TEMPERATURE REGULATION OF FLOWERING IN WOODLAND STRAWBERRY

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# Temperature regulation of flowering in Woodland Strawberry

Strawberries are perennial, flowering plants of genus *Fragaria*, in the family Rosaceae. Woodland Strawberry (wild *F. vesca*) is the most widely distributed natural species in the northern hemisphere and a new promising model plant for the study of flowering in perennial plants. Classified as a seasonal flowering short day (SD) plant, wild *F. vesca* initiates flowering in the autumn, flowers in spring and forms fruits in summer followed by a vegetative growth phase until the next autumn. The perpetual flowering genotype *Hawaii-4* flowers continuously throughout the season. Flowering is regulated by ambient signals such as photoperiod and temperature. The photoperiod x temperature interaction was found to play a vital role in flowering initiation in *Fragaria*. SD *F. vesca* can behave as a day-neutral, short day plant or non-flowering plant depending on different ambient temperature. Flower initiation takes place regardless of photoperiod when *F. vesca* is put at 9°C, but only flower under SD at 15°C and no flowering is observed when the ambient temperature is higher than 21°C. The experiment was designed to observe flower induction and explore the role of few flowering genes in the temperature regulation of flowering at long day (LD) condition. The expression of key flowering genes *FT*, *SOC1* and *TFL1* were analyzed in wild *F. vesca* and transgenic lines in *F. vesca* background. The results showed low temperature (11°C) promotes flowering in SD *F. vesca*. *FT* was down-regulated by 11°C and negatively correlated with flowering in *F. vesca*, in contrast to findings in other SD plants. *SOC1* may act downstream of *FT*, it represses flowering in SD *F. vesc* and may affect vegetative growth by interacting with GA pathway. Although *FT* functions as an integrator gene in flowering, there may be an *FT*-independent pathway in SD *F. vesca*. *SOC1* integrates signals from different pathways; it might be the candidate gene mediating signals from the thermosensory pathway. *TFL1*, identified as *SEASONAL FLOWERING LOCUS (SFL)* in SD *F. vesca*, represses flowering and is regulated by temperature.

**Avainsanat — Nyckelord**
*Fragaria vesca*, flowering gene, temperature, interaction, *FT*, *TFL1*

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Supervisors Dr. Timo Hytönen & Dr. Takeshi Kurokura
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1 INTRODUCTION

Strawberries are perennial, flowering plants of genus *Fragaria*, in the rose family *Rosaceae* which includes also many other economically important crops such as apple, pear and rose. The genus *Fragaria* consists of diploid (2n = 2x = 14), tetraploid (2n = 4x = 28), hexaploid (2n = 6x = 42), and octoploid (2n = 8x = 56) species, in total of at least 15 recognized species (Hancock 1990).

*Fragaria* is a broadly adapted genus. Among all the species, the diploid woodland strawberry (*F. vesca* L.) is most widely distributed, inhabiting North and South America, Europe and Asia (Hancock 1990). *F. vesca* is becoming an increasingly popular model species for *Rosaceae* as it has small genome. Its genetic linkage map has been constructed by Sargent et al. (2004), the high efficiency transformation method is available and described by Oosumi et al. (2006). *F. vesca* is classified as seasonal flowering short-day (SD) plant, but also perpetual flowering long-day (LD) plant accessions are known (Guttridge 1985, Sønsteby 2007a). The cultivated strawberries are octoploid species with perpetual flowering trait and complex inheritance which hinders further detailed genetic research (Shaw & Famula 2005). In Finland, the annual production of strawberry amounts to 10 286 tons, ranking as the most important berry crop (FAO 2010).

In SD *F. vesca*, the *TERMINAL FLOWERING1 (TFL1)* homolog was identified to correspond to the *SEASONAL FLOWERING LOCUS (SFL)*, which was indicated to be the single gene regulating the difference between seasonal and perpetual flowering (Iwata et al. 2012, Koskela et al. 2012). A 2-bp deletion of the *TFL1* homolog is responsible for the perpetual flowering trait. Koskela et al. (2012) further showed that the photoperiodic control of *TFL1* causes seasonal flowering habit in SD *F. vesca*.

Photoperiod and temperature are the two main factors influencing flowering process (Sønsteby & Heide 2007b). Heide and Sønsteby (2007) showed that interaction of photoperiod and temperature plays a vital role in regulating flowering in SD *F. vesca*. Although the photoperiodic regulation in SD *F. vesca* is a heated research field, the study of temperature regulation of flowering at molecular level is yet lacking. This study tried to reveal the molecular regulation of flower induction at low temperature which can facilitate a better understanding of the flowering mechanisms involved in temperature regulation.
2 LITERATURE REVIEW

2.1 The physiology of strawberry

Strawberry is well adapted to a diverse array of climates (Hancock 1990). It is well known to classify strawberry into two distinct flowering types: seasonal flowering (Junebearing) and perpetual flowering (everbearing) (Brown & Wareing 1965). Most cultivars grown in Europe are seasonal flowering types. Seasonal flowering strawberries are induced to flower in autumn by SD and cool temperature (Brown & Wareing 1965, Darrow 1966), whereas perpetual flowering strawberries show continuous flowering under LD (Darrow 1966).

Strawberry is a rosette plant (Fig. 1). The vegetative growth involves the formation of crowns and runners (Darrow 1966). The thick stem with short internodes of strawberry is named as a crown. During the vegetative growth, the aboveground stolons called runners are formed from the axillary buds of the crown (Konsin et al. 2001). The axillary buds can develop into either runners or axillary crowns, depending on the growing environment (Darrow 1966). In seasonal flowering strawberry, runners are produced from axillaries in the early vegetative stage, whereas the formation of axillary leaf rosettes called branch crowns becomes dominant under SD in autumn (Darrow 1966, Konsin et al. 2001, Taylor 2002, Hytönen et al. 2004).

Seasonal flowering strawberry enters reproductive phase in autumn and flower initiation occurs in the shoot apex of the main crown as well as in branch crowns which are competent for floral development (Jahn & Dana 1970, Hytönen et al. 2004). Strawberries have a sympodial growth habit which means that the vegetative growth of the crown is terminated upon floral induction, the topmost axillary buds, however, continue vegetative growth and produce branch crowns (Brown & Wareing 1965, Darrow 1966). In contrast to seasonal flowering strawberries, perpetual flowering strawberry produces only few runners, which could be a result of the continuous flowering habit and differentiation of axillary buds to branch crowns (Sønsteby & Heide 2007b).
Figure 1 Schematic representation of the development in SD *F. vesca* grown under LD followed by SD. Strawberry has very short internodes. In each node, one leaf and axillary bud are formed. During the vegetative growth under LD an axillary bud differentiates into a runner. In SD, branch crowns are formed. The apical meristems of the main crown and branch crowns are induced to flower in autumn, after a certain of SD cycles. Then the topmost axillary bud continues the development of the main crown. Figure modified from Brown & Wareing 1965 and Hytönen 2009.

2.2 Environmental regulation of flowering in strawberry

Photoperiod and temperature are the two main which control flowering in both seasonal and perpetual flowering strawberry (Hytönen et al. 2004, Sønstebø & Heide 2006, Sønstebø & Heide 2007b). Other factors such as water supply and mineral nutrition can limit the flowering as well, but with very little impact (Guttridge 1985).
2.2.1 Seasonal flowering strawberry

Seasonal flowering strawberries are considered to be facultative short-day (SD) plants (Guttridge 1985). They show an antagonistic vegetative and reproductive development which is regulated by photoperiod and temperature, for example, floral induction terminates the vegetative growth of the crown (Brown & Wareign 1965, Heide & Sønsteby 2007).

During vegetative growth, photoperiod regulates axillary bud differentiation. Long photoperiod (>14-16h) and high temperature (>17-20°C) promote the formation of runners in SD strawberry (Brown & Wareing 1965, Hytönen 2009), whereas SD conditions and high light intensity promote crown branching which can give rise to more floral meristems in flowering phase (Hytönen et al. 2009, Wagstaffe & Battey 2004).

In SD garden strawberries, short photoperiod in autumn induces flower initiation at the apical meristem and LDs in summer promote the vegetative growth of new shoots in the uppermost nodes (Heide 1977, Battey et al. 1998, Battey 2000, Heide & Sønsteby 2007). There is intraspecies variation of critical photoperiods (11 to 16 hours) and number of SD cycles (7 to 23) among different cultivars (Guttridge 1985). However, in some cultivars, temperature less than 15°C can cause flower initiation regardless of photoperiod (Guttridge 1985). In contrast, temperature above 24 to 30°C or under 9°C will not induce flowering either in SD or LD (Ito & Saito 1962, Sønsteby & Heide 2006).

The interaction of temperature and photoperiod plays a major role in the regulation the flowering of SD *F. vesca*. Heide and Sønsteby (2007) showed the interaction of photoperiod and temperature on five Norwegian *F. vesca* accessions originating from different latitudes. They were all induced to flower at 9°C regardless of the photoperiod. Flowering was inhibited when the temperature was as high as 21°C. At optimum conditions (15°C, 10-h SD), flower induction occurs with a minimum exposure period of 4 weeks and SD was required. Similar situation was found in SD *F. vesca* of British origin. They initiate flowering in a narrow temperature range (10-15°C) regardless of photoperiod, although there is a delay of 3 to 4 weeks of flowering in LD conditions (Brown & Wareing 1965, Battey et al. 1998). Although their work was a milestone in indicating the interaction of temperature and photoperiod on flowering, further study at
molecular level would reveal a better understanding of flowering mechanisms regulated by the interaction.

2.2.2 Perpetual flowering strawberry

The classification of perpetual flowering genotypes either as day-neutral (DN) or long-day (LD) plants has been a debating topic (Nishiyama & Kanahama 2002, Sønsteby & Heide 2007a). In most early studies, the perpetual flowering genotypes and cultivars were perceived as DN because of the negligible effect of photoperiod on flowering time (Guttridge 1985). Recent studies, however, showed that LD and high temperature can promote flowering both in perpetual flowering cultivars of garden strawberry and in accessions of SD F. vesca (Sønsteby & Heide 2007a, Hytönen 2009).

In the study of Sønsteby and Heide (2007b), EB cultivar ‘Elan’ showed a LD promotion of flowering in the temperature range of 15 - 27°C. Based on further research of other five EB cultivars, Sønsteby & Heide 2007a concluded that EB strawberries are obligatory LD plants at high temperature 27°C, facultative LD plants at intermediate temperature 15°C to 21°C, and DN plants at temperature below 10°C.

Hytönen (2009) and Mouhu et al. (2009) showed that perpetual flowering F. vesca produced only 5 to 8 leaves before terminal inflorescence under LD conditions, but 4 to 5 more leaves were formed before flowering when plants were placed under SD at 18°C for 5 weeks. Taken together, LD promotes flowering in perpetual flowering accessions of F. vesca, in contrast to seasonal flowering accessions.

2.3 Genetic control of flowering in model species

In the annual LD plant Arabidopsis, four genetic flowering pathways have been characterized in detail: photoperiod, vernalization, autonomous and gibberellin pathways. In addition, light quality and ambient temperature pathways may regulate flowering in some conditions. The photoperiod, vernalization and ambient temperature pathways which mediate the effect of environmental signals are explained as follows.

2.3.1 Photoperiod pathway

In the photoperiodic pathway, the circadian clock controls daily rhythms of the duration of photoperiod. CONSTANS (CO) was identified to mediate flowering by measuring the
day length (Suárez-López et al. 2001). CO activates floral integrators FLOWERING LOCUS T (FT) and SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1) which promote flowering (Samach et al. 2000). FT is the primary and major target of CO, it is expressed in the leaves in response to photoperiod (Wigge et al. 2005, Corbesier et al. 2007). The activation of FT expression is followed by the movement of FT protein from leaves to shoot apical meristem through phloem (Corbesier et al. 2007). In the shoot apex, FT protein forms a complex with the bZIP protein FD which is abundant and provides a spatial specificity for FT (Wigge et al. 2005). The FT/FD complex then up-regulates floral meristem identity gene APETALA1 (AP1) (Wigge et al. 2005), and regulates FRUITFUL (FUL) and SEPALALATA 3 (SEP3) accumulation (Teper-Bammolker & Samach 2005).

SOC1 is expressed mainly in developing leaves and meristems (Lee & Lee 2010). SOC1 acts downstream of CO and might also be the primary target of FT (Wigge et al. 2005, Corbesier et al. 2007). SOC1 was identified by Samach et al. (2000) as a gene whose loss of function results in delayed flowering in 35S::CO background. It was also identified to be an important integrator in photoperiod pathway, vernalization and autonomous pathway (Lee et al. 2000).

TERMINAL FLOWER 1 (TFL1) which belongs to the same Phosphatidylethanolamine-Binding Protein (PEBP) family with FT (Ratcliffe et al. 1998, Hanano & Goto 2011). They share 71% amino acid residues but play antagonistic role in mediating flowering (Kobayashi et al. 1999). Also TFL1 was also identified to interact with FD to suppress flowering (Hanano & Goto 2011). The expression of TFL1 mRNA is limited to the axillary meristems during vegetative phase. TFL1 protein, however, acts as a mobile signal and controls shoot meristem identity at a short distance (Conti & Bradley 2007, Sohn et al. 2007). Flower induction up-regulates TFL1 in the shoot apex to maintain the indeterminate inflorescence meristem (Shannon & Meeks-Wagner 1991, Conti & Bradley 2007). Thus, TFL1 is a negative regulator of transition from vegetative to reproductive phase, and from inflorescence meristem to floral meristem.

The mechanism found in Arabidopsis has been proved to be conserved in short day plant rice. In rice, Heading date 1 (corresponding to CO) suppresses Heading date 3a (corresponding to FT) under LD, thus inhibiting flowering (Izawa et al. 2002, Hayama & Coupland 2004, Hayama et al. 2003). Under SD, Hd1 is expressed during the night which enables Hd1 to induce Hd3a expression and promote flowering (Hayama &
Coupland 2004). Hd3a protein, as FT protein in Arabidopsis, acts as a mobile flowering signal in rice (Tamaki et al. 2007) and interacts with 14-3-3 proteins in the apical cells of shoots to activate transcription of AP1 homologue in rice (Taoka et al. 2011).

2.3.2 Vernalization pathway

The promotion of flowering by a period of winter cold exposure is called vernalization. In Arabidopsis, there are two types of accessions: summer-annuals which can flower rapidly without vernalization, and winter-annuals that show extreme late-flowering unless vernalized. A single dominant locus FRIGIDA (FRI), conferring vernalization habit of winter-annual, was identified from certain crosses of winter-annuals and summer-annuals (Napp-Zinn 1987). Further genetic studies revealed another flower repressor gene FLOWERING LOCUS C (FLC) as a target of FRI (Michaels & Amasino 2001). FRI and FLC function synergistically, loss-of-function of either genes eliminates the late-flowering phenotype (Johanson et al. 2000, Michaels & Amasino 1999).

The molecular nature of vernalization was revealed by cloning of FLC (Michaels & Amasino 1999). FLC encodes a MADS-box transcriptional regulator that represses flowering. Furthermore, FRI positively regulates the expression of FLC to the level to inhibit flowering, but vernalization strongly represses FLC and overcomes the effect of FRI (Michaels & Amasino 1999, Sheldon et al. 1999). The vernalization induced FLC suppression is mitotically stable in all tissues. The length of cold treatment and the level of FLC down-regulation are quantitatively related (Sheldon et al. 2000). Thus FLC is the central regulator of flower induction by vernalization (Sheldon et al. 2000).

Although the role of FLC and FRI seem to be conserved in other crucifers (Osborn et al. 1997), genetic analyses in spring and winter varieties of wheat and barley revealed the antagonistically acting dominant alleles VRN1 and VRN2 (Tranquilli & Dubcovsky 2000, Yan et al. 2004). VRN1 promotes spring growth habit in wheat and barley, whereas VRN2 is necessary for a winter growth habit – indicating that VRN2 has the analogous role to FLC (Yan et al. 2004).

As an integrator from different pathways, SOC1 acts downstream of late flowering gene FRI and FLC as its overexpression promoted flowering in winter annuals that highly expressed FRI FLC (Lee et al. 2000). Searle et al. (2006) showed that SOC1 was regulated by CO and FLC, through different regions of SOC1 promoter sequence. The
RT-PCR results suggested the expression of *FLC* in phloem can reduce the expression level of *FT* and *SOC1* in the leaves (Fig. 2) (Searle et al. 2006).

**Figure.2** Flowering-related genes in the photoperiod, ambient temperature, vernalization and autonomous pathways in Arabidopsis. *CO* acts upstream of *FT* and *SOC1* and mediates flowering in photoperiod pathway. Ambient temperature affects floral integrator genes by regulating genes such as *FCA*, *FVE* and *SVP*. *FLC* is involved both in vernalization and autonomous pathways. Floral integrator genes *FT* and *SOC1* activate floral meristem identity genes *AP1*, *FUL*, and *LFY* to initiate flowering. Arrows indicate positive regulation and bars negative. Figure modified from Hytönen (2009) and Koskela (2009).

### 2.3.3 Ambient temperature pathway

Most of the molecular genetic studies of Arabidopsis focused on photoperiod or vernalization pathways, whereas much less is known about how plants respond to ambient temperature at molecular level, which is getting increasingly important taking
into consideration of the changes of global climate (Fitter & Fitter 2002). It was also found that the onset of flowering has been advanced due to the spring temperature raise in temperate zones (Fitter & Fitter 2002). The complex mechanisms involved in the perception of ambient temperature also make it worthy of equal attention as other pathways (Samach & Wigge 2005, Blázquez et al. 2003).

Blázquez et al. (2003) proposed a thermosensory pathway controlling flowering time. \(FCA\) and \(FVE\) promote flowering in response to increasing ambient temperature (Blázquez et al. 2003). By comparing the flowering time of mutants to wild-type, it was identified that \(FVE\) and \(FCA\) respond to ambient temperature mostly through a \(FLC\)-independent pathway (Blázquez et al. 2003). Thus, \(FCA\) and \(FVE\) may be involved in regulating flowering through a thermosensory pathway (Blázquez et al. 2003, Kim et al. 2004), whereas \(FLC\) is involved in the vernalization pathway (Dennis & Peacock 2007).

\(SHORT\) \(VEGETATIVE\) \(PHASE\) (\(SVP\)) was identified to mediate temperature signals downstream of \(FCA\) and \(FVE\) (Lee et al. 2007). \(SVP\) protein negatively regulates \(FT\) expression by binding to \(FT\) promoter. The expression of \(FT\) was reduced at low temperature, but \(SOC1\) was only slightly down-regulated (Blázquez et al. 2003). Decreased \(FT\) level is a cause for the delayed flowering at low temperature, but there might be another pathway, since \(ft\) loss-of-function mutant still responds to ambient temperature (Blázquez et al. 2003).

Balasubramanian et al. (2006) found that higher temperature strongly induced flowering in Arabidopsis in the absence of photoperiodic cues. They identified \(FT\) as the mediator of thermal induction for flowering and proposed that temperature regulates flowering in two ways: one is through floral repressor \(FLC\), and the other one is the activation of \(FT\) independently of photoperiod. \(CO\), however, is not involved in the thermal induction.

### 2.4 Genetic regulation of flowering in strawberry and other perennials

The classical crossing experiment of Brown and Wareign (1965) demonstrated that in \(F.\) \(vesca\) flowering (seasonal and perpetual) and runnering (non-runnering and runnering) segregated separately and showed Mendelian inheritance, indicating the characters can be regulated by different genes, although in most genotypes they show a clear relation of antagonism. Seasonal flowering and runnering are controlled by dominant genes \(Seasonal\) \(Flowering\) \(locus\) (\(SFL\)) and \(Runnering\) \(locus\) (\(RL\)),
respectively, whereas the corresponding recessive alleles contribute to perpetual flowering and non-runnering characters (Albani et al. 2004).

Typically perennial plants undergo a transition from vegetative growth to flowering every year. The transition depends on the differential behaviour of meristems on a single perennial plant. Flowering in perennial plants is consecutive after induction, but vegetative growth is maintained at the same time. The differential behaviour of meristems assures some remain in the vegetative stage but others start floral transition (Albani & Coupland 2010).

Perennial plants share conserved key flowering genes with annual plants, although the gene regulation in perennial plants differs from the annual ones (Albani & Coupland 2010). Endo et al. (2005) found that Citrus \( FT \) ortholog induced early flowering and reduced the generation time. The \( FT \) homolog (\( PtFT1 \)) in \( Populus \) trees not only play a role in regulating flowering time in spring, but also control growth cessation in the fall – together with \( PtCO \) (Böhlenius et al. 2006). Furthermore, the transcript abundance of \( PtFT2 \) increases as poplar (\( Populus \) spp.) transits from juvenile phase to reproduction phase (Hsu et al. 2006). Taken together, \( PtFT1 \) and \( PtFT2 \), as homologs of \( FT \), are functionally diverged. Analysis of \( PtFT1 \) and \( PtFT2 \) revealed the role of \( PtFT1 \) in promoting flowering by responding to winter cold, but \( PtFT2 \), activated by LD and high temperature, promotes vegetative growth and inhibits bud set (Hsu, et al. 2011).

SD \( F. \) vesca has been seen as a model plant for perennial plants of Rosaceae family (Folta & Davis 2006, Sargent et al. 2004, Hytönen & Elomaa 2009). It has a small diploid genome of 240Mb, and a 3 to 4 months generation time. A genetic map for \( F. \) vesca has been constructed with almost 350 markers covering ca. 570 cM and the whole genome has been sequenced, which supports further research in the species (Battey 2000, Folta & Davis 2006, Sargent et al. 2004, Shulaev et al. 2011)

Mouhu et al. (2009) identified the homologs of Arabidopsis flowering time genes in \( Fragaria \) by EST sequencing analysis. They found 66 candidate flowering genes from all know flowering pathways but some key genes such as \( FT \) were not found. Mouhu et al. (2009) also compared the expression of selected flowering time genes in SD and perpetual flowering accessions, but no major differences were found. The strong activation of \( AP1 \) in flowering-induced perpetual flowering accessions enabled them to propose \( AP1 \) as a marker gene for floral induction.
In SD *F. vesca*, there are two *FT*-like genes, namely *FvFT1* and *FvFT2* (Shulaev et al. 2011, Koskela et al. 2012). *FvFT1* is expressed in old leaves of SD *F. vesca* and exclusively under LD (Koskela et al. 2012). Genomic synteny is conserved only around *FvFT1* and Arabidopsis *FT*, indicating that *FvFT1*, but not *FvFT2*, is the homolog gene of *FT*.

The *F. vesca* homolog of the Arabidopsis *SOC1* was also cloned in the study of Mouhu et al. (2009). Later Mouhu et al. (unpublished data 2012) tested whether the function of *FvSOC1* is conserved with Arabidopsis *SOC1* by over-expressing *FvSOC1* in the Arabidopsis T-insertion line, and it was found that *FvSOC1* is still functional as a floral activator in Arabidopsis (Mouhu et al. unpublished data 2012). Mouhu et al. (unpublished data 2012) also showed that *FvSOC1* is photoperiodically regulated, acts downstream of *FvFT* and represses flowering in the presence of *FvTFL1*.

The *TFL1* homolog was found to repress flowering in apple and rose (Kotoda et al. 2006; Iwata et al., 2012). Iwata et al. (2012) found the perpetual flowering *F. vesca* genotype had a 2-bp deletion in the *TFL1* homolog, compared to the seasonal flowering genotype. They proposed this 2-bp deletion *TFL1* homolog causes a frame-shift which leads to the production of a non-functional protein and results in perpetual flowering. The functional evidence provided by Koskela et al. (2012) further showed a 2-bp deletion in *FvTFL1* reverses photoperiodic regulation of flowering from seasonal flowering SD type to perpetual flowering LD type, which results in *FvFT1*-dependent LD flowering. For the first time, Koskela et al. (2012) reported that *FvTFL1* is involved in the photoperiod control of flowering in SD *F. vesca*. As a floral repressor, *FvTFL1* plays a key role in regulating the seasonal cycling between vegetative and reproductive phases.

3 OBJECTIVES

This master’s thesis aimed to explore the molecular control of temperature dependent flower induction in woodland strawberry. The specific aims were: 1) to find out the regulation of low temperature in flower induction in woodland *F. vesca* and LD genotype Hawaii-4, and the phenotypic changes in wild type and transgenic lines; 2) to find out the correlation between low temperature and regulation of flowering genes *FvFT1*, *FvSOC1* and *FvTFL1*; and 3) to analyse the role of *FvFT*, *FvSOC1* and *FvTFL1*
in the temperature regulation of flowering by gene expression analysis, with transgenic lines of *SOC1* and *TFL1*.

### 4 MATERIALS AND METHODS

#### 4.1 Plant material, growth conditions and phenotyping

The experiment included SD *F. vesca* (PI551792), perpetual flowering accession Hawaii-4 (PI551572) and CaMV 35S promoter driven overexpression lines of *SOC1* and *TFL1* in PI551792 (Table 1).

In November 2011, runner cuttings were collected from the mother plants grown in LD conditions at 21°C in the greenhouse. The collected cuttings were rooted in 10 x 10 cm plastic pots in commercial potting medium (Kekkilä, Tuusula, Finland). They were put in a greenhouse maintained at 21°C and 18-h LD condition for 18 days, and during the first week, plants were covered with a layer of plastic. When the plants had four to five unfolded leaves, the planned number of plants as indicated in Table 1 was moved to 11°C for 16 or 32 days. After the exposure, plants were returned to 18°C for observation. The remaining were kept under LD at 18°C.

**Table 1.** Plant materials included in the study and the number of plants used in the greenhouse experiments. Except SD *F. vesca* and Hawaii-4, two lines of *SOC1*-ox were used: 3.2.11.5 and 3.2.11.10, they were marked as *SOC1*-ox#5 and *SOC1*-ox#10 in following thesis, two lines of *TFL1*-ox were also used: 3.10.19.4 and 3.10.19.5. They were marked as *TFL1*-ox#4 and *TFL1*-ox#5 in following thesis.

<table>
<thead>
<tr>
<th>Plant Material</th>
<th>line</th>
<th>No. of plants (11°C)</th>
<th>No. of plants (18°C)</th>
<th>Total No.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>F. vesca</em></td>
<td>26</td>
<td>10</td>
<td></td>
<td>36</td>
</tr>
<tr>
<td>Hawaii-4</td>
<td>5</td>
<td>16</td>
<td></td>
<td>21</td>
</tr>
<tr>
<td><em>SOC1</em>-ox</td>
<td>3.2.11.5</td>
<td>9</td>
<td>10</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>3.2.11.10</td>
<td>8</td>
<td>10</td>
<td>18</td>
</tr>
<tr>
<td><em>TFL1</em>-ox</td>
<td>3.10.19.4</td>
<td>8</td>
<td>10</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>3.10.19.5</td>
<td>9</td>
<td>10</td>
<td>19</td>
</tr>
</tbody>
</table>
The 18-h LD condition was established by illuminating plants 12-h daily with High-Pressure Sodium (HPS) lamps (120 µmol/m²/s) followed by an 6-h daylength extension with incandescent lamps (10 µmol/m²/s). During treatments, the plants were fertilized weekly with fertilizer solution, and after all the plants were moved to 18 °C, they were fertilized twice a week.

Ten *F. vesca* plants were moved back to 18 °C after 16, and 32 days of low temperature treatment, in order to analyze the number of days needed for flower induction at 11 °C. All other plants were moved to 18 °C after 32 days of low temperature treatment.

The effect of treatments on the number of runners, branch crowns, petiole length and flowering time were observed. Runners were counted weekly and removed afterwards until the 13th week. Branch crowns were counted at the 3rd, 7th and 13th week after the beginning of the treatments. The youngest petiole was marked in the beginning of experiment in all plants and petiole length was measured at the 5th week. The flowering time was recorded when the flower was fully open.

### 4.2 Gene expression analysis

#### 4.2.1 RNA isolation

Triplicate leaf samples were collected as biological replicates in the beginning of the treatment and after 8, 16 and 32 days at low temperature. Middle leaflets of the youngest fully open leaf were pooled from 3 to 4 plants in one sample in a 15ml conical tube. The shoot apex samples were taken from the same plant groups at 0, 16 and 32 days. All samples were stored at -80 °C before extracting RNA.

The leaf sample was crushed with a glass rod, around 0.5ml crushed samples were put into 2.0ml eppendorf tubes with locking lid containing two stainless steel grinding balls per tube. Samples in 2.0ml eppendorf tubes were grinded in the oscillating mill (MM40 Retsch® GmbH, Germany) at 28 r/s. The samples were kept frozen by dipping into liquid nitrogen occasionally during the grinding process. Shoot apex samples containing 3-4 apices were grinded by using the same protocol.

The protocol of RNA extraction for pine tree method was used (Monte & Somerville 2002). The RNA extraction buffer (Table 2) was added with 2 % β-mercaptoethanol and
heated to 65 °C. 800 µl of extraction buffer was mixed with ground tissue and vortexed briefly.

Table 2. The contents of RNA Extraction Buffer.

<table>
<thead>
<tr>
<th>Concentration in the buffer</th>
<th>Concentration of the reagents (M)</th>
<th>Measured amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 % CTAB</td>
<td></td>
<td>10 g</td>
</tr>
<tr>
<td>2 % PVP</td>
<td></td>
<td>10 g</td>
</tr>
<tr>
<td>100 mM Tris-HCl</td>
<td>1 M</td>
<td>50 ml</td>
</tr>
<tr>
<td>25 mM EDTA</td>
<td>0.5 M</td>
<td>25 ml</td>
</tr>
<tr>
<td>2.0 M NaCl</td>
<td>5 M</td>
<td>200 ml</td>
</tr>
<tr>
<td>0.5 g/L spermidine</td>
<td></td>
<td>0.25 g</td>
</tr>
<tr>
<td>Sterile, distilled water</td>
<td></td>
<td>225 ml</td>
</tr>
</tbody>
</table>

The mixture was centrifuged for 2 minutes and 700 µl of the upper phase was removed to a new 1.5 ml eppendorf tube. Equal amount of chloroform:isoamyl alcohol (24:1 v/v) was added, the sample was mixed gently and centrifuged (12 000 rpm) for five minutes in a table top centrifuge at room temperature, and 500 µl of the upper aqueous phase was pipetted into a new tube. The chloroform:isoamyl alcohol extraction was repeated, and 360 µl of upper layer phase was removed into a fresh tube. Afterwards RNA was precipitated overnight at 4 °C with 120 µl 8M LiCl (1/3 of the sample volume). The sample was centrifuged (12 000 rpm) for 20 minutes at 4 °C. The pellet was dissolved into warm 500 µl SSTE (1.0 M NaCl, 0.5% SDS, 10 mM Tris-HCl pH8.0 and 1 mM EDTA pH8.0) and centrifuged with equal amount of chloroform:isoamyl alcohol (12 000 rpm) for 10 minutes at 4 °C again. 450 µl of the upper phase was pipetted into a new tube and mixed with 1000 µl freezer cold ethonal. After being precipitated for 30 minutes at -70 °C, the sample was centrifuged (14 000 rpm) for 20 minutes at 4 °C. RNA pellet was then briefly dried and resuspended in 12 µl of RNase free water. Sample was kept on ice for at least 30 minutes (or overnight at 4 °C) before the concentration was measured.

RNA concentrations and purity were determined by the ratio of UV absorbance at 260nm and 280nm. Using a 1:40 dilution, the UV absorbance (A230, A260, A280) was
measured with a spectrophotometer GeneQuant 1300 (GE Healthcare, Chalfont St. Giles, UK). The quality of extracted RNA was checked for degradation on 1% agarose gel in 0.5 x TBE buffer with 0.1 µg/ml ethidium bromide (EtBr). The electrophoresis buffer also contained 0.1 µg/ml EtBr. Only samples with clear 18S and 28S RNA bands were selected for cDNA synthesis (Figure 3). RNA extraction was repeated in the RNA samples which showed degradation as the marked arrows in Figure 3.

![Figure 3](image-url)

**Figure 3** Intact RNA and two degraded RNA samples. 1 µl of sample RNA was run on gel beside the GeneRuler™ DNA ladder. The 18S and 28S RNA bands are clearly visible in most RNA samples, except in the marked samples which showed lower molecular weight smear.

### 4.2.2 cDNA synthesis and RT-PCR

The cDNAs were synthesized from total RNA using Superscript III Reverse Transcriptase kit (Invitrogen, Carlsbad, US). A mastermix of Oligoanchor dT18-VN (50 µM) and dNTP Mix (2.5 µM each) was made in a nuclease-free microcentrifuge tube. 2 µl of the mastermix was added into each PCR tubes. The total amount of RNA (1 µg) was calculated according to the measured concentration and added into the PCR tubes. To make the final volume 14 µl, RNase free water was added separately after calculation.

The 14 µl mixture was heated to 65 °C with the PCR machine for five minutes, and was transferred immediately on ice for incubation for one minute. Afterwards the contents of the tubes were collected by brief centrifugation. A mastermix containing components listed in Table 3 were made and 6 µl was added into each tube. The mixture was mixed by pipetting gently up and down. The samples were incubated at 50 °C for 60 minutes, and the reaction was inactivated by heating at 70 °C for 15 minutes. The product was put into -20 °C freezer until next step.
Table 3. Components added for cDNA synthesis.

<table>
<thead>
<tr>
<th>Components</th>
<th>Amount (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 x First-strand Buffer</td>
<td>4</td>
</tr>
<tr>
<td>0.1 M DTT</td>
<td>1</td>
</tr>
<tr>
<td>Superscript III RT (200 units/µl)</td>
<td>0.7</td>
</tr>
<tr>
<td>RNase free water</td>
<td>0.3</td>
</tr>
<tr>
<td>add. 6</td>
<td></td>
</tr>
</tbody>
</table>

The synthesized cDNAs were diluted into 160 µl and used as templates for real-time PCR. Mixtures of 5 µl of template cDNA, 7.5 µl of LightCycler 480 SYBR Green I Master Kit (Roche Diagnostics, Indianapolis, US) and a primer mix of *FT*, *TFL1*, *SOC1* or *MSII* (3µM each for forward and reverse primers, see Table 4 for sequence) were used to perform 15 µl real-time PCR reactions in a 384-well plate. The quantitative RT-PCR was carried out with Light Cycler 480 real-time PCR system (Roche Diagnostics). The reaction condition was set as follows:

Cycle 1 Pre-incubation: 1 repeat at 95° C for 10 min
Cycle 2 Amplification: 45 repeats
  Step 1: 95° C for 10 sec
  Step 2: 59° C for 10 sec
  Step 3: 72° C for 10 sec

*MSII* (*Multicopy Suppressor of Ira 1*) was used as the normalization gene due to its stable expression in *F.vesca* (Mouhu et al., 2009).

Table 4. PCR primers used in real-time RT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>FT</em></td>
<td>CAATCTCTTGCCGAAAAACT</td>
<td>TGAGCTCAACCTTCCAAG</td>
</tr>
<tr>
<td><em>SOC1</em></td>
<td>ACTTGCTGGTTCTTTTCC</td>
<td>GAGCTTTCCCTGGAGAGAGA</td>
</tr>
<tr>
<td><em>TFL1</em></td>
<td>CTGGCACCACAGATGCTACA</td>
<td>AACGCGAGCAACAGGAAC</td>
</tr>
<tr>
<td><em>MSII</em></td>
<td>TCCCCACACCTTTGATGGCA</td>
<td>ACACCATCAGTCTCTGCAAG</td>
</tr>
</tbody>
</table>
4.2.3 Data analysis

The quantitative RT-PCR analysis gave Ct and Tm values. Ct value was determined by the PCR cycle represented as the intensity of fluorescence so that the exponential phase of the fluorescence density curve crosses the threshold. The threshold is calculated automatically by the qPCR analysis software (LightCycler 480 Software version 1.5). The higher the Ct value is, the less the mRNA is present in the samples. Ct values in this experiment varied from 15-35. MSI1 was set as the endogenous control or reference gene, as it is expressed stably in all samples. The SD F.vesca sample collected in the beginning of the experiment was set as the calibrator, to which all other samples were compared. The relative quantification (RQ) of the calibrator is 1.

The RQ is the fold change compared to the calibrator. It is calculated with the formula:

$$ RQ = 2^{-(\Delta C_t \text{ sample} - \Delta C_t \text{ calibrator})} $$

in which

$$ \Delta C_t = C_t \text{ test gene} - C_t \text{ endogenous control}. $$

A RQ of 2 means the gene is 2 times more expressed than the calibrator; a RQ of 0.5 means the gene is 2 times less expressed. Usually when the value of RQ is out of the range of $0.5 \rightarrow 2$, the expression of that gene is significant compared to the calibrator.

4.3 Statistical analysis of observation data

Analysis of variance (ANOVA) was used to compare the means of runners in the 5th week of TFL1-ox and SOC1-ox lines, and petiole length in all plant groups. The statistical analyses were carried out with SPSS software (Statistical Package for the Social Sciences, version 17.0, SPSS Inc., Chicago, IL, USA).
5 RESULTS

5.1 Temperature control of flowering

Wild type SD *F.vesca* was exposed to 11°C for 16 and 32 days under LD separately. After the exposure, plants were put to 18°C for observation. As shown in Table 5, all plants flowered after 32-day 11°C treatment, 16 days of exposure was enough for 90% floral initiation, although there was a delay of ~38 days in flowering compared with 32 days of exposure. There was no floral induction in wild type SD *F.vesca* at 18°C.

The LD accession Hawaii-4, as an *tfl1* mutant (Koskela et al. 2012), flowered continuously under LD regardless of the temperature difference. All plants showed uniform flowering both at 11°C and 18°C, with an obvious delay in flowering time at 11°C. ANOVA analysis showed the flowering time at 11°C and 18°C was significantly different ($p < 0.05$).

In contrast to SD *F.vesca* and Hawaii-4, flowering responses in several overexpression lines were similar. None of the *FvTFL1* and *FvSOC1* overexpression lines did flower at either 11°C or 18°C.

Table 5. Flowering plants (%) and days to first open flower of different genotypes. Plants were grown under 18h LD and exposed to 11°C for 32 days, with control plants at 18°C. Values are mean ± SE.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Temperature treatment</th>
<th>Flowering plants (%)</th>
<th>No. of days to first open flower</th>
<th>No. of treated plants</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>F. vesca</em></td>
<td>18°C</td>
<td>0</td>
<td>&gt;120</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>11°C-16d</td>
<td>90</td>
<td>117.6 ± 6.9</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>11°C-32d</td>
<td>100</td>
<td>78.2 ± 1.2</td>
<td>16</td>
</tr>
<tr>
<td>Hawaii-4</td>
<td>18°C</td>
<td>100</td>
<td>46.9 ± 0.7</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>11°C</td>
<td>100</td>
<td>73.4 ± 4.8</td>
<td>5</td>
</tr>
<tr>
<td>TFL1-ox#4</td>
<td>18°C</td>
<td>0</td>
<td>&gt;120</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>11°C</td>
<td>0</td>
<td>&gt;120</td>
<td>8</td>
</tr>
<tr>
<td>TFL1-ox#5</td>
<td>18°C</td>
<td>0</td>
<td>&gt;120</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>11°C</td>
<td>0</td>
<td>&gt;120</td>
<td>9</td>
</tr>
<tr>
<td>SOC1-ox#5</td>
<td>18°C</td>
<td>0</td>
<td>&gt;120</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>11°C</td>
<td>0</td>
<td>&gt;120</td>
<td>9</td>
</tr>
<tr>
<td>SOC1-ox#10</td>
<td>18°C</td>
<td>0</td>
<td>&gt;120</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>11°C</td>
<td>0</td>
<td>&gt;120</td>
<td>8</td>
</tr>
</tbody>
</table>
5.2 Temperature control of axillary bud differentiation

Low temperature affected axillary bud differentiation in SD *F.vesca*. From the second week onwards, there was a difference between runner formation of SD *F.vesca* put at 11°C and 18°C (Fig. 4A). The 2nd week and 5th week time point correspond to 16 days and 32 days treatment. The cumulative curve of runners of SD *F.vesca* at 11°C stayed at the same level, indicating the differentiation of axillary buds into runners was repressed. After the SD *F.vesca* plants were transferred to 18°C at the 5th week time point, the runner formation was not promoted by high temperature but still repressed. The axillary buds of control plants at 18°C differentiated into runners constantly from the first week to the end of the experiment.

All the overexpression lines were treated with 11°C for 32 days; after which they were transferred to 18°C for observation. Similarly, the cumulative number of runners in both *TFL1*-ox lines showed a decrease in low temperature (11°C) compared to that in higher temperature (18°C) (Fig. 4B & C). The axillary bud differentiation into runners of *TFL1*-ox lines was repressed when they were at 11°C. One week after returning to 18°C, however, the axillary buds were strongly promoted to be differentiated into runners, as shown in Figure 3B. ANOVA analysis showed at the 5th week time point the number of runners at 11°C differed significantly different from that of 18°C (*p* = 0.002), and more significant than the difference on the 13th week (*p* = 0.004). Low temperature repressed axillary bud differentiation into runners in *TFL1*-ox lines, but the effect can be compensated by following 18°C treatment.

On the other hand, the axillary bud differentiation into runners in *SOC1*-ox lines was only slightly slowed down by low temperature. There was no significant difference in runner formation in *SOC1*-ox lines between 11°C and 18°C treatments at the 5th (*p* = 0.052) or the 13th week time point (*p* = 0.085).
Figure 4: Cumulative number of runners in wild type SD F. vesca (A), TFL1-ox lines (B) and SOC1-ox lines (C), at 11°C and 18°C, LD. Number of plants used for each line was described in Table 1. Values are mean.
In contrary to runner formation, the number of branch crowns was greatly enhanced by low temperature (Fig. 5). Runner formation was repressed in SD *F.vesca* treated at 11°C, while the branch crowns were formed at an increasing rate throughout the experiment. The control plants grown under 18°C started to produce branch crowns when they were moved to and kept at 18°C long enough, no flower initiation occurred.

Similar to SD *F.vesca*, the axillary buds differentiation in *TFL1*-ox lines was strongly regulated by low temperature and they differentiated into branch crowns at 11°C, indicating that *FvTFL1* is not involved in the control of axillary bud differentiation. Although low temperature promoted branch crown formation in *TFL1*-ox lines, the formation rate was slowed down by 18°C from the 7th week, which was in contrast to SD *F.vesca*.

![Figure 5](image_url)

**Figure. 5** Cumulative number of branch crowns formed in wild type SD *F.vesca* and *TFL1*-ox lines at 5th, 7th and 13th week (C). Number of plants used for each line was described in Table 1. Values are mean.
5.3 Temperature control of petiole elongation

The youngest leaves were marked in the beginning of the experiment and the petiole length of these leaves was measured in the end of the experiment. Flower induction is associated with reduced vegetative growth, usually characterized by the decrease in petiole elongation (Konsin et al. 2001). As shown in Table 6, petiole length elongation was significantly reduced by 11°C in all plant groups except SOC1-ox lines.

Although the petiole length in Hawaii-4 and TFL1-ox lines at 11°C was significantly different from that of 18°C, the significance within groups at 11°C was different. The petiole length of Hawaii-4 and TFL1-ox#5 was significantly shorter than in SD F.vesca, but not in TFL1-ox#4. There was no significant difference of the petiole length within various plant lines at 18°C.

Table 6. The petiole length (cm) of different plant groups at 11°C and 18°C, 18-h LD conditions. Values are mean ± SE. Petiole length are data collected at the 5th week of treatment. Significance values between groups of 11°C and 18°C are marked in the right column. Significant difference within groups is compared to F.vesca and marked with asterisk (*).

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Petiole length</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>11°C</td>
<td>18°C</td>
</tr>
<tr>
<td><em>F. vesca</em></td>
<td>7.65 ± 0.21</td>
<td>10.3 ± 0.55</td>
</tr>
<tr>
<td>Hawaii-4</td>
<td>6.56 ± 0.37*</td>
<td>10.6 ± 0.36</td>
</tr>
<tr>
<td><em>TFL1-ox#4</em></td>
<td>7.51 ± 0.27</td>
<td>9.91 ± 0.18</td>
</tr>
<tr>
<td><em>TFL1-ox#5</em></td>
<td>7.01 ± 0.18*</td>
<td>10.08 ± 0.27</td>
</tr>
<tr>
<td><em>SOC1-ox#5</em></td>
<td>8.94 ± 0.18*</td>
<td>10.06 ± 0.48</td>
</tr>
<tr>
<td><em>SOC1-ox#10</em></td>
<td>8.88 ± 0.19*</td>
<td>9.49 ± 0.52</td>
</tr>
</tbody>
</table>
5.4 The effect of temperature on the expression of flowering genes

5.4.1 The expression of flowering genes in leaves

To study the role of SD *F.vesca FT1*, its expression patterns were analyzed in leaves of SD *F.vesca*, Hawaii-4 and transgenic lines. As demonstrated in Figure 6A, *FvFT1* was strongly down-regulated (by about 100 times) in SD *F.vesca* during 11°C treatment, and the down-regulation was obvious at each time point. However, *FvFT1* expression was invariable in perpetual flowering Hawaii-4.

The expression of *FT1* mRNA in *SOC*-ox lines showed a decreasing trend, although there was slight difference between the two *SOC*-ox lines. There was high variation between the replicates, which makes it difficult to make conclusions about the expression pattern. However, clearly *SOC*-ox affected the expression of *FT1* in SD *F.vesca*. *TFL1*-ox lines mimicked SD *F.vesca*, and a strong down-regulation of *FT1* was observed (Fig. 6A).

As is shown in Fig. 6B, expression of *SOC1* in SD *F.vesca* and perpetual flowering Hawaii-4 was differently regulated, as that of *FT1*. In SD *F.vesca*, *SOC1* was only down-regulated by about 2-fold, in contrast to 100 times down-regulation of *FT1* by 11°C. In contrast, *SOC1* expressed in Hawaii-4 was up-regulated also by 2-fold, whereas *FT1* level almost stayed at the same level at 11°C in Fig. 6A.

The expression of *SOC1* in *SOC1*-ox lines was high in all time points and down-regulated by 11°C. However, the down-regulated level at 32-day was still several fold higher than in the wild type *F.vesca*. There was a slight down-regulation of *SOC1* in *TFL1*-ox lines at 0-16 days 11 °C treatment, but an up-regulation at 32-day almost brought the level up to the same of 0-day expression (Fig. 6B).
Figure 6 Expression of FT1, SOC1 and TFL1 normalized to MSII, in the leaves of *F. vesca*, Hawaii-4 and transgenic line overexpressing TFL1 and SOC1 in *F. vesca* background. Data are relative quantification for biological triplicates in each sample, with the 0-day *F. vesca* samples as calibrator. nd indicates no data.
As a floral repressor gene, the expression of *F.vesca TFL1* mRNA was mainly clustered in the shoot apex under LD (Koskela, et al. 2012). Also in this study, the expression level of *TFL1* is generally low in leaves as shown in Fig. 6C.

There was an overall increasing trend of *TFL1* expression in SD *F.vesca*, Hawaii-4 and *SOC1*-ox lines. The up-regulation in all these three groups was around 2-fold compared to the 0-day sample. In contrary to all the other lines, the expression of *TFL1* in the *TFL1*-ox lines was highly up-regulated in leaves in all time points (Fig. 6C).

### 5.4.2 The expression of flowering genes in shoot apex

The regulation of *SOC1* in the shoot apex of SD *F.vesca* and Hawaii-4 was quite similar to that in the leaves. As shown in Fig. 7A, *SOC1* was down-regulated in SD *F.vesca*, whereas up-regulated in Hawaii-4. There was a slight up-regulation in SD *F.vesca* at 32-day apex samples compared to 16-day samples, however.

The level of *SOC1* in the apex of overexpression lines was kept high and not affected by low temperature treatment. In the shoot apex of *TFL1*-ox lines, *SOC1* expression level was comparable to SD *F.vesca*, and about 2-fold down-regulation was observed at 11 °C.

In the apex of SD *F.vesca*, *TFL1* mRNA was about the same level in 0- and 16-day, and down-regulated at 32-day (Fig. 7B). The expression of *TFL1* was around 4 times down-regulated at 32-day compared to 0-day. In the apex of *SOC1*-ox lines, *TFL1* expression almost stayed at the same level in all time points. Not surprisingly, *TFL1* was highly up-regulated in *TFL1*-ox lines.

The floral meristem identity genes *AP1* and *FUL* were slightly down-regulated in SD *F.vesca* in plants treated for 16 days at 11°C. At 32-day apex samples however, *AP1* and *FUL* were highly expressed (Fig. 8). In the *TFL1*-ox lines, *AP1* and *FUL* mRNA were kept at a low level, and negatively correlated to their expression in wild *F.vesca*. Similarly, in the *SOC1*-ox lines, *AP1* and *FUL* levels were low correlating with their flowering phenotypes. The expression of *AP1* was down-regulated, in correlation with increasing *TFL1* expression levels in *SOC1*-ox lines as shown in Fig. 7B. *AP1* and *FUL* expression levels in Hawaii-4 were also low from 0 to 32 day under 11°C treatment, in comparison with the level in SD *F.vesca*. 
Figure 7 Expression of $SOCl$ (A) and $TFL1$ (B) normalized to $MSII$ in the shoot apex of $F.vesca$, Hawaii-4 and transgenic line overexpressing $TFL1$ and $SOCl$ in $F.vesca$ background. Data are relative quantification for biological triplicates in each sample, with the 0-day $F.vesca$ samples as calibrator.
Figure 8 Expression of AP1 and FUL normalized to MSI1, in the shoot apex of F.vesca, Hawaii-4 and overexpressing lines. Data are relative quantification for biological triplicates in each time point, with the 0-day F.vesca samples as calibrator.
The results in this study confirm that Finnish SD *F. vesca* accession acts as a day-neutral plant at 11°C under LD. This is in agreement with earlier studies carried out by Heide & Sønsteby (2007) that five distant Norwegian *F. vesca* populations were all induced to flower at 9°C, and earlier findings by Battey et al. (1998) and Brown & Wareing (1965). After the research of Heide & Sønsteby (2007), which proved temperature and photoperiod interact to control flowering, most studies have focused on the photoperiodic regulation of flowering at molecular level (Mouhu et al., 2009; Koskela et al., 2012). However, less is known about ambient temperature regulation. This study demonstrates that low temperature regulates the vegetative growth in SD *F. vesca*, characterized by the axillary bud differentiation into branch crowns at 11°C and reduced petiole length. *TFL1* is not involved in regulation of vegetative growth while *SOC1* alters vegetative development and may interact with the gibberellin pathway (Mouhu et al. unpublished data). This study also showed that *FvFT1* is strongly down-regulated by low temperature and negatively correlated with floral induction in SD *F. vesca*. The possible candidate output gene of ambient temperature signalling could be *SOC1*. Expression of *FvSOC1* in the leaves and shoot apices of SD *F. vesca* correlated with that of *FvFT1*, indicating its function could be downstream of *FvFT1*. *FvSOC1* might repress flowering by activating the floral repressor gene *FvTFL1*, as the up-regulation of *FvTFL1* mRNA was found in the shoot apices in *SOC1*-ox lines at 11°C. *FvTFL1* is strongly down-regulated by temperature and its down-regulation is required for floral induction SD *F. vesca*.

### 6.1 Temperature affects the vegetative growth of SD *F. vesca*

The effect of temperature on axillary bud differentiation and petiole elongation was studied. Low temperature repressed the differentiation of axillary buds into runners in SD *F. vesca*, and the suppression was constant even after SD *F. vesca* was moved back to 18°C at the 5th week time point. In contrast branch formation was greatly enhanced. High temperature greatly promoted runner formation (Fig. 4). The study of Heide & Sonsteby (2007) also showed the great enhancement of runner formation at high temperature in SD *F. vesca*. The results in this study indicate that, similar to photoperiod (Darrow 1936, Heide 1977) temperature regulates the axillary bud differentiation.
As the vegetative growth and flowering are generally oppositely, or antagonistically regulated by temperature (Heide 1977), the overexpression lines of flowering genes TFL1 and SOC1 were also observed for their axillary bud differentiation. The runner formation in TFL1-ox lines was repressed at 11°C as in SD F.vesca, but the repression was not constant and runner formation was strongly enhanced one week after transfer to 18°C (Fig. 4). In addition, TFL1-ox lines at 11°C produced more branch crowns than at 18°C (Fig. 5). Furthermore, the petiole length of TFL1-ox lines at 11°C was significantly reduced compared to that of 18°C, similarly with in SD F.vesca (Table 6). The results suggest that, firstly TFL1-ox is not, or at least not directly, involved in the temperature regulation of axillary bud differentiation and petiole elongation. Secondly, as a floral repressor, TFL1 overexpression inhibits the transition from vegetative to reproductive phase (Bradley et al. 1997, Koskela et al. 2012). Therefore, although axillary bud differentiation into branch crowns was enhanced by low temperature in TFL1-ox lines, runner formation was also promoted by high temperature after plants were moved to 18°C.

In this study, SOC1-ox lines produced runners continuously (Fig. 4), formed rather long petiole length which was significantly different from that of SD F.vesca at 11 °C, and no branch crowns at all. The results indicate that FvSOC1 affected vegetative growth in SD F.vesca since its overexpression obviously altered the vegetative growth. The role of SOC1 in vegetative growth has been mostly studied in the model plant Arabidopsis. During vegetative phase of Arabidopsis, SOC1 is expressed in leaves independently of FT (Melzer et al. 2008, Schimid et al. 2003). Melzer et al. (2008) found soc1 ful double mutant in Arabidopsis enhanced aerial rosette formation, stem growth and repeated reversion to vegetative growth. SOC1 protein is thought to function as a regulator in organogenesis during vegetative growth (Lee & Lee 2010).

It was hypothesized that FvSOC1 may regulate vegetative development in SD F.vesca by affecting the gibberellin (GA) pathway (Mouhu et al. unpublished data, Shalit et al. 2009). GA is necessary for the runner initiation in strawberry and the absence of GA leads to formation of branch crowns (Hytönen et al. 2009). GA also plays a role in regulating the petiole growth in strawberry (Wiseman & Turnbull 1999). Taken together, the distinctive long petiole length and continuous runner formation in SOC1-ox lines could be GA-dependent. In the study of Mouhu et al. (unpublished data), the F.vesca homologs of GA biosynthetic genes GA20ox (GA20-oxidase) and GA3ox (GA3-oxidase) were activated in SOC1-ox plants, but down-regulated in the RNAi lines. In
Arabidopsis, \textit{SOC1} is involved in mediating GA responses to flowering (Searle et al. 2006), but how \textit{SOC1} affect GA biosynthesis and further regulate vegetative growth still remains an open question.

6.2 Low temperature promotes flowering in SD \textit{F.vesca}, 16 days was enough for floral induction

The observed flowering phenotypes at 11°C indicate that low temperature plays an important role in regulating flowering in SD \textit{F.vesca}. Under LD, SD \textit{F.vesca} was induced to flowering at 11°C, but no flowering was initiated at all at 18°C (Table 5). The five \textit{F.vesca} populations in the experiment of Heide & Sønsteby (2007) showed \textit{F.vesca} only flower in SD at 18°C. Results in this study also suggest that \textit{F.vesca} needs a critical photoperiod less than 18h to induce flowering at 18°C.

Heide & Sønsteby (2007) found that 4 weeks of exposure to SD at 15°C was required for floral induction in \textit{F.vesca}. This study also tried to identify the critical time period required for floral induction under 11°C, LD. SD \textit{F.vesca} was induced to flower only after 16 days of exposure at 11°C, although the flowering was obviously delayed compared to the 32 days exposure (Table 6). The results showed a critical length of no more than 16 days was required for floral induction under 11°C, LD. The critical floral induction period is different when SD \textit{F.vesca} is placed under different conditions.

6.3 \textit{FvFT1} is the main but not a single output of ambient temperature signalling in SD \textit{F.vesca}

In SD \textit{F.vesca}, there are more than one \textit{FT}-like genes – \textit{FvFT1} and \textit{FvFT2} (Shulaev et al. 2011, Koskela et al. 2012). Genomic synteny is conserved with Arabidopsis \textit{FT} only around \textit{FvFT1} which is highly expressed in old leaves (Shulaev et al. 2011, Koskela et al. 2012). \textit{FvFT1} was found to present an expression peak during night under LD, but no expression under SD (Koskela et al. 2012), which means \textit{FvFT1} is expressed in a negative correlation with flower induction. In this study, all the plants were put in LD condition, and the tested \textit{FT} gene was \textit{FvFT1}. \textit{FvFT1} mRNA expression was strongly down-regulated by low temperature and negatively correlated with flowering in SD \textit{F.vesca} (Fig. 6). This is in accordance with the findings by Koskela et al. (2012), in whose research the down-regulation of \textit{FvFT1} under SD correlated with flower induction. The result was contrasted with \textit{FT} homolog mRNA expression in other SD
plants such as rice (Tamaki et al. 2007). In SD plant rice, Tamaki et al. (2007) showed that the FT ortholog gene Hd3a also encodes the Hd3a protein which moves from leaves to meristem to induce flowering. Hd3a mRNA accumulates mainly in leaf blade under inductive SD conditions, which is similar to FT expression in Arabidopsis under LD.

In perpetual flowering genotype Hawaii-4, FvFT1 expression stayed almost at the same level at 11°C (Fig. 6), and there was a delayed flowering of Hawaii-4 at 11°C compared to plants at 18°C (Table 5). So FvFT1 might be positively correlating with flowering induction in Hawaii-4, as in the LD plant Arabidopsis. In Arabidopsis, FT is not expressed under SD, but activated when plants are exposed to LD (Corbesier et al. 2007, Imaizumi et al. 2003). Koskela et al. (2012) also identified the role of FvFT1 in activating floral identity genes in LD accession Hawaii-4.

In SD F.vesca, although FvFT1 was highly expressed under LD at 0-day, low temperature (11°C) strongly down-regulated its expression before plants were induced to flower at 32-day when floral meristem genes AP1 and FUL were strongly up-regulated (Fig. 6 & 8), indicating that FvFT1 is unlikely an effective flowering activator. Given that the wild type SD plants treated under 11°C LD conditions flowered without the expression of FT homologous gene raises the question that, what then could be the flowering signal?

In Arabidopsis, the signals from ambient temperature are integrated by FT (Kobayashi et al., 1999). The thermosensory pathway mediator SVP, which acts downstream of FCA and FVE, binds to FT and negatively regulates its expression (Lee et al., 2007). In SD F.vesca, however, the answer could be that FvFT1 may not be the single output gene in the ambient temperature signaling pathway (Fig. 9). SOC1 could be another candidate as an ambient temperature output gene in SD F.vesca, since the function of soc1 mutants is additive to ft mutants (Samach et al. 2000, Lee et al. 2007). SOC1 integrates signals from different pathways – the photoperiod pathway, the autonomous pathway and gibberellin pathway (Borner et al., 2000). The hypothesis in SD F.vesca is supported by the findings of Li et al. (2008) in Arabidopsis. Li et al. (2008) found that SVP, the mediator of ambient temperature, modulates not only the expression of FT in the leaves, but also forms a complex with FLC and bind to the promoter of SOC1 directly for transcriptional repression in leaf and shoot apex. Furthermore, the SVP-FLC complex affects SOC1 expression more strongly than FT. FLC is absent in SD F.vesca,
but a similar mechanism including different regulators might be present. Another possible redundant gene could be \textit{FvFT2}, which is exclusively expressed in flower buds of wild type \textit{F.vesca} under SD (Koskela et al. 2012). However, all of these hypotheses need more research to support.

\textbf{6.4 \textit{FvSOC1} may act downstream of \textit{FvFT1} and repress flowering in SD \textit{F.vesca}}

In SD \textit{F.vesca}, \textit{FvSOC1} expression in the leaves and apices was in accordance with the \textit{FvFT} expression and down-regulated in general by low temperature (Fig. 6 & 7). However, the expression of \textit{FvSOC1} in the leaves and apices of SD \textit{F.vesca} and in Hawaii-4 was oppositely regulated, as that of \textit{FvFT1} (Fig. 6 & 7). Mouhu et al. (unpublished data) found the silencing of \textit{FvFT1} lead to down-regulation of \textit{FvSOC1} and they hypothesized that the regulation between \textit{FT} and \textit{SOC1} is conserved between annual Arabidopsis and perennial SD \textit{F.vesca}. Taken together, \textit{FvSOC1} may act downstream of \textit{FvFT1} in SD \textit{F.vesca} – it acts as a floral repressor in SD \textit{F.vesca} and an activator in LD accession Hawaii-4.

The overexpression of \textit{SOC1} homolog in SD \textit{F.vesca} kept the plants at continuous vegetative development under flower inductive condition 11°C, LD, indicating \textit{SOC1-ox} lines were not sensitive to ambient temperature regarding flower induction. Mouhu et al. (unpublished data) found that \textit{FvSOC1} overexpressed in Arabidopsis promoted flowering, suggesting the function is conserved with \textit{AtSOC1}. However, the finding that \textit{FvSOC1} represses flowering in SD \textit{F.vesca} implies it may have different targets or interacting partners compared to Arabidopsis.

Furthermore, \textit{FvSOC1} may repress flowering by activating the repressor \textit{FvTFL1} to prevent flowering. \textit{FvTFL1} was identified as a major floral repressor in SD \textit{F.vesca} (Koskela et al. 2012). Mouhu et al. (unpublished data) provided functional evidence showing that the function of \textit{FvSOC1} depends on \textit{FvTFL1}. \textit{FvSOC1} and \textit{FvTFL1} are localized in the overlapped region in the shoot apex of SD \textit{F.vesca} under LD, and \textit{FvSOC1} activates flowering rather weakly in the \textit{FvTFL1} mutant Hawaii-4. \textit{FvTFL1} expression was up-regulated in \textit{FvSOC1-ox} lines and down-regulated in \textit{FvSOC1-RNAi} lines of SD \textit{F.vesca} background (Mouhu et al. 2012 unpublished data).

In this study, the up-regulation of \textit{FvTFL1} mRNA in \textit{SOC1-ox} lines was found in the shoot apices of SD \textit{F.vesca} at 11°C (Fig. 6 & 7), suggesting \textit{FvSOC1} may prevent the
down-regulation of \textit{FvTFL1} at low temperature and repress flowering similar to the photoperiodic regulation. Whether the activation is direct or not, however, needs more research.

6.5 \textit{FvTFL1} represses flowering and is regulated by temperature in SD \textit{F.vesca}

The role of \textit{FvTFL1} has to be considered in revealing the flowering mechanism of SD \textit{F.vesca} in response to ambient temperature. \textit{TFL1} was found to be a floral repressor in \textit{Arabidopsis} (Bradley et al. 1997, Ratcliffe et al. 1998), and its homologs were also identified to regulate flowering seasonality in perennial plants such as rose, tomato and \textit{Populus} (Iwata et al. 2012, Pnueli et al. 1998, Mohamed et al. 2010). In this study, the \textit{AP1} and \textit{FUL} expression in the shoot apex of \textit{TFL1}-ox lines were low compared to SD \textit{F.vesca} and were not affected by low temperature during the time interval tested (Fig. 8), indicating that \textit{FvTFL1} represses floral meristem identity genes.

There was strong temperature regulation of \textit{FvTFL1} in the shoot apices but not in the leaves of SD \textit{F.vesca} (Fig. 6 & 7). At 0-day of 11°C treatment, \textit{FvTFL1} was highly expressed in the shoot apex, whereas the expression was strongly down-regulated to a weak level at 32-day (Fig. 6 & 7). However, at 32-day floral meristem identity genes \textit{AP1} and \textit{FUL} were strongly up-regulated for floral induction (Fig. 8). The results suggest that \textit{FvTFL1} is regulated by temperature and the down-regulation of \textit{FvTFL1} is necessary for floral induction in SD \textit{F.vesca} at 11°C. In the study of Koskela et al. (2012), genotype Hawaii-4 flowered continuously under LD condition due to its nonfunctional \textit{FvTFL1} gene, as was the flowering trait in mutated \textit{FvTFL1} overexpressing lines. Similarly here, Hawaii-4 flowered continuously under LD regardless of the temperature difference.

Koskela et al. (2012) showed a strong photoperiodic regulation of \textit{FvTFL1} in the shoot apex of SD \textit{F.vesca}. SD \textit{F.vesca} requires SD for induction of flowering due to the photoperiodic regulation of \textit{FvTFL1}, but \textit{FvTFL1} is absent in perpetual flowering Hawaii-4. Furthermore, the RNAi silencing of \textit{FvTFL1} resulted in LD flowering trait in SD \textit{F.vesca}.

As \textit{SEASONAL FLOWERING LOCUS (SFL)}, \textit{TFL1} may respond to both photoperiod and temperature. Strasser et al. (2009) suggested \textit{TFL1} is one of the target genes in the genetic pathway regulating flowering in response to ambient temperature. The results
from this study showed that the expression of *FvFT1* and *FvSOC1* in the *FvTFL1*-ox lines mimic those in wild *F.vesca*, indicating the constitutive expression of *FvTFL1* does not alter the pathway that involves either *FT* or *SOC1*.

*TFL1* is homologous gene of *FT* but play antagonistic functions in flowering regulation. Both of them can interact with the FD bZIP transcription factor and form a complex which can either induce or repress flowering (Abe et al. 2005, Wigge et al. 2005, Ahn et al. 2006). In Arabidopsis, temperature induced flowering is mediated by *FT*. *FT* is probably mediated by autonomous pathway genes *FCA* and *FVE*, independently of *FLC* (Blázquez et al. 2003, Balasubramanian et al. 2006). Recent research also revealed *PHYTOCHROME INTERACTING FACTOR4* (*PIF4*) regulates *FT* in a temperature-dependent way (Kumar et al. 2012). Actually Blázquez et al. (2003) had indicated an *FT*-independent flowering pathway in response to ambient temperature in Arabidopsis, since they found *ft* loss-of-function mutated plants were still responding to ambient temperature.

![Diagram of temperature regulation of flowering in *F.vesca*.](image)

**Figure. 9** Model showing the temperature regulation of flowering in SD *F.vesca*. Arrows indicate activation, and bars indicate repression.
In SD *F. vesca*, Koskela et al. (2012) proposed that *FvTFL1* might overcome the function of activator *FvFT1* to repress flowering under LD. But this hypothesis was not convincible since *F. vesca* grown under SD showed down-regulation of both *TFL1* and *FT*. They further suggested an *FvFT1*-independent pathway regulating flowering in SD *F. vesca*. Results in this study also lead to a similar hypothesis: as SD *F. vesca* was induced to flower at 11°C LD but also showed down-regulation of both *FvTFL1* and *FvFT1* (Fig. 9). More research needs to be done to identify the *FT*-independent pathway.
7 CONCLUSION

This master thesis showed that in SD *F.vescia*, ambient temperature alters the expression of *FT*, *SOC1* and *TFL1* homologs. It might be that *FT*, *SOC1* and *TFL1* homologs act in a linear pathway in SD *F.vesca*. The role of *FvFT1* and *FvSOC1*, however, is much more complex regarding their response to ambient temperature. The results in this study suggest *FT* may not be the single output of thermosensory pathway, an *FT*-independent pathway may regulate flowering during ambient temperature signaling.

Temperature and photoperiod crosstalk in plants is becoming increasingly important as their interaction was proved to help the plants to monitor ambient surroundings and adapt to environmental change. This study is a small step on exploring the temperature dependent flowering regulation in SD *F.vesca* using a molecular approach. The role of flowering genes identified in the perennial model plant might be useful in studying the flowering mechanisms in octoploid strawberry, and further help the practical cultivation and breeding.

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