SD F. vesca, the regulation of FoTFL1 contributes to the cycling of vegetative and reproductive phases, and our gene expression results support this hypothesis. Whereas FoTFL1 was down-regulated and FoAP1/FUL was up-regulated in the apex of the primary shoot under SD, subsequent LD conditions restored high FoTFL1 and low FoAP1/FUL mRNA levels in the apices of new vegetative axillary shoots. Earlier studies have shown that strawberries are able to initiate flowers at the apex of the primary shoot and axillary shoots, which have reached competence to flower (Arney, 1953; Hytönen et al., 2004; Hytönen and Elomaa, 2011). Therefore, we hypothesize that the down-regulation of FoTFL1 under SD in the autumn allows flower induction to occur in the apex of the primary shoot and older axillary shoots. However, its up-regulation in the LDs of spring is crucial for perennialism, because it prevents further floral initiation in the newly emerged axillary shoots until SD conditions return. Our results on FoTFL1 and earlier findings that seasonal changes in the expression of two Populus FT-like genes regulate the perennial growth cycle in Populus (Hsu et al., 2011) highlight the importance of CETs proteins in the control of perennialism. Also, in A. alpina, TFL1 contributes to perennialism by regulating the age-dependent sensitivity of meristems to vernalization; but transient silencing of the FLC ortholog, PEP1, by vernalization has a major role in the cycling between vegetative and generative phases in this species (Wang et al., 2009, 2011).

In contrast to FoTFL1, which is broadly expressed in the shoot apex (Fig. 4B), Arabidopsis TFL1 is weakly expressed in the axillary meristems and lower part of the apical meristem during the vegetative phase and is activated in the inflorescence meristem (Bradley et al., 1997; Ratcliffe et al., 1999). This intriguing difference is probably associated with differences in inflorescence development. In Arabidopsis, TFL1 is activated in the inflorescence meristem in order to allow indeterminate flowering in the apex of the primary shoot under SD, subsequent LD conditions restored high FoTFL1 and low FoAP1/FUL mRNA levels in the apices of new vegetative axillary shoots. Earlier studies have shown that strawberries are able to initiate flowers at the apex of the primary shoot and axillary shoots, which have reached competence to flower (Arney, 1953; Hytönen et al., 2004; Hytönen and Elomaa, 2011). Therefore, we hypothesize that the down-regulation of FoTFL1 under SD in the autumn allows flower induction to occur in the apex of the primary shoot and older axillary shoots. However, its up-regulation in the LDs of spring is crucial for perennialism, because it prevents further floral initiation in the newly emerged axillary shoots until SD conditions return. Our results on FoTFL1 and earlier findings that seasonal changes in the expression of two Populus FT-like genes regulate the perennial growth cycle in Populus (Hsu et al., 2011) highlight the importance of CETs proteins in the control of perennialism. Also, in A. alpina, TFL1 contributes to perennialism by regulating the age-dependent sensitivity of meristems to vernalization; but transient silencing of the FLC ortholog, PEP1, by vernalization has a major role in the cycling between vegetative and generative phases in this species (Wang et al., 2009, 2011).
growth of the raceme (Bradley et al., 1997; Ratcliffe et al., 1999), whereas the production of a cymose inflorescence with a terminal flower in strawberry (Jahn and Dana, 1970) would be expected to require the silencing of FvTFL1 in the apical meristem.

**FvTFL1 Overcomes the Function of the LD-Specific Floral Activator FvFT1**

The CO/FT module controls photoperiodic flowering in both annual and perennial plants (Suárez-López et al., 2001; Hayama et al., 2003; Böhlenius et al., 2006), and FT is thought to be a universal flowering signal (Turck et al., 2008; Turnbull, 2011; Wigge, 2011). In concordance with studies in other plants, we observed that the expression of FvFT1, the likely ortholog of FT, correlated with flowering in the LD accession Hawaii-4. Furthermore, strong reduction in the expression of FvAP1/FUL genes in FvFT1 RNAi plants as well as their late-flowering phenotype suggested that FvFT1 controls flower induction in *F. vesca* LD accessions (Fig. 7). However, in the SD *F. vesca*, FvFT1 was highly expressed in noninductive LD, which contrasts with findings in other SD plants (Hayama et al., 2003, 2007; Kong et al., 2010). Our data suggest that in this genotype, FvTFL1 expression in the shoot apex may overcome the function of FvFT1 as an activator of flowering. FT and TFL1 are closely related proteins whose opposite function is caused by differences in the external loop (Ahn et al., 2006). A recent study showed that FT forms a so-called “florigen activation complex” together with FD and 14-3-3 proteins (Taoka et al., 2011). Because TFL1 homologs in different species can also bind FD and 14-3-3 (Pnueli et al., 2001; Purwestri et al., 2009; Hanano and Goto, 2011), FvTFL1 may inhibit flowering by competing for binding partners with FvFT1. This is consistent with the regulation of flowering in tomato (*Solanum lycopersicum*), in which the balance between the expression of FT and TFL1 homologs controls flowering (Salit et al., 2009). However, in SD *F. vesca*, both FvFT1 and FvTFL1 are downregulated under flower-inductive conditions. Therefore, we hypothesize that flower induction in SD *F. vesca* takes place via an FvFT1-independent mechanism, whereas in Hawaii-4, FvFT1 functions as a LD-specific floral promoter in the absence of functional FvTFL1. On the other hand, we cannot exclude the possibility that FT is involved in the LD activation of FvTFL1, which is photoperiodically regulated only in the shoot apex (Fig. 4D; Supplemental Fig. S6B). As the perception of photoperiod is thought to occur in the leaves (An et al., 2004; Corbesier et al., 2007), a systemic signal may be required for the photoperiodic control of FvTFL1. FT is a good candidate for such a signal, because it is emerging as a general photoperiodic signaling molecule in diverse plant species (Wigge, 2011). Furthermore, Hecht et al. (2011) have shown evidence that leaf-expressed FT may mediate the photoperiodic control of another CETS family gene in the pea (*Pisum sativum*) shoot apex. Further studies are needed to resolve the function of FvFT1 in SD *F. vesca* and to establish which mechanism activates floral meristem identity genes after the down-regulation of FvTFL1.

**A Model of the Regulation of Flowering and Growth Cycles by *F. vesca* TFL1 and FT Homologs**

Based on our studies, we propose a model (Fig. 8) in which the flowering of SD *F. vesca* accessions is repressed through LD-activated FvTFL1 expression, which overrides the floral activator function of FvFT1. When photoperiods drop below a critical level during autumn, FvTFL1 mRNA levels decrease, floral meristem identity genes are up-regulated, probably through an FvFT1-independent pathway, and floral development begins. In the following spring and summer, FvTFL1 is activated again in new axillary shoots and keeps them vegetative until inductive conditions return. In contrast, the absence of functional FvTFL1 in LD accessions leads to FvFT1-mediated flower induction and continuous flowering under LDs. More studies are needed to explore how this model generated in the diploid *Fragaria* can be translated to the more complex octoploid cultivated strawberry, which shows similarities in the environmental control of flowering with *F. vesca* (Heide, 1977; Heide and Sonstebey, 2007; Sonstebey and Heide, 2007, 2008; Bradford et al., 2010).

The timing and duration of flowering season are traits of importance to crop production because they are major factors contributing to yield potential and the length of the harvest season. Natural variation in TFL1 homologs has been important for the domestication and crop improvement of several annual crops (Turnbull, 2011), and our study shows the importance of TFL1 in the perennial context. As TFL1 homologs exist in all angiosperms (Karlgren et al., 2011) and their function as floral repressors is conserved between plant families (Shannon and Meeks-Wagner, 1991; Foucher et al., 2003; Kotoda et al., 2006; Mohamed et al., 2010; Imamura et al., 2011), our findings are of general significance for understanding the regulation of flowering- and perennial-specific traits in a wide range of economically important crops.

**MATERIALS AND METHODS**

**Plant Material**

*Fragaria* vesca accession (PI551792) and *F. vesca* f. semperflorens Hawaii-4 (PI551572) were obtained from the National Clonal Germplasm Repository. The Arabidopsis (*Arabidopsis thaliana*) *fht-2* mutant (Nottingham Arabidopsis Stock Centre identifier N3091) in the Landsberg erecta background was used in the genetic complementation test.

**Growth Conditions and Phenotyping**

*F. vesca* plants were grown in greenhouse rooms equipped with darkening curtains. Standard LD growth conditions were 18 h of high-pressure sodium...
light (approximately 150 μmol m⁻² s⁻¹) at 18°C. In photoperiodic experiments at 18°C, 12 h of high-pressure sodium light (approximately 150 μmol m⁻² s⁻¹) was provided for all plants (SD), and under LD, 6 h of day extension was given as low-intensity incandescent light (approximately 8 μmol m⁻² s⁻¹).

Arabidopsis plants were grown under LD (16 h of light at 22°C) or SD (10 h of light at 22°C) in a growth chamber. Light was provided by high-pressure sodium lamps (approximately 180 μmol m⁻² s⁻¹). Flowering time was measured either as the number of leaves initiated before the first inflorescence or the number of days from sowing to the first open flower.

Statistics

The χ² goodness-of-fit test was performed on the flowering data from the F2 and BCI mapping populations. The critical value at α = 0.05 was 3.841 for all χ² tests.

Genotyping and Mapping

DNA extraction was done using a previously described protocol (Albani et al., 2004). The markers used for mapping are listed in Supplemental Table S1. Linkage maps were constructed as described earlier (Sargent et al., 2004). The F2 mapping population was characterized for FoTFL1 using TFL1-6FAM primers (Supplemental Table S1).

Identification of Single-Nucleotide Polymorphism Markers by Genome Resequencing

DNA of SD F. vesca (PI551792) was extracted (Albani et al., 2004) and treated with RNase A. A genomic DNA library was generated using the Genomic DNA Sample Prep Kit (Illumina), and 30-bp reads were sequenced using the Illumina GAIIx flow cell. Image analysis and base calling were done by OLB version 1.6.0 software and sequence alignment by CASAVA GERALD version 1.6.0. A default chastity threshold of 0.6 was used for purity filtering. All sequence reads with two or fewer mismatches were aligned with 801,610 bp of the genomic reference sequence of Hawaii-4 between markers V8p98 and V8p278 (Supplemental Fig. S1) using Bowtie version 0.12.2 and Readaligner version 2010_4rc2 software, and the resulting .sam files were viewed by Tablet version 1.10.05.21 in order to find single-nucleotide polymorphisms. Resequencing and related bioinformatics were carried out at Biomedicum Genomics, University of Helsinki.

Expression Analysis

Total RNA was extracted, cDNA was synthesized, and real-time PCR was performed as described earlier (Mouhu et al., 2009). FoMSI1 was used as a stable control gene (Mouhu et al., 2009) for normalization. The number of biological replicates is indicated in each figure legend. Three technical replicates were performed for each sample. Real-time PCR primers are listed in Supplemental Table S1.

Plasmid Constructs

Plasmid constructs for overexpressing FoTFL1 or FvTFL1 were created according to Gateway Technology with Clonase II (Invitrogen). For overexpression of TFL1, the primers used to amplify cDNA from SD F. vesca (PI551792) and sfl mutant Baran Solemacher (PI551507) were 5'-AAAAAGCAGGCTCTG-TACACACCCCTTCCTCCTCTCTCCTCTC-3' (attB1) and 5'-AGAAAGCTGGGTCC-TCCCTGCAAGTGCCCTTA-3' (attB2). For the double-stranded FoTFL1 RNAi construct, the fragment was amplified from SD F. vesca cDNA with primers 5'-AAAAAGCAGGCTCTGTTGGCCTTGCCACTCTCG-3' (attB1) and 5'-AGAGCAGTCGGCTTCGATGCTCGCCAAACC-3' (attB2). For creating FoFT1 RNAi constructs, cDNA from SD F. vesca (PI551792) was amplified with primers 5'-AAAAAGCAGGCTCTGTTGGCCTTGCCACTCTCG-3' (attB1) and 5'-AGAGCAGTCGGCTTCGATGCTCGCCAAACC-3' (attB2). The destination vectors were p7WG2D for overexpression and PKC7GWIG2D(B) for RNAi (Karimi et al., 2003).

Transformation

Arabidopsis plants were transformed with Agrobacterium tumefaciens strain GV3101 by the floral dip method (Zhang et al., 2006). Strawberry transformations were performed as described earlier (Oosumi et al., 2006). Plants of the T1 or T0 generation were analyzed in Arabidopsis and F. vesca, respectively.

In Situ Hybridization

In situ hybridization was performed on the apex of the main shoot as described previously (Kurokura et al., 2006). The probe template fragment was amplified by reverse transcription-PCR using the primers shown in Supplemental Table S1 and cloned into pDrive cloning vector (Qiagen). Sense and antisense probes were synthesized from T7 or SP6 promoters.

Sequence data from this article can be found in the GenBank data library under accession numbers JN172097 and JN172098.

Supplemental Data

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

Supplemental Figure S1. Fine-mapping of SFL.

Supplemental Figure S2. Deletion in FoTFL1 cosegregates with SFL in the SD F. vesca × F. vesca f. sensperfoliens BCI population.

Supplemental Figure S3. Phenotypes of 10-month-old LD-grown Hawaii-4 and P35S: FoTFL1 lines.

Supplemental Figure S4. FoTFL1 expression in P35S: FoTFL1 transgenic lines.

Supplemental Figure S5. FoTFL1 complements the Arabidopsis tfl1-2 mutant phenotype.

Supplemental Figure S6. Expression analysis of FoTFL1.

Supplemental Figure S7. Effect of photoperiod on the expression of floral meristem identity genes.

Supplemental Figure S8. F. vesca LD accession Hawaii-4 flowers continuously after LD induction in both SD and LD conditions.

Supplemental Figure S9. Expression of FoFT1 in LD-grown P35S: FoFT1 RNAi lines.

Supplemental Table S1. Primers used in this study.

ACKNOWLEDGMENTS

We thank L. Sareilainen for technical assistance in genetic transformation and J.P.T. Valkonen, O. Junttila, and M. Mattsson for critical reading of the manuscript.

Received March 5, 2012; accepted May 3, 2012; published May 7, 2012.

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FeTFL1 Controls Photoperiodic Flowering

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Koskela et al.


