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Genetic and Environmental Control of Flowering in Wild and Cultivated Strawberries

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GENETIC AND ENVIRONMENTAL CONTROL OF FLOWERING IN WILD AND CULTIVATED STRAWBERRIES

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ACADEMIC DISSERTATION

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ABSTRACT

Strawberries (*Fragaria* sp.) belong to the large family of Rosaceae that includes commercially important crop plants such as apple, pear, peach and roses. The economic impact of these species is huge and breeders around the world are striving to keep up with consumers’ demands on novelty produce. At the same time, climate change is having an impact on the onset of flowering especially in species that are grown in temperate climates. As flowering is a prerequisite for yield formation, it is extremely important to gain an insight on how the environmental factors, most importantly photoperiod and temperature, affect the timing of flowering in Rosaceous species.

Although studying flowering responses directly in the cultivated species could provide immediate practical applications, it is often not feasible due to e.g. complex genomics of the species, large plant size or long juvenile period. The woodland strawberry *Fragaria vesca* (L.) has arisen as a convenient model plant for strawberries and the entire Rose family. It is a diploid species and therefore has a less complex genome than the cultivated octoploid strawberry.

The work described here begun by elucidating the molecular identity of *SEASONAL FLOWERING LOCUS* (*SFL*), a locus controlling the switch from seasonal to continuous flowering habit in woodland strawberry. *SFL* was identified as the woodland strawberry orthologue of *TERMINAL FLOWER1* (*FvTFL1*) based on its location on the strawberry genome and similarity to its *Arabidopsis* counterpart, *TFL1*. In woodland strawberry, *FvTFL1* was shown to be photoperiodically regulated, and it was demonstrated that the continuous flowering habit is caused by a mutation at *FvTFL1*. In the following experiments, altered regulation of *FvTFL1* was associated with the unique vernalisation requirement in the artic *F. vesca* accession Alta-1, suggesting a previously uncharacterised function for a *TFL1* orthologue.

The findings on *FvTFL1* were extended to cultivated strawberry. It was demonstrated that *F. × ananassa* homologue of *TFL1* (*FaTFL1*) also represses flowering, and that differences in the regulation of *FaTFL1* were associated with different flowering times in strawberry cultivars. The finding that *FaTFL1* is a major determinant in the flowering response of cultivated strawberry provides breeders with a new breeding target; producing cultivars with lowered *FaTFL1* expression level could expand the flowering and fruiting season of strawberries.

These results clearly demonstrate the feasibility of the model plant approach and also highlight the importance of fundamental research. The knowledge gained on fundamental genetic pathways in model plants can be transferred to crop plants, in which similar genetic studies would be impossible or at least extremely complex, slow and costly to perform.
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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following publications, which are referred to in the text by their Roman numerals (I–III). The publications are reprinted with permission from their original publishers.


AUTHORSHIP STATEMENT

In (I), EAK carried out genetic linkage analysis for the F2 population, designed and produced the FoTFL1 vector constructs for transformation into plants, did the transformations and analyzed the transgenic plants. RNA extraction and RT-qPCR was done by EAK, KM and MR. MR designed, produced and analyzed the FT-RNAi transgenic plants. MCA carried out genetic linkage analysis for the BC population. TK designed and performed the in situ hybridizations. Manuscript was written by EAK, TH and KM with input from all the authors.

In (II), the growth experiments were designed and carried out by EAK, AS, TH and OMH. EAK genotyped the F2 population, extracted RNA and performed RT-qPCR analysis. TK made the crosses between Alta-1 and the other parents. TT and TH designed, carried out and analysed the GBS experiment and phenotyping of woodland strawberry accessions. Manuscript was written by EAK and TT with input from all the authors.

In (III), the growth experiments were designed and carried out by EAK, AS, TH and OMH. EAK did RNA extractions and RT-qPCR analysis. Plant transformations were done using the vectors constructed in (I) by HF and MVH, who also carried out the initial analyses of the transgenic plants. Growth experiments for the transgenic plants were carried out by TH. Manuscript was written by EAK and TH with input from all the authors.
1 INTRODUCTION

Strawberries (Fragaria sp.) belong to the large family of Rosaceae that includes commercially important crop plants such as apple, pear, peach and roses. The economic impact of strawberry cultivation and trade is huge; in the year 2013, the worldwide production of strawberries exceeded 7.5 million metric tonnes with an estimated gross value of 13.7 billion euros (FAOSTAT, 2015). However, strawberry production is not without problems. As temperature has a large effect on flowering time of strawberries, progressing climate change is bound to have an effect on many strawberry growing areas. To meet these environmental challenges, plant breeders need tools for developing new cultivars that are suitable for growing in specific environments. Research on genetics and genomics of strawberry could provide such tools for more accurate selection. For instance, identification of genes with major effects on physiological responses could provide new breeding targets for tailored flowering time. Also, development of molecular markers linked with specific genes could speed up the breeding process significantly.

Strawberries show a large variation in their environmental responses. Most diploid and octoploid strawberries are short day (SD) plants that are induced to flower in autumn and bear flowers the following spring (Darrow, 1966). However, some accessions and cultivars are everbearers that produce new inflorescences throughout summer. This exceptional response has attracted a lot of attention, as it can serve as a source for environmental adaptation.

This PhD thesis studies the genetic mechanisms controlling flowering in both diploid and octoploid strawberries. The first article elucidates the molecular identity of the SEASONAL FLOWERING LOCUS (SFL), a major locus controlling the switch from SD to everbearing flowering type in woodland strawberry, and shows that SFL encodes for an F. vesca orthologue of the floral repressor TFL1. The second article presents a previously uncharacterised role for a TFL1 orthologue in the unique vernalisation response observed in an arctic diploid strawberry accession Alta-1. In the third article, the results obtained from studies with the diploid woodland strawberry are extended to the octoploid strawberry, and the role of FaTFL1 in environmentally regulated flowering in the octoploid strawberry is investigated.
2 REVIEW OF THE LITERATURE

Luckily for plant breeders, diversity in the *Fragaria* genus is large. *Fragaria* includes 27 acknowledged taxa with ploidy levels ranging from diploid to decaploid and covering a wide distribution over most of Eurasia and extending down to Peru in South America (Staudt, 2009; Njuguna et al., 2013). The *Fragaria* species with by far the widest distribution range is the diploid woodland strawberry *F. vesca*, which is spread over Eurasia and northern America (Staudt, 2009; Liston et al., 2014). Most diploid and tetraploid strawberry species are native to southern Asia, which has been considered the place of origin of the most recent common ancestor of the *Fragaria* species of today (Njuguna et al., 2013; Johnson et al., 2014). The only hexaploid strawberry species *F. moschata* is native to Europe and has been cultivated there for centuries (Liston et al., 2014). The octoploid *F. chiloensis* grows on a narrow coastal strip in western North America and central and southern Chile, has been cultivated in Chile for over thousand years (Hancock et al., 1999), while the octoploid Virginian strawberry *F. virginiana* is native to Northern America (Darrow, 1966; Liston et al., 2014). Natural hybrids between strawberry species have been repeatedly reported, even between species with different ploidy levels (Bringhurst, 1990). Interspecies hybridization has also led to the birth of the cultivated strawberry, *Fragaria × ananassa* (Darrow, 1966).

2.1 HISTORY OF THE CULTIVATED STRAWBERRY

From the human point of view, the cultivated strawberry *Fragaria × ananassa* is evidently the most important species of *Fragaria*. As a species, the cultivated strawberry is very young; it originated as an interspecific cross between the octoploids *F. chiloensis* and *F. virginiana* in a French garden some time between 1714 and 1766. The maternal *F. chiloensis* was brought to Europe from Chile in 1714 by a French spy, Captain Frézier (Darrow, 1966). However, all the five imported plants turned out to be female and did not produce any fruit due to lack of pollenisers. When interplanted with *F. virginiana*, which had been introduced earlier from Northern America, the plants produced fruit and *F. chiloensis* became the major strawberry species cultivated in Europe (Darrow, 1966; Hancock et al., 1999). As time passed, strawberry seedlings with unusually large fruit and red flesh began to appear in European gardens. In 1766, botanist Duchesne determined these seedlings to be hybrids of *F. chiloensis* and *F. virginiana*, and the new species was named as *F. × ananassa* (Darrow, 1966).

The origins of the everbearing trait in cultivated strawberry can be traced back to several independent sources. The first described everbearing cultivar ‘Gloede’s Seedling’ was introduced in France in 1866. Although it was freely
runnering, and thus easy to propagate clonally, it was not commercially successful (Darrow, 1966). Other early everbearing cultivars developed in Europe are thought to originate from interspecific crosses between diploid everbearing strawberries and large-fruited octoploid SD cultivars (Darrow, 1917), but it is doubtful whether the parentage is correctly reported. Everbearing cultivars with European origin met only limited success, except for ‘Sans Rivale’, which was the leading everbearer grown in France in the 1960’s (Darrow, 1966). The first successful everbearer of American origin is ‘Pan American’ introduced in 1902. ‘Pan American’ originated from a chance seedling or clonal mutation of the SD cultivar ‘Bismarck’, and it appears in the parentage of many modern day everbearing cultivars (Darrow, 1917; Darrow, 1966). The most recent source of the everbearing trait is a native population of *F. virginiana* ssp. *glauca* in the Wasatch Mountains in Utah (Bringhurst and Voth, 1980). This germplasm introduction has resulted in the release of many commercially important everbearing strawberry cultivars suitable for growing in North America (Ahmadi et al., 1990).

Most strawberry cultivars are descendants of the early European cultivars (Darrow, 1966), although germplasm introductions from wild progenitor species have been made occasionally (Bringhurst and Voth, 1980; Hancock et al., 2002; Hancock et al., 2010). As a result of a limited number of founding parents, modern strawberry cultivars have narrow genetic diversity. For example, Dale and Sjulin (1990) examined pedigree data of 134 North American cultivars released between 1960 and 1987 and discovered that these cultivars originated from just 17 maternal founding clones. More recently, Honjo et al. (2009) detected only three maternal sources of chloroplast DNA in a study that included 75 strawberry cultivars from around the world. Loss of diversity seems to have accelerated as a result of modern breeding programmes; both Gil-Ariza et al. (2009) and Horvath et al. (2011) showed that modern American and European cultivars have much lower degree of diversity than old European cultivars introduced before 1930. It is therefore vital that the genetic basis of strawberry breeding programmes is widened to avoid further losses of diversity.
2.2 PHYLOGENETIC RELATIONSHIPS WITHIN FRAGARIA

As *F. × ananassa* is such an important crop, special interest lies in identifying the diploid ancestors of the octoploid species. Cytological studies (Bringhurst, 1990) proposed octoploid genomic composition of AAA’A’BBB’B’, suggesting an allopolyploid history and subgenomic contribution from two diploid ancestral species. The work of Bringhurst (1990) also suggested that the octoploid genomes are highly diploidized, an observation which has been later confirmed in several studies (Ashley et al., 2003; Rousseau-Guetin et al., 2008).

The earliest report on *Fragaria* phylogeny based on molecular evidence came from a restriction fragment length polymorphism (RFLP) study on chloroplast DNA (Harrison et al., 1997). However, the authors reported only limited amount of variation in their samples and were unable to ascertain phylogenetic relationships at a high resolution. Potter et al. (2000) used both chloroplast and nuclear sequence data to study phylogenetic relationships of 14 *Fragaria* species, and concluded that *F. vesca* and *F. nubicola* are the diploid species most closely related to the polyploid species.

The most recent efforts to examine the ancestry of octoploid strawberries use thousands of loci, either using targeted sequence capture (Tennessen et al., 2014) or SNP marker arrays (Sargent et al., 2016). Both groups arrived at the conclusion that one of the octoploid subgenomes originates from an *F. vesca*-like ancestor and one from *F. iinumae*, and the two groups also agreed that all the subgenomes display disomic inheritance. However, whereas Tennessen et al. (2014) identified the two remaining subgenomes to be *F. iinumae*-like, Sargent et al. (2016) suggested an unidentified subgenomic ancestor. Irrespective of the ancestral origins of the two subgenomes, *F. vesca* appears to be a close relative of the cultivated strawberry, making the use of *F. vesca* as a model species plausible.
2.3 STRAWBERRY PHYSIOLOGY

Strawberry life cycle

All strawberry species share similar life histories; they are small, perennial herbs capable of both sexual reproduction via flowering and clonal growth via stolon (or runner) formation (Johnson et al., 2014). Strawberry rosette crown consists of a thick stem with short internodes. The stem terminates in main shoot apex, which is sheltered by leaf bases (Figure 1A). Each leaf axil is accompanied by an axillary bud, which may either remain dormant or develop into runners, new branch crowns or inflorescences (Brown and Wareing, 1965; Darrow, 1966). Fates of the axillary meristems depend on environmental conditions, which define the yearly growth cycles of generative and vegetative growth.

Figure 1. Plant structure and seasonal growth cycles in strawberry. A) A LD-grown vegetative strawberry plant with several branch crowns. Runners have been removed. Magnification illustrates a single axillary branch crown; B) Typical seasonal growth cycle of woodland strawberry grown in temperate zone. Flowering occurs in late spring. Vegetative growth is continued from young branch crowns that were not induced in autumn. The axillary meristems of these branch crowns produce runners during the summer months, and new branch crowns towards autumn. In autumn, the main shoot apical meristem and the apical meristems of the oldest axillary branch crowns develop into inflorescence meristems, which complete their development the following spring.
After overwintering, plants start a period of active growth manifested by outgrowth of leaf petioles and inflorescences (Figure 1B). Flowering occurs in spring, after which plants enter a phase of active vegetative growth, characterized by ample runner production. Runner production ceases towards autumn and axillary buds develop into branch crowns. Crown branching is promoted by shortening days in autumn and is essential for the perennial growth habit of strawberries, as the young branch crowns continue the vegetative growth of the plant. During floral induction, the apical meristem of the main shoot develops into inflorescence meristem, which overwinters and finishes its development the following spring (Brown and Wareing, 1965; Darrow, 1966).

**Physiological responses to photoperiod and temperature in the cultivated strawberry**

Perhaps the most important physiological response to environmental conditions in strawberry from the point of view of yield formation is flower induction, and it has been extensively studied in cultivars of *F. × ananassa*. Most strawberry cultivars are SD plants, or June-bearers, although also everbearing cultivars (Darrow and Waldo, 1934) and photoperiod-insensitive day-neutral cultivars (Brinthurst and Voth, 1980) have been described. Everbearing cultivars originate from old European and American cultivars introduced in the beginning of the 1900s (Darrow, 1966; Hancock et al., 1999), whereas day-neutral cultivars originate from an introduction of *F. virginiana* ssp. *glauca* into the cultivated strawberry germplasm (Brinthurst and Voth, 1980).

The everbearing and day-neutral *F. × ananassa* cultivars have been historically classified into distinctive flowering types (Durner et al., 1984), although recent studies have shown that flowering in both types is actually promoted by long days (LDs) (Nishiyama and Kanahama, 2002; Sonstbye and Heide, 2007). The confusion in the literature about flowering types of the cultivated strawberry is probably caused by the strong photoperiod × temperature interaction; a cultivar may appear day-neutral at one tested temperature, but turn out to have an obligatory photoperiod requirement at another temperature. For clarity, the term “everbearer” is used in this work to refer to strawberry cultivars showing a perpetual flowering phenotype, irrespective of the origin of the everbearing trait. Moreover, perpetual flowering strawberries were referred to as everbearers already by Darrow (1917), and therefore the term is also historically justified.

SD cultivars are induced to flower when the daylength falls below a certain critical limit in autumn. The critical daylength is heavily dependent on temperature; flowering is inhibited in all photoperiods at high temperatures of above 24°C, short days promote flowering at intermediate temperatures between 14 and 20°C whereas cooler temperatures induce flowering
Physiological responses of the diploid F. vesca to environmental conditions are notably similar to the responses observed in cultivars of octoploid strawberry. Most naturally occurring F. vesca accessions are SD plants (Heide and Sønsteby, 2007), although also everbearing flowering types have been characterized (Brown and Wareing, 1965). The photoperiodic response of SD accessions is strongly dependent on temperature; flowering is inhibited under all photoperiods at temperatures above 21°C, short days promote flowering at independently of photoperiod (Heide, 1977; Durner et al., 1984; Sønsteby and Nes, 1998; Manakasem and Goodwin, 2001). Cultivars have marked differences in their responses to photoperiod and temperature; for example, early cultivars bred for growing at high altitudes in Norway flower photoperiod-independently even at 18°C whereas cultivars developed for Middle European conditions require SDs of 13 hours at the same temperature (Heide, 1977).

In contrast to SD cultivars, everbearing cultivars form new inflorescences throughout the summer and produce more than one crop per growing season. Flowering in everbearing cultivars is controlled by an interaction of photoperiod and temperature; everbearers have an obligatory LD requirement at the high temperature of 27°C, LDs promote flowering at intermediate temperatures between 15 and 21°C, and cool temperature of 9°C delays flowering irrespective of the photoperiod (Nishiyama and Kanahama, 2002; Sønsteby and Heide, 2007).

The number of apical meristems that can be induced to form an inflorescence is directly dependent on the number of branch crowns on the plant. Therefore, branch crown formation has a large effect on subsequent yield potential, and in some cases, the high yield potential of everbearing strawberries has been attributed to the higher number of branch crowns per plant (Camacaro et al., 2002). Crown branching is under photoperiodic control and it is promoted by SDs in SD cultivars (Konsin et al., 2001; Hytönen et al., 2004). Photoperiodic control of branch crown formation in everbearing strawberries has been studied much less, although it appears that LDs may advance branch crown formation at least in some everbearing cultivars (Taylor, 2002).

Runner formation is generally promoted by LDs and high temperature in both SD and everbearing cultivars, although there are cultivar-specific differences (Heide, 1977; Konsin et al., 2001; Sønsteby and Heide, 2007; Bradford et al., 2010). Moreover, many everbearing cultivars produce only limited numbers of runners, making their nursery propagation difficult and hindering their utility for commercial production (Camacaro et al., 2002; Bradford et al., 2010).
intermediate temperatures between 15 and 18°C, whereas cooler temperatures induce flowering independently of photoperiod (Figure 2A; Heide and Sønsteby, 2007). Flowering in the everbearing types of *F. vesca* is promoted by LDs and high temperature (Figure 2B; Sønsteby and Heide, 2008; Mouhu et al., 2009), similarly to the situation in cultivated strawberry.

Figure2. Typical flowering responses in seasonal flowering and everbearing *F. vesca* genotypes. Seasonal flowering woodland strawberry genotypes (A) flower at cool temperature nearly daylength-independently (dark grey and light grey circles for SD and LD, respectively; the degree of circle filling depicts the approximate percentage of flowering plants), while high temperatures (white circle) inhibit flowering. Flowering in everbearing genotypes (B) is promoted by LDs and SDs inhibit flowering especially at very high temperature. Data adapted from Heide and Sønsteby (2007), Sønsteby and Heide (2008) and Rantanen et al. (2015).

Runner formation in *F. vesca* is promoted by LDs and high temperature (Heide and Sønsteby, 2007), similarly to the cultivated strawberry. Many everbearing *F. vesca* accessions do not form runners at all (Brown and Wareing, 1965), unless they are subjected to SDs at high temperature (Sønsteby and Heide, 2008).

An accession of the diploid *F. vesca* displays a physiological response not characterised in the cultivated strawberry, or in any other strawberry species studied to date. When Heide and Sønsteby (2007) studied Norwegian *F. vesca* populations, they discovered an accession in the northern Norway that could not be induced to flower even after a 15-week exposure to SDs at 9°C. The accession, named Alta-1 after its place of origin, required at least 5 weeks at 2°C, with longer cold duration accelerating flowering. Alta-1 also flowered in field conditions, albeit two weeks later than the other Norwegian populations included in the experiment (Heide and Sønsteby, 2007). These results suggested that Alta-1 may possess an adaptation to artic conditions, preventing premature flowering in spring.
2.4 PATHWAYS TO FLOWERING

Although the physiology of flowering and vegetative development have been extensively studied in strawberries, much less is known about the molecular pathways controlling these events. Molecular level studies have been conducted mostly in model plant Arabidopsis, in which both endogenous and environmentally regulated flowering pathways have been identified. Endogenous flowering pathways are regulated by hormones and carbohydrate assimilates. Environmental factors that affect flowering are photoperiod and temperature; temperature regulates flowering via ambient temperature and vernalisation pathways (Huijser and Schmid, 2011). Although Arabidopsis is only distantly related to strawberry, several genetic components of the flowering pathways in Arabidopsis have been identified also in F. vesca (Mouhu et al., 2009), indicating that similar mechanisms control at least some of the aspects of plant development in these two species. Moreover, studies in other plant species, e.g. rice and poplar, have shown that some parts of the molecular machinery controlling flowering are conserved in even distantly related species (Andrés and Coupland, 2012). Therefore, knowledge on flowering pathways and comparisons of the pathways between plant species are vital tools for understanding the mechanisms controlling flower induction in F. vesca.

Photoperiodic pathway

Photoperiodism, or promotion of flowering by a specific daylength, was characterized already in 1920 by Garner and Allard. Although the molecular basis of photoperiodism was starting to be deciphered from the 1990s onwards, a model for photoperiodic control of flowering was proposed already in 1936. Bünning (1936) suggested that plants sense seasonal changes by coupling the observed daylength with an intrinsic circadian rhythm. According to this model, an endogenous circadian clock generates a rhythm that is sensitive to light only at certain times of the day. When this light-sensitive period coincides with daylight, flowering responses are promoted in LD plants and inhibited in SD plants. Later on, this model known as external coincidence model was refined by Pittendrigh and Minis (1964), who proposed that light could also affect the circadian rhythm by entraining the circadian clock.

Work on the molecular machinery controlling photoperiodic flowering in Arabidopsis has shown that the model of Bünning was quite accurate. It is now known that an intricate machinery consisting of dozens of genes generates a steady circadian rhythm with a period of approximately 24 hours (Bouché et al., 2016). One of the outputs of the circadian clock is the rhythmic expression pattern of CONSTANS (CO), a CCT domain transcription factor required for the LD-dependent promotion of flowering (Putterill et al., 1995; Sawa et al.,
In addition to transcriptional regulation, CO is regulated at the protein level by different mechanisms at different times of the day. CO protein expressed at the end of a LD is stabilized by interaction with FLAVIN-BINDING, KELCH-REPEAT, F-BOX1 protein (FKF1; Nelson et al., 2000; Song et al., 2012), whereas morning expressed CO is degraded by the action of phytochrome B (PHYB; Valverde et al., 2004). During darkness, CO protein is degraded by the CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1)–SUPPRESSOR OF PHYA (SPA) ubiquitin ligase complex (Andrés and Coupland, 2012).

Perception of photoperiod takes place in leaves, and all the genes involved in the circadian clock and regulation of CO are expressed in leaf vascular tissues (Andrés and Coupland, 2012). However, inflorescences are initiated at the shoot apical meristem. The mobile signal linking these tissues is FLOWERING LOCUS T (FT; Koornneef et al., 1991; Corbesier et al., 2007), a member of the CETS protein family (Kardailsky et al., 1999). In LDs, the CO protein expressed in phloem upregulates FT directly by binding to its promoter (An et al., 2004; Tiwari et al., 2010). The FT protein is actively exported from the phloem companion cells to sieve elements (Liu et al., 2012) and is transported to the shoot apex.

In the shoot apex, FT forms a complex with the bZIP transcription factor FLOWERING LOCUS D (FD; Abe et al., 2005). The FT–FD complex acts as a transcriptional activator, whose targets include the MADS box transcription factor SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1; Samach et al., 2000) and the meristem identity genes APETALA1 (AP1; Abe et al., 2005), LEAFY (LFY; Moon et al., 2005) and FRUITFUL (FUL; Tepfer-Bannolker and Samach, 2005). Once meristem identity genes are expressed, the shoot apical meristem is irreversibly committed to flowering (Hempel et al., 1997).

Regulation of flower induction in the shoot apex is complex, as also flowering inhibitive factors play roles in flower induction. One of the floral repressors acting in the shoot apex is TERMINAL FLOWER1 (TFL1; Shannon and Meeks-Wagner, 1991), another member of the CETS protein family that bears high resemblance to FT (Kobayashi et al., 1999). In Arabidopsis, TFL1 is required for maintaining inflorescence meristem indeterminacy, and mutations at TFL1 cause early flowering and development of a terminal flower (Shannon and Meeks-Wagner, 1991). TFL1 exerts its action by preventing the expression of AP1 and LFY in the inflorescence meristem (Liljegren et al., 1999), and the expression of TFL1 is reciprocally repressed by AP1 and LFY (Liljegren et al., 1999; Kaufmann et al., 2010). TFL1 cannot bind to DNA on its own, and it requires an interacting partner to repress flowering. It was recently shown that TFL1 competes with FT for binding to FD, and these interactions are mediated by 14-3-3 proteins (Ho and Weigel, 2014).

The components of the photoperiodic pathway are fairly conserved across diverse plant species, although modifications to the regulation of the pathway genes do exist. In the SD plant rice, the photoperiodic flowering response is
controlled by Heading date 3a (Hd3A) and Heading date1 (Hd1), genes that are homologous to Arabidopsis FT and CO, respectively (Kojima et al., 2002). The rice Hd1 represses flowering in LDs by inhibiting the expression of Hd3A, causing a photoperiodic flowering response opposite to that of Arabidopsis (Hayama et al., 2003). The Hd3A protein produced in SDs interacts with 14-3-3 proteins and a rice FD homologue (OsFD1) in the shoot apex and this complex activates the expression of OsMADS15, a rice AP1 homologue (Taoka et al., 2011).

The function of FT as a universal floral promoter is conserved, as the gene has been shown to promote flowering in the SD plants rice (Kojima et al., 2002) and cucurbits (Lin et al., 2007), in LD plants Arabidopsis (Kardailsky et al., 1999) and barley (Yan et al., 2006) and many perennial species, including poplar (Böhlenius et al., 2006) and apple (Tränkner et al., 2010). It appears likely that the components of the photoperiodic pathway could be conserved in Fragaria as well, although regulation of the individual components is likely to differ between the annual LD plant Arabidopsis and the perennial SD plant F. vesca.

**Vernalisation pathway**

Some plants require an extended period of cold before they are able to respond to other flowering-promoting stimuli. This process is termed vernalisation and it was characterised in cereals as early as 1857 by Klippar (Chouard, 1960). Later on, vernalisation requirement has been shown to exist in many annual plant species belonging to several plant families, including Brassicaceae (Arabidopsis thaliana; Napp-Zinn, 1953), Amaranthaceae (Beta vulgaris; Chroboczek, 1934) and Solanaceae (Hyoscyamus niger; Melchers, 1936). In all these plant species, the requirement for vernalisation changes the life cycle of the species from summer annual (completing the entire life cycle within one growing season) to a biennial, or winter annual form, which requires a period of cold before being able to receive flowering-inductive stimuli.

The molecular machinery controlling the vernalisation process has been studied in detail only in Arabidopsis, temperate grasses and beet. In winter-annual Arabidopsis accessions, a MADS box transcription factor FLOWERING LOCUS C (FLC) represses flowering in non-vernalized plants and is upregulated by FRIGIDA (FRI) prior to vernalisation (Michaels and Amasino, 1999). FLC delays flowering by directly repressing several genes required for floral promotion. These genes include FT in the leaves and SOC1 and FD in the shoot apex (Hepworth et al., 2002; Searle et al., 2006). As vernalisation proceeds, the FLC locus is epigenetically and stably silenced by trimethylation at lysine 27 of histone 3 (Wood et al., 2006). Silencing of FLC allows for the LD-dependent upregulation of FT and SOC1, which then promote flowering as described for the photoperiodic pathway.
The winter growth habit in temperate grasses is caused by interactions at three loci, VRN1, VRN2 and VRN3. VRN1 is the grass orthologue of the meristem identity gene AP1 (Yan et al., 2003), while VRN3 is orthologous to FT (Yan et al., 2006). VRN2 encodes for a CCT domain protein that does not have close homologues in Arabidopsis, but is downregulated by vernalisation and SDs similarly to FLC (Yan et al., 2004). In non-vernalised plants VRN2 delays flowering by repressing VRN1 and VRN3. Vernalisation causes downregulation of VRN2, allowing for the subsequent LDs to promote the expression of VRN3, which in turn upregulates VRN1 (Yan et al., 2006). The identity of the repressor is not the only element different between Arabidopsis and grass vernalisation pathways. In wheat, the three genes form a regulatory feedback loop not characterised in Arabidopsis, as VRN1 has been shown to downregulate VRN2. Moreover, the high level of VRN1 expression in leaf tissues contrasts the expression pattern of Arabidopsis AP1, which is almost exclusively expressed in flowering induced shoot apical meristems (Yan et al., 2003).

In cultivated beet, the vernalisation response is determined by interactions of a pseudo-response regulator gene BOLTING TIME CONTROL1 (BvBTC1), and two beet homologues of FT, BvFT1 and BvFT2 (Pin et al., 2012). BvFT2 has the same flowering-promoting function as Arabidopsis FT, whereas BvFT1 has evolved into a floral repressor (Pin et al., 2010). In annual beet, flowering is promoted in LDs through upregulation of BvBTC1, which in turn leads to repression of BvFT1 and upregulation of the floral promoter BvFT2. In biennial beet, the BvBTC1 gene harbors a large 28 kb insertion in its promoter region, making the gene non-responsive to LDs. Accessions with this insertion require a long period of cold to sufficiently induce BvBTC1 expression to promote flowering (Pin et al., 2012).

The vernalisation pathways of Arabidopsis, temperate cereals and beet include different components. However, all these pathways contain a repressor expressed at a high level prior to vernalisation, and silenced as vernalisation proceeds, and the vernalisation pathways in these three species involve genes homologous to the floral promoter FT.
Although both ambient temperature and vernalisation pathways mediate responses to changes in temperature, they are fundamentally different. The onset of the vernalisation response requires weeks (Chouard, 1960), whereas plants respond to changes in ambient temperature typically in a matter of days (Balasubramanian et al., 2006). The term ambient temperature refers to a non-stressful temperature range, which in Arabidopsis lies between 12 and 27°C (Wigge, 2013). The effects of ambient temperature on flowering differ from species to species; in Arabidopsis, elevating the temperature by 4°C from 23 to 27°C can accelerate flowering under SDs (Balasubramanian et al., 2006). In contrast, elevating temperature from 18°C to 25°C has been shown to delay flowering in Boechera stricta, a perennial relative of Arabidopsis (Anderson et al., 2011).

The molecular components of the ambient temperature pathway include both positive and negative regulators. FT, a positive regulator of the photoperiodic pathway, is also a component of the ambient temperature controlled flowering pathway. Regulation of FT by ambient temperature occurs at multiple levels. Low temperature induces changes in chromatin conformation and makes the FT promoter less accessible to transcription (Kumar and Wigge, 2010). This is caused by an increased incorporation of histone variant H2A.Z into nucleosomes instead of the regular H2A histones. Incorporation of H2A.Z histone makes DNA pack more tightly. At elevated temperature, H2A.Z is evicted from nucleosomes and the FT promoter is accessible to transcription factors (Kumar and Wigge, 2010). One of these transcription factors is PHYTOCHROME INTERACTING FACTOR4 (PIF4), a bHLH transcription factor that binds to the FT promoter to advance flowering at high temperatures in SDs (Kumar et al., 2012).

In LDs, high temperature triggered flowering is regulated via interactions between the MADS box transcription factors SHORT VEGETATIVE PHASE (SVP) and FLOWERING LOCUS M (FLM). SVP is a floral repressor that forms homodimers and is effectively degraded at high temperature (Lee et al., 2013). FLM is subject to temperature-dependent alternative splicing; at low temperature, the flowering-repressive splice variant FLM-β prevails. However, at higher temperatures the splice variant FLM-δ, which is impaired in its DNA binding capacity, is more abundant (Posé et al., 2013). Both splice variants can form heterodimers with SVP, and they compete for binding to SVP to regulate flowering in an antagonistic manner. At low temperature, the SVP-SVP and SVP-FLM-β complexes actively prevent flowering by downregulating their target genes which include SOC1 and possibly FT. At higher temperatures the splice variant FLM-δ inhibits binding of the protein complexes to DNA, thus regulating flowering in a dominant-negative manner (Lee et al., 2013; Posé et al., 2013).

Also other genes have been shown to play a role in the ambient temperature response in Arabidopsis. Strasser et al. (2009) found that delayed flowering


caused by low ambient temperature could be at least partially alleviated by mutations at \textit{EARLY FLOWERING3 (ELF3)} and \textit{TFL1}, and that the double mutant \textit{elf3 tfl1} was insensitive to temperature. The authors suggested that \textit{TFL1} represses flowering at low temperature, and that \textit{ELF3} and \textit{TFL1} act on two independent pathways; \textit{ELF3} is regulated through \textit{PHYB}, whereas \textit{TFL1} is regulated through cryptochromes (Strasser et al., 2009).

The molecular components of the ambient temperature pathway have not been studied extensively in other plants than \textit{Arabidopsis}. However, studies in several species such as Chinese narcissus (\textit{Narcissus tazetta} var. \textit{Chinensis}; Li et al., 2013), chrysanthemums (\textit{Chrysanthemum} sp.; Nakano et al., 2013) and Satsuma mandarin (\textit{Citrus unshiu}; Nishikawa et al., 2007) suggest the involvement of \textit{FT} homologues in ambient temperature dependent flowering; in all these species, ambient temperature-induced flowering is associated with an increased transcription of \textit{FT}-like genes.

\textit{Genetic control of flowering in Fragaria}

In \textit{F. x ananassa}, studies into the genetic control of flowering have yielded somewhat conflicting results. The early studies on the genetics of flowering suggested that the everbearing trait is controlled by a single dominant locus (Ahmadi et al., 1990; Sugimoto et al., 2005), whereas later it was proposed that the everbearing character is controlled by several QTL (Weebadde et al., 2008). Recently, two independent studies showed that the everbearing trait in the cultivated strawberry is controlled by a dominant QTL located on linkage group 4 (LGIV), and that the same QTL controls also the running trait (Gaston et al., 2013; Castro et al., 2015). Moreover, Honjo et al. (2015) showed by genetic complementation that the everbearing trait is caused by the same locus in both older everbearers with European or American origin and in cultivars with \textit{F. virginiana} ssp. \textit{glauca} germplasm in their ancestry. Also the locus identified by Honjo et al. (2015) is likely located on the LGIV of the cultivated strawberry.

The difficulty of studying the genetics of the everbearing trait in cultivated strawberry with a complex octoploid genome was recognised very early. Therefore, diploid everbearing \textit{F. vesca} accessions have been used for elucidating the genetic basis of the everbearing trait as early as the 1960s. All everbearing accessions (historically nominated \textit{F. vesca} f. \textit{semperflorens}) are thought to have originated from the same natural source in the Alps, and for that reason are often called “Alpine” strawberries in older literature (Darrow, 1966). The everbearing trait in woodland strawberry has been shown to be recessive, and genetic complementation experiments have demonstrated that the trait is indeed caused by the same gene in the studied everbearing accessions (Brown and Wareing, 1965). This locus was subsequently nominated the \textit{SEASONAL FLOWERING LOCUS (SFL); Albani et al., 2004}). Studies into phenotypic segregation ratios revealed that the everbearing trait
and runnering are controlled by two independent loci (Brown and Wareing, 1965), the everbearing trait located on diploid *Fragaria* linkage group 6 (LGVI), and the runnering locus on linkage group 2 (LGI; Sargent et al., 2004). Based on different chromosomal locations, it therefore seems that the genetic basis of the everbearing character is different in diploid and octoploid strawberries.

The genetic nature of *SFL* in woodland strawberry has been subject to speculation from the 1960s. Brown and Wareing (1965) reasoned that the perpetual flowering trait arises as a result of a mutation at *SFL*, which was thought to encode for a floral inhibitor. It has been proposed that *SFL* could be a gene homologous to the vernalisation pathway gene *FLC* (Battey, 2000). However, no *FLC* homologs have been found in the Rosaceae EST database containing more than 500 000 sequences (Mouhu et al., 2009).

Other candidates for *SFL* have included a *CO*-like gene and GA pathway genes, *CO* because of the photoperiodic flowering response in strawberry (Mouhu et al., 2009) and GA pathway genes because GA application inhibits flowering in strawberries (Guttridge and Thompson, 1964). However, the genomic location of strawberry *CO* does not match with that of *SFL* (Stewart, 2007). GA pathway gene are also unlikely candidates, as no differences in the expression of GA pathway-related genes between everbearing and SD genotypes have been observed (Mouhu et al., 2009).

Studies in other perennial species propose that *SFL* may encode for a CETS gene. In hybrid poplar, it has been shown that a poplar orthologue of *TFL1*, a gene called CENTRORADIALIS-LIKE1 (*CENL1*), is downregulated as a response to SDs (Ruonala et al., 2008), and silencing poplar *CENL* genes results in early flowering (Mohamed et al., 2010). Moreover, a *TFL1* homologue of apple, *MdTFL1*, is involved in maintenance of the vegetative phase (Kotoda and Wada, 2005), and silencing *MdTFL1* leads to very early flowering (Kotoda et al., 2006).
The CETS family

As seen in the previous chapters, the CETS genes play important roles in plant development. Interestingly, CETS proteins share similarities with human Raf kinase inhibitor proteins (RKIPs), which have been associated with cell differentiation and cell-cycle arrest (Yeung et al., 1999). The CETS gene family in Arabidopsis contains genes with important roles in plant development; in addition to the florigen FT and the floral repressor TFL1, the Arabidopsis genome contains TWIN SISTER OF FT (TSF), ARABIDOPSIS CENTRO RADIALIS (ATC), BROTHER OF FT AND TFL1 (BFT) and MOTHER OF FT AND TFL1 (MFT) (Kobayashi et al., 1999). MFT is associated with seed germination and release from seed dormancy (Footit et al., 2011), ATC and BFT are floral repressors and functionally redundant with TFL1 (Yoo et al., 2010; Huang et al., 2012) and TSF acts as a floral promoter redundantly with FT (Yamaguchi et al., 2005).

TSF shares high amino acid similarity with FT, expression of TSF is promoted in the phloem in LDs and TSF overexpressing plants flower early resembling the phenotype of FT overexpressors (Michaels et al., 2005; Yamaguchi et al., 2005). Moreover, TSF responds to changes in ambient temperature in the same manner as FT, and the gene has been shown to be a major target of the ambient temperature regulated flowering pathway (Lee et al., 2013). However, tsf single mutants do not display a flowering time related phenotype under LD conditions, and it was recently shown that TSF has a poorer ability to move from phloem to the shoot apex and is less stable than FT (Jin et al., 2015). It is thus possible that TSF has a less significant biological function than FT.

The amino acid sequence of BFT resembles FT more than TFL1, and BFT expression is upregulated by LDs similarly to FT and TSF (Yoo et al., 2010). However, BFT overexpression results in late flowering and floral defects that resemble those caused by ectopic TFL1 expression. Modifications in BFT expression level also change plant architecture by altering the rate of terminal to axillary inflorescence development (Yoo et al., 2010). Therefore, BFT appears to control inflorescence meristem identity redundantly with TFL1.

ATC is named after Antirrhinum CEN, with which ATC shares high protein-level similarity. Although ATC can functionally complement the tfl1 mutation when constitutively expressed, its expression pattern in vascular tissues suggests a different biological role (Mimida et al., 2001). Indeed, it has been shown that ATC mRNA is a mobile floral repressor expressed in leaves in SDs. Similarly to FT and TFL1, also the ATC protein is capable of interacting with FD and regulating the same downstream targets (Huang et al., 2012). Long-distance transport from leaves to the shoot apex was also shown for chrysanthemum anti-florigenic FT/TFL1 family protein (CsAFT). Moreover, CsAFT expression was induced after exposure to a night-break, a treatment that inhibits flowering in chrysanthemums (Higutchi et al., 2013).
3 AIMS OF THE STUDY

Gaining an insight into molecular networks regulating flowering is essential for developing cultivars better adapted to the changing environment. As the cultivated strawberry is genetically complex, the less complicated woodland strawberry was used as a model plant. Therefore, the broad objective of this study was to identify genes regulating flower induction in woodland strawberry and study how environmental factors affect the action of these genes. The second objective was to utilize the genetic information from studies with woodland strawberry and examine how the homologous genes function in cultivated strawberry.

Specific aims for the original publications were:

I Identification and molecular characterisation of the gene residing at the \textit{SEASONAL FLOWERING LOCUS (SFL)} in woodland strawberry

II Elucidation of the role of \textit{TERMINAL FLOWER1} in the vernalisation response of arctic woodland strawberry accession Alta-1

III Elucidation of the role of \textit{TERMINAL FLOWER1} in the control of flowering in cultivated strawberry
4 MATERIALS AND METHODS

Methods used in this thesis are summarised in Table 1 and described in more detail in respective publications. Methods used by co-authors are indicated in parentheses.

Table 1. Methods used in this thesis.

<table>
<thead>
<tr>
<th>Method</th>
<th>Publication</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA synthesis</td>
<td>I, II, III</td>
</tr>
<tr>
<td>Crossing populations</td>
<td>I, (II)</td>
</tr>
<tr>
<td>DNA extraction</td>
<td>I, II</td>
</tr>
<tr>
<td>Gateway™ vector construction</td>
<td>I</td>
</tr>
<tr>
<td>Genotyping-by-sequencing analysis</td>
<td>(II)</td>
</tr>
<tr>
<td>Genetic transformation</td>
<td>I, (III)</td>
</tr>
<tr>
<td>Genotyping</td>
<td>I, II</td>
</tr>
<tr>
<td>Growth experiments</td>
<td>I, II, III</td>
</tr>
<tr>
<td>in situ hybridisation</td>
<td>I</td>
</tr>
<tr>
<td>Linkage analysis</td>
<td>I, (I)</td>
</tr>
<tr>
<td>Marker design</td>
<td>I, II</td>
</tr>
<tr>
<td>RNA extraction</td>
<td>I, II, III</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>I, II, III</td>
</tr>
</tbody>
</table>

Plant materials and experimental conditions

To identify the SFL via positional cloning, two cross populations were produced; an F2 population resulting from a cross between a seasonally flowering *F. vesca* accession 'Punkaharju' (National Clonal Germplasm Repository accession PI551792, abbreviated as Punk-1) and an everbearing accession 'Hawaii-4' (H4), and a back-cross (BC) population between a seasonal *F. vesca* and everbearing *F. vesca f. semperflorens*. The F2 population consisted of 978 seedlings, whereas the BC population included 2996 seedlings. The seedlings were germinated and grown in LDs at 18°C and then subjected to a SD treatment for 5 weeks. After the SD treatment, flowering (presence/absence) was scored in LDs. In addition, *F. vesca*, H4 and *Arabidopsis tfl1-2* mutant in the Landsberg erecta (Ler) background were subjected to daylength treatments described in detail in (I).

The effects of low temperature were studied in the seasonal *F. vesca* accession Punk-1 and the vernalisation requiring Alta-1 accession from northern Norway. The plants were subjected to several low temperature treatments detailed in (II). An F2 population resulting from a cross between Alta-1 and 'Hawaii-4' carrying the *FvTFL1* hairpin construct was produced to study the effect of non-functional and silenced *FvTFL1* on the vernalisation response.
To study the effect of TFL1 on the flowering response in cultivated strawberry, five F. × ananassa cultivars (Table 2) were subjected to daylength (SD/LD) and temperature (9–21ºC) treatments described in detail in (III).

**Table 2.** Plant materials used in the experiments.

<table>
<thead>
<tr>
<th>Species</th>
<th>Accession name</th>
<th>Accession number</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. thaliana</td>
<td>Landsberg erecta</td>
<td>tfl1-2</td>
<td>N3091</td>
</tr>
<tr>
<td>F. × ananassa</td>
<td>Alaska Pioneer</td>
<td>PI551796</td>
<td>Natural Resources Institute Finland (Luke)</td>
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<tr>
<td>F. × ananassa</td>
<td>Elsanta</td>
<td>PI551579</td>
<td>Natural Resources Institute Finland (Luke)</td>
</tr>
<tr>
<td>F. × ananassa</td>
<td>Glima</td>
<td>na</td>
<td>Natural Resources Institute Finland (Luke)</td>
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<tr>
<td>F. × ananassa</td>
<td>Honeoye</td>
<td>PI551588</td>
<td>Natural Resources Institute Finland (Luke)</td>
</tr>
<tr>
<td>F. × ananassa</td>
<td>Polka</td>
<td>na</td>
<td>Natural Resources Institute Finland (Luke)</td>
</tr>
<tr>
<td>F. vesca</td>
<td>Punkaharju</td>
<td>PI551792</td>
<td>NCGR</td>
</tr>
<tr>
<td>F. vesca</td>
<td>Hawaii-4</td>
<td>PI551721</td>
<td>NCGR</td>
</tr>
<tr>
<td>F. vesca</td>
<td>Alta</td>
<td>na</td>
<td>A. Sonsteby (NIBIO Norway)</td>
</tr>
</tbody>
</table>

**Transgenic experiments**

Plasmid vectors for plant transformation were constructed as described in (I) and transformed into plant tissues via Agrobacterium-mediated transformation. Transformation was done by the floral dip method (Zhang et al., 2006) for Arabidopsis, by the protocol of Oosumi et al. (2006) for F. vesca and by the protocol of Fischer et al. (2014) for F. × ananassa. In total, four different constructs were transformed into four genotypic backgrounds (Table 3).

In addition to direct transformation, the FvTFL1 hairpin construct for silencing FvTFL1 was utilized for producing the Alta-1 x 'Hawaii-4' F2 population. The paternal 'Hawaii-4' parent carried the FvTFL1 hairpin construct to promote flowering in the F1 generation.

**Table 3.** The vectors used and transgenes introduced into different plant genotypic backgrounds.

<table>
<thead>
<tr>
<th>Background</th>
<th>Vector</th>
<th>Transgene</th>
<th>Publication</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. thaliana Ler tfl1-2</td>
<td>pK7WG2D-2 (overexpression)</td>
<td>Functional FvTFL1</td>
<td>I</td>
</tr>
<tr>
<td>F. × ananassa 'Elsanta'</td>
<td>pK7WG2D2D-II (RNAi)</td>
<td>FvTFL1 hairpin</td>
<td>III</td>
</tr>
<tr>
<td>F. vesca 'Hawaii-4'</td>
<td>pK7WG2D2 (overexpression)</td>
<td>Functional FvTFL1</td>
<td>I</td>
</tr>
<tr>
<td>F. vesca 'Hawaii-4'</td>
<td>pK7WG2D2 (overexpression)</td>
<td>Mutated FvTFL1</td>
<td>I</td>
</tr>
<tr>
<td>F. vesca 'Hawaii-4'</td>
<td>pK7WG2D2D-II (RNAi)</td>
<td>FvTFL1 hairpin</td>
<td>I, II</td>
</tr>
<tr>
<td>F. vesca 'Hawaii-4'</td>
<td>pK7WG2D2D-II (RNAi)</td>
<td>FvFT1 hairpin</td>
<td>I</td>
</tr>
<tr>
<td>F. vesca 'Punkaharju'</td>
<td>pK7WG2D2 (overexpression)</td>
<td>Functional FvTFL1</td>
<td>I</td>
</tr>
<tr>
<td>F. vesca 'Punkaharju'</td>
<td>pK7WG2D2 (overexpression)</td>
<td>Mutated FvTFL1</td>
<td>I</td>
</tr>
<tr>
<td>F. vesca 'Punkaharju'</td>
<td>pK7WG2D2D-II (RNAi)</td>
<td>FvTFL1 hairpin</td>
<td>I</td>
</tr>
</tbody>
</table>
Genotyping and mapping

Genetic mapping was used in (I) and (II) to identify genes associated with qualitative flowering responses. DNA extraction was done following the protocol of Doyle and Doyle (1990), scaled down to fit in a 1.5 ml tube. The markers used for genotyping are described in (I) and (II). Linkage maps were constructed as described by Sargent et al. (2004).

RT-qPCR

RNA for real-time quantitative PCR (RT-qPCR) was extracted according to a protocol described earlier (Mouhu et al., 2009). In (II) and (III), RNA samples were treated with rDNase (Macherey-Nagel GmbH, Düren, Germany) according to manufacturer’s recommendations. cDNA was synthesized and RT-qPCR run as described in respective publications. *MSI1* was used as a stable reference gene and relative expression ratios were determined following the ΔΔCt method (Pfaffl, 2001).
5 RESULTS AND DISCUSSION

5.1 SFL ENCODES FOR AN ORTHOLOGUE OF TFL1 IN F. VESCA (I)

Genetic mapping in F2 and BC populations indicated that the SFL was located within a narrow mapping window of 248 kb in F. vesca LGVI (I, Figures S1 and S2). This genetic interval contained the F. vesca homologue of TFL1 (FvTFL1). FvTFL1 was selected as the most promising candidate, because homologues of TFL1 have been shown to repress flowering in both annual (Arabidopsis; Bradley et al., 1997; Hanano and Goto, 2011) and perennial (Malus × domestica; Kotoda et al., 2006) species. Sequencing FvTFL1 from the everbearing cross parents showed that both had a 2 base pair deletion in the first exon of the gene, causing a frameshift and resulting in a putatively nonfunctional protein product. These results were in full concordance with the data of Iwata et al. (2012), who some time earlier showed that the everbearing trait was associated with a disruption in the reading frame of TFL1 homologues in both F. vesca and rose.

According to Iwata et al. (2012), the loci controlling continuous flowering in rose and strawberry are orthologous and named the loci RoKSN and FvKSN, respectively. However, the same gene had been named as FvTFL1 already earlier by Mimida et al. (2012). Moreover, both the conserved synteny around FvTFL1 (Figure 2) and Arabidopsis TFL1 and phylogenetic relationships (Mimida et al., 2012) suggest that the genes are true orthologues, supporting consistent nomination of FvTFL1 for the F. vesca orthologue.

![Figure 3. Synteny around the TFL1 locus in woodland strawberry and Arabidopsis. A 600 kb window of Fragaria (top) and Arabidopsis (bottom) genomes show synteny around the regions surrounding FvTFL1 and TFL1. Syntenous regions were visualized using GEvo in the CoGe platform (Lyons and Freeling, 2008). Green blocks represent gene models and pink lines connect high-scoring sequence pairs identified by BlastZ. Orange color in the Fragaria genome represents unsequenced regions.](image-url)
If *FvTFL1* was indeed a floral repressor activated under LDs, we would expect to detect *FvTFL1* mRNA in the shoot apical meristems of non-induced plants. Indeed, we found *FvTFL1* transcripts over the entire apical meristem of LD-grown non-induced Punk-1 plants (I, Figure 4). Moreover, *FvTFL1* expression level in shoot apical meristems responded to photoperiodic conditions; the gene showed gradual downregulation in SDs and was again upregulated upon returning the plants to LDs (I, Figures 4 and 5). Photoperiodic regulation of a *TFL1* orthologue has not been observed in other species studied so far; in Arabidopsis, *TFL1* is developmentally regulated (Bradley et al., 1997; Ratcliffe et al., 1998), and the pea *PsTFL1c* does not exhibit environmental or developmental regulation, although the loss of *PsTFL1* leads to early flowering (Foucher et al., 2003).

The role of *FvTFL1* as a floral repressor was confirmed by overexpressing the functional and mutated alleles of *FvTFL1* in the everbearing H4 background. Plants that overexpressed mutated and putatively non-functional *FvTFL1* flowered continuously, resembling wild type H4 plants, while the plants carrying the overexpressed functional *FvTFL1* remained vegetative for at least 10 months (I; Figure 1). Moreover, silencing functional *FvTFL1* by RNAi in the SD-requiring Punk-1 background removed the SD requirement, causing the LD-grown transgenic plants to flower at the same time as SD-treated wild type Punk-1 plants (I, Figure 2). These data indicate that the mutated everbearing allele indeed results in a non-functional protein, and that the functional *FvTFL1* is a floral repressor that causes the seasonal flowering habit.

The finding that the *SFL* encodes for a homologue of the floral repressor *TFL1* is not surprising. In the monocot maize (*Zea mays*), overexpression of *TFL1*-like genes results in delayed flowering time (Danilevskaya et al., 2010), and in pea (*Pisum sativum*), the loss of *PsTFL1c* results in early flowering (Foucher et al., 2003). In Rosaceous fruit crops, *TFL1* controls also the length of the juvenile phase; silencing *TFL1* in pear (*Pyrus communis L.*) and apple has resulted in accelerated life cycles, thus speeding up breeding processes (Flachowsky et al., 2012; Freiman et al., 2012). It is possible that *FvTFL1* has a similar function in maintaining the juvenile phase, as flower induction shown by *FvAP1* upregulation occurs earlier in H4 seedlings than in Punk-1 seedlings grown under inductive photoperiodic conditions (I, Figure S7).
5.2 PHOTOPERIOD REGULATES *FVTFL1* EXPRESSION AND FLOWERING (I)

After identifying *FvTFL1* as *SFL*, we were interested in finding out how the gene functions to regulate the photoperiodic flowering response. Therefore, expression of *FvTFL1* and its putative downstream targets, the floral meristem identity genes *FvAP1* and *FvFUL1* were examined in shoot apical meristems of both wild type and transgenic plants grown under different photoperiodic conditions. In all cases, downregulation of *FvTFL1* was associated with higher levels of *FvAP1* and *FvFUL1* mRNAs and with earlier flowering time (I). The correlation between *FvAP1* upregulation and subsequent flowering corroborated the findings of Mouhu et al. (2009) who suggested that *FvAP1* could be used as a marker for floral initiation.

*FT* proteins have been identified as major mobile floral promoters in a range of species (Turck et al., 2008), and we were interested in elucidating the role of *F. vesca* orthologue of *FT* in photoperiodic control of flowering. The *F. vesca* genome harbors three *FT* homologues (Mimida et al., 2012). Tissue-specific expression patterns and conserved synteny with the Arabidopsis orthologue (Figure 3; I, Figure 6) suggested that *F. vesca* *FT1* was the most likely orthologue of *FT* and was studied further. The function of *FvFT1* as a floral promoter was confirmed by silencing the gene in the H4 background; flowering was delayed in *FvFT1*-silenced plants (I, Figure 7).

Expression analysis showed that *FvFT1* expression correlated with flowering only in H4, where LDs promoted *FvFT1* expression and early flowering. In Punk-1, LDs also resulted in an elevated *FvFT1* transcript level but no flowering occurred (I, Figure 6). These data led us to build a model according to which functional *FvTFL1* represses flowering under LDs in the SD-requiring Punk-1 accession. In the LD accession H4, the lack of functional *FvTFL1* leads to rapid *FvFT1*-mediated flowering. How *FvTFL1* overrides the function of *FvFT1* in Punk-1 to repress *FvAP1* and *FvFUL1* could be explained by competitive binding to protein complexes; it is possible that *FvTFL1* has a
higher binding affinity to FD than FvFT1 does. Such a mechanism has been suggested in rose, where the presence of RoKSN weakened the interaction between RoFD and RoFT (Randoux et al., 2014).

The photoperiodic model for flower induction was further refined by Mouhu et al., (2013), who showed that \textit{FvTFL1} is regulated via the \textit{F. vesca} orthologue of \textit{SOC1} (\textit{FvSOC1}), and that \textit{FvSOC1} expression in turn is affected by \textit{FvFT1}. \textit{FvSOC1} is involved in the photoperiodic regulation of both reproductive and vegetative aspects of growth; constitutive \textit{FvSOC1} expression in transgenic lines results in production of runners, whereas silencing \textit{FvSOC1} by RNAi results in inflorescence formation. The authors also showed that altering \textit{FvTFL1} transcription did not notably change the expression of \textit{FvSOC1} (Mouhu et al., 2013). These data helped to explain why the formation of branch crowns and stolons were not affected in the transgenic \textit{FvTFL1} plants (I, Figure 3). According to the results of Mouhu et al. (2013) and (I), \textit{FvSOC1} and \textit{FvTFL1} are components of the same photoperiodic flowering pathway, with \textit{FvSOC1} controlling aspects of vegetative development, but also flowering via regulation of \textit{FvTFL1}. As \textit{FvTFL1} putatively acts downstream of \textit{FvSOC1}, changes in \textit{FvTFL1} expression affect only the flowering response, and not patterns of vegetative development.
5.3 ALTERED REGULATION OF FVTFL1 IS ASSOCIATED WITH THE UNIQUE VERNALISATION RESPONSE IN ALTA-1 (II)

When comparing flowering responses of six Norwegian woodland strawberry accessions, Heide and Sønsteby (2007) inadvertently found an accession which appeared to require winter before being able to flower. This Alta-1 accession would not flower in SDs or LDs at temperatures ranging from 9 to 21°C, and when tested under field conditions, it flowered considerably later than the other populations (Heide and Sønsteby, 2007). Exposing Alta-1 to 2°C for a minimum of five weeks induced flowering in a proportion of the plants, and it was concluded that, unlike any other strawberry accession studied to date, Alta-1 possesses a unique vernalisation requirement.

Seasonal flowering cycle in altered in Alta-1 (II)

Alta-1 appears to have an altered seasonal flowering cycle (Heide and Sønsteby, 2007), and therefore it was of interest to elucidate whether the correlation between flowering time and seasonal expression patterns of flowering-related genes would differ in Alta-1 and Punk-1 plants grown under natural conditions. The flowering time observations on the field were consistent with the observations of Heide and Sønsteby (2007); Alta-1 flowered considerably later and continued to produce new inflorescences for longer than Punk-1 (II, Figure 2).

Expression analysis of FvTFL1 in shoot apex samples of field grown Alta-1 and Punk-1 plants collected monthly from August to December and again in May showed that the expression of FvTFL1 was indeed higher in Alta-1 at all tested timepoints (II, Figure 1A). Moreover, downregulation of FvTFL1 by shortening days and cooling temperature occurred at a slower rate in Alta-1 than in Punk-1 (II, Figure 1A and S2). The meristem identity gene FvAP1 was upregulated in shoot apices of Punk-1 already in October, whereas in Alta-1, upregulation of FvAP1 was not observed before May (II, Figure 1C).

The expression patterns of FvTFL1 and FvAP1 suggested that floral induction had not taken place in field-grown Alta-1 plants in December. To confirm these observations, Alta-1 and Punk-1 plants were taken to greenhouse in December and their flowering was observed under LD (18°C) conditions. In concordance with elevated FvAP1 expression, all Punk-1 plants flowered uniformly after 31 days in the greenhouse, whereas only 20% of Alta-1 plants flowered after approximately 51 days (II, Table S1). The observed late and incomplete flowering of Alta-1 together with FvAP1 expression data confirmed that the plants had not been flowering-induced under field conditions, and that the greenhouse conditions were not favorable for floral induction.
Alta-1 requires exceptionally low temperatures to fulfil the vernalisation requirement (II)

The work of Rantanen et al. (2015) indicated that the photoperiod-independent flowering observed in Punk-1 at cool temperature of 10°C is correlated with downregulation of *FvTFL1*. Therefore, we were interested to see whether *FvTFL1* regulation was altered in the vernalisation-requiring Alta-1. To study this, runner propagated Alta-1 plants were exposed to natural SDs at 4°C for 0 to 15 weeks, followed by flowering observations in LDs at 20°C. These conditions were not favorable for floral induction, as none of the plants flowered even after 15 weeks at 4°C (data not shown) and *FvTFL1* was not downregulated in the shoot apices of plants sampled at any time point (II, Figure 3).

To clarify the conditions required for fulfilling the vernalisation requirement of Alta-1, we subjected Punk-1 and Alta-1 plants to continuous LDs, three weeks of SDs and to low fluctuating temperature (±2°C) for five or ten weeks. In addition, Alta-1 plants were subjected to a constant temperature of 0°C for five to ten weeks. As expected, neither accession flowered in LDs, whereas Punk-1 was induced to flower by a three week exposure to SDs, and also by the low temperature treatments. In contrast, SDs and low temperature treatments induced ample runner production in Alta-1 (II, Figure S3B), which is considered diagnostic of active vegetative growth (Konsin et al., 2001; Hytönen et al., 2004). Only an exposure to low temperature for ten weeks could induce flowering in a proportion of Alta-1 plants (II, Table 1, Table S2). The expression of *FvAP1* correlated with the flowering observations; the gene was upregulated in Punk-1 exposed to SDs and low temperature, whereas no upregulation was observed in Alta-1 by the end of the low temperature treatments (II, Figure 3C).

Alta-1 is induced to flower by LDs and cool temperature (II)

As per definition, vernalisation results in acquired competence to flower, but it does not induce flowering *per se* (Chouard, 1960). Based on the results presented in (II), the Alta-1 accession has a true vernalisation response; the accession requires low temperature to downregulate *FvTFL1* but this process does not induce flowering in itself. The seasonal experiments suggested that Alta-1 is induced to flower in spring, when the days are relatively long (14–16 h) and temperature is around 10°C (II, Figure 1S). This prompted us to hypothesise that Alta-1 requires LDs and cool temperature after vernalisation to induce flowering. This hypothesis was tested in an experiment where field-grown, naturally vernalised Alta-1 plants were brought to greenhouse in January and grown at either 10 or 20°C in LDs for five weeks. Flowering was observed only in plants subjected to 10°C (II, Figure 4C and Table 2), corroborating our hypothesis that cool temperature and LDs are
needed to induce flowering in Alta-1. However, *FvTFL1* was upregulated after five weeks at both 10 and 20°C (II, Figure 4A). At this point, it remains unclear how the plants exposed to 10°C were induced to flower although *FvTFL1* was upregulated in the shoot apices collected after five weeks at 10°C. It is possible that upregulation of *FvTFL1* was faster at 20°C than at 10°C and that floral induction at 10°C occurred within a narrow time window during the first weeks in LDs at 10°C.

**FvTFL1 expression is required for the vernalisation response (II)**

As our data suggested that *FvTFL1* was involved in the vernalisation response of Alta-1, we wanted to gain experimental confirmation to this hypothesis. We crossed the vernalisation requiring Alta-1 with a transgenic H4 line carrying a single copy of the *FvTFL1*-RNAi construct described in (I) to obtain an F1 population in which approximately half of the progeny carried the transgene and half were non-transgenic (II; Figure S7). As H4 is homozygous for nonfunctional *FvTFL1* (I), and Alta-1 is homozygous for functional *FvTFL1*, we did not expect the non-transgenic F1 plants to flower. As expected, none of the non-transgenic plants in the F1 population flowered in LDs (data now shown), whereas F1 plants carrying the *FvTFL1*-RNAi construct showed a reduction in *FvTFL1* transcript level and flowered early in LDs, (II, Figure 5). A similar flowering pattern was observed in the F2 population produced from seeds of a GFP positive F1 plant; the plants carrying the transgene or homozygous for the H4-derived nonfunctional *FvTFL1* flowered in LDs (II; Table S3). These data convincingly show that *FvTFL1* expression is required for the vernalisation response.

Based on these results, it was impossible to say whether the gene causing the vernalisation response was *FvTFL1* itself, or an upstream regulator of *FvTFL1*. To test for the presence of a novel *FvTFL1* regulator, we subjected the non-transgenic F2 plants to SDs for six weeks, after which flowering was observed in LDs. If the vernalisation response was caused by an upstream regulator of *FvTFL1*, the gene would be likely to segregate in the F2 population, resulting in SD-promoted flowering phenotype in a proportion of the F2 plants. However, no flowering was observed in the non-transgenic F2 population, suggesting that the vernalisation response is controlled by a single dominant gene that may be *FvTFL1* itself, or a gene located in the proximity of *FvTFL1* on chromosome 6. It can be speculated that the vernalisation response in Alta-1 may be caused by a mutation at *FvTFL1* promoter region. Although a gain-of-function mutation seems unlikely, it is not without precedent. In beet, it was recently shown that a large 28 kb mutation at the promoter of key flowering gene *BvBTC1* can cause a vernalisation response (Pin et al., 2012). The vernalisation response in beet is an adaptation to northern climate, as it appears in northern accessions of *Beta vulgaris* ssp. *maritima*, the putative ancestor of the cultivated beet (Pin et al., 2010). Within *Fragaria* genus, Alta-
1 is the only known example of an accession requiring vernalisation. We phenotyped a total of 67 northern European accessions for SD-induced flowering, and were unable to find accessions that would not flower after an exposure to inductive conditions (II, Table S4). Moreover, genome-wide genotyping data from 78 accessions (II, Figure 7) showed that Alta-1 is more closely related to other accessions from the Alta region in northern Norway than to accessions from elsewhere in northern Europe. This suggests that the mutation causing the Alta-1 phenotype is a local one, and has arisen relatively recently. The finding also supports the notion that vernalisation requirement has evolved independently in individual plant lineages (e.g. Ream et al., 2012).

*The photoperiodic FvFT1–FvSOC1 pathway is regulated similarly in Punk-1 and Alta-1 (II)*

*FvFT1* and *FvSOC1* are photoperiodically regulated in Punk-1, with LDs upregulating and SDs downregulating their expression (I; Mouhu et al., 2013). Similarly to Punk-1, *FvFT1* expression was downregulated by SDs in Alta-1 (II, Figure S4), and we were unable to find differences in *FvSOC1* expression between the two accessions in a range of both natural (II, Figure 1) and controlled conditions (II, Figure 3). As previous experiments with transgenic plants have suggested that *FvTFL1* expression is activated by *FvFT1* and *FvSOC1* in LDs (I; Mouhu et al., 2013), we were surprised to see upregulation of *FvTFL1* in SDs in Alta-1 (II, Figure 4A and S4). These data suggested that unknown factor(s) upregulate *FvTFL1* independently of the *FvFT1–FvSOC1* pathway.

This suggests that the vernalisation response in Alta-1 is controlled by a mechanism different from that described in the perennial Brassicaceae model species *Arabis alpina*, in which the regulation of *AaSOC1* is involved in the vernalisation response (Wang et al., 2011). In Alta-1, the vernalisation response is likely regulated via altered expression of *FvTFL1*, thus adding one more aspect to the multitude of processes that *TFL1* homologues may control.
5.4 **TFL1 IS A FLORAL REPRESSOR IN F. × ANANASSA (III)**

The results discussed so far provide strong evidence for the role of *FvTFL1* in the environmentally controlled flowering response in woodland strawberry (I), and show that changes in the expression patterns of *FvTFL1* may even change the entire life history of a plant (II). However, these studies were conducted in the diploid model plant *F. vesca*, in which the molecular control of flowering could be different from that of the more economically important cultivated strawberry *F. × ananassa*. Therefore, it was of interest to elucidate whether the knowledge gained from studies on molecular control of flowering in woodland strawberry could be extended to the cultivated strawberry.

**Silencing FaTFL1 leads to early flowering (III)**

To study the function of *TFL1* in cultivated strawberry, we silenced *FaTFL1* in the SD cultivar ‘Elsanta’ by using the same *FvTFL1*-RNAi hairpin construct as was used for silencing *FvTFL1* in woodland strawberry. Flowering in LD-grown primary transgenic lines F138 and F139 occurred in approximately 78 and 125 days, respectively, whereas no flowering was observed in the wild type ‘Elsanta’ plants grown in LDs (II, Figure S3). Although the flowering times of the LD-grown transgenic lines may appear late, they are actually comparable to the flowering times observed for seed-propagated everbearing cultivars grown under continuous flowering inducing LD conditions (Table 4; Sønsteby and Heide, 2007). It has been noted that *in vitro* propagated plants display morphological features that resemble those observed in juvenile seed propagated plants, and that this juvenile period lasts for four to five weeks (Huxley and Cartwright, 1994). Therefore, it is possible that the relatively late flowering observed in the transgenic lines was a juvenility effect caused by *in vitro* propagation.

**Table 4.** Flowering time of everbearing F1 cultivars ‘Elan’, ‘Milan’ and ‘Tarpan’, and transgenic ‘Elsanta’ plants with silenced TFL1-RNAi grown under continuous LD conditions. Flowering time is expressed as days after sowing.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Days to flowering ± standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elsanta TFL1-RNAi F138</td>
<td>78.4 ± 5.9</td>
</tr>
<tr>
<td>Elsanta TFL1-RNAi F139</td>
<td>125.3 ± 18.8</td>
</tr>
<tr>
<td>Elan</td>
<td>104.9 ± 9.2</td>
</tr>
<tr>
<td>Milan</td>
<td>109.3 ± 10.7</td>
</tr>
<tr>
<td>Tarpan</td>
<td>110.02 ± 9.2</td>
</tr>
<tr>
<td>Elsanta</td>
<td>&gt;150</td>
</tr>
</tbody>
</table>
The effect of reduced FaTFL1 expression on the seasonal growth cycle of the cultivated strawberry was evaluated using clonally propagated runner plants. In this emulated seasonal cycle, plants of wild type ‘Elsanta’ and the transgenic line F138 were first exposed to natural SDs in an unheated greenhouse, followed by a chilling period at 6°C for eight weeks. After the artificial “winter”, the transgenic line showed an early and continuously flowering phenotype, producing new inflorescences until the end of the experiment, whereas the wild type ‘Elsanta’ flowered later and produced only a few inflorescences (II, Figure 1).

Promotion of flowering by reducing TFL1 expression in strawberry is in line with the results obtained from studies with other Rosaceous species; in pear and apple, introduction of a TFL1-RNAi construct has led to greatly accelerated flowering, hastened inflorescence development and shortened juvenile period (Freiman et al., 2012; Flachowsky et al., 2012). However, there are also species-specific differences; development of a terminal flower is typical of pears and apples with silenced TFL1, while no differences in inflorescence development were observed in transgenic cultivated strawberry lines. Moreover, TFL1-silenced transgenic apples typically died or stopped their vegetative growth after flowering, displaying a life cycle more typical of annual than perennial plants (Flachowsky et al., 2012). An explanation for this difference may lie in the contrasting growth patterns of pome fruits and strawberries. In pears and apples that follow a monopodial growth pattern (Costes et al., 2014), TFL1 may be required in the maintenance of vegetative meristems. Strawberries grow sympodially, with the vegetative apical meristem having strong dominance over axillary buds located in lower nodes (Heide et al., 2013; Costes et al., 2014). When the strawberry apical meristem is converted to an inflorescence meristem, the uppermost axillary meristem continues vegetative growth of the crown and resumes apical dominance (Heide et al., 2013). Therefore, it may be that the fate of axillary meristems in strawberries is controlled by factors other than FvTFL1, and that silencing FvTFL1 affects only the identity of the apical meristem.

Our data shows that FaTFL1 functions as a floral repressor in the cultivated strawberry and silencing this gene leads to a flowering phenotype resembling that of everbearing cultivars, but does not affect runner development. However, the gene does not appear to cause everbearing flowering in the cultivars used in recent mapping studies, as these studies have located the everbearing locus on F. × ananassa LGIV (Gaston et al., 2013; Castro et al., 2015; Honjo et al., 2015), and based on the conserved macrosynteny between F. × ananassa and F. vesca (Rousseau-Guetin et al., 2008), FaTFL1 should be located in LGVI. These results lead Gaston et al. (2013) to state that the genetic control of flowering in diploid and octoploid strawberries is different. However, given that Gaston et al. (2013) utilized poorly transferable AFLP markers, it is difficult to say what kind of genetic landscape their QTL region represents.
Castro et al. (2015) and Honjo et al. (2015) used transferable SSR markers for mapping the everbearing trait. What is noteworthy, the two studies shared a common marker and were able to find a linkage between the marker on LGIV and the everbearing locus in four different mapping populations. Interestingly, ongoing work in our research group suggests a QTL in LGIV of woodland strawberry involved in the control of flowering time (Samad et al., manuscript in preparation), providing support for the idea that the two strawberry species have similar genetic pathways regulating flowering time.

Irrespective of the genetic identity of the previously characterised flowering time QTL in cultivated strawberry, our results on the role of \textit{FaTFL1} as a floral repressor provide a new perspective for strawberry breeding. Modification of \textit{FaTFL1} expression either by conventional means or via transgenesis enables tailoring of flowering time in strawberries without affecting vegetative growth.

\textit{Photoperiod regulates} \textit{FaTFL1} \textit{expression in cultivated strawberry (III)}

At intermediate temperature range, photoperiod-dependent downregulation of \textit{FvTFL1} is a prerequisite to floral induction in woodland strawberry (I), and the gene has been shown to mediate temperature regulated flowering responses in woodland strawberry (Rantanen et al., 2015). To investigate the role of \textit{FaTFL1} in environmental responses of the cultivated strawberry, gene expression patterns of flowering-related genes and their correlation with flowering time were studied in five strawberry cultivars.

Photoperiodic responses at 18°C were studied using the cultivars ‘Honeoye’, ‘Polka’ and ‘Alaska Pioneer’. The cultivars were selected based on their different flowering responses; according to USDA National Plant Germplasm System, ‘Honeoye’ is an early mid-season cultivar, ‘Alaska Pioneer’ is reportedly an everbearer, whereas ‘Polka’ flowers approximately one week later than ‘Honeoye’ under Nordic conditions (Hytönen and Richterich, personal communication).

Photoperiodic flowering responses were investigated in the three cultivars exposed to LDs or SDs at 18°C for 6 weeks. No flowering was observed in ‘Honeoye’, ‘Alaska Pioneer’ or ‘Polka’ plants grown in LDs, and in SDs ‘Honeoye’ flowered nearly two weeks earlier than the other two cultivars (II, Table 1). In all cultivars, SDs downregulated \textit{FaTFL1} and upregulated the meristem identity gene \textit{FaFUL1} (II, Figure 2). Downregulation of \textit{FaTFL1} in SDs was strongest in the early-flowering cultivar ‘Honeoye’ and mildest in the late cultivar ‘Polka’ (II, Figure 2). However, downregulation of \textit{FaTFL1} occurred also in LDs in cultivars ‘Honeoye’ and ‘Alaska Pioneer’, although no flowering was observed and the meristem identity gene \textit{FaFUL1} was not expressed in LD-grown plants (II, Figure 2). It is possible that another LD-activated repressor delayed flowering in these two cultivars. The two strawberry \textit{CENTRORADIALIS-LIKE (CEN-L)} genes could be candidates for such a repressor. Although the \textit{CEN-L} genes have not been studied in detail in
strawberry, research in *Arabidopsis* and chrysanthemum suggest a role for CEN homologs in photoperiod-dependent floral repression (Huang et al., 2012; Higutchi et al., 2013).

The discrepancy between *FaTFL1* downregulation and flowering phenotype could also be explained by the results obtained by Bradford et al. (2010), who found that flowering in ‘Honeoye’ was not inhibited but only delayed by two weeks in LDs at 17°C. It is therefore possible that LD-grown ‘Honeoye’ and ‘Alaska Pioneer’ could have eventually flowered if observations had been continued for a longer time.

A similar photoperiod-independent reduction in *FaTFL1* expression has been observed in a Japanese SD cultivar ‘Nyoho’ (Nakano et al., 2015). A possible explanation for these observations could be that *FaTFL1* expression in some cultivars is controlled in an age-dependent manner. High *TFL1* expression has been shown to correlate with juvenile phase in *Lolium perenne* (Jensen et al., 2001) and citrus (Pillitteri et al., 2004).

* Cultivar-dependent differences in *FaTFL1* regulation are correlated with flowering time (III)

Interactions of photoperiod and temperature were studied in SDs and LDs at temperatures of 9, 15 and 21°C using two strawberry cultivars with different temperature responses. ‘Elsanta’ has been reported an obligatory SD cultivar that is not induced to flower by cool temperatures (Sønsteby and Heide, 2006), whereas ‘Glima’ flowers photoperiod-independently at temperatures below 21°C (Heide, 1977). Our results corroborated the earlier findings, as ‘Elsanta’ did not flower in LDs at any tested temperature, whereas ‘Glima’ flowered readily in LDs between temperatures of 9 and 15°C, and a large proportion of the plants were induced to flower even at 21°C (II, Table 2, Figure 3).

*FaTFL1* expression was generally correlated with the flowering response; in ‘Elsanta’, SDs downregulated *FaTFL1* and induced flowering at all tested temperatures. In ‘Glima’, *FaTFL1* transcript levels were lower than in ‘Elsanta’, and photoperiod had an effect on *FaTFL1* expression only in LDs at 21°C, correlated with delayed flowering under these conditions (II, Figure 5). The only environmental conditions under which *FaTFL1* downregulation did not correlate with flowering were LDs at 9°C in the cultivar ‘Elsanta’. It is possible that, at such a cool temperature, *FaTFL1* is a stronger floral repressor than at higher temperatures. Cool temperature has been shown to render *Arabidopsis TFL1* a more potent floral repressor (Hanano and Goto, 2011; Kim et al., 2013; Strasser et al., 2009), and a similar mechanism could be at work in the cultivated strawberry.

The observed discrepancy between *FaTFL1* expression and flowering under some of the experimental conditions could also arise from the incapability of the RT-qPCR primers to capture the expression of all the *FaTFL1* homoeologs. As demonstrated by Tennessen et al. (2014) and Sargent et al. (2016), one of
the subgenomes of the cultivated strawberry is *F. vesca*-like and one is *F. iinumae*-like. An examination of *TFL1* sequences from the putative diploid ancestors of cultivated strawberry reveals SNPs at the primer binding sites of the *TFL1*-specific RT-qPCR primers (data not shown), possibly biasing our estimation on *FaTFL1* expression. Preferential expression of one of the subgenomes has been reported in the natural allotetraploid upland cotton (*Gossypium hirsutum*; Hovav et al., 2008). It may therefore be that our RT-qPCR assay was not able to capture physiologically relevant changes in the expression of the most *F. iinumae*-like *FaTFL1* homoeolog.

The patterns of expression of *FaTFL1* were notably similar to those observed in woodland strawberry; in *F. vesca*, the temperature-dependent flowering response is mediated by *FvTFL1*, which is downregulated photoperiod-independently below the critical limit of 13°C (Rantanen et al., 2015). It could be argued that the differences in environmental responses between these two cultivars arise from different critical temperature limits; in ‘Glima’, the critical limit for photoperiod-independent floral induction is exceptionally high, as flowering is induced in both SDs and LDs at 21°C, whereas in ‘Elsanta’ the critical limit would be exceptionally low. It is also noteworthy, that the highest temperature used in the experiment did not cause photoperiod-independent upregulation of *FaTFL1*, contrasting with the findings of Rantanen et al. (2015), who observed that a temperature of 23°C upregulated *FvTFL1* in woodland strawberry in both LDs and SDs. However, the results of Sønsteby and Heide (2006) indicate, that 27°C is required for photoperiod-independent floral inhibition in ‘Elsanta’. It therefore seems likely that the highest temperature used in the current experiment was not high enough to cause photoperiod-independent upregulation of *FaTFL1* in the cultivars used.

In addition to woodland strawberry, flowering is correlated with downregulation of *TFL1* in several other Rosaceous species, including apple (Hättasch et al., 2008), peach (Chen et al., 2013), Japanese apricot (Esumi et al., 2010) and rose (Iwata et al., 2012). The data presented here demonstrate that downregulation of *FaTFL1* in the cultivated strawberry is generally correlated with floral induction, and the gene responds to changes in both photoperiod and temperature in a cultivar-dependent manner.
The photoperiodic FT1–SOC1–TFL1 pathway is conserved between diploid and octoploid strawberries (III)

In woodland strawberry, the photoperiodic pathway involving the upregulation of \( FvFT1 \) and \( FvSOC1 \) is activated in leaf tissues in LDs (I; Mouhu et al., 2013). \( FvSOC1 \) in turn upregulates \( FvTFL1 \), thus inhibiting flowering in LDs (Mouhu et al., 2013). To determine whether \( FT1 \) and \( SOC1 \) homologues show similar expression patterns in cultivated strawberry, we studied the expression of these genes in five cultivars grown under different photoperiods and temperatures. In the tested cultivars, \( FaFT1 \) was upregulated in leaf tissue by LDs, as was \( FaSOC1 \) (II, Figure 2, 4 and 5). Also the diurnal expression pattern of \( FaFT1 \) in leaf tissue (II, Figure S4) was similar to that of \( FvFT1 \) (I, Figure 6) with extremely low expression in SDs and a peak in expression in the middle of the dark period in LDs. The results on \( FaFT1 \) expression agree with the data of Nakano et al. (2015) and Nakajima et al. (2014), who showed that \( FaFT1 \) is upregulated in the leaf in LDs. However, Nakano et al. (2015) were not able to detect clear photoperiod-dependent differences in \( FaSOC1 \) expression in the cultivar ‘Nyoho’. The cultivars included in our experiments (II) showed much higher expression of \( FaSOC1 \) in LDs than SDs already after two weeks of photoperiodic treatments (II, Figure 2). The results of Nakano et al. (2015) are, however, in agreement with the data shown by Lei et al. (2013), who could not observe clear downregulation of \( FaSOC1 \) after two weeks in SDs in the cultivar ‘Camarosa’. It therefore appears that strawberry cultivars may have differences in photoperiodic regulation of \( FaSOC1 \), but it is impossible to say how these differences correlate with flowering time without direct comparisons between cultivars.

As discussed earlier, altering \( FvTFL1 \) expression in woodland strawberry does not affect \( FvSOC1 \) expression or patterns of vegetative development, i.e. runner and branch crown formation. The results obtained from transgenic cultivated strawberry lines were very similar to those obtained from \( F. vesca \); the expression of \( FaSOC1 \) in \( TFL1 \)-RNAi lines of cultivated strawberry were not notably different from the wild type (II, Figure 1), and the transgenic lines produced approximately the same number of runners as the wild type plants (II, Figure 1). These results suggest that the role of \( TFL1 \) as a floral repressor is conserved between the diploid and octoploid strawberries and render further support to the idea of using woodland strawberry as a model species for studying the genetics of environmental responses in strawberries.
6 CONCLUSIONS

The results discussed above provide a significant improvement to our understanding of the genetic control of flowering in strawberries. Identification of SFL as an F. vesca orthologue of TFL1 opened a route to study the photoperiodic control of flowering at the molecular level. However, identification of FvTFL1 as the major gene controlling flowering in the woodland strawberry would have been much more difficult without the knowledge gained from studies in the annual model plant Arabidopsis. The process of utilising information from a model species to identify genes controlling similar physiological processes in less-well studied species emphasises the importance of in-depth fundamental studies in model plants.

CETS genes are involved in a multitude of processes in the plant kingdom, ranging from the control of seed germination to the control of meristem identity and yearly growth cycles. Our results on the connection between the vernalisation response and FvTFL1 regulation in Alta-1 add yet another process that a TFL1 orthologue may control, highlighting the adaptive importance of the gene family. Despite the multitude of processes that TFL1 seems to control, there is not much information available on the identity of genes regulating TFL1. Future research should therefore be aimed at elucidating the genetic components that cause the observed differential regulation of TFL1 in strawberries.

The finding that TFL1 homologues control flowering also in the cultivated strawberry could open up new avenues for breeding for environmental adaptation in this important crop. Earlier studies have located the everbearing trait in the cultivated strawberry on the F. × ananassa LGIV, and shown that the same locus controls also runner production. However, FaTFL1 resides on LGVI, and changes in its expression pattern do not affect runnering in the cultivated strawberry. FaTFL1 could therefore offer a novel target for breeding for modified flowering time, without adverse effects on runner formation.

In the model plant Arabidopsis, the photoperiodic and temperature-controlled flowering pathways are integrated at the regulation of FT and SOC1. According to the data discussed here, FT1 and SOC1 may play less pronounced roles in the control of flowering in strawberries, and instead, TFL1 has evolved into the main floral pathway integrator gene. The identity of components that control the expression of TFL1 remain an active topic for further studies.
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