**TERMINAL FLOWER1** is a breeding target for a novel everbearing trait and tailored flowering responses in cultivated strawberry (*Fragaria × ananassa* Duch.)

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**Summary**

The effects of daylength and temperature on flowering of the cultivated octoploid strawberry (*Fragaria × ananassa* Duch.) have been studied extensively at the physiological level, but information on the molecular pathways controlling flowering in the species is scarce. The flowering pathway has been studied at the molecular level in the diploid short-day woodland strawberry (F. vesca L.), in which the *FLOWERING LOCUS T1* (*FvFT1*)–*SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*FvSOC1*)–*TERMINAL FLOWER1* (*FvTFL1*) pathway is essential for the correct timing of flowering. In this work, we show by transgenic approach that the silencing of the floral repressor *FaTFL1* in the octoploid short-day cultivar ‘Elsanta’ is sufficient to induce perpetual flowering under long days without direct changes in vegetative reproduction. We also demonstrate that although the genes *FaFT1* and *FaSOC1* show similar expression patterns in different cultivars, the regulation of *FaTFL1* varies widely from cultivar to cultivar and is correlated with floral induction, indicating that the transcription of *FaTFL1* occurs at least partially independently of the *FaFT1–FaSOC1* module. Our results indicate that changing the expression patterns of *FaTFL1* through biotechnological or conventional breeding approaches could result in strawberries with specific flowering and runnering characteristics including new types of everbearing cultivars.

**Introduction**

The cultivated strawberry (*Fragaria × ananassa* Duch.) is an agronomically important crop species grown under a wide range of environmental conditions. One aim in strawberry breeding programmes is to extend the cropping season. This can be achieved by breeding for early and late cultivars or everbearing cultivars, that is cultivars that flower perpetually throughout the growing season. Understanding the genetic mechanisms controlling flower induction in strawberry could help breeders in developing new cultivars with the desired flowering characteristics.

The physiology of flowering in cultivated strawberry (*Fragaria × ananassa* Duch.) has been extensively studied for nearly a century. Already the early work with the so-called June-bearing strawberries revealed that these are facultative short-day (SD) plants, with floral induction taking place in SDs at temperatures above 15°C, behaving in day-neutral manner at lower temperatures and flowering only poorly or not at all at high (>24°C) temperatures (Darrow and Waldo, 1934). However, different cultivars show variable environmental responses (Bradford et al., 2010; Heide, 1977), and some cultivars have an obligatory SD requirement for flower induction (Sønsteby and Heide, 2006). Environmental factors regulate also vegetative development; photoperiod and temperature control the fate of axillary meristems, which develop either into stolons (runners) or axillary leaf rosettes called branch crowns. Generally, branch crown development in SD cultivars is enhanced by environmental conditions favouring floral induction, and stolon formation is promoted by long days (LDs) and high temperature (Heide, 1977; Hytönen et al., 2004; Konsin et al., 2001; Mouhu et al., 2013).

Some strawberry cultivars flower perpetually and do not require SDs or low temperature for flowering. The flowering behaviour of these cultivars has been under debate, and this group has sometimes been nominated everbearers, day-neutrals or LD plants, depending on the cultivars and experimental conditions used. Recently, it was convincingly shown that, in terms of flower induction, these strawberries are indeed obligatorily LD plants at high temperature (27°C), quantitative LD plants at intermediate temperature and truly day-neutrals only at cool (9°C) temperature (Sønsteby and Heide, 2007). As in SD strawberries, the flowering response in LD strawberries is

dependent on the interaction of photoperiod and temperature in a cultivar-specific manner, which may be the reason for confusion found in the literature. The vegetative responses in LD strawberries are more varying; stolon formation is promoted by high temperature and either by SDs or LDs, depending on the cultivar (Sønsteby and Heide, 2007).

Although flowering in the cultivated strawberry has been studied in detail at the physiological level, only few reports on the genetics of flowering in this species exist. Studies aimed at elucidating the genetic basis of the everbearing character via genetic mapping have reached varying results. The earliest reports suggested that the trait is controlled by a single dominant locus (Ahmadi et al., 1990; Sugimoto et al., 2005), while later it was proposed that the everbearing character is controlled by several QTLs (Weebadde et al., 2008). Two recent mapping studies by Gaston et al. (2013) and Castro et al. (2015) have identified a major QTL controlling both the everbearing and running traits, located on LGIVb-f of the cultivated strawberry. Unfortunately, these two studies used different markers and mapping populations and it is therefore not possible to determine whether the QTLs are the same. Moreover, these studies did not suggest candidate genes for the everbearing trait, and there are no studies confirming the function of any flowering-related gene in the cultivated strawberry.

As the genetics in the octoploid cultivated strawberry are notoriously complex, the closely related diploid wild strawberry F. vesca L. has been used in functional genetic studies for elucidating the molecular pathways controlling flowering in Fragaria. In F. vesca, both SD and LD genotypes exist and the flowering responses of these two genotypes are similar to those observed in the cultivated strawberry. SD genotypes initiate flowers photoperiodically at low temperatures, are obligatory SD plants at temperatures between 13 and 20°C and are inhibited to flower at higher temperatures (Heide and Sønsteby, 2007; Rantanen et al., 2015). In contrast, flowering in the LD genotypes is promoted by LDs and temperature above 18°C, and delayed by SDs at cool (11°C) and high (27°C) temperatures (Mouhu et al., 2009; Sønsteby and Heide, 2008). The comparable physiological responses in F. vesca and the cultivated strawberry suggest that the principles of the molecular control of flowering in the two strawberry species are similar, and therefore, the use of the diploid F. vesca as a model species is plausible.

The genetics of the everbearing trait in F. vesca was studied already in the 1960s by Brown and Waring (1965), who found that the everbearing character was caused by a recessive single gene termed SEASONAL FLOWERING LOCUS (SFL). More recently, it was shown by Koskela et al. (2012) that the recessive everbearing trait is caused by a lack-of-function mutation in the coding sequence of a F. vesca homologue of TERMINAL FLOWER 1 (FvTFL1), a gene that represses flowering in a range of species, including Arabidopsis (Ohshima et al., 1997), apple (Malus × domestica; Kotoda et al., 2006; Flachowsky et al., 2012), roses (Rosa sp.; Iwata et al., 2012) and maize (Ze a mays; Danilevskaya et al., 2010). It was further shown that the expression of FvTFL1 is controlled by the photoperiodic pathway and is activated under LDs by F. vesca SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (FvSOC1; Mouhu et al., 2013). FvSOC1 is in turn promoted by Fragaria FLOWERING LOCUS T1 (FvFT1), which is expressed in leaf tissues exclusively under LDs (Koskela et al., 2012; Rantanen et al., 2014). Also an F. vesca homologue of CONSTANS (FvCO) has been identified, but its role in the photoperiodic regulation of FvFT1 is unclear (Rantanen et al., 2014). The FvFT1–FvSOC1–FvTFL1 pathway is activated under LDs in both SD and LD genotypes but leads to different flowering responses. In SD genotypes, LDs up-regulate the expression of functional FvTFL1, which overrides the floral activator function of FvFT1 and flowering is allowed only under SDs when FvFT1 is down-regulated. In contrast, the absence of functional FvTFL1 leads to FvFT1-mediated floral induction in LD genotypes grown under LDs (Koskela et al., 2012; Rantanen et al., 2014). In both genotypes, the floral meristem identity genes F. vesca APETALA1 (FvAP1) and FRUITFULL (FvFUL1) are up-regulated at the time of floral induction (Koskela et al., 2012; Mouhu et al., 2009, 2013).

The temperature-controlled flowering pathway in the diploid strawberry has been studied recently by Rantanen et al. (2015), who showed that the effects of photoperiodic and temperature pathways converge at the regulation of FvTFL1. In F. vesca, cool temperature (<13°C) down-regulates FvTFL1 independently of photoperiod and SDs are required for floral induction only at intermediate temperatures (14–18°C). At high temperature (23°C), flowering is prevented because FvTFL1 is up-regulated independently of the photoperiodic pathway by an unknown activator.

In this work, we take advantage of the knowledge on molecular pathways regulating flowering in the diploid F. vesca and use functional and gene expression analysis to explore whether the pathways function similarly in the octoploid cultivated strawberry. We provide functional evidence of the role of F. × ananassa homologue of TFL1 as a floral repressor and show that FaTFL1 regulation is associated with floral induction under a range of environmental conditions. Our results suggest that FaFT1 and FaSOC1 mRNA levels in the cultivated strawberry respond similarly to changes in environmental conditions as the photoperiodic pathway genes FvFT1 and FvSOC1 in the diploid F. vesca, but their expression does not always correlate with the FvTFL1 mRNA levels.

**Results**

**Silencing FaTFL1 in ‘Elsanta’**

It has been demonstrated in F. vesca that the silencing of FvTFL1 eliminates the SD requirement for flowering (Koskela et al., 2012). To elucidate whether TFL1 is a floral repressor also in the cultivated strawberry, we transformed the short-day cultivar ‘Elsanta’ using TFL1-RNAi construct described by Koskela et al. (2012). The first transformation experiments resulted in several putative transgenic shoots originating from independent transformation events. Two shoots were positively tested for the presence of transgenic DNA sequences by PCR (Figure S1). Both shoots were clonally propagated to obtain the transgenic lines F138 and F139. These lines were found to contain at least one copy of the transferred T-DNA by Southern hybridization (Figure S1).

Silencing of FaTFL1 in the two transgenic lines was further confirmed by quantitative RT-PCR. To reliably compare the expression level of FaTFL1 between the wild type and the transgenic plants, we needed to obtain vegetative apical tissues. As the transgenic plants flowered very early in LDs, we decided to use young runner apices without visible flower buds. In runner apices of LD-grown F138 and F139, FaTFL1 expression was strongly down-regulated, whereas FaTFL1 mRNA was expressed at a high level in wild-type ‘Elsanta’ (Figure 1a). Expression of the floral integrator gene FaSOC1 putatively upstream of FaTFL1 on
Function of FaTFL1 in the cultivated strawberry

The photoperiodic flowering pathway was not significantly affected in the transgenic lines (Figure 1b).

The genome of *F. vesca*, a putative diploid progenitor species of *F. × ananassa*, harbours seven genes belonging to the same PEB (phosphatidyl ethanolamine-binding) protein family as *FvTFL1* (Mimida *et al.*, 2012). Therefore, it was of interest to confirm that the *TFL1*-RNAi construct introduced into ‘Elsanta’ would silence only the transcripts orthologous to *TFL1*. We searched the Kazusa DNA Research Institute’s database for *F. × ananassa* coding sequences and identified three coding sequences with similarity to the *TFL1*-RNAi fragment. Phylogenetic analysis revealed that these sequences were the *F. × ananassa* homologues of *TFL1*, *CENTRORADIALIS1* (CEN1) and CEN2 (Figure S2a). As FaCEN2 showed nearly 80% identity with the *TFL1*-RNAi fragment (Figure S2a), we decided to examine whether the *TFL1*-RNAi construct would silence also FaCEN2. However, we were unable to detect notable changes in the expression level of FaCEN2 between the wild-type ‘Elsanta’ and the transgenic lines (Figure S2b).

Flowering phenotypes of the transgenic lines were assessed under LDs at 18°C. The transgenic line F138 started flowering 78.4 ± 5.9 days after moving the plants to the greenhouse, whereas ‘Elsanta’ remained vegetative in LDs. Some of the F139 plants started flowering already *in vitro* (Figure S3a). When the line F139 was grown under greenhouse conditions (LD, 18°C), flowering was observed after approximately 125 days. No floral buds were observed in the wild-type ‘Elsanta’ plants after 150 days (Figure S3b).

As null mutation of *FvTFL1* causes continuous flowering in the woodland strawberry (Koskela *et al.*, 2012), we tested the seasonality of flowering in ‘Elsanta’ *TFL1*-RNAi line F138 by subjecting the plants to the artificial seasonal cycle in the greenhouse (see Experimental procedures). After SD and chilling periods, 60% of F138 plants flowered on 2nd of February, within 26 days of greenhouse forcing, whereas wild-type ‘Elsanta’ started to flower 2 weeks later (Figure 1c and e). ‘Elsanta’ flowered only for a short period and all plants produced only 1 or 2 inflorescences. F138, however, continuously produced new inflorescences until the end of the experiment showing that the silencing of cultivated strawberry *TFL1* homologue causes continuous flowering in ‘Elsanta’. Both ‘Elsanta’ and F138 produced equal number of runners by the end of the experiment, although the first runners were observed 1 week earlier in ‘Elsanta’ than in F138 (Figure 1d).

Photoperiodic responses in cultivars ‘Alaska Pioneer’, ‘Honeoye’ and ‘Polka’

To explore the photoperiodic regulation of key flowering time genes, three cultivars, ‘Honeoye’, ‘Polka’, and ‘Alaska Pioneer’, were selected based on their reportedly different flowering times. According to USDA National Plant Germplasm System, ‘Honeoye’ is considered an early mid-season cultivar. Under Nordic conditions, ‘Honeoye’ is an early cultivar flowering approximately 1 week earlier than the mid-season cultivar ‘Polka’ (Hyttönen and Richterich, personal communication). ‘Alaska Pioneer’ was selected because it has been classified as an everbearing cultivar by USDA, but in our hands it requires SDs for flowering. Photoperiodic responses of these cultivars were studied in plants exposed to SDs (12 h of light) or LDs (18 h of light) at 18°C for 6 weeks. No flowering was observed in LDs, and in SDs, ‘Honeoye’ was the first to flower, followed by ‘Alaska Pioneer’ and ‘Polka’ (Table 1).

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Photoperiod</th>
<th>Flowering plants</th>
<th>Days to anthesis ± SD</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>(h)</td>
<td>N (%)</td>
<td></td>
</tr>
<tr>
<td>Honeoye</td>
<td>SD 12</td>
<td>17 100</td>
<td>54.4 ± 3.5</td>
</tr>
<tr>
<td></td>
<td>LD 18</td>
<td>10 0</td>
<td>&gt;80</td>
</tr>
<tr>
<td>Alaska Pioneer</td>
<td>SD 12</td>
<td>12 100</td>
<td>65.2 ± 5.0</td>
</tr>
<tr>
<td></td>
<td>LD 18</td>
<td>12 0</td>
<td>&gt;80</td>
</tr>
<tr>
<td>Polka</td>
<td>SD 12</td>
<td>14 85.7</td>
<td>65.8 ± 6.8</td>
</tr>
<tr>
<td></td>
<td>LD 18</td>
<td>10 0</td>
<td>&gt;80</td>
</tr>
</tbody>
</table>

Table 1 Flowering of short-day (SD) and long-day (LD) grown plants of three strawberry cultivars. The plants were exposed to the specified daylengths at 18°C for 6 weeks and observed for flowering in LDs for the following 10 weeks. Days to anthesis was calculated from the end of daylength treatments. N = number of plants.
FaSOC1 was expressed at a higher level in LDs than SDs (Figure S4b). Under SDs, there was no clear rhythm in FaSOC1 expression, but in LDs the gene was slightly up-regulated in the morning. In the shoot apex, on a longer time span, FaSOC1 was down-regulated after 2 weeks in SDs and remained low under short-day conditions in all three cultivars (Figure 2a–c). When the plants were returned to LDs, expression levels of FaSOC1 were restored to almost the same levels as in the LD-treated plants.

FaTFL1 expression showed more diverse patterns in the three cultivars analysed. In the beginning of the experiment, FaTFL1 was expressed very strongly in the shoot apex tissues of ‘Honeoye’. In this cultivar, the floral repressor FaTFL1 was gradually down-regulated in both LDs and SDs, although down-regulation occurred at a faster rate in SDs (Figure 2d). Similar trends were found also in ‘Alaska Pioneer’, although the reduction in the FaTFL1 mRNA level under LD was weaker than in ‘Honeoye’ (Figure 2e). Only the cultivar ‘Polka’ showed a constant level of FaTFL1 expression in LDs and the down-regulation of the gene in SDs (Figure 2f). The floral meristem identity gene FaFUL1 was up-regulated only in the shoot apices of SD-treated plants 4 weeks after the end of the SD treatment, concordant with the flowering observations (Figure 2g–i; Table 1).

Interaction of photoperiod and temperature in ‘Elsanta’ and ‘Glima’

Next, we were interested in determining how the interaction of photoperiod and temperature affects flowering and the expression of flowering-related genes in the cultivated strawberry. The cultivars ‘Elsanta’ and ‘Glima’ were selected for these treatments based on their different flowering responses; ‘Glima’ flowering readily in LD at temperatures below 21 °C (Heide, 1977), whereas ‘Elsanta’ is an obligatory SD plant and does not initiate flowers in LDs even at temperatures as low as 9 °C (Sønsteby and Heide, 2006). The experimental plants were subjected to SDs and LDs at three temperatures for 5 weeks. No flowering

**Figure 2** Expression of FaSOC1, FaTFL1 and FaFUL1 in the shoot apices of three strawberry cultivars. (a–c) Expression of FaSOC1; (d–f) expression of FaTFL1; (g–i) expression of FaFUL1 in three strawberry cultivars grown in SDs or LDs. (a), (d) and (g) ‘Honeoye’; (b), (e) and (h) ‘Alaska Pioneer’; (c), (f), (i) ‘Polka’. Grey bars present SD (short day) and light bars LDs (long days). W = weeks under photoperiodic treatments; 6W + 4W LD = 6 weeks under photoperiodic treatments followed by 4 weeks under LDs. Error bars indicate ± SD (n = 3).
Flowering of ‘Elsanta’ and ‘Glima’ grown under short days (SDs) or long days (LDs) at different temperatures. The plants were treated under different photoperiods and temperatures for 35 days, after which they were moved to greenhouse (LD, 20°C) for flowering observations. Days to anthesis was calculated from the end of the treatments. n = 12.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Photoperiod (h)</th>
<th>Temperature (°C)</th>
<th>Flowering plants (%)</th>
<th>Days to anthesis</th>
<th>No. of inflorescence/plant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glima</td>
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<td>9</td>
<td>100</td>
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</tr>
<tr>
<td></td>
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<td>4.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>21</td>
<td>100</td>
<td>24.0</td>
<td>4.4</td>
</tr>
<tr>
<td></td>
<td>LD 20</td>
<td>9</td>
<td>100</td>
<td>27.7</td>
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<td></td>
<td>21</td>
<td>60</td>
<td>58.7</td>
<td>0.8</td>
</tr>
<tr>
<td>Elsanta</td>
<td>SD 10</td>
<td>9</td>
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<td>41.8</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
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<td>34.1</td>
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</tr>
<tr>
<td></td>
<td>LD 20</td>
<td>9</td>
<td>0</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
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Probability level of significance (ANOVA) Source of variation

<table>
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<th>Photoperiod (A)</th>
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<td>Cultivar (C)</td>
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<tr>
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<td>0.003</td>
</tr>
<tr>
<td>A x B x C</td>
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</table>

The effects of temperature, photoperiod and cultivar as well as their interactions were found to be statistically significant at \( \alpha = 0.05 \) (Table 2).

To understand the effects of daylength and temperature on the regulation of flowering-related genes, leaf and shoot apex samples of ‘Glima’ and ‘Elsanta’ were collected after 30 days under experimental conditions. In leaf tissues, FaFT1 expression was strongly down-regulated by SDs, being nearly undetectable in the SD samples (Figure 4a–b). However, temperature had an effect on FaFT1 expression in both cultivars under LDs, the expression being highest at 15°C. FaSOC1 expression in leaf tissue was down-regulated by SDs, and no obvious differences between cultivars and temperature treatments were observed (Figure 4c–d).

In the shoot apices of both cultivars, FaSOC1 expression was strongly down-regulated by SDs, similarly as in the leaf samples (Figure 5a–b). Temperature did not affect FaSOC1 expression levels in the apices of ‘Glima’, whereas in ‘Elsanta’ FaSOC1 mRNA was slightly less abundant in the LD/15°C samples than in the other LD samples. In addition to using our own primers for quantitative RT-PCR, we measured FaSOC1 relative expression with the primers described by Nakano et al. (2015). The expression patterns with the two primer pairs were nearly identical (Figure 5a–b).

The expression levels of FaTFL1 were generally concordant with the flowering data. In ‘Glima’ (Figure 5c), expression of FaTFL1 was lower than in ‘Elsanta’ (Figure 5d) in all treatments. Daylength had an effect on FaTFL1 expression only at 21°C, where LDs up-regulated FaTFL1 in correlation with delayed flowering (Figure 5, Table 2). In ‘Elsanta’, SDs down-regulated FaTFL1 at all tested temperatures, concordant with flowering phenotypes. However, FaTFL1 transcript level in ‘Elsanta’ was quite low also in LDs at 9°C, although no flowering was observed in this treatment.

Figure 3 Percentage of flowering plants of ‘Glima’ and ‘Elsanta’. Clonally propagated plants of ‘Glima’ and ‘Elsanta’ were exposed to the specified conditions for 5 weeks, and flowering was then recorded under LD conditions at 20°C as days from the end of treatments. n = 12.
Discussion

In this report, we show how the silencing of a key floral repressor FaTFL1 leads to daylength-independent flowering in the cultivated strawberry. We provide evidence that the expression of FaFT1 and FaSOC1 in the different cultivars of the cultivated strawberry responds similarly to environmental conditions as the FvFT1 and FvSOC1 genes in the diploid F. vesca, but the regulation of FaTFL1 does not always coincide with the changes in the FaFT1 and FaSOC1 mRNA levels. We show that the regulation of FaTFL1 expression in the cultivated strawberry cultivars is diverse and is associated with the varying flowering responses of the cultivars.

TFL1 is a floral repressor in the cultivated strawberry

Homologues of TFL1 have been shown to act as repressors of flowering in a wide range of species including Arabidopsis (Bradley et al., 1997; Hanano and Goto, 2011), Malus × domestica (Flachowsky et al., 2012; Kotoda et al., 2006), Arabis alpina (Wang et al., 2011) and F. vesca (Koskela et al., 2012). In Arabidopsis, mutations at TFL1 cause early flowering and conversion of the inflorescence meristem into floral meristem, leading to formation of the terminal flower (Bradley et al., 1997). Similarly, in Arabis alpina, silencing AaTFL1 leads to formation of terminal flowers and also reduces the juvenile phase (Wang et al., 2011). Moreover, disruption of TFL1 expression in apple reduces the duration of both the juvenile and vegetative phases and results in very early flowering (Kotoda et al., 2006). The gene is also reported to have a role in the control of yearly growth cycles in perennial plants such as Lombardy poplar (Populus nigra var. italica; Igasaki et al., 2008), roses (Iwata et al., 2012) and F. vesca (Koskela et al., 2012). In F. vesca, FvTFL1 is photoperiodically regulated by the LD-activated FvFT1–FvSOC1 flowering pathway at temperatures between 13 and 18°C, leading to the repression of flowering in LDs (Mouhu et al., 2013; Rantanen et al., 2015). At cool temperatures, FvTFL1 is repressed daylength

Figure 4 Expression of photoperiodically regulated genes in leaves of ‘Glima’ and ‘Elsanta’. (a–b) Expression of FaFT1 in leaves of ‘Glima’ (a) and ‘Elsanta’ (b); (c–d) expression of FaSOC1 in leaves of ‘Glima’ (c) and ‘Elsanta’ (d) grown under different daylength and temperature conditions for 30 days. Error bars indicate ± SD (n = 3).

Figure 5 Expression of photoperiodically regulated genes in the shoot apices of ‘Glima’ and ‘Elsanta’. (a–b) Expression of FaSOC1 in ‘Glima’ (a) and ‘Elsanta’ (b); (c–d) expression of FaTFL1 in ‘Glima’ (c) and ‘Elsanta’ (d) grown under different daylengths and temperatures. Error bars indicate ± SD (n = 3).
independently and flowering is induced in both SDs and LDs, whereas high temperatures promote FvTFL1 expression and inhibit flowering (Rantanen et al., 2015). To investigate the role of FaTFL1 in repression of flowering in the cultivated strawberry, we analysed transgenic ‘Elsanta’ plants with silenced TFL1.

In F. vesca, silencing of FvTFL1 leads to daylength-independent flowering but is not involved in the photoperiodic control of vegetative development, that is development of runners (Koskela et al., 2012). Similarly, in the transgenic ‘Elsanta’ lines with TFL1-RNAi construct, silencing of TFL1 abolished seasonal regulation of growth cycles causing daylength-independent perpetual flowering (Figure 1c and e), but did not affect the formation of runners in the transgenic lines (Figure 1d). These data provide strong evidence that FaTFL1 functions as a floral repressor, which has no direct effect on the vegetative reproduction in the cultivated strawberry. The absence of connection between TFL1 and vegetative vigour seems to be strawberry specific; in many other Rosaceous crop plants, including pear (Freiman et al., 2012) and apple (Flachowsky et al., 2012; Kotoda et al., 2006), reducing TFL1 expression results in plants with greatly reduced vegetative growth.

Results from transgenic experiments in F. vesca indicated that FvSOC1 separately promotes runner formation through the activation of the gibberellin pathway and represses flowering by activating FvTFL1 expression. However, FvTFL1 has no effect on the expression of FvSOC1 or runner formation (Koskela et al., 2012; Mouhu et al., 2013). Similarly, the silencing of FaTFL1 in ‘Elsanta’ did not change FaSOC1 expression. Further studies are needed to reveal whether FaSOC1 also functions as a branching point in the genetic pathway controlling vegetative and generative reproduction in the cultivated strawberry. Based on our findings, flowering time in the cultivated strawberry could be extended by reducing FaTFL1 expression either by means of conventional breeding, through transgenic procedures or genome editing (Xiong et al., 2015) without direct consequences in vegetative reproduction. Poor runner production in current everbearing cultivars (Sønsteby and Heide, 2007), which was reported to be caused by the same major QTL as the perpetual flowering habit itself (Gaston et al., 2013), is limiting their vegetative propagation in nurseries. Based on our data, the production of novel everbearing cultivars based on FaTFL1 silencing could possibly solve this problem.

Altered patterns of FaTFL1 expression may contribute to the different flowering responses in the cultivated strawberry

TFL1 down-regulation has been shown to correlate with the expression of floral meristem identity genes and subsequent flowering in several perennial Rosaceous species, namely the diploid strawberry F. vesca (Koskela et al., 2012), M. domestica (Hättañas et al., 2008) and Rosa sp. (Iwata et al., 2012). Although the gene appears to have an important role in the control of flowering, the variation in its expression patterns within a certain species has not been studied. In this work, we analysed the variation in FaTFL1 expression patterns in five octoploid strawberry cultivars. The results show striking cultivar-dependent differences in the regulation of FaTFL1 expression by environmental conditions.

FaTFL1 down-regulation correlated with flowering in cultivars ‘Polka’, ‘Glima’ and ‘Elsanta’. By contrast, down-regulation of FaTFL1 was detected under both daylengths in the early cultivar ‘Honeoye’ and in ‘Alaska Pioneer’ that flowered eleven days later, although the gene was expressed at a higher level in LDs than SDs at all time points (Figure 2). It appears contradictory that FaTFL1 was down-regulated in both SDs and LDs in these cultivars, although no flowering in LDs was observed (Table 1), and FaFUL1 was up-regulated only in SDs (Figure 2g–h). However, earlier experiments with ‘Honeoye’ have shown that the cultivar can be induced to flower daylength independently at 17°C, with LDs delaying flowering (Bradford et al., 2010). It is therefore possible that flowering could have eventually occurred in ‘Honeoye’ and ‘Alaska Pioneer’ also in LDs had the plants been observed for a longer time. Moreover, a recent study by Nakano et al. (2015) demonstrated similar daylength-independent down-regulation of FaTFL1 in the Japanese octoploid strawberry cultivar ‘Nyoho’. The authors suggested that the observed down-regulation of FaTFL1 in ‘Nyoho’ could be due to high initial levels of FaTFL1 caused by the conditions where the experimental plants were prepared, that is under high natural temperature. It seems likely that the initial level of FaTFL1 mRNA was high in the young ‘Honeoye’ and ‘Alaska Pioneer’ plants used in the experiment, but this cannot be caused by high temperatures or high light conditions as the plants were prepared during winter under controlled greenhouse conditions. A more likely explanation, which should be studied further, is that the level of FaTFL1 transcript is extremely high in young plants of these cultivars and is reduced in an age-dependent manner. Age-dependent reduction in TFL1 mRNA has been earlier described in the perennial A. alpina (Wang et al., 2011), in which TFL1 expressed in the shoot apical meristem blocks flowering of young plants exposed to vernalization.

In F. vesca, FvTFL1 is down-regulated and flowering induced independently of the photoperiod below a critical limit of 13°C, whereas high temperature (23°C) represses flowering by up-regulating FvTFL1 (Rantanen et al., 2015). Previous experiments with the cultivated strawberry cultivars have suggested that temperature may control flowering through a similar mechanism, although critical temperature limits differ widely depending on the cultivar. Sønsteby and Heide (2006) have shown that even temperature as low as 9°C is not sufficiently low for induction of flowering in ‘Elsanta’, and temperature of 27°C is required for repressing flowering in SDs. In contrast, ‘Glima’ induces flowers independent of daylength at 18°C, and temperature of 24°C is not high enough for complete floral repression (Heide, 1977). Our results are in line with the earlier physiological studies, as SDs down-regulated FaTFL1 and induced flowering in ‘Elsanta’ at all tested temperatures (the highest being 21°C), whereas in ‘Glima’, FaTFL1 mRNA levels were lower in LDs than in ‘Elsanta’ and flowering occurred in all photoperiod–temperature combinations (Table 2 and Figure 5). However, LD caused a partial floral repression at the highest temperature, which was associated with increased FaTFL1 mRNA level compared to SD. Contradictory to the observations in F. vesca, cool temperature of 9°C did not induce flowering in ‘Elsanta’ although FaTFL1 was down-regulated. It is possible that although the level of FaTFL1 in ‘Elsanta’ under the LD/9°C treatment was low, it was still sufficient to repress flowering in this cultivar. Several studies in Arabidopsis have shown that TFL1 is a stronger repressor of flowering under cool temperature (Hanano and Goto, 2011; Kim et al., 2013; Strasser et al., 2009), and the same could be true in the cultivated strawberry. Another possibility is that the FaTFL1 homeologs not detected by our RT-qPCR primers were expressed at a higher level. Future research with subgenome specific primers should be carried out to show whether the homeologs are expressed at different levels.
Is the FaFT1–FaSOC1–FaTFL1 pathway present in the cultivated strawberry?

The long-day activated photoperiodic flowering pathway has been well characterized in *F. vesca*. In *F. vesca*, LDs activate *FvFT1* expression in the leaf leading to *FvSOC1* activation in the shoot apex. By contrast, SDs strongly down-regulate *FvFT1* expression in the leaf and cause a gradual down-regulation of *FvSOC1* in the shoot apex leading to the reduction in *FvTFL1* mRNA levels and flowering induction (Koskela et al., 2012; Mouhu et al., 2013).

To elucidate whether the LD-activated *FT1–SOC1* pathway shows similar expression patterns in the cultivated strawberry, we studied the expression of these genes in several strawberry cultivars. In all tested cultivars, *FaFT1* was expressed in leaf tissues exclusively in LDs (Figures 4 and S4). Moreover, the diurnal rhythm of *FaFT1* in ‘Honeoye’ showed a similar pattern to the rhythmical expression of *FvFT1* observed in *F. vesca* (Koskela et al., 2012). Interestingly, *FaFT1* expression in the cultivated strawberry is regulated also by temperature, the expression being highest at intermediate temperature (Figure 4). Similar results were reached in *F. vesca* by Rantanen et al. (2015), who showed that *FvFT1* is regulated by ambient temperature, although no correlation between the flowering response and *FvFT1* expression was observed between the temperatures. Our results on *FaFT1* regulation in cultivated strawberry support the recent reports by Nakano et al. (2015) and Nakajima et al. (2014), who reported higher *FaFT1* expression in LDs than SDs in the Japanese cultivars ‘Nyoho’ and ‘Tochiotome’, respectively. However, Nakano et al. (2015) did not detect clear daylength-dependent regulation of *FaSOC1*, in contrast with our finding that *FaSOC1* is down-regulated in SDs in both leaves and apical tissues (Figures 2, 4 and 5). We first hypothesized that the discrepancy could be caused by different primers used for quantitative RT-PCR. When we tested the *FaSOC1* primers of Nakano et al. (2015), the expression patterns were nearly identical to those obtained with our own primers (Figures 5 and S5). It therefore appears that the observed lack of photoperiodic regulation of *FaSOC1* in the Japanese cultivar ‘Nyoho’ reflects a true difference in *FaSOC1* regulation as compared to European strawberry cultivars.

In general, we detected lower *FaTFL1* expression levels in SD- than in LD-grown shoot apices except in ‘Glima’ indicating that, similarly to *F. vesca* (Mouhu et al., 2013), the photoperiodic control of *FaTFL1* is present in the cultivated strawberry. In many cases, however, the changes in the *FaTFL1* mRNA levels did not clearly reflect the changes in the expression of *FaFT1* and *FaSOC1* indicating that other mechanisms are involved in the regulation of *FaTFL1*. This is clearly true also in *F. vesca*, in which strong coincidence in the photoperiodic control of *FvSOC1* and *FvTFL1* only occurs in quite narrow temperature range (Rantanen et al., 2015). Our results suggest that the identification of the genetic variation causing variable *TFL1* expression patterns in strawberries may open new possibilities for tailoring flowering responses.

Conclusions

The results presented here suggest that *FaTFL1* acts as a floral repressor in the cultivated strawberry, and its down-regulation is correlated with subsequent floral induction in most cases. Cultivars exhibit large differences in the regulation of *FaTFL1*; some cultivars may down-regulate *FaTFL1* in an age-dependent manner, whereas in other cultivars *FaTFL1* down-regulation is more dependent on environmental conditions. As the diverse expression patterns do not seem to arise from differential activity of the photoperiodically controlled *FaFT1–FaSOC1* pathway, the elucidation of other upstream regulators of *FaTFL1* or functional allelic variation in the *FaTFL1* locus could provide valuable information for targeted plant breeding. Decreasing *FaTFL1* expression levels either via conventional breeding or using transgenic approaches could result in novel everbearing or earlier flowering cultivars without direct effect on vegetative reproduction through runners.

Experimental procedures

Plant transformation

For plant transformation, auxillary shoot cultures of the octoploid strawberry (*F. × ananassa Duch.*) cultivar ‘Elanta’ were used. The plant material was propagated *in vitro* on shoot proliferation medium containing MS salts and vitamins (Murashige and Skoog, 1962) supplemented with 0.1 mg/L 6-benzylaminopurine (BAP), 0.01 mg/L indole-3-acetic acid (IAA), 30 g/L sucrose and 0.45% Difco Bacto-agar (Difco, Heidelberg, Germany). Plants were grown in a culture chamber (16 h light at 21°C and 8 h dark at 16°C) and subcultured every 3–4 weeks. Plant transformation was performed using the *Agrobacterium tumefaciens* strain EHA105 complemented with the binary plasmid vector pK7GWG2DJI-TFL1-RNAi (Koskela et al., 2012) as described by Fischer et al. (2014). Regenerated meristems were excised 9–12 weeks postinoculation. Regenerated shoots were propagated and subcultured on shoot proliferation medium with 500 mg/L timetin and 300 mg/L kanamycin at 16 h of light at 21°C and 8 h of darkness at 16°C. Rooted plantlets (*n = 5* for each transgenic line) were grown in 5 cm plastic pots in the greenhouse at LD conditions at 18°C.

PCR-based detection of transgenic sequences

Genomic DNA was extracted from *in vitro* leaves of the transgenic lines and wild-type ‘Elanta’ using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). PCR was performed in 25 μL volume containing 10 ng DNA, 1× DreamTaq™ buffer, 0.2 mM deoxynucleoside triphosphates (dNTPs), 0.5 μM of each primer and 0.5 U DreamTaq™ DNA polymerase (MBI Fermentas, St. Leon-Roth, Germany). The PCR started by initial denaturation at 94°C for 4 min, followed by 33 cycles of 30 s of denaturation at 94°C, 1 min of annealing at 56°C and 1.5 min of extension at 72°C, and a final extension at 72°C for 7 min. PCR was performed using a MyCycler™ thermocycler (Bio-Rad, Hercules, CA). Primers used for detecting the *nptII* marker gene and the chimeric *TFL1* hairpin construct are listed in Table S1.

Plant materials, experimental conditions and sampling

Plants for all experiments were propagated clonally from runner plants of LD-grown mother plants. In the artificial seasonal cycle experiment with wild-type ‘Elanta’ and *TFL1*-RNAi line F138, the clonally propagated plants (*n = 10*) were grown for 1 month in LDs in the greenhouse, after which they were subjected to natural SDs in an unheated greenhouse in Helsinki from 25 September 2014 to 13 November 2014. Then, greenhouse temperature was set to 6°C for the following 56 days (1344 chilling hours) to release dormancy. After chilling, plants were forced in the greenhouse under 18-h photoperiod. Temperature was first set to 12°C and then gradually increased to 18°C during the first 2 weeks. Inflorescences with open flowers and runners were counted and removed weekly.
For the environmental treatments, runner-propagated plants of ‘Alaska Pioneer’, ‘Honeoye’ and ‘Polka’ were first grown in LDs (18 h light, 18°C) for 3 weeks. Plants were then exposed to either SD (12 h, 18°C) or LD (18 h, 18°C) conditions for 6 weeks and then moved to LD at 18°C for flowering observations. For analysing temporal changes in gene expression, three biological replicates of apex samples were collected at ZT9 0, 2, 4 and 10 weeks after the beginning of the daytime treatments, each sample containing the main apex of three individual plants. Leaf samples for analysing circadian rhythmic expression in cultivar ‘Honeoye’ were collected 3 weeks after the beginning of daylength treatments at 4, 8, 12, 16, 20, 24 and 28 h after the ‘first’ subjective dawn. Leaf samples consisted of the middle leaflet of a fully opened leaf, and three biological replicates were collected.

Clonally propagated plants of cultivars ‘Glima’ and ‘Elsanta’ were rooted directly into 10 cm pots and grown in a greenhouse in LDs (20 h) at 20°C for 4 weeks. The plants were exposed to combinations of temperature (9°C, 15°C and 21°C) and daylength (SD = 10 h, and LD = 20 h) for 5 weeks, after which the plants were moved to LD at 20°C for flowering observations. Using low-intensity (7 µmol quantum/m²/s) incandescent light for daylength extension, the daily integral differed only by 0.5% between the daylength treatments. Leaf and apex samples for RNA extractions were collected at noon (+2 h) on day 30 with three biological replicates. Each leaf replicate was pooled from the youngest fully open leaves of 4 plants, and each apex sample contained the main shoot apices of four plants. All samples were immediately frozen in liquid nitrogen and stored at −80°C.

RNA extraction and quantitative RT-PCR

Apex samples were milled in Retsch MM400 ball mill (Retsch GmbH, Düsseldorf, Germany) at 28/second for 30 s. Leaf samples were ground in liquid nitrogen using a mortar and a pestle. RNA was extracted using the CTAB-extraction buffer based pine tree method (Monte and Somerville, 2002). cDNA was synthesized from 1 µg total-RNA using Superscript III reverse transcriptase (Invitrogen, Thermo Fisher Scientific, MA). RT-PCRs were performed with three technical replicates and three biological replicates with SYBR Green I master mix (Roche Deutschland Holding GmbH, Mannheim, Germany) run in Lightcycler 480 instrument (Roche). Quantitative RT-PCR was run in a total volume of 10 µL with final concentration of 1 × SYBR Green Master Mix I (Roche) and 0.45 µM primers. The RT-qPCR profile is described in Figure S6. A total of 3.5 µL of diluted cDNA (final volume 150 µL) was used in each reaction. Relative expression levels were calculated by ΔΔCT method (Pfaffl, 2001) with FaMSI1 (Fragaria × ananassa homologue of MULTICOPY SUPPRESSOR OF IRA1) as normalization gene. Primers used for RT-qPCR are listed in Table S1.

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References


Supporting information

Additional Supporting information may be found in the online version of this article:

**Table S1** Primer sequences used in the experiments.

**Figure S1** Evaluation of transgenic strawberry clones for the presence of the transferred DNA sequences.

**Figure S2** Specificity of silencing FaTFL1 using the FvTFL1-RNAi construct.

**Figure S3** Flowering phenotype of the transgenic line F139.

**Figure S4** Circadian rhythms of FaFT1 and FaSOC1.

**Figure S5** FaSOC1 expression in ‘Glima’ and ‘Elsanta’ shoot apices.

**Figure S6** RT-qPCR programme used for analyzing gene expression.

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