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Regulation of Vegetative and Generative Reproduction in the Woodland Strawberry

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REGULATION OF VEGETATIVE AND GENERATIVE REPRODUCTION IN THE WOODLAND STRAWBERRY

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Abstract

Unlike annual plants, perennials have repeated cycling between the vegetative and generative stages. Studying the balance between these two phases would enable breeders to produce higher quality crops. The woodland strawberry is used as a model to study developmental patterns in perennials because it has a wide geographical distribution, a small sequenced genome, and a number of available natural mutants, which provide excellent resources for physiological, molecular and genetic studies. This thesis investigated the genetic and environmental coordination of shoot apical meristem (SAM) and axillary meristem (AXM) fates in woodland strawberry. In woodland strawberry, SAM forms an inflorescence after flower induction, whereas AXMs can differentiate either into runners or branch crowns that are able to form additional inflorescences.

Genetic mapping and the experiments using transgenic lines and natural accessions with contrasting environmental responses showed that a number of genes regulated the balance between vegetative and generative development in woodland strawberry. In general, cool temperature or short days (SD) induced flowering and promoted AXM differentiation to branch crowns, while warm temperature and long days (LD) promoted runner formation. High levels of *FvTERMINAL FLOWER1* (*FvTFL1*) expression in *FvTFL1* overexpression lines and NOR1 accession inhibited flowering at temperatures of 10-22°C in both SD and LD, but the environmental control of AXM fate was not affected in these plants indicating that environment influenced AXM differentiation irrespective of flowering. In the seasonal flowering genotype, *FvSUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*FvSOC1*) was observed to quantitatively increase runner formation.

The photoperiodic control of flowering and AXM fate was studied in more detail using *FvCONSTANS* (*FvCO*) and *FvFLOWERING LOCUS T1* (*FvFT1*) transgenic lines. These studies

showed that FvCO controls the expression of *FvFT1,* and they both have a major role in the control of the balance between the vegetative and generative development in SD and LD.

Genetic mapping studies under differing environments identified five QTLs that, together, explained about half of the observed flowering time variance in the mapping population, and two additional QTLs were identified for the number of branch crowns explaining about 20% of variance. The flowering time QTL on LG6 colocalized with *FvTFL1,* and one of the QTL regions on LG4 that controlled both flowering time and AXM fate was close to the *PFRU*, a previously identified locus in the commercial strawberry. Among the previously unknown loci, two flowering time QTLs on LG7 colocalized with putative flowering time genes *FvEARLY FLOWERING 6 (FvELF6)* and *FvCENTRORADIALIS1 (FvCEN1)*, a homolog of *FvTFL1*. Furthermore, a gene encoding TCP transcription factor and a homolog of *DORMANCY ASSOCIATED MADS BOX (DAM)* were identified as candidate genes in QTL regions controlling AXM fate on LG4 and LG5, respectively. This study shed new light into the genetic and environmental control of AXM and SAM fates providing new means to control the balance between vegetative and generative reproduction under different environmental conditions.

Contents

List of original publications

The thesis is based on two publications and one manuscript which are referred to in the text in Roman numerals (I – III):

I. **Samad, S.**, Koskela, E., Hytönen, T. 2017. "Regulation of axillary meristem fate in woodland strawberry." [Manuscript]

II. Kurokura, T., **Samad, S.**, Koskela, E., Mouhu, K., Hytönen, T. 2017. "Fragaria vesca CONSTANS controls photoperiodic flowering and vegetative development". *J. Exp. Bot*. 68(17): 4839–4850, doi.org/10.1093/jxb/erx301

III. **Samad S.**, Kurokura, T., Koskela, E., Toivainen, T., Patel, V., Mouhu, K., Sargent D.J., Hytönen, T. 2017. "Additive QTLs on three chromosomes control flowering time in woodland strawberry. (*Fragaria vesca* L.)". *Hort. Res*. 4, 17020–, doi:10.1038/hortres.2017.20

Authorship statement

In (I), the experiments were planned and designed by SS, EK and TH. SS had the main responsibility in phenotyping observations and statistical analysis. EK coordinated the study and participated with phenotyping and statistical analysis. The manuscript was written by SS, EK and TH.

In (II), the experiment was designed by TH and TK. SS took part in the phenotypic observations with EK and TK. RT-qPCR were performed by SS and TK. KM generated the transgenic lines while TK was involved in phylogenetic analysis. TH supervised the study. TH together with TK wrote the final manuscript.

In (III), SS and TK shared first authorship of the manuscript. TK and TH were involved in experimental design and phenotypic observations. TK also did candidate searches and analyzed the data. SS carried out the genetic mapping, designed and optimizing primers, prepared the samples for genotyping-by-sequencing (GBS) analysis. DS participated with QTL mapping and GBS analysis. The F1 cross, DNA extraction and hereditary analysis was conducted by KH. The F2 mapping population was developed by EK. EK was also involved in marker development and phenotypic observations. VP was involved with whole genome sequence analysis. TT analyzed GBS data and SNP mining. TH supervised the work and wrote the final manuscript with SS and TK.

1 INTRODUCTION

Plants are sessile organisms and thus, the reproductive success of plants relies on consistently observing and responding to their environment (Bernier and Périlleux, 2005). Therefore, plants have developed complex molecular networks and systems to monitor and integrate various internal and external cues such as temperature, photoperiod (Tan and Swain, 2006) and also hormones (Levy and Dean, 1998).

Plant growth is dependent on the formation of undifferentiated meristematic cells that form new organs. The shoot apical meristem (SAM) is such a group of cells that is found at the shoot apex. The SAMs in the annuals and perennials behave differently when plant transits to generative stage (Battey, 2000; Thomas *et al.*, 2000). In annual plants the commitment to generative phase is permanent and flowering followed by senescence is the last stage in its life cycle. In these plants, the SAMs in all shoots enter generative phase at the same time, known as monocarpic growth habit. Polycarpic perennial plants have reiterative vegetative and generative stage and may return to vegetative stage after flowering (seasonal flowering) or may continuously flower after induction (perpetual flowering) (Brown and Wareing, 1965; Darrow, 1966). For a plant to be termed perennial, at least one meristem remains vegetative for the next season (Battey, 2000; Thomas *et al.*, 2000).

Most perennial plants have economical values and produce edible fruits, which make up a significant part of the daily diets. Therefore, understanding the balance between vegetative and generative phase and the underlying mechanisms is a key ingredient in breeding higher quality crops. With the climate changing becoming more unpredictable, to acquire this knowledge has become even more important because temperature has an impact on flowering and by extension on the yield.

1.1 Vegetative stage

The vegetative stage of a plant begins from germination until the onset of sexual reproduction. During this stage, plants produce vegetative structures, increase in size and mass while at the same time remain insensitive to floral induction. The primary shoot axis of the plant is formed during embryonic development and is defined by the shoot apical meristem (SAM) (Sussex, 1989). In dicots such as *Arabidopsis*, the SAM is centralized that is in between the cotyledons. Whereas in monocots like rice and maize, the SAM forms at the base of a single cotyledon. The sides of the SAM produce leaf primordia and a small region of stem cells grow between the SAM and the primordia known as the axillary meristems (AXM). New axes grow and produce additional AXM, which are reiterated as modules or phytomer. Each phytomer is composed of a number of vegetative structures such as stem, nodes bearing leaves or leaf-like structures, internode and AXM. The various forms of architecture in plants are influenced by the number of phytomers, their production and the relationship between the different components (Sussex, 1989; Bennett and Leyser, 2006), which coordinates the initiation, differentiation and development of different organs from branches to inflorescence (Costes *et al.*, 2014; Janssen *et al.*, 2014).

1.1.1 Vegetative development

The meristematic tissue is very flexible and is organized throughout the lifecycle of the plant; it can remain dormant or form vegetative structures such as leaves and branches or it can become determinate and form inflorescence (Kerstetter and Hake, 1997). The fate of the meristem can also be influenced by environmental cues, such as day length and temperature. This ensures that flowering only occurs during favorable conditions (Griffiths and Halliday, 2011).

In some plants, the vegetative growth can further be divided into juvenile and adult vegetative stages. In tree species, the juvenile stage can extend for a number of years before the plant becomes matured. During the juvenile stage, the plant lacks reproductive competence even during favorable environment while during the adult phase, the plant can transition into generative stage if induced (Sussex, 1989). The AXM can record phase changes because lateral buds during the vegetative phase are vegetative and the same is true for juvenile and adult phases, where AXM under each stage produces phase specific shoots. For example the annual model, *Arabidopsis* produces small round shaped leaves during the juvenile phase, larger narrower leaves during the adult stages and branches after floral transition. (Grbić and Bleecker, 2000; Poethig, 2003). In the pea, the AXM develops at the nodes along the stem, while in *Arabidopsis* there is a delay in the formation of AXM and thus some nodes may lack AXM.

1.1.2 Genetic control of vegetative development

Several genetic pathways regulate the vegetative state of the plant and one of the main group fall under the TEOSINTE BRANCHED 1, CYCLOIDEA, PCF1 (TCP). The TCP transcription factors are a small family of transcription factors specific to plants that play vital role in regulation and proliferation of plant growth and development (Kieffer *et al.*, 2011). TCP factors have been studied in many plants and have shown to affect branching, leaf and flower development and also development through the hormone pathways (Aguilar-Martínez *et al.*, 2007; Kieffer *et al.*, 2011). TCP transcription factors have two gene clades, THE CIN-like clade is involved in lateral organs and the CYC/TB1 clade that controls the AXM development (Wei *et al.*, 2016). *TB1* in maize inhibits branching and a single homolog was found to be conserved in monocots. While in dicots like *Arabidopsis,* multiple orthologs have been identified such as *BRANCHED1* (*BRC1*) and

BRANCHED2 (*BRC2*) that inhibit AXM outgrowth (Doebley *et al.*, 1995; Aguilar-Martínez *et al.*, 2007; Kieffer *et al.*, 2011; Kebrom *et al.*, 2013).

Plant hormones are mobile molecules that regulate plant development in minute concentrations and bind to specific receptor proteins. Thus, the spatial and temporal concentration of hormones is important for them to interact with receptors (Frébort *et al.*, 2011). Auxin and its role has been known in a phenomena known as ”apical dominance”, which is the predominant growth of the main axil while inhibiting axillary bud outgrowth. (Thimann and Skoog, 1933). Cytokinin (CK) hormones promote cell division and axillary bud outgrowth; thus, is antagonistic to auxin (Werner *et al.*, 2001). A study in pea (*Pisum sativum* L.) also proposed that auxin inhibits CK biosynthesis in the stem nodal (Tanaka *et al.*, 2006).

Gibberellins (GA) also promote cell division, elongation (Mutasa-Göttgens and Hedden, 2009), stimulate seed germination and control phase changes such as floral transition (Gupta and Chakrabarty, 2014). GA is regulated by endogenous cues such as auxin and environmental signals such as light and temperature. These cues can directly influence GA metabolism or alter the accumulation of growth repressors such as DELLA proteins therefore, affecting GA function (Sun, 2008). GA promotes plant growth by regulating DELLA proteins that repress GA activity by directly binding to the promoters of GA-regulated genes (Mutasa-Göttgens and Hedden, 2009). In *Arabidopsis,* GA deficient mutants displayed a dwarf phenotype and gibberellin-insensitive (GAI) mutant abundantly produced axillary shoots (Koornneef and van der Veen, 1980; Wilson and Somerville, 1995); conversely in the woody perennial *Jatropha curcas*, GA is needed to facilitate CK to promote lateral bud outgrowth through putative homologs of BRC1 and BRC2 (Ni *et al.*, 2015).

1.2 Generative reproduction

The transition from vegetative to generative phase is a vital survival strategy in plants. This typically occurs in the SAM and is coordinated by both internal and external signals. At adult phase, a certain endogenous balance is attained, favorable signals such as photoperiod and temperature are perceived in the leaves and a mobile signal from the leaves is transmitted the SAM (Wellensiek, 1964; Aukerman and Amasino, 1998). The signal induces the plant to converse the vegetative SAM to an inflorescent meristem that forms the flower primordia, forming the precursor for flowers and flowering shoots and the process is called floral transition (Kerstetter and Hake, 1997).

After attaining maturity, the plant cycles between the vegetative and generative stages by forming vegetative or generative structures from the AXM. In *Arabis alpina*, the cycling between the two stages is controlled by *PERPERTUAL FLOWERING 1 (PEP1)*, which is an orthologue of *FLOWERING LOCUS C (FLC)*, a floral repressor in *Arabidopsis*. *PEP1* is a MADS-box transcription factor that is epigenetically regulated by histone modifications (Wang *et al.*, 2009). In *Arabidopsis*, after the terminal meristem becomes generative, the lateral vegetative meristems also produce inflorescence branches. However the opposite is also true in some perennial species like the tomato, where the lateral meristems form inflorescence while the primary meristem remain vegetative (Poethig R. S., 1990). In Poplar, the terminal shoot remains indeterminate through the lifecycle but matured trees develop AXM after winter dormancy (Mohamed *et al.*, 2010). Since the axillary meristems can produce either the vegetative or generative structures, there is a balance between the two developmental stages and it is hypothesized that vegetative structures prevent flowering (Geber, 1990; Bonser and Aarssen, 2006).

Molecular regulation of flowering has been studied in details in *Arabidopsis*, which has several genetic pathways to flower. The main pathways to flower are photoperiod, temperature,

autonomous, GA and vernalization pathways. These pathways converge at genes known as floral integrators, which include floral promoters FLOWERING LOCUS T (FT) and SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1) (Blázquez *et al.*, 1998; Nilsson *et al.*, 1998; Hayama *et al.*, 2004). These integrators are then responsible for the activation of the meristem identity genes such as *APETALA1* (AP1) (Kaufmann *et al.*, 2010), which initiate flowering.

1.2.1 The photoperiodic pathway

One of the most important environmental signal for perennial plants is photoperiod, which is the measure of daylength. Photoperiod is the only accurate seasonal cue because it follows the same pattern every year (Andrés and Coupland, 2012). Garner and Allard in 1920 were the first to demonstrate how photoperiod could manipulate flowering and classified plants according to their reaction to daylength. Long day (LD) plants flower when they are exposed to photoperiod which exceeds a certain threshold. While short day (SD) plants flower when the length of the night exceeds a critical threshold (Jarillo *et al.*, 2008; Fujiwara *et al.*, 2008; Blackman, 2017). Day neutral plants on the other hand, are photoperiod insensitive and flower both in LD and SD conditions (Sønsteby and Heide, 2007).

The photoperiodic pathway (Figure 1) is one of the most studied flowering pathways, which has been characterized in the LD *Arabidopsis*. In *Arabidopsis*, the photoperiodic control of flowering time is connected to the circadian clock, which regulates the oscillation of output genes and synchronizes it to approximately 24 hrs in order to match the daily light hours (Fujiwara *et al.*, 2008). In 1936, Bünning acknowledged that there was a relationship between light and the circadian clock that initiated flowering. This theory was later developed and termed external

coincidence by Pittendrigh who stated that the circadian clock cycles and the plant's sensitivity to light changes in different phases of the clock cycle (Pittendrigh and Minis, 1964).

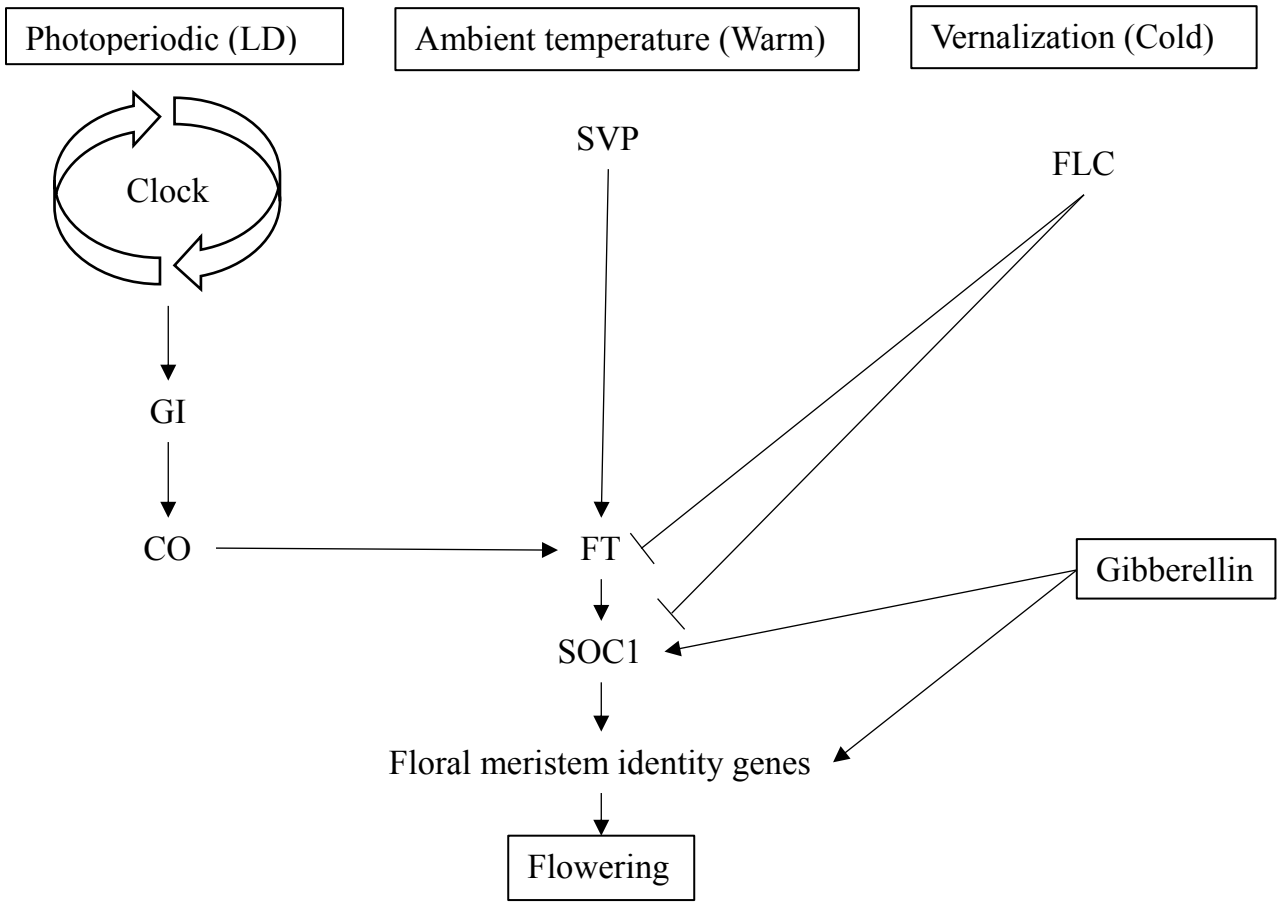

Figure 1 A simplified model of the main flowering pathways in Arabidopsis. The arrows indicate activation while the bars represent repression.

One of the key players of the pathway is *CONSTANS* (CO), which encodes a zinc finger transcription factor and is regulated both at transcriptional as well as at post-transcriptional levels (Putterill *et al.*, 1995). *CO* mRNA builds up in the leaves and the protein functions as an activator of *FT* during LDs but not SDs (Yanovsky and Kay, 2002; Sawa and Kay, 2011). This is because during LDs, *CO* expression coincides with light conditions when the CO protein is stabilized (Imaizumi *et al.*, 2005; Sawa *et al.*, 2007). Under LD, CYCLING DOF FACTOR1 (CDF1) peaks

first during the day and binds to the promoter of *CO* to represses its expression. GIGANTEA (GI) accumulates next and forms a complex with CDF1, which also prevents *CO* accumulation. Finally in the late afternoon, FLAVIN BINDING KELCH REPEAT F-BOX1 (FKF1) reaches its peak and forms a complex with GI to degrade the CDF proteins, allowing the accumulation of *CO* mRNA (Sawa and Kay, 2011).

Furthermore, a stable CO protein is only formed in the presence of light in the afternoon in LDs. The CO protein is degraded in the dark by proteasome activity of an E3 ubiquitin ligase encoded by *CONSTITUTIVE PHOTOMORPHOGENIC 1* (*COP1*). This regulates the CO protein levels, enabling it to accumulate only when plant is exposed to long photoperiod in order to induce flowering by activating FT (Corbesier *et al.*, 2007; Li, 2011).

Once activated by CO, the FT protein moves to the SAM, where it binds to form a complex between 14-3-3 protein and *FLOWERING LOCUS D* (*FD*) (Kobayashi *et al.*, 1999; Abe *et al.*, 2005). The complex then stimulates flowering by up regulating floral identity genes *AP1, LEAFY* (*LFY*) and *FRUITFULL* (*FUL*) genes (Albani and Coupland, 2010).

FT is the universal floral inducing signal in many plants and belongs to the member of the phosphatidylethanolamine-binding protein (PEBP) family. The PEBP family has diverse functions involving signaling pathways, growth and differentiation and contain both floral promoters and repressors (Hanzawa *et al.*, 2005).

The PEBP family also consists of another important gene, *TERMINAL FLOWER1* (*TFL1*) (Shannon and Meeks-Wagner, 1991). *TFL1* is a floral repressor and has an antagonistic function to that of *FT* (Hanzawa *et al.*, 2005). Low expression of *TFL1* is found in the lower parts of the apical meristem during the vegetative stage and the protein moves to the apex to repress flowering. In *Arabidopsis*, *TFL1* is up-regulated after floral induction to continue to maintain the

indeterminate inflorescence meristem (Pidkowich *et al.*, 1999). Mutations in the *Arabidopsis TFL1* can reduce the length of the vegetative stage by converting the *Arabidopsis* inflorescence to determinate. While in both primary and lateral shoots, the vegetative phase is extended by overexpressing *TFL1* (Alvarez, 1992).

SOC1 codes for a MADS box transcriptional factor (Lee and Lee, 2010), which is activated by the interaction of *FT* and *FD* and it has several repressors in the apex (Lee *et al.*, 2007; Immink *et al.*, 2012). *SOC1* expression in SAM is one of the earliest markers involved in the floral transition pathway (Albani and Coupland, 2010). *AP1* is a floral identity gene that codes for a MADS box transcription factor. *AP1* is involved in the floral differentiation (Abe *et al.*, 2005), and its activation indicates the end of the floral transition and the start of the development of flowers (Wellmer and Riechmann, 2010; Gómez-Ariza *et al.*, 2015). Kaufmann (2010) speculated that *AP1* directly or indirectly reduces the expression of *TFL1* by binding to the 3' end of the gene.

Although it is said that the flowering pathways are conserved across most species and that most perennials have similar genes to that of *Arabidopsis*; however, some of these play a different function in perennial plants or may have two or more homologues or paralogues playing the same function. One such example is the perennial poplar (*Populus spp*.), which has two *FT* paralogs (Ruiz-García *et al.*, 1997), *FT1* and *FT2*, that show different temporal expression patterns and functions. *FT1* initiates generative development upon perceiving winter, while *FT2* promotes vegetative growth as the days get longer and warmer (Hsu *et al.*, 2011).

1.2.2 The temperature pathway

Temperature also has a strong role in influencing flowering through the vernalization (Sung and Amasino, 2005) and ambient temperature pathways. Vernalization is a cold requirement by certain

plants to induce flowering while small fluctuations in the surrounding temperature are controlled through the ambient temperature pathway (Wigge, 2013).

Natural winter annual accession of *Arabidopsis* requires winter chilling period prior to flowering and functional alleles of two genes, *FRIGIDA* (FRI) and *FLC*. FRI activates FLC and the protein binds to the gene sites of *FT* and *SOC1,* inhibiting flowering. Vernalization regulates this repression through epigenetic factors such as histone modifications by repressing FLC expression and in annuals, this downregulation is stable even after plants are moved to warm conditions (Napp-Zinn, 1987; Michaels and Amasino, 2001; Amasino, 2004; He and Amasino, 2005).

The key factor in the vernalization pathway is the regulation of FLC. This requires other factors such as VERNALIZATION INSENSITIVE 3 (VIN3), which is a plant homeodomain protein. VIN3 expression increases with the duration of the cold exposure, which correlates to the degree of *FLC* inhibition (Sung and Amasino, 2004). During vernalization, *FLC* levels drops through the increase in trimethylation at the lysine 27 residue of the histone 3 tail (H3K27me3) at the FLC locus by the interaction of VIN3 and POLYCOMB REPRESSION COMPLEX2 (PRC2) and this enables flowering during subsequent LD (Bastow *et al.*, 2004; Jeong *et al.*, 2009).

In addition to that, the ambient temperature pathway also regulates *FT* and *SOC1* (Balasubramanian *et al.*, 2006). The temperature regulation in flowering has also been studied in the annual *Arabidopsis,* where warm temperature results in early flowering, while cooler temperatures lead to delayed flowering responses (Balasubramanian *et al.*, 2006; Jarillo and Piñeiro, 2011). On the other hand, an increase in temperature in *Boechera stricta*, a perennial relative of *Arabidopsis* delays flowering (Anderson *et al.*, 2011).

Further research has also shown an epigenetic mode of regulation (Kumar and Wigge, 2010; Ito *et al.*, 2012). Recent research in *Arabidopsis* has shown that there is epigenetic regulation involved

in this pathway. One such study shows that production of FT is influenced through changes in the chromatin by histone complex. Under low temperatures, the FT promoter is restricted via chromatin compression by H2A.Z, a variant of the normal H2A. However at higher temperature, H2A.Z is removed, enabling transcription factors to access the FT promoter (Kumar and Wigge, 2010). One such transcription factor activated at high temperature is PHYTOCHROME INTERACTING FACTOR4 (PIF4), a bHLH transcription factor that binds to the FT promoter and induces early flowering under SDs (Kumar *et al.*, 2012).

In *Arabidopsis* there are five FLC-related loci named *MADS AFFECTING FLOWERING* genes (MAF 1–5), which are involved in the temperature regulation of flowering. MAF1 has also been named FLM and acts as a flowering repressor (Ratcliffe *et al.*, 2001). Under LD, flowering under high temperature is controlled through the interaction of MADS box transcription factors SHORT VEGETATIVE PHASE (SVP) and FLM. The floral repressor SVP, is degraded under high temperatures(Lee *et al.*, 2013), while FLM was thought to undergo temperature –dependent alternative splicing to regulate the flowering responses under the differing temperatures. It was suggested that both variants bind and compete in an antagonistic manner to form a heterodimer with SVP. However, the balance was shifted towards the floral promoter FLM-δ, under high temperatures and towards FLM-β, the repressor form under low temperatures (Posé *et al.*, 2013). Under low temperatures, the SVP-SVP and SVP-FLM-β complexes delay flowering by downregulating SOC1 and FT. Whereas at higher temperatures, the SVP-FLM-δ complex is not able to repress flowering due to the inability to bind to FT and SOC1 promoters (Lee *et al.*, 2013; Posé *et al.*, 2013). Recent research using CRISPR/Cas9 to create specific exon deleted lines showed that the role of FLM-δ in floral regulation is negligible (Capovilla *et al.*, 2017).

1.3 Strawberry, an economically important berry

One of the most commercially important soft fruit is strawberry (*Fragaria*), which has a worldwide market and has been included in The International Treaty on Plant Genetic Resources, Annex 1 (Hummer *et al.*, 2011). Hence, examining traits which are important to increase and produce a better yield has become the focus for many studies. It was recorded that in 2014, the worldwide production of strawberries exceeded 8.1 million metric tons, out of which Europe produced about 20%. China and USA were the top producers, producing 3.1 million and 1.3 million tons respectively (FAO Statistics Division 2016, 2016).

Strawberry is a perennial plant of the genus *Fagaria* of the Rosaceae family, subfamily Rosoideae (Staudt, 2006). The Rosaceae family consists of 3000 species in about 90 genera (Illa *et al.*, 2011) and consists of fruit trees like *Malus* (apple) and *Pyrus* (pear); stone fruits from the *Prunus* such as peach and cherry; berries such as *Fragaria* (strawberry) and ornamentals such as Rosa (rose) (Dirlewanger *et al.*, 2002; Cabrera *et al.*, 2009; Shulaev *et al.*, 2011; Longhi *et al.*, 2014). The members of the family are both phenotypically as well as genetically diverse with differences in plant habit, fruit type and also in chromosome numbers which range from x = 7 to x = 17 (Illa *et al.*, 2011).

The *Fragaria* genus has 27 known taxa, which range from the diploid to decaploid and it also has a number of natural hybrids (Njuguna *et al.*, 2013). Many of the diploids and tetraploids are found in Asia, near the Sea of Japan and the Sino-Himalayan region (Darrow, 1966; Hummer *et al.*, 2011; Njuguna *et al.*, 2011; Liston *et al.*, 2014).

The cultivated strawberry *F.* × *ananassa ssp. ananassa* Duchesne ex Rozier is an octoploid species (Njuguna *et al.*, 2011). The history of the cultivated strawberry is traced to Europe during the mid-1700s, where a coincidental cross between octoploid species *F. chiloensis* (Mill.) and *F. virginiana*

(Duch.) took place forming an allo-octoploid (2n =8x = 56) species called *Fragaria* × *ananassa* Duch., which is now commercialized (Rousseau-Gueutin *et al.*, 2008; Gil-Ariza *et al.*, 2009; Bassil *et al.*, 2015; Mahoney *et al.*, 2016).

The family is a vital plant family in the temperate region and the fruits have been an important source of food since ancient times and eaten in various forms from jams to juices and this gives rise to many combinations of flavours and textures thus, a higher consumer choice (Dirlewanger *et al.*, 2002). Over time, selections and domestication of the plants have given rise to large fleshy fruits making them different from their wild relatives.

1.4 The woodland strawberry, a perennial model

The diploid *Fragaria vesca* has been actively researched on because it is one of the progenitors of the commercial octoploid strawberry (Gil-Ariza *et al.*, 2006). It has been proposed as a model plant for molecular analysis of perennial crops as well as that of the Rosaceae family, because it is easier to study due to its small diploid genome of 219 Mbp (Ruiz-Rojas *et al.*, 2010; Shulaev *et al.*, 2011). Moreover, it is easy to grow and propagate from seed, is quick to reproduce because it has a short generation time and moreover, it is easily transformed by Agrobacterium tumefaciens (Oosumi *et al.*, 2006; Ruiz-Rojas *et al.*, 2010). *Fragaria* has diverse phenotypes (Sargent *et al.*, 2004*b*), genotypes (Hummer *et al.*, 2011) and a vast distribution spreading across boreal Eurasia, North America and introduced into Japan and the Hawaiian Archipelagos (Hummer *et al.*, 2011; Njuguna *et al.*, 2011), which makes it a unique model that is easily available in different environmental conditions. It also has naturally occurring mutants to study early flowering and also runnerless accessions. Furthermore, it also has genotypes which induce flowering in short days (SD) such as *F. vesca* L. and also long days (LD) for example Fragaria *vesca semperflorens* Hawaii and 'Baron Solemacher' (Darrow and Waldo, 1932; Mouhu *et al.*, 2009) and it can form viable hybrids from crosses between different species within its taxa to produce new varieties (Schulze *et al.*, 2011).

1.5 Strawberry physiology

In *Fragaria*, the stem is a thick rootstock with short internodes and this together with the terminal bud is called a crown. *Fragaria* produces trifoliate leaves arranged in a rosette and each leaf axil has an axillary bud that can differentiate into runner (Figure 2), which is a long shoot made of two long internodes and ending with a leaf rosette (daughter plant) or can form a new leaf rosette known as branch crown (Figure 2). Daughter plants are genetically identical to the mother plants and can be utilized for vegetative reproduction (Hytönen and Elomaa, 2011).

Figure 2 Picture of woodland strawberry under vegetative condition. Picture on the left shows (1) a runner, (2) the daughter plant (3) leaf. On the right (4) circulates a branch crown.

Vegetative development is vital for plants to continue growing and in strawberries this also allows asexual propagation through runners. The vegetative development is perceived by growth rate, which can be measured by observing the leaf size, petiole length and increase in runner production (Durner and Poling, 1985).

Runnering and petiole length is regulated through environmental conditions such as temperature, photoperiod, hormones, nutrient and their interactions. In most accessions like the SD *Fragaria vesca* accession, LD and high temperature increases the rate of AXM producing runners, while SD promote branch crowns. Crown branching indirectly increases cropping potential by increasing the number of meristems that can transition to inflorescence meristems and produce flowers. The effect of the environment on the plant varies due to the vast diversity within the genus (Pure *et al.*, 1973; Heide *et al.*, 2013).

Once the plant is induced to flower, AXMs produces branch crowns and the apical meristem terminates with a primary flower and this is proceeded with two lateral branches which each terminates with a secondary flower. Each primary branch gives rise to two secondary branches and each terminates with a tertiary flower (Guttridge, 1985).

There are four stages to flowering namely, induction, initiation, differentiation and development. Induction occurs in the leaf, when FT relays the signal that causes the formation of a floral bud in the meristem while initiation summarizes the physiological and morphological changes happening in the meristem post induction. This is followed by the formation of floral organs known as the differentiation stage while the production of flowers is the final developmental step (Durner and Poling, 1985).

Floral initiation occurs in the shoot apex of the main crown. The inflorescence are formed from terminal apical meristems, after which the control of crown extension is taken over by the uppermost lateral bud, which becomes dominant over other lateral crowns (Heide *et al.*, 2013).

In both the garden strawberry and woodland strawberry, flowering is controlled by a complex photoperiod x temperature interaction. Flowering occurs independently of photoperiod at low temperatures, at SD (typically less than 14 hrs of day light) during intermediate temperatures

(between 14℃ – 20 ℃) and is repressed during high temperatures (>21℃). These critical temperatures and daylength vary based on accessions and cultivars (Heide and Sønsteby, 2007).

The alpine strawberry *F. vesca var. semperflorens* Duch. is unique as it requires LDs at intermediate (15-21℃) and high temperatures (> 26℃) to flower (Darrow, 1936; Sønsteby and Heide, 2007), while at low temperatures (< 9℃), photoperiod has a quantitative effect on flowering in some accessions. Flowering was also reported to be suppressed in these genotypes under SD with increasing temperature (Sønsteby and Heide, 2008; Heide *et al.*, 2013).

In *Fragaria*, flowering is antagonistic to runner formation because when induced to flower, the runner production reduces in both genotypes. The perpetual flowering genotypes have poor runner production probably because these are induced to flower at an early age (Sønsteby and Heide, 2007). Both traits can be manipulated by environmental cues such as photoperiod and temperature and their interaction thus, allowing for both genetic and phenotypic studies of growth and development as a model plant (Sønsteby *et al.*, 2013).

1.6 Genetic and molecular studies in strawberry

New varieties of strawberry are frequently being introduced into the market and stable methods to identify them is essential for breeders especially when these varieties are clonally propagated. In addition to that, genetic markers are also used for exploring strawberry genetic variation (Sargent *et al.*, 2004*b*; Brunings *et al.*, 2010), diversity (Gil-Ariza *et al.*, 2006; Njuguna *et al.*, 2011) and form molecular maps (Weebadde *et al.*, 2008; Gaston *et al.*, 2013; Castro *et al.*, 2015; Honjo *et al.*, 2016). Due to the synteny in the *Rosaceae* family, markers (Vilanova *et al.*, 2008; Zorrilla-fontanesi *et al.*, 2011; Gar *et al.*, 2011; Longhi *et al.*, 2014) as well as the whole genome sequencing (WGS) approach has also been used for comparative studies (Jung *et al.*, 2012).

Many commercially important traits, such as flowering time, fall under regions in the genome where genetic differences can be quantified and it is hypothesized that the genetic difference is related to the given trait and thus, known as quantitative traits (QTL) (Salazar *et al.*, 2013). By quantifying the genetic variation, the trait of interest can be analyzed. Hence, the increased interest in the construction of linkage maps using molecular markers to identify these genetic variations, linking them to a phenotype, which can be quantified, to hypothesize the locations of genes of interest. A number of researchers have constructed genetic maps for *Fragaria* (Sargent *et al.*, 2003, 2004*a*, 2006*a*, 2007, 2008; Hadonou *et al.*, 2004; Nier *et al.*, 2006; Govan *et al.*, 2008; Zorrilla-fontanesi *et al.*, 2011; Illa *et al.*, 2011; Mahoney *et al.*, 2016) thus, providing available resources for experimenting and breeding companies.

Since the commercial strawberry is an octoploid species, the diploid woodland strawberry has been used as an alternative to studying genes in strawberry. In the past, many maps have used various PCR based markers (Ashley *et al.*, 2003; Sargent *et al.*, 2003, 2004*a*, 2006*b*, 2007, 2011; Nier *et al.*, 2006; Rousseau-Gueutin *et al.*, 2008) and these were later used to anchor the reference genome

(Shulaev *et al.*, 2011). With the advancement of technology, high-throughput maps have been generated using next generation sequencing methods (Bassil *et al.*, 2015; Sargent *et al.*, 2016).

Flowering and runnering were thought to be inversely controlled by a single locus however, Brown and Wareign (1965) showed through simple genetics, that two separate loci, then called seasonal flowering locus (SFL) and runnering locus (R) controlled the processes (Brown and Wareing, 1965; Hytönen and Elomaa, 2011). The *FvTFL1* gene characterized by Koskela, *et al.* (2012) co-localizes with the SFL and *FvTFL1* was characterized to be a strong floral repressor that maintains the vegetative phase of the plant and integrates environmental signals to maintain the identity of the meristem.

Homologs of *Arabidopsis* photoperiodic pathway genes are present in woodland strawberry genome (Mouhu *et al.*, 2009). However, the same genes play different roles in the different genotypes (Mouhu *et al.*, 2013). Recent research has shown that the photoperiodic pathway (*FvFT1*- *FvSOC1*- *FvTFL1*) is intact in the seasonal flowering genotype through LDs; although, the same pathway results in varying flowering phenotypes for the perpetual flowering mutant (Mouhu *et al.*, 2013; Rantanen *et al.*, 2015; Koskela *et al.*, 2016). In the SD seasonal flowering accession *Fragaria vesca* (FIN56), under LD, FvFT1 activates FvSOC1 which in turn upregulates *FvTFL1* inhibiting flowering. Under cool temperatures, *FvTFL1* is repressed irrespective of the photoperiod by an unknown factor and thus flowering is induced, while at 14-18°C, SD is required to downregulate *FvTFL1* and induce flowering. At higher temperatures flowering is suppressed irrespective of photoperiod because *FvTFL1* is upregulated (Rantanen *et al.*, 2015; Koskela *et al.*, 2016). Recent reports discussed that in a perpetual flowering accession *F. vesca* f. *semperflorens* Hawaii (H4), LD also promotes *FvFT1* which in turn upregulates *FvSOC1,* but due the presence

of a nonfunctional *FvTFL1*, both *FvFT1* and *FvSOC1* promote flowering (Rantanen *et al.*, 2014; Koskela *et al.*, 2016).

Research has also shown that in the SD seasonal flowering background overexpression of *FvSOC1* was able to manipulate the vegetative characteristics such as continuous runnering behavior, while silencing *FvSOC1* reduced runner formation, independently of the photoperiod. Previous studies have shown that *FvSOC1*, a homolog of the *Arabidopsis SOC1,* regulates the expression of several GA biosynthetic and signaling genes (Mouhu *et al.*, 2013).

GA is involved in cell growth and development through cell division and elongation (Mutasa-Göttgens and Hedden, 2009) and has previously been reported to be regulated by photoperiod (Hytönen *et al.*, 2009). The GA pathway is made up of complex regulation of the biosynthesis and deactivation of GA through multiple steps. Recently through fine mapping studies in Yellow Wonder (YW), a *Fragaria vesca* mutant, a deletion in the active site of *FvGA20ox4* a gene encoding GA biosynthetic enzyme, was found and this gene was identified as a likely candidate of R. The mutation gives rise to dormant shoots or shoots bearing inflorescence but not runners, which can be reversed by the application of GA (Tenreira *et al.*, 2017).

Recent ENU mutagenesis screen of the YW plants have found a mutant that regains the runnering ability and was called suppressor of runnerless (srl). Using bulk segregate mapping-by-sequencing of the F2 population, the causal gene was mapped at the end of LG4. A putative DELLA gene from the *F. vesca* genome, previously named as *FveRGA1* (Kang *et al.*, 2013), was identified as a candidate gene. This gene exhibited a nonsynonymous mutation close to a stop codon in the *srl* mutant (Caruana *et al.*, 2017).

2 OBJECTIVES

Strawberry is an economically important crop species, and with the changing climate, it has become more important to understand the environmental control of strawberry development at the genetic and molecular level. This thesis was designed to analyze the roles of known flowering genes in the control of the balance between vegetative and generative reproduction in strawberry using woodland strawberry as a model. It covers aspects of molecular genetics, plant transformation, plant architecture, phenotyping under different temperatures and photoperiods, marker development and genetic mapping. The thesis is based on three publications that address following specific aims:

I. To investigate the environmental and molecular control of the balance between vegetative and generative development in the seasonal flowering woodland strawberry.

II. To study the role of *FvCO* in the control of flowering and vegetative development in woodland strawberry.

III. To identify candidate genes involved in the control of vegetative and generative development through QTL mapping in order to facilitate the selection and breeding of new varieties of strawberry that will exploit early and late season dips in traditional strawberry production, where the market price is higher.

3 Materials and methodology

Table 1 summaries the methods utilized in the thesis. Detailed methodology is explained in the respective publications. Methods used by co-authors are in parenthesis.

Table 1: List of methods used in this thesis

Methodology	Publication
Bioinformatic analysis	(II)
cDNA synthesis	II, (II)
Crossing populations	(III)
DNA extraction	(III)
Gateway™ vector construction	(I), (II)
Genetic mapping	III
Genetic transformation	(I), (II)
Genotyping-by-sequencing analysis	(III)
Growth experiments	I, II
Marker design	III
Plant architecture	I, II
Phylogenetic analysis	(II)
RT-qPCR	II, (II)
Shoot architecture	I, II

3.1 Plant materials and experimental conditions

Two separate experiments were constructed using a seasonal flowering accession and a perpetual flowering accession. The wild F. *vesca* accession 'Punkaharju' (National Clonal Germplasm Repository accession PI551792, abbreviated as FIN56) is a seasonal flowering SD accession, which has been used in prior investigation and thus, had available transgenic lines. The F. *vesca* (L.) var. *semperflorens* (Duch.) Staudt 'Hawaii-4' ('H4') is a perpetual flowering LD plant, which had also been used in previous studies and therefore, seeds were available for *FvCO* transgenic lines that were characterized in this thesis. Table 2 and 3 summaries the plant material used and past publications in which these were introduced.

Table 2: The lines used in the publication I.

Genotypes	Line	Publication
FIN56	WT	PI551792
NOR1	WT	(Heide and Sønsteby, 2007)
SOC1-OX	*FvSOC1*-OX7	(Mouhu *et al.*, 2013)
	FvSOC1-OX11	(Mouhu *et al.*, 2013)
	FvSOC1-OX12	(Mouhu *et al.*, 2013)
SOC1-RNAi	*FvSOC1* RNAi3	(Mouhu *et al.*, 2013)
TFL1-OX	*FvTFL1*-OX2	Unpublished data
	FvTFL1-OX3	Unpublished data

FIN56 and its transformants were used to study the effects of varying photoperiod and temperature on the vegetative and generative pattern in strawberry (Table 2). H4 and *FvCO* RNAi and overexpression lines were used to study the role of *FvCO* in the control of generative and vegetative development in diploid perpetual flowering strawberry.

For the FIN56 experiment, young runner cuttings from mother plants grown in non-inductive conditions were propagated and kept in the LD greenhouse for two weeks before being moved to the growth room for a further 1.5 weeks to acclimate to the different condition prior to the start of the environmental treatments. During the environmental treatments, plants were subjected to three different temperatures (low =10 °C, intermediate = 18 °C high =22 °C) in two photoperiods (SD = 12hr, LD = 16 hr). The details of the experimental layout is elaborated in (I).

For the H4 experiment plants were grown from seeds, which were germinated on petri dishes and GFP+ plants were potted in the greenhouse under LD. At 2-3 leaf stage, the plants were moved to the growth room and allowed to acclimatize for a further 1.5 weeks before the start of the treatments. Both treatments had the same temperature of about 18 °C under different photoperiods (SD = 12hr, LD = 16 hr). Details of the experimental design are described in (II).

For (III), an available H4 × FIN56 crossing population, which was previously used for map-based cloning of *FvTFL1* (Koskela *et al.*, 2012), was used to explore QTLs linked to flowering and vegetative traits. However, only a subset of the population was used consisting of 335 seedlings of various flowering time.

3.2 Plant architecture

During and after the environmental treatments, newly opened leaves were marked weekly with a number to trace the fate of the axillary bud in each leaf axil. For the perpetual flowering H4, plants were broken down after the flowering date was recorded and the leaf axil that produced a runner was marked with “R”, those that formed branch crowns were marked as “BC” while those that produced the inflorescence were marked as “F”. Some leaf axils remained dormant and these were marked as “0”. For the seasonal flowering plants, the fate of the axillary buds was observed every

1-2 weeks by marking the newly opened leaves and the fate of the axillary buds were recorded similar to H4 plants.

3.3 Genotyping and mapping

Additional genes, to the known and studied genes were mined using genetic mapping in (III). A number of previously published SSR were used to form a basic map which was filled with segregating SNPs designed to flank the QTL regions and finally, 186 seedlings were used for high-density mapping using genotyping-by-sequencing (GBS) analysis. The details of the markers used and the analysis are described in (III).

3.4 cDNA synthesis and RT qPCR

For (I) and (II) RNA was extracted as described by (Mouhu *et al.*, 2009) and then treated with rDNAse (Macherey-Nagel GmbH, Düren, Germany) according to the manufacturers protocol. cDNA was synthesized followed by real-time quantitative PCR (RT-qPCR), details of which can be found in (I) and (II). The reference gene used was MULTICOPY SUPRESSOR OF IRA1 (MSI1) and the calculations were done using ΔΔCt method (Pfaffl, 2001).

4 RESULTS AND DISCUSSION

4.1 Control of meristem fate in woodland strawberry (I)

Strawberry is a perennial rosette plant that can reproduce both sexually though seeds and asexually through runners that produce daughter clones. In the vegetative state, SAM produces new leaves and one AXM in the axil of each leaf. The AXM can either remain dormant or form either runners or branch crowns. After floral induction, SAM of both the main and branch crowns produces a determinate inflorescence and the total number of inflorescence per plant depends on the number of branch crowns. Since a single meristem can differentiate into one structure, there is a trade-off as to which structure is formed and this is regulated through a complex interaction between the developmental, environmental and genetic components (Guttridge, 1960; Hytönen and Elomaa, 2011). The ability to manipulate this balance would be an important tool for growers who would like to either improve flowering and consequently yield or increase the efficiency of clonal propagation.

4.1.1 Environmental regulation of AXM fate (I)

We studied environmental regulation of axillary bud differentiation in a diploid woodland strawberry that is used as a model for the cultivated strawberry, which has a more complex octoploid genome. To investigate the response of AXM to different temperatures and photoperiods, a seasonal flowering accession FIN56 was grown at three different temperatures of 10°C, 16 °C and 22°C under SD and LD (I).

At cool temperature, AXMs were insensitive to photoperiod, actively initiating branch crown formation and 100% of the plants flowered under SD, while 73.3% flowered under LDs. At intermediate temperature, however, AXM differentiation was photoperiodically regulated, where

LDs favored runner production, whereas flower inductive SDs promoted branch crown formation. About 66.7% plants flowered under SDs and 20% of the plants under LDs also flowered. Since flowering is known to affect branch crown formation, we compared flowering and non-flowering plants in both SD and LD. Under SDs, the plants that flowered had more branch crowns as compared to runners, while under LDs plants that flowered had about the same number of runners and branch crowns (Figure 3). Those that did not flower, actively produced runners over branch crowns irrespective of the photoperiod. This showed a connection between photoperiodic and developmental regulation, where runner production was favored under LDs, while branch crowns were abundant under SDs in flowering plants; however, non-flowering plants produced runners over branch crowns.

At warm temperature, AXM promoted runner formation and branch crown production was significantly reduced. Flowering was inhibited, similar to previous studies by Rantanen *et al.* (2015), who showed that high temperature upregulated *FvTFL1* expression independently of photoperiod to repress flowering. The floral inhibition caused by *FvTFL1* is the likely reason for the AXM to differentiate into runners instead of branch crowns. However, an unknown factor increases *FvTFL1* under SD at high temperature.

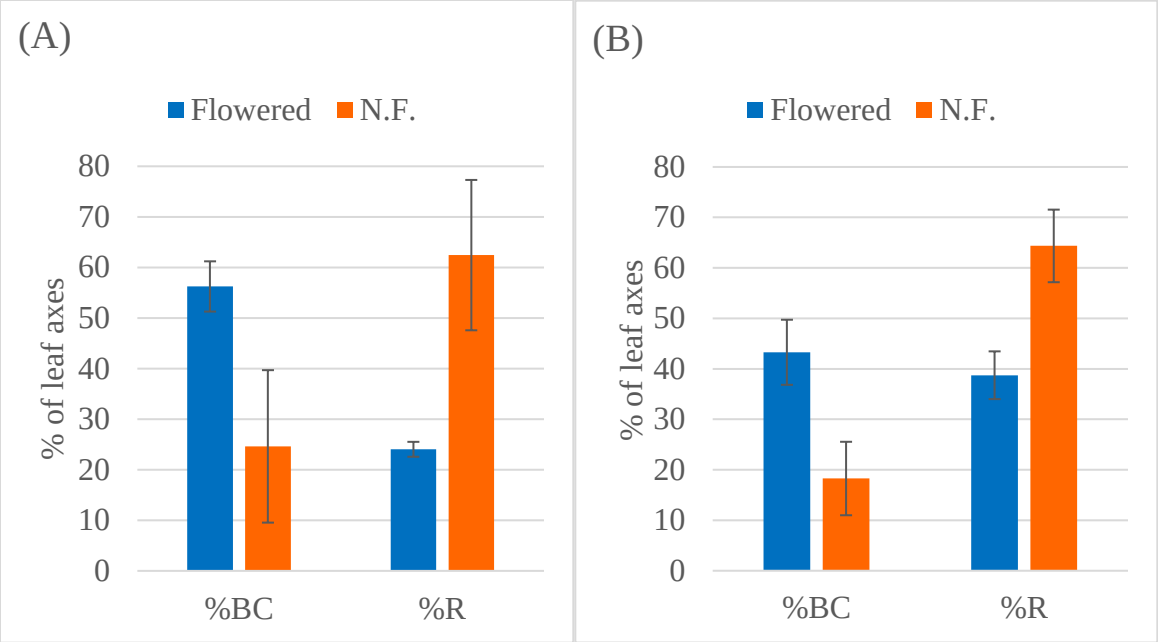

Figure 3 The effect of flowering on AXM differentiation in FIN56 in (A) SD (N = 5-10) and (B) LD (N= 6- 9) at intermediate temperature (16 °C). The average percentage of AXM that differentiated into either branch crown (BC) or into runners (R) is shown. Error bars represent standard errors, N = 5-10. It is to be noted that some AXM also remained dormant.

Studies in FIN56, could not clearly separate the environmental and developmental regulation of AXM differentiation. Therefore, genotypes that were vegetative under these conditions were used in the study. These included plants overexpressing *FvTFL1*, which is a strong floral repressor involved in both photoperiodic and thermal regulation of flowering in woodland strawberry (Koskela *et al.*, 2012; Mouhu *et al.*, 2013; Rantanen *et al.*, 2015) and NOR1 (Heide and Sønsteby, 2007), a Norwegian accession that requires obligatory vernalization prior to flowering due to the high expression of *FvTFL1* mRNA (Koskela *et al.*, 2017).

At intermediate temperature the *TFL1* overexpressing lines and NOR1 demonstrated a photoperiodic response in AXM differentiation. Similar to FIN56, LDs promoted runnering, while

SDs resulted in an increase in branch crown formation. Temperature also regulated the differentiation of AXM. The *TFL1*-OX and NOR1 plants behaved similar to FIN56 that is by increasing branch crown production at cool temperature and favoring runner production at warm temperature. In summary, *FvTFL1* has little or no direct role in the differentiation of AXM into runners in the conditions tested in this study, which is in agreement with previous reports (Koskela *et al.*, 2012, 2016). Taken together, this illustrates that environmental conditions controlled axillary bud differentiation independently of flowering. Moreover, more studies concentrating on temperature control of AXM differentiation are needed to make a definitive conclusion.

4.1.2 Genetic control of AXM fate (I)

FT1 activates the MADS-box transcription factor, *SOC1* that functions as a floral activator in both SD and LD plants (Menzel *et al.*, 1996; Lee *et al.*, 2000, 2004). Previous research illustrated that in woodland strawberry under LDs, *FvFT1* activates *FvSOC1* that in turn activates *FvTFL1* to inhibit flowering in FIN56 (Figure 3). In addition to that, *FvSOC1* promotes runner formation through the GA pathway (Mouhu *et al.*, 2013). In this study, the role of *FvSOC1* in AXM differentiation under different conditions was tested using previously generated transgenic lines. *FvSOC1* overexpressing lines showed a quantitative preference to runner production, where a higher *FvSOC1* transgene level increased the frequency of AXM differentiation into runners as compared to weaker expressing lines. The strongest overexpression line, *FvSOC1*-OX12 produced runners at all temperatures and no branch crowns were observed in this line. An increase in *SOC1* levels resulted in all the lines to become insensitive to photoperiods and abundantly produce runners except at cool temperatures, where some branch crowns were observed in the weaker *SOC1*-OX lines. Previous studies reported that at cool temperature and SD, *FvSOC1* and *FvTFL1*

expression levels in FIN56 drop (Koskela *et al.*, 2012; Mouhu *et al.*, 2013) and similar observations were found in cultivated strawberry (Nakano *et al.*, 2015; Koskela *et al.*, 2016). This indicates that the runner production in strawberries is dependent on *SOC1* levels in different conditions and that SD and cool temperature are indications of the forthcoming winter to initiate the AXM differentiation into branch crowns instead of runners and to prepare the plant to flower the following spring.

Silencing the *SOC1* expression on the other hand, did not completely abolish runner formation but the RNAi lines stopped runnering earlier than FIN56 as observed also by Mouhu *et al.* (2013). This indicates that there is a parallel pathway that is likely promoting AXM differentiation into runners independently of *FvSOC1*. This study and also Rantanen *et al* (2015) showed that in the *FvSOC1* RNAi lines, flowering was not observed at high temperatures. Taken together, it is hypothesized that an unknown factor inhibits flowering by increasing *FvTFL1* mRNA level and promotes runner formation under SD at high temperature independently of *SOC1*. More research needs to be done to find out how this temperature is perceived, how the signal is integrated and how the genes are regulated based on this information.

Hytönen *et al*. (2009) illustrated that GA promoted AXM to form runner in cultivated strawberry and Mouhu *et al.* (2013) showed the involvement of *FvSOC1* in the activation of the GA pathway genes in the woodland strawberry including a number of GA20ox. Recent studies fine-mapped the R-locus and identified a mutation in the candidate gene *FvGA20ox4* that made the enzyme inactive. Expression analysis detected that the gene was expressed in the AXM and in developing runners (Tenreira *et al.*, 2017).

Recently a runnering mutant was identified from the runnerless YW accession through chemical mutagenesis and was called *suppressor of runnerless* (*srl*). A DELLA gene named *FveRGA1* (Kang

et al., 2013) was identified as a candidate gene through QTL mapping using the M2 population. It was shown that the *srl* mutant occurred due to a nonsense mutation in the DELLA gene that enabled runnering in runnerless YW, showing that *FveRGA1* regulates AXM differentiation in strawberry (Caruana *et al.*, 2017). The DELLA proteins are repressors of GA-responsive growth, that are degraded by the E3 ubiquitin ligase in response to GA binding to GID1 receptors (Harberd *et al.*, 2009; Sun, 2010; Fukazawa *et al.*, 2015). These results prove that GA is needed for AXMs to differentiate into runners and a simplified flow diagram is illustrated in Figure 4 below.

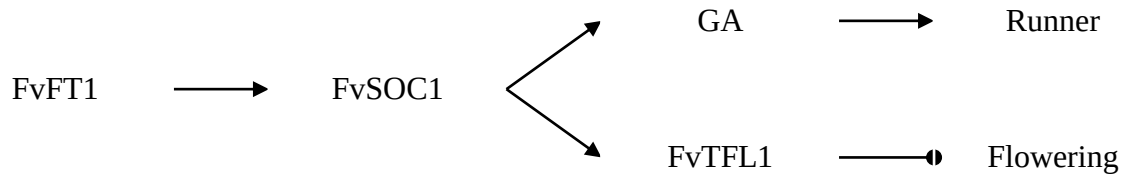

Figure 4 Role of SOC1 in the control of flowering and AXM differentiation under LDs in the woodland strawberry. The arrows indicate activation, while the arrow head with a broken circle indicates repression. FvFT1 activates *FvSOC1* which influences the fate of the AXM through the GA pathway to form runners and also controls flowering through *FvTFL1*.

The morphology of the commercial octoploid strawberry is similar to that of the diploid woodland strawberry. There is a lot of research done on many different cultivars (Gaston *et al.*, 2013; Heide *et al.*, 2013; Koskela *et al.*, 2016) that has revealed cultivar-specific thresholds controlling the physiological and developmental response to environmental changes. Like in the woodland strawberry, seasonal flowering cultivars of the cultivated strawberry are induced to flower in SDs or at cool temperatures, whereas high temperature inhibit flowering (Darrow and Waldo, 1932; Waldo and Darrow, 1932; Ito and Saito, 1962; Darrow, 1966; Bradford *et al.*, 2010; Durner, 2015).

The control of AXM differentiation in the cultivated strawberry is also similar to the woodland strawberry, where branch crown formation is promoted in floral inductive conditions whereas runnering is antagonistic to branch crown formation (Heide, 1977; Konsin *et al.*, 2001). Thus, the knowledge gained from the studies in the woodland strawberry can be applied to that of the commercial strawberry.

4.2 Photoperiodic control of meristem fate in perpetual flowering woodland strawberry (II)

4.2.1 FvCO is the only Group Ia COL protein in woodland strawberry (II)

The photoperiodic pathway revolves around the LD activation of FT protein in the leaves by a stable CO protein. This CO-FT module is conserved in many plants however, the outcome differs between SD and LD species (Hayama *et al.*, 2003). Under LDs in the facultative LD *Arabidopsis*, CO activates FT to induce flowering. However, in rice a SD plant, CO represses flowering under LDs (Hayama *et al.*, 2004).

Since photoperiod regulates flowering and also AXM in strawberry (I), this study was conducted to understand the role of *CO* in the photoperiodic pathway of the woodland strawberry. Phylogenetic analysis of the *FvCO*-like sequences found in the *F. vesca* whole- genome v1.1 assembly (Shulaev *et al.*, 2011) revealed only a single amino acid sequence that grouped together Group Ia COL proteins. This protein was previously named FvCO (Shulaev *et al.*, 2011).

4.2.2 *FvCO* controls flowering phenotype (II)

FvCO overexpressing and RNAi transgenic lines were generated in the LD accession Hawaii-4 (PI551572; National Clonal Germplasm Repository, Corvallis, OR; called H4 hereafter). H4 is a perpetual flowering mutant that has a two bp deletion in the first axon of the *FvTFL1* gene that results in a non-functional *FvTFL1* protein and thus, abolishes the SD requirement to flower. In this accession, *FvFT1* and *FvSOC1* promote flowering under LDs, whereas in the SD accessions, flowering is inhibited under LDs because *FvTFL1* is upregulated by *FvFT1* through *FvSOC1*.

The AXM differentiation into either runners or branch crowns as well as the flowering time was investigated in the H4 (WT) and in *FvCO* and *FvFT1* transgenic lines under LD and SD at 20–22 °C. H4 produced more runners under SDs than under LDs. The balance between runner production and branch crown formation was photoperiodically regulated also in the *FvCO*-OX lines. SDs promoted runner formation, but a higher percentage of branch crowns was observed in the overexpression lines than in H4 especially under LDs. Despite the difference in AXM differentiation under SD and LD, flowering was observed under both photoperiods for the overexpressing lines, although slightly later under SDs than LDs. When moved to floral inductive LD conditions, H4 continued runner formation for a longer period of time and thus produced more runners than *FvCO*-OX. The overexpressing lines flowered earlier than H4 and the average number of inflorescences produced was also higher in the overexpressing lines than in H4. Similarly, overexpressing *FvFT1* caused extreme early flowering (Rantanen *et al.*, 2015) and no runners were observed in *FvFT1*-ox plants (T. Hytönen, personal communication).

FvCO RNAi-silenced lines were insensitive to photoperiod. They formed more runners in both photoperiods and only a few branch crowns, flowered significantly later especially in LDs, and produced fewer inflorescences as compared to H4 (II), similar to the previously studied *FvFT1* RNAi lines (Koskela *et al.*, 2012; Rantanen *et al.*, 2014).

Our results (II) coincided with previous results and showed that silencing either *FvCO* or *FvFT1* delayed flowering, while overexpressing either *FvCO* or *FvFT1* (Rantanen *et al.*, 2014) advanced flowering suggesting that *FvCO* regulates *FvFT1*. Gene expression analysis of *FvCO* transgenic lines confirmed that *FvCO* activates *FvFT1* especially under LD conditions, and previous research showed that the highest *FvFT1* mRNA level was observed at intermediate temperature at 16 °C as compared to 13 and 23 °C (Rantanen *et al.*, 2015). The expression levels of the genes downstream

of *FvFT1* such as *FvSOC1* and *FvAP1* correlated with the flowering data. In comparison to the H4, *FvCO*-OX lines showed an upregulation in the *FvSOC1* and *FvAP1* expression, while the RNAi lines had reduced expression of *FvSOC1* and untraceable amounts of *FvAP1* mRNA under both photoperiods.

In H4, *FvSOC1* mRNA level negatively correlated with runner production unlike in the SD accession (I). Therefore, it is proposed that in H4, AXM differentiation is developmentally regulated through floral induction and *FvSOC1* has a minor role in the process. Upon floral transition the uppermost AXMs differentiate into new branch crowns, providing new meristems for inflorescence formation and hence, indirectly reduces runner formation (Hytönen *et al.*, 2004). However, *FvFA20ox4* that is activated by *FvSOC1,* at least in the leaves, is also needed for runner formation in perpetual flowering accessions of the woodland strawberry (Mouhu *et al.*, 2013; Tenreira *et al.*, 2017). Recent studies have reported that due to a deletion in the active site of *FvFA20ox4*, a gibberellin (GA) 20-oxidase (GA20ox) encoding gene, results in a runner-less phenotype. The mutation inhibits the production of runners and makes the AXM either dormant or form branch crowns. Another possibility is that similar to the SD accession FIN56, high temperature (22 °C) that was used in the experiments could have prevented flowering and promoted runner formation in H4 in SDs independently of *FvSOC1*. Therefore, further studies at a lower temperature is required to understand the photoperiodic regulation of the balance between vegetative and generative development in H4.

4.2.3 The photoperioidic rhythm (II)

In *Arabidopsis*, the external coincidence model suggests that *CO* expression peaks during the evening and under LDs this peak coincides with and external factor; i.e. light, resulting in the

activation of FT (Suárez-López *et al.*, 2001). To understand the photoperiodic control of *FvCO* and *FvFT1*, their expression patterns were studied in a 24 hr period. *FvCO* showed a single peak at dawn under both photoperiods in both the perpetual flowering LD (H4) and the seasonal flowering SD (FIN56) accessions. However, two peaks were observed in the *FvFT1* expression (Koskela *et al.*, 2012) and only the morning peak coincided with *FvCO* expression.

In the *FvCO*-OX lines under LDs, the diurnal expression of *FvFT1* was disrupted and the expression of *FvFT1* was always higher than that of H4 WT from 0 to 16 hrs after dawn while under SDs, *FvFT1* mRNA peaked twice at 4 and 12 hrs after dawn. The *FvCO* RNAi plants had low or undetectable *FvFT1* expression levels throughout the cycle. This shows that although *FvFT1* rhythm does not overlap with that of *FvCO*, functional *FvCO* is needed to activate *FvFT1* at both time points. Although the second *FvFT1* peak was slightly higher than the first in H4; however, the first peak was slightly higher in FIN56, the rhythm was similar in both accessions.

Previous studies demonstrated that the height of the peaks in strawberry is dependent on the light conditions (Rantanen *et al.*, 2014). Thus, darkness experiments were conducted that revealed that the *FvCO* peaks in darkness and that the light regulates its expression. Interestingly, the downregulation of *FvCO* by light at dawn is similar to that shown in the SD plant *Chenopodium rubrum* (Drabešová *et al.*, 2014). However, it was also observed that *FvCO* peak is at a different phase than the *CO* peak in *Arabidopsis,* and the dawn phase suggests similarities to other Group 1a COL genes showing a convergent evolutionary model. However, more research is needed to make any definite conclusions.

4.3 Environmental influence on the fate of meristems is an interplay between several QTL (III)

Exploring out-of-season production is an economically important topic for plant breeders. Previous mapping studies have concentrated on perpetual flowering varieties in both the octoploid, *Fragaria×ananassa Duch* and the diploid, F. vesca (Weebadde *et al.*, 2008; Iwata *et al.*, 2012; Koskela *et al.*, 2012; Gaston *et al.*, 2013; Castro *et al.*, 2015; Perrotte *et al.*, 2016); however, early flowering in strawberry has not been given much attention. One of the ways to investigate unexplored areas on genomes, is through genetic mapping (Ehrenreich *et al.*, 2009; Fan *et al.*, 2010; Sadok *et al.*, 2013; Zhang *et al.*, 2013; Bielenberg *et al.*, 2015), and recent development in molecular markers and bioinformatics tools has enabled efficient fine mapping (Bassil *et al.*, 2015; Mahoney *et al.*, 2016).

After successfully finding and functionally characterizing *FvTFL1* in an F2 cross between the perpetual flowering accession, *F. vesca f. semperflorens* 'Hawaii-4' (H4) and the seasonal flowering accession *F. vesca* subsp. *vesca* (FV) (denoted H4×FV) (Koskela *et al.*, 2012), the same mapping population was further analyzed to search for QTLs influencing flowering time and axillary bud differentiation. However, only plants which possessed at least one allele from the FV parent at the *FvTFL1* locus were included because homozygous for "H4" at that locus produced perpetual flowering phenotypes. SSR and SNP markers were developed and used for initial mapping. The map was then saturated by genotyping by sequencing (GBS)-derived SNP markers and the resultant map proved reliable when compared with the physical positions of markers from *Fvb* genome assembly (Tennessen *et al.*, 2014).

To show that the QTLs found were robust, data of selected individuals was analyzed under three different environments: a field experiment, a greenhouse experiment, in which the plants were

induced to flower in the field followed by phenotypic observations in the greenhouse and a growth chamber experiment. Based on the observations from the greenhouse experiment, a subset of 16 extremely early and 16 extremely late lines were selected for the growth chamber experiment. Several overlapping additive QTLs on linkage groups (LG), 4, 6 and 7 were found for flowering time in woodland strawberry. However, the field experiment only revealed a QTL on LG4. The QTL on LG6 mapped to the *FvTFL1* region (Koskela *et al.*, 2012) while one QTL was mapped close to the previously identified *PFRU* locus on LG4 (Gaston *et al.*, 2013; Castro *et al.*, 2015; Honjo *et al.*, 2016).

Based on the QTL on LG4 that was found close to the bx083 maker in both the greenhouse and field experiments and has previously been identified as one of the markers close to the *PFRU* locus (Perrotte *et al.*, 2016), we propose another role for *PFRU* in controlling early flowering trait in woodland strawberry. However, the other QTL upstream of *PFRU* on LG4 was observed only in specific environments.

The study discovered previously undocumented QTLs located near the marker BFaCT044 on LG7 linked to flowering, which is probably environmentally controlled as they were only detected in the greenhouse and growth chamber experiments but not the field. Alleles at the QTLs on LG4 and LG7 were analyzed and showed additive effects on flowering time and revealed that the "H4" was dominant and even a single allele from that parent delayed flowering response while homozygous "FV" alleles at *FvTFL1* also caused delayed flowering phenotype.

Previous studies have proposed two candidate genes, *FvFT2* and *FvCDF2*, for the *PFRU* locus, which are regulators of photoperiodic flowering (Perrotte *et al.*, 2016). CDFs in *Arabidopsis* are known to repress *CO* and *FT* during the morning (Song *et al.*, 2012). However, in *Fragaria*, *FvFT2* is mostly expressed in flowers and fruits (Kang *et al.*, 2013).

A candidate for the QTL on LG7 was identified as *FvELF6*, which in *Arabidopsis* has an epigenetic function of regulating chromatin through histone demethylation (Jiang *et al.*, 2007; Jeong *et al.*, 2009). This candidate seems plausible since chromatin regulation through methylation is a known function in flowering control (Jeong *et al.*, 2015) and a QTL on LG2 that co-localizes with ELF6 in almond has been shown to be related to heat requirement for flowering (Sánchez-Pérez *et al.*, 2012).

TFL1/CEN has been shown to act as a floral repressors in many species (Fan, 2010; Romeu *et al.*, 2014) including *Fragaria* (Koskela *et al.*, 2012). In this study two QTLs have been linked to that gene class, one on LG6, which has been functionally validated by Koskela *et al*. (2012) and another peak on LG7 which co-locolized with *FvCEN1*.

In addition to that, two QTLs on LG4 and LG5 indirectly affected the yield by controlling branch crown and runner production. The QTL on LG4 co-localized with the previously published *PFRU* locus (Gaston *et al.*, 2013) and was downstream of the flowering time QTL from this study. It can be hypothesized that the two QTLs on LG4 may control AXM differentiation into runners or branch crowns and flowering. However, further research is needed to derive conclusions.

A *TCP* transcription factor, *FvTCP7* (Wei *et al.*, 2016) was located close to the LG4 marker and identified as a potential candidate. Previous research has revealed that *FvTCP7* is similar to the *Arabidopsis TCP14* and *TCP15*, which regulate cell proliferation in young internodes, developing leaves and floral tissues and the mechanism varies depending on the tissue (Kieffer *et al.*, 2011). This is an interesting candidate considering that runners and branch crowns are shoots with long or short internodes, respectively, and recent research in strawberry showed that it is highly expressed in vegetative tissues such as runners, flower buds and matured flowers (Wei *et al.*, 2016).

Two homologs of DAM and SVP that function as floral repressors in *Arabidopsis* and are associated with dormancy in the Rosaceae family (Hartmann *et al.*, 2000; Bielenberg *et al.*, 2008; Fan *et al.*, 2010; Sánchez-Pérez *et al.*, 2012) were identified near the LG5 QTL. Strawberries do not undergo true dormancy, but under SDs in autumn runner formation is reduced and chilling is required to recommence development (Heide *et al.*, 2013).

Further studies are needed to functionally characterize the candidate genes presented in this study and to reveal their roles in the control of flowering and axillary meristem differentiation. Especially the analysis on the role of *FvDAM* , *FvTCP7* and PFRU in AXM differentiation and their connections to the *FvSOC1* and GA pathway (I) (Hytönen *et al.*, 2009; Mouhu *et al.*, 2013) would increase the understanding of the pathways involved in the control of AXM differentiation and flowering.

5 CONCLUSIONS

Strawberries can reproduce both sexually and asexually through runners. Many studies have explored the genetic and molecular control of flowering in the woodland strawberry (Brown and Wareing, 1965; Albani *et al.*, 2004; Sønsteby and Heide, 2008; Koskela *et al.*, 2012; Mouhu *et al.*, 2013; Rantanen *et al.*, 2014, 2015), but less is known about the control of vegetative development (Smeets and Kronenberg, 1955; Smeets, 1982; Mouhu *et al.*, 2013; Caruana *et al.*, 2017; Tenreira *et al.*, 2017).

The role of temperature and photoperiod in the fate of AXM is summarized in Figure 5. As observed previously in the control of flowering, this study found that temperature overrides photoperiod in the control AXM fate. Cool temperature or SDs enhanced branch crown formation and induced flowering, while at warm temperature and LDs, AXMs differentiated into runners and SAM remained vegetative irrespective of photoperiod. (Heide and Sønsteby, 2007; Rantanen *et al.*, 2015). However, using plants with high *FvTFL1* expression levels, we found that the environmental regulation of AXM fate is at least partially independent of the SAM fate.

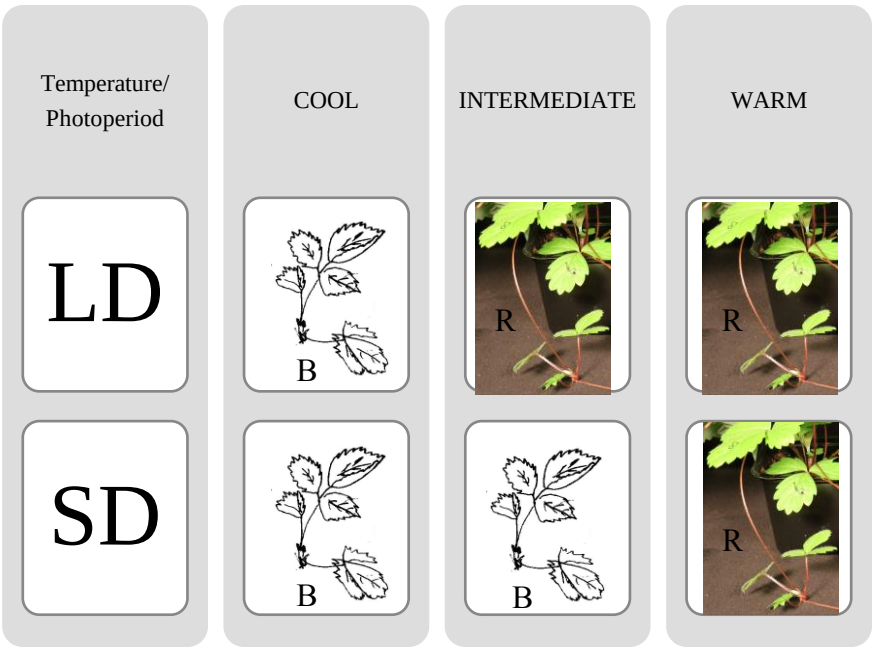

Figure 5 Interaction of photoperiod and temperature in the control of AXM differentiation. The "B" represents an increase in branch crown formation while the "R" represents an increase in runner formation at each temperature and photoperiod.

Although detailed molecular studies are needed to make firm conclusions, we propose an updated hypothetical model for the control of AXM fate in seasonal flowering woodland strawberry (Figure 6). In LD conditions, *FvCO-FvFT1* pathway is activated, and FvFT1 upregulates *FvSOC1* that has a quantitative effect on AXM fate. FvSOC1 promotes AXM to differentiate into runners by activating *FvGA20ox4* which leads to the accumulation of bioactive GA_1 and the degradation of FvRGA1 DELLA proteins. In parallel, FvSOC1 also activates *FvTFL1* to repress flowering. Under SDs, however, the photoperiodic pathway is downregulated, which changes the fates of SAM and AXM. Temperature controls flowering independently of the photoperiodic pathway by affecting the expression of *FvTFL1*, but the mechanism mediating the temperature regulation of AXM fate is unknown. Using QTL mapping, we found several QTLs related to early flowering and branch

crown formation and identified new candidate genes. Putative roles of these candidate genes in the environmental regulation of AXM and SAM fates require functional studies. Detailed knowledge on the molecular control of AXM and SAM fates is crucial for breeders to be able to develop high-yielding cultivars that can also be propagated vegetatively though runners.

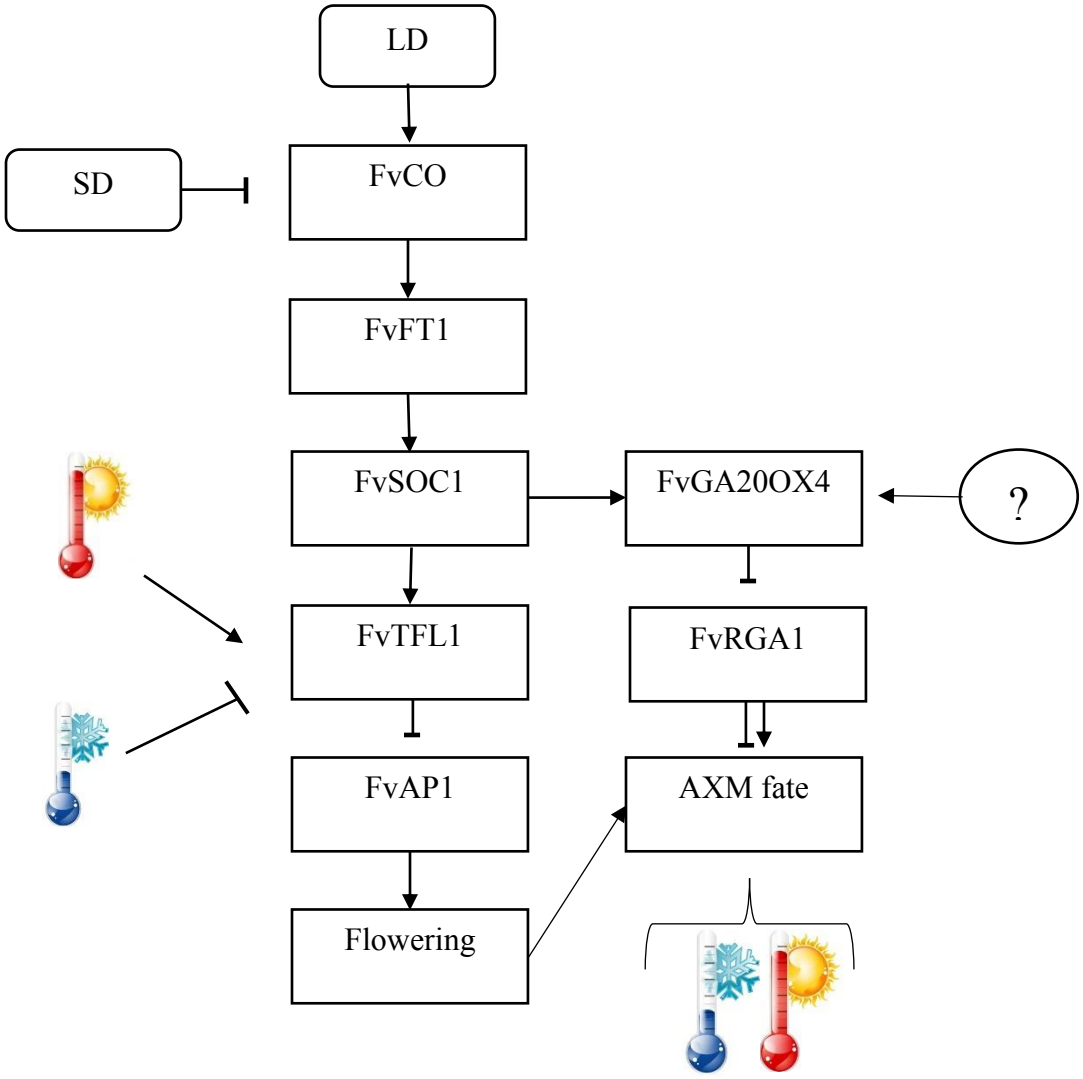

Figure 6 Model illustrating the environmental regulation of flowering and runner formation in strawberry. Arrows indicate activation and line indicate repression.

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Regulation of axillary bud fate in woodland strawberry

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Abstract

Strawberry forms terminal inflorescences from shoot apical meristems (SAM), while axillary meristems (AXM) can differentiate to either stolons or axillary leaf rosettes called runners and crown branches, respectively. Runners are important for the clonal propagation of the plant, while the number of branch crowns affects the maximum number of inflorescences per plant and, consequently, potential yield. The aim of this study was to use available transgenic lines of woodland strawberry to understand AXM differentiation under different photoperiods and temperatures. Our results were in line with previous studies showing that temperature overrides the effect of photoperiod on AXM differentiation in the seasonal flowering accession. Photoperiodic and temperature regulation of AXM fate persisted in non-flowering plants with high expression level of *Fv TERMINAL FLOWERING1 (FvTFL1)* that encodes a floral repressor. This finding suggested that AXM and SAM fates are not always connected, and that FvTFL1 does not directly control the fate of the AXM. Moreover, the *Fragaria* homolog of *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (FvSOC1*) had a quantitative effect in enhancing runner production likely through the gibberellin (GA) biosynthesis pathway. Taken together, SAM and AXM fates can be separately controlled, which provides means to affect the balance between flowering and vegetative reproduction.

Introduction

The woodland strawberry, *Fragaria vesca* L. is a perennial plant that is closely related to the economically important garden strawberry (*F.* × *ananassa* Duch.). Since the woodland strawberry is a diploid species that is easy to genetically manipulate, it is used as a model for the octoploid garden strawberry (Gil-Ariza *et al.*, 2006; Shulaev *et al.*, 2011). In addition, the woodland strawberry has many phenotypically and genotypically diverse accessions that are useful for studying the genetic basis of economically-important traits (Shulaev *et al.*, 2011).

Strawberries are perennial rosette plants with short internodes and trifoliate leaves. During vegetative stage, the shoot apical meristem (SAM) of the crown (the stem of the leaf rosette) forms internodes and one leaf and axillary bud at each node. The bud can remain dormant or depending on the conditions, form either branch crown (the stem of the axillary leaf rosette) or stolon called runner. Runners are long vegetative shoots with two long internodes terminating at a daughter plant. A second axillary bud of the runner continues runner elongation while the first axillary bud may produce another runner but may also remain dormant. Strawberry forms determinate inflorescences from the SAM of the main crown and the branch crowns (Hytönen, 2009). Since the number of inflorescences depends on the number of branch crowns, there is a strong trade-off between flowering and runner formation.

Extensive number of physiological studies have shown that photoperiod and/or temperature regulate flowering and runner formation in strawberries (Reviewed by Heide *et al.*, 2013). Typically, seasonal flowering genotypes require short days (SDs) to induce flowering at intermediate temperature range of about 14–20°C. At cool temperatures of 9–13°C, however, flower induction occurs irrespective of the photoperiod, whereas temperatures above 20°C prevent

flower induction. The woodland strawberry exhibits a strict temperature limit for flower induction (Heide and Sønsteby, 2007; Rantanen *et al.*, 2015), whereas in the garden strawberry, temperature thresholds vary between cultivars with some northern cultivars having a lower threshold than others (Hartmann, 1947; Heide, 1977; Durner, 1984; Stewart and Folta, 2010).

In 1965, Brown and Wareing demonstrated that two separate genetic loci were responsible for seasonal flowering and runner formation, *SEASONAL FLOWERING LOCUS* (*SFL*) and *RUNNERING LOCUS* (*RL* or *R*), respectively, and mutations in these loci lead to perpetual flowering and runnerless phenotypes (Brown and Wareing, 1965; Albani *et al.*, 2004). The *SFL* locus was found to co-localize with the strawberry homolog of the gene encoding a floral inhibitor, *TERMINAL FLOWER1* (*FvTFL1*) (Koskela *et al.*, 2012), while *R* was mapped onto linkage group II (Sargent *et al.*, 2004). Recent fine mapping revealed a gene encoding a gibberellin 20-oxidase (*FvGA20ox4*) as a candidate gene for *R*. This gene was found to be expressed in axillary meristems, and it was shown that the gene is mutated in runnerless genotypes, leading to a disruption at the active site of the enzyme (Tenreira *et al.*, 2017).

Studies in the annual model Arabidopsis have revealed several genetic pathways that regulate generative development. The floral integrators, FLOWERING LOCUS T (FT) belonging to the phosphatidylethanolamine-binding protein (PEBP) family and the MADS box transcription factor SUPPRESSOR OF THE OVEREXPRESSION OF CONSTANS1 (SOC1) converge different external and internal signals to activate a group of genes known as floral identity genes including *APETALA1* (*AP1*) and *LEAFY* (Koornneef *et al.*, 1991; Weigel *et al.*, 1992; Mandel and Yanofsky, 1995; Simon *et al.*, 1996; Samach *et al.*, 2000). Both photoperiod and temperature regulate the expression of *FT* in leaves, and FT protein moves to the SAM to induce flowering. Another PEBP,

TFL1, plays an opposite role as a floral repressor specifically affecting the floral identity genes (Mandel and Yanofsky, 1995; Liljegren *et al.*, 1999; Kaufmann *et al.*, 2010).

Similar to Arabidopsis, woodland strawberry homologs of FT and SOC1 promote flowering in perpetual flowering genotypes in long days (LDs) (Koskela *et al.*, 2012; Mouhu *et al.*, 2013; Rantanen *et al.*, 2014). Seasonal flowering genotypes, however, show opposite photoperiodic response because FvSOC1, activated by leaf-expressed FvFT1, upregulates the floral repressor *FvTFL1* in the SAM (Koskela *et al.*, 2012; Mouhu *et al.*, 2013). Flower induction occurs in SDs after the silencing of these genes (Koskela *et al.*, 2012; Mouhu *et al.*, 2013; Rantanen *et al.*, 2015). Cool temperatures below 13°C also induce flowering by silencing *FvTFL1* independently of the photoperiod, whereas temperatures above 20°C prevent flower induction by activating *FvTFL1* through an unknown mechanism (Rantanen *et al.*, 2015). Mouhu *et al*. (2013) showed that FvSOC1 affects also the expression of several GA pathway genes including *FvGA20ox4* and mediates the photoperiodic signal to control the differentiation of the axillary meristems (AXM) into runners or branch crowns. FvTFL1, however, does not have direct effect on the photoperiodic control of AXM differentiation (Koskela *et al.*, 2012). Although knowledge on the photoperiodic control of AXM differentiation is emerging, not much is known about the role of temperature. Therefore, the objective of this study was to investigate the regulation AXM differentiation to runners and branch crowns under different environmental conditions using available transgenic lines of woodland strawberry.

Materials and Method

Plant material

Experiments were conducted using SD accession of *Fragaria vesca* (PI551792; National Clonal Germplasm Repository, Corvallis, OR; called FIN56 hereafter) and previously studied transgenic genotypes in the FIN56 background overexpressing *FvSOC1* or *FvTFL1* under the *cauliflower mosaic virus 35S* promoter (summarized in Supplement Table S1). In addition, a single *FvSOC1*-RNAi was studied. Supplemental table S1The experimental material also included NOR1, a Norwegian vernalisation-requiring *F. vesca* accession (Heide and Sønsteby, 2007; Koskela *et al.*, 2017). Runner cuttings of FIN56, NOR1 and the transgenic genotypes were propagated from vegetative mother plants grown under LD conditions. The runner cuttings were potted and grown under LD conditions in the greenhouse for three weeks before moving to the growth room (LDs, 22□C), two weeks prior to the start of the treatments. Old leaves and runners were removed and the plants were given fertilizer (Kekkilä Oy, Vantaa, Finland) every week.

Growth conditions

All temperature experiments were conducted under both SD (12 hours of light) and LD (18 hours) conditions. The temperature treatments were split into two experiments (Exp-1 and Exp-2) in growth chambers equipped with light-emitting diodes (LEDs; AP67, Valoya, Finland) with a light intensity was 200 $\mu mol^{-2}s^{-1}$. Exp-1 consisted of two temperatures, cool (daytime: 12℃ and night: 10℃) and warm temperature (daytime: 22℃ and night: 20℃) and was conducted during the winter season 2016–2017. Exp-2 was conducted at intermediate temperature (daytime: 18℃ and night: 16℃) and was done during spring 2017. Plants were kept in the respective conditions for five weeks, after which all the plants were moved to greenhouse (LDs, 20℃) for further observations.

These plants were illuminated with high pressure sodium (HPS) lamps (Airam 400W, Kerava, Finland) at a PPFD of 120 $\mu mol^{-2}s^{-1}$ to supplement natural light for 18 h daily.

Phenotypic observations

Fates of leaf axillary meristems (AXMs) of the main crown were recorded every two weeks as dormant, producing a branch crown or a runner. AXM fates were observed for a total of nine weeks in Exp-1 and eight weeks in Exp-2. Flowering was recorded as presence of a visible terminal inflorescence.

Statistical analyses

Exp-1 was carried out as a 2 * 2 * 8 factorial design with photoperiod (SD and LD), temperature (cool and warm) and genotype (eight genotypes listed in Table S1) as factors. Exp-2 was carried out as a 2 * 7 factorial design with photoperiod (SD and LD) and genotype as factors. All statistical analyses were done using R (R Core Team, 2016). Response variables were the proportion of AXMs producing branch crowns, the proportion of AXMs producing runners and the proportion of flowering plants. Proportion data on branch crowns and runners was analyzed by fitting a generalized linear model with binomial regression and logit link, with photoperiod, temperature and genotype as main effects and also considering their interactions.

Substantial collinearity was found between the variables, and this was found to inflate standard errors, leading to very high p-values and increased type II error risk especially for interaction terms in the binomial regression models. Therefore, only the main effects (photoperiod, temperature and genotype) from the complete models are reported. Data were separated by temperature and pairwise differences between proportions (AXMs with runners or branch crowns out of the total number of AXMs) were inspected by pairwise.prop.test function in the R stats package using Holm

correction to adjust for multiple testing. All the statistical analyses were done using R version 3.0.2 with the Rstudio interface version 1.1.442 (RStudio Core Team, 2016).

Results

Strawberries exhibit a strong trade-off between flowering and vegetative reproduction through runners. To understand this trade-off, we studied environmental and genetic control of flowering and AXM differentiation to runners and branch crowns in woodland strawberry accessions FIN56 and NOR1 that has contrasting seasonal growth cycles and in several transgenic lines.

Environmental control of vegetative and generative growth in FIN56

SDs and/or cool temperatures typically induce flowering and enhance branch crown formation, while LDs and warm temperatures promote runner formation in the natural accessions of the woodland strawberry (Heide and Sønsteby, 2007; Rantanen *et al.*, 2015), as well as in cultivars of garden strawberry (Konsin *et al.*, 2001; Bradford *et al.*, 2010). We first explored the effect of photoperiod and temperature on flowering and AXM fate in FIN56, a Finnish woodland strawberry accession that has been used as a model for studying flowering behavior in strawberries (e.g. Koskela *et al*., 2012; Mouhu *et al*., 2013; Rantanen *et al*., 2015).

Results from our current experiments were in line with the earlier findings. At intermediate temperature, flowering and AXM fate in FIN56 were photoperiodically regulated; LD conditions favored runner production, while SDs promoted branch crown formation and flowering (Table 1 and Figure 1). At warm temperature, a high percentage of AXMs produced runners in both photoperiods and flowering was not observed (Figure 2). At cool temperature, the opposite was

observed as flowering and branch crown formation were promoted and runner production reduced irrespective of photoperiod (Figure 3).

Photoperiod and temperature have direct effects on AXM fate.

Flowering is usually associated with an increase in branch crown formation, and this was the case also in FIN56 in our study. In order to explore the direct effect of photoperiod and temperature on AXM fate, we studied genotypes with higher-than-normal *FvTFL1* expression including NOR1 and *FvTFL1* overexpressing lines, in which flowering is inhibited or strongly delayed under a range of environments (Koskela *et al.*, 2012, 2017; Rantanen *et al.*, 2015). In the current experiment at intermediate temperature, none of the genotypes with higher-than-normal *FvTFL1* expression flowered (Table 1). However, in terms of AXM differentiation, all the lines responded to photoperiod similarly to the wild type FIN56, with SDs promoting branch crown formation and LDs enhancing runner production (Tables S5 and S6). In most cases, higher-than-normal *FvTFL1* expression did not affect AXM fate compared with FIN56, although the line *FvTFL1*-OX2 produced slightly more runners and fewer branch crowns than the wild type FIN56 in LDs.

Similarly, FIN56 at cool temperature promoted branch crown formation, while warm temperature enhanced runner production in NOR1 and *FvTFL1*-OX lines, and in both cases, photoperiod had no additional effect (Figures 2 and 3, Tables S3 and S4). However, the genotypes with higher-than-normal *FvTFL1* expression level had a tendency of producing more runners and less branch crowns than FIN56 at both cool and warm temperature, and in many cases these differences were also statistically significant (Figures 2 and 3, Tables S7–S10). No flowering was observed in *FvTFL1*-OX2 line, and only a few NOR1 plants flowered at cool temperature in LDs, whereas almost half of the *FvTFL1*-OX4 plants flowered at cool temperature in both photoperiods (Table 1). It is

187 notable, that photoperiod and temperature regulated the AXM fate in NOR1 and *FvTFL1*-OX2
188 plants without changes in SAM fate (Table 1; Figures 1 – 3), demonstrating that branch crown
189 formation is not always a direct result of floral initiation, but is promoted by the same
190 environmental conditions as floral initiation.

191 The role of FvSOC1 in determination of axillary bud fate

192 The role of FvSOC1 has been previously studied by Mouhu *et al*. (2013), who showed that the
193 overexpression of *FvSOC1* leads to enhanced runner production, reduced branch crown formation,
194 floral inhibition and insensitivity to photoperiod, whereas the silencing of *FvSOC1* has opposite
195 effect on flowering and AXM fate. Since these studies were carried out only at intermediate
196 temperature (18°C), we performed a more detailed analysis on the environmental responses of
197 *FvSOC1* transgenic lines.

198 Our results at intermediate temperature corroborate earlier findings. In the current experiment,
199 overexpression of *FvSOC1* at intermediate temperature inhibited flowering, promoted runner
200 production and nearly abolished branch crown formation in both SDs and LDs (Figure 4). Warm
201 temperature enhanced the effect of *FvSOC1* overexpression, as the formation of branch crowns
202 was completely inhibited with almost all AXMs developing into runners (Figure 5). At cool
203 temperature, the effect of *FvSOC1* overexpression on AXM fate and flowering was less
204 pronounced, and there were notable genotype-dependent differences. The strongest *FvSOC1*
205 overexpressing genotype (FvSOC1-OX12) produced only runners and did not flower. In contrast,
206 the milder genotypes had up to 50% of AXMs developing into branch crowns (Figure 6).
207 Moreover, cool temperature was partially able to override the inhibitive effect of *FvSOC1*

overexpression on flowering in the milder overexpression genotypes, resulting in a small percentage of flowering plants (Table 1).

We also studied FvSOC1-RNAi line at cool and warm temperatures in both SD and LD. Silencing *FvSOC1* did not change flowering characteristics nor the patterns of vegetative development as compared to the wild type FIN56 in these conditions (Table 1, Figures 5 & 6; Figure S2).

Discussion

Strawberries can reproduce both generatively and vegetatively through runners. Since AXMs can differentiate to either runners or branch crowns that are able to bear flowers, AXM differentiation has a major role in controlling the balance between flowering and runnering (Hytönen *et al.*, 2004; Mouhu *et al.*, 2013; Tenreira *et al.*, 2017). Growers want to control this balance in both fruit production and clonal propagation of plant materials, and therefore, detailed knowledge on the control of SAM and AXM fates are needed. In both the garden strawberry (Darrow, 1936; Hartmann, 1947; Guttridge, 1960; Pure *et al.*, 1973; Durner and Poling, 1985; Le Miere *et al.*, 1998; Sønsteby and Heide, 2007; Bradford *et al.*, 2010) and the woodland strawberry (Heide and Sønsteby, 2007; Sønsteby and Heide, 2008; Koskela *et al.*, 2012, 2017; Mouhu *et al.*, 2013; Rantanen *et al.*, 2014, 2015), temperature and photoperiod are known to control both SAM and AXM fates, but floral initiation also affects AXM differentiation. Here, we report detailed analysis on the photoperiodic and temperature regulation of AXM/SAM fates in woodland strawberry and demonstrate that these environmental signals can control AXM fate independently of SAM state.

Environmental control of SAM and AXM fates are tightly connected in strawberry

Previous studies in woodland strawberry flowering responses to different temperatures and photoperiods showed that seasonal flowering accessions required SDs for flower induction at intermediate temperatures between 13–20°C. At lower temperatures, flower induction occurred independently of photoperiod, whereas higher temperatures inhibited flower induction (Heide and Sønsteby, 2007; Rantanen *et al.*, 2015). In this study, we found that temperature had a strong effect on AXM differentiation in seasonal flowering accession FIN56, with cool temperature promoting branch crown formation and warm temperature enhancing runnering independently of photoperiod. Photoperiod, however, had an effect at intermediate temperature, where SD increased the number of branch crowns compared with LDs, as previously reported by Mouhu *et al*. (2013). Notably, a high percentage of AXMs differentiated to branch crowns instead of runners in flower-inductive conditions. This indicated that SAM and AXM fates were tightly connected in FIN56 at different environmental conditions. Previous studies showed similar environmental responses in SAM and AXM fates also in cultivated strawberry, but temperature limits of the photoperiodic response varied between cultivars (Heide, 1977; Konsin *et al.*, 2001). This tight connection between SAM and AXM fates has been hindering studies on direct environmental effects on AXM fate.

Independent control of AXM and SAM fates

Independent genetic control of SAM and AXM fates was shown as early as 1960's, when Brown and Wareing (1965) reported the crosses between wild type and runnerless perpetual flowering

mutants and showed that different recessive mutations caused these traits. Recent results showed that runnerless genotypes contain a 9-bp deletion in *FvGA20ox4* gene that affects the active site of the encoded enzyme (Tenreira *et al.*, 2017) and a 2-bp deletion in *FvTFL1* was confirmed as a causal mutation for perpetual flowering using transgenic lines (Koskela *et al.*, 2012). Further analysis in *FvTFL1*-OX lines also showed that both runner and branch crown formation were similar to wild type FIN56 at intermediate temperature (Koskela *et al.*, 2012). Our findings suggested a separate environmental control of SAM and AXM fates in woodland strawberry.

Since *FvTFL1-OX* lines remained vegetative in the conditions used in our study (Rantanen *et al.*, 2015), we utilized them to explore direct effect of different photoperiods and temperatures on AXM fate. In addition, we studied NOR1, an arctic accession that required long period of vernalization to become competent to flower (Heide and Sønsteby, 2007; Koskela *et al.*, 2017). We found that, although almost all NOR1 and *FvTFL1*-OX2 plants remained vegetative in all treatments, cool temperature and SDs at intermediate temperature enhanced crown branch formation in these genotypes, whereas LDs and high temperature favored runner production. These results demonstrated that, although SAM fate has been suggested to affect AXM fate directly in strawberries (Costes *et al.*, 2014), cool temperature or SDs at intermediate temperature can trigger branch crown formation also independently of the SAM fate in woodland strawberry.

Genetic control of AXM fate

The function of FvSOC1 at least partially explains the tight connection between SAM and AXM fates. In LDs, FvSOC1 represses flower induction by activating *FvTFL1* and promotes runner formation by affecting the expression of GA biosynthetic and/or signaling genes (Mouhu *et al.*,

2013). Previous results, however, suggested that FvSOC1 does not have a major role in the temperature regulation of flowering (Rantanen *et al.*, 2015).

We attempted to test a previously unknown role of FvSOC1 in the temperature regulation of AXM differentiation using transgenic lines. Overexpression of *FvSOC1* strongly promoted runner formation in all temperature-photoperiod conditions tested, and only the weakest line produced a relatively high percentage of branch crowns at cool temperature (Figures 4–6), indicating that *FvSOC1* has a quantitative effect on AXM differentiation, and that certain threshold of *FvSOC1* expression is needed to completely prevent branch crown formation. Mouhu *et al*. (2013) showed that the effect of FvSOC1 on AXM differentiation likely depends on GA, and recent molecular studies confirmed the major role of GA in the control of AXM fate by showing that the mutation in *FvGA20ox4* leads to runnerless phenotype (Tenreira *et al.*, 2017), and that a single DELLA protein, FveRGA1, mediates the effect of GA to control AXM fate (Caruana *et al.*, 2017; Li *et al.*, 2017). Interestingly, FvSOC1 was shown to activate the expression of *FvGA20ox4* in leaves at intermediate temperatures (Mouhu *et al.*, 2013), indicating that FvSOC1 may control AXM fate through GA biosynthetic pathway in these conditions. Further gene expression analyses on *FvSOC1* transgenic lines are needed to confirm the regulation of *FvGA20ox4* by FvSOC1 in AXM in different conditions.

RNAi silencing of *FvSOC1*, in contrast, affected neither AXM nor SAM fate at cool or high temperatures compared to wild type FIN56 (Figures 5 and 6). Strikingly, most AXMs differentiated to runners at high temperature in RNAi plants, although, according to Mouhu *et al*. (2013), runner formation was strongly reduced in RNAi lines at intermediate temperature in both SD and LD. In parallel, RNAi silencing of *FvSOC1* led to photoperiod-independent flower induction at intermediate temperature, but no flowering was observed at 22°C because of strong activation of

293 *FvTFL1* by an unknown molecular mechanism (Mouhu *et al.*, 2013; Rantanen *et al.*, 2015). We
294 hypothesize that this mechanism could also activate runner formation directly through GA pathway
295 or indirectly by preventing flowering, but further studies are needed to test this idea.

296 Although molecular mechanism mediating the temperature regulation of AXM fate remains an
297 enigma, the photoperiodic controlling mechanisms are emerging. Studies in perpetual flowering
298 woodland strawberry genotype H4 have shown that *FvCO* and *FvFT1* are involved in the
299 photoperiodic control of SAM and AXM fates (Koskela *et al.*, 2012; Mouhu *et al.*, 2013; Kurokura
300 *et al.*, 2017), which is in line with previous studies that have shown the role of CO-FT module as
301 a general photoperiod-sensing system that controls, for example, flowering time, bud set in trees
302 and tuberization in potato (Böhlenius *et al.*, 2006; Hsu *et al.*, 2006; Corbesier *et al.*, 2007; Navarro
303 *et al.*, 2011).

304 Taken together, cool and high temperatures override the effect of photoperiod in the control of
305 AXM fate in seasonal flowering woodland strawberry, but the molecular control of these
306 temperature responses remain largely unknown. However, we propose following hypothetical
307 model of the photoperiodic control of AXM fate for further studies. *FvSOC1*, which is a key player
308 in the photoperiodic control of AXM fate (Mouhu *et al.*, 2013), is activated in AXM in LD
309 conditions by the leaf-expressed photoperiodic pathway that include *FvCO* and *FvFT1*. FvSOC1
310 in turn activates the expression of *FvGA20ox4* that may encode a rate-limiting enzyme in the GA
311 biosynthetic pathway, and high GA_1 level in AXM triggers the degradation of FveRGA1 DELLA
312 proteins to induce runner formation. In SDs, however, the pathway upstream of FveRGA1 is
313 downregulated leading to branch crown formation.

314

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Figures and tables

Table 1 Percentage of plants in which inflorescence were visible before the cut-off point at week 9 per treatment. Plants (n = 9–19) were grown under cool (daytime: 11ºC and night: 10°C), intermediate (18/16ºC) or warm (22/20°C) temperatures in 16-h long days (LD) or 12-h SDs.

	Cool		Intermediate
	LD	SD	LD
FIN56	80.0	71.4	20.0
FvTFL1-OX2	0.0	0.0	0.0
FvTFL1-OX4	46.7	43.8	0.0
NOR1	14.3	0.0	0.0
FvSOC1-OX7	7.1	0	0.0
FvSOC1-OX11	12.5	6.3	0.0
FvSOC1-OX12	0.0	0.0	0.0
FvSOC1-RNAi3	72.7	70.0	n/a

n/a – not analyzed; warm temperature data is not shown as none of the plants flowered

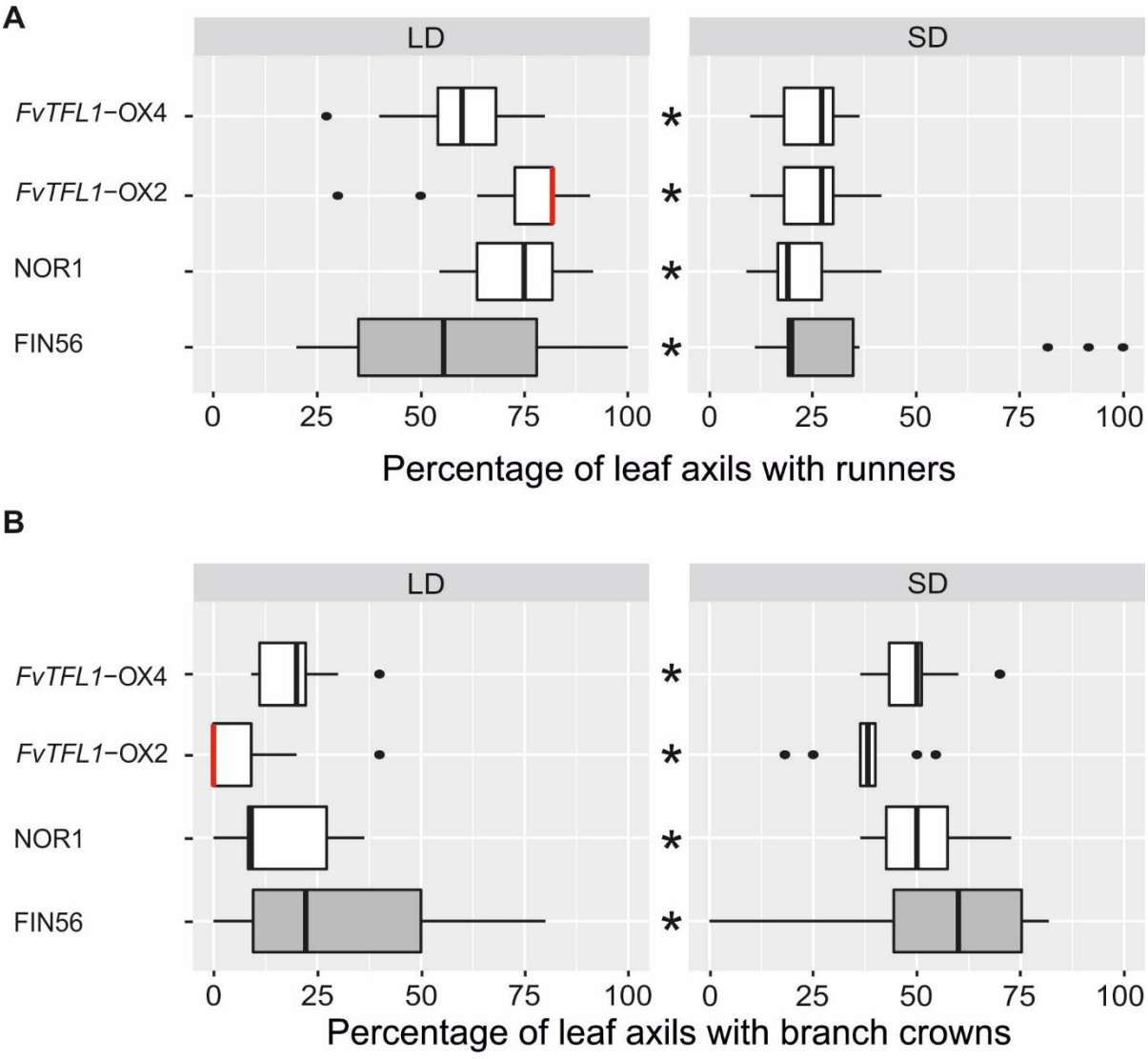
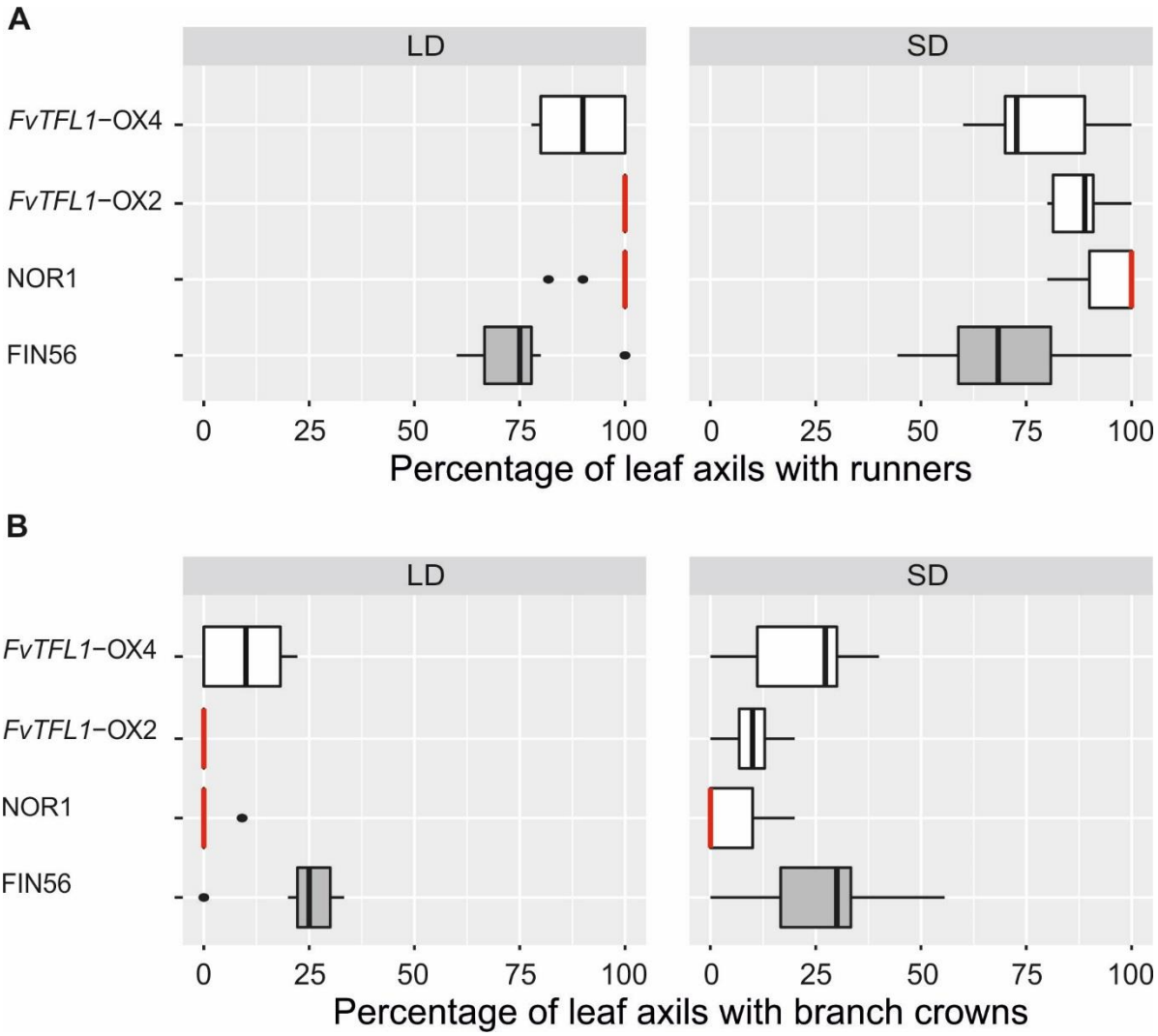
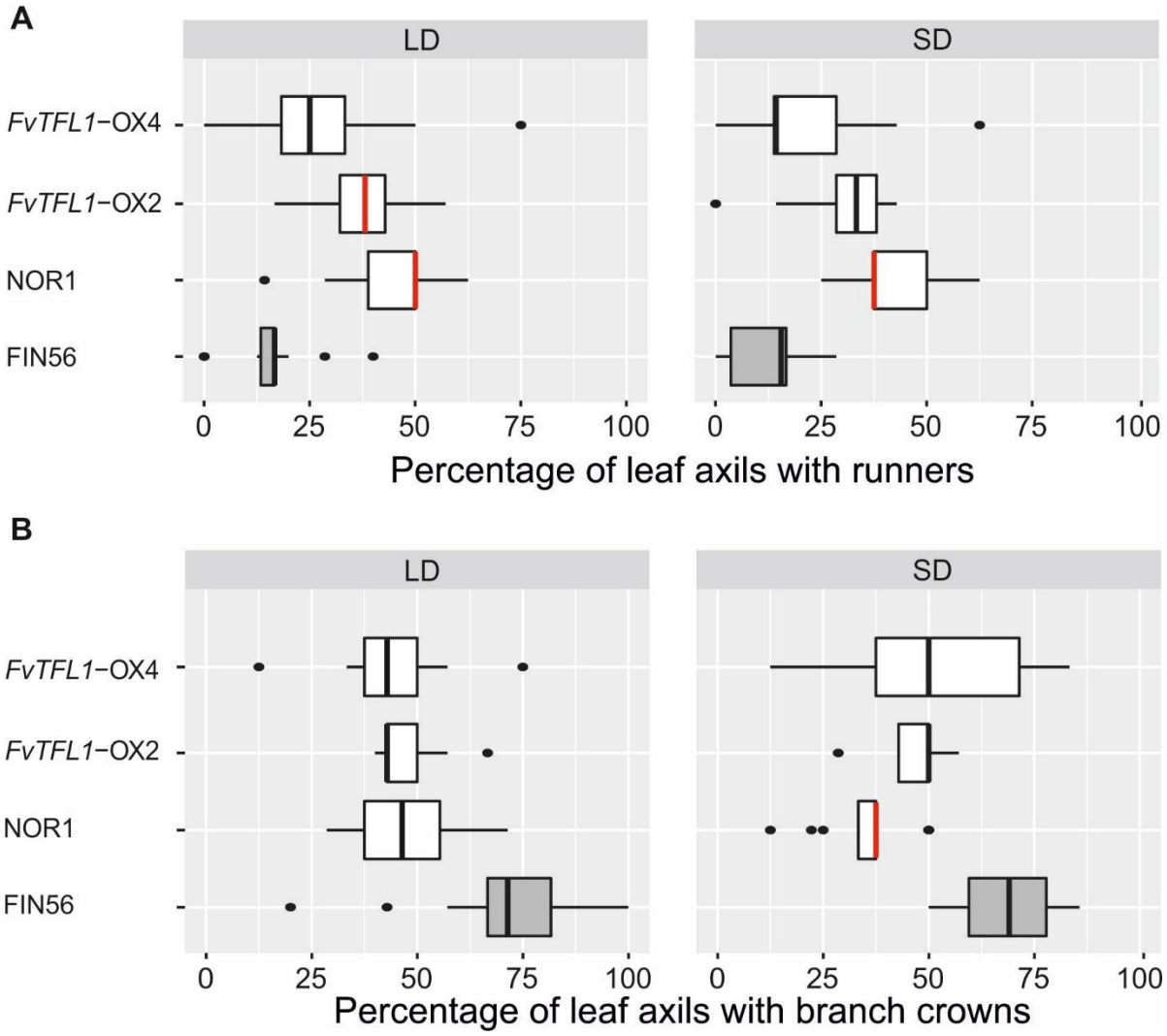


Figure 1 Percentage of axillary meristems producing either runners (A) or branch crowns (B) per genotype grown at intermediate temperature (daytime: 18ºC and night: 16°C), where LD and SD indicate 16-h long days and 12-h short days, respectively. The ends of the box represent the lower and upper quartiles, so that the box spans the interquartile range with the median marked with a solid vertical line. The whiskers represent the highest and lowest values observed in each category while the dots represent outliers. Medians marked in red denote genotypes which differed significantly from FIN56 under a given photoperiod, while asterisks denote significant differences between photoperiods within a genotype at $p < 0.05$ ($n = 13–18$).



453

454 Figure 2 Percentage of axillary meristems producing either runners (A) or branch crowns (B) per
455 genotype grown at warm temperature (daytime: 22ºC and night: 20ºC), where LD and SD indicate
456 16-h long days and 12-h short days, respectively. The ends of the box represent the lower and
457 upper quartiles, so that the box spans the interquartile range with the median marked with a solid
458 vertical line. The whiskers represent the highest and lowest values observed in each category while
459 the dots represent outliers. Medians marked in red denote genotypes which differed significantly
460 ($p < 0.05$) from FIN56 under a given photoperiod ($n = 9–16$).



461

462 Figure 3 Percentage of axillary meristems producing either runners (A) or branch crowns (B) per
463 genotype grown at cool temperature (daytime: 11ºC and night: 10ºC), where LD and SD indicate
464 16-h long days and 12-h short days, respectively. The ends of the box represent the lower and
465 upper quartiles, so that the box spans the interquartile range with the median marked with a solid
466 vertical line. The whiskers represent the highest and lowest values observed in each category while
467 the dots represent outliers. Medians marked in red denote genotypes which differed significantly
468 ($p < 0.05$) from FIN56 under a given photoperiod ($n = 12–16$).

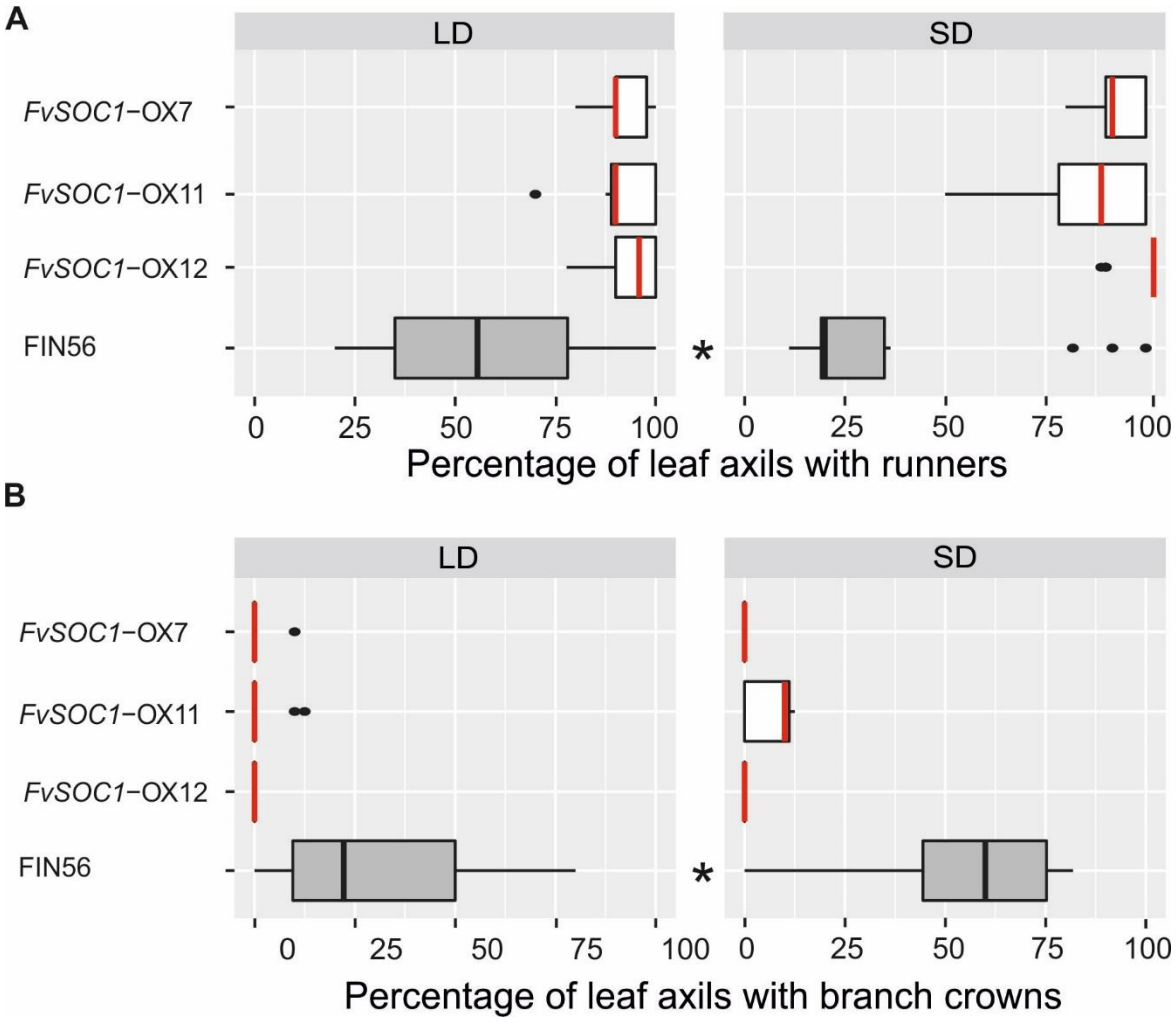


Figure 4 Percentage of axillary meristems producing either runners (A) or branch crowns (B) per genotype grown at intermediate temperature (daytime: 18ºC and night: 16ºC), where LD and SD indicate 16-h long days and 12-h short days, respectively. The ends of the box represent the lower and upper quartiles, so that the box spans the interquartile range with the median marked with a solid vertical line. The whiskers represent the highest and lowest values observed in each category while the dots represent outliers. Medians marked in red denote genotypes which differed significantly ($p < 0.05$) from FIN56 under a given photoperiod ($n = 15–19$).

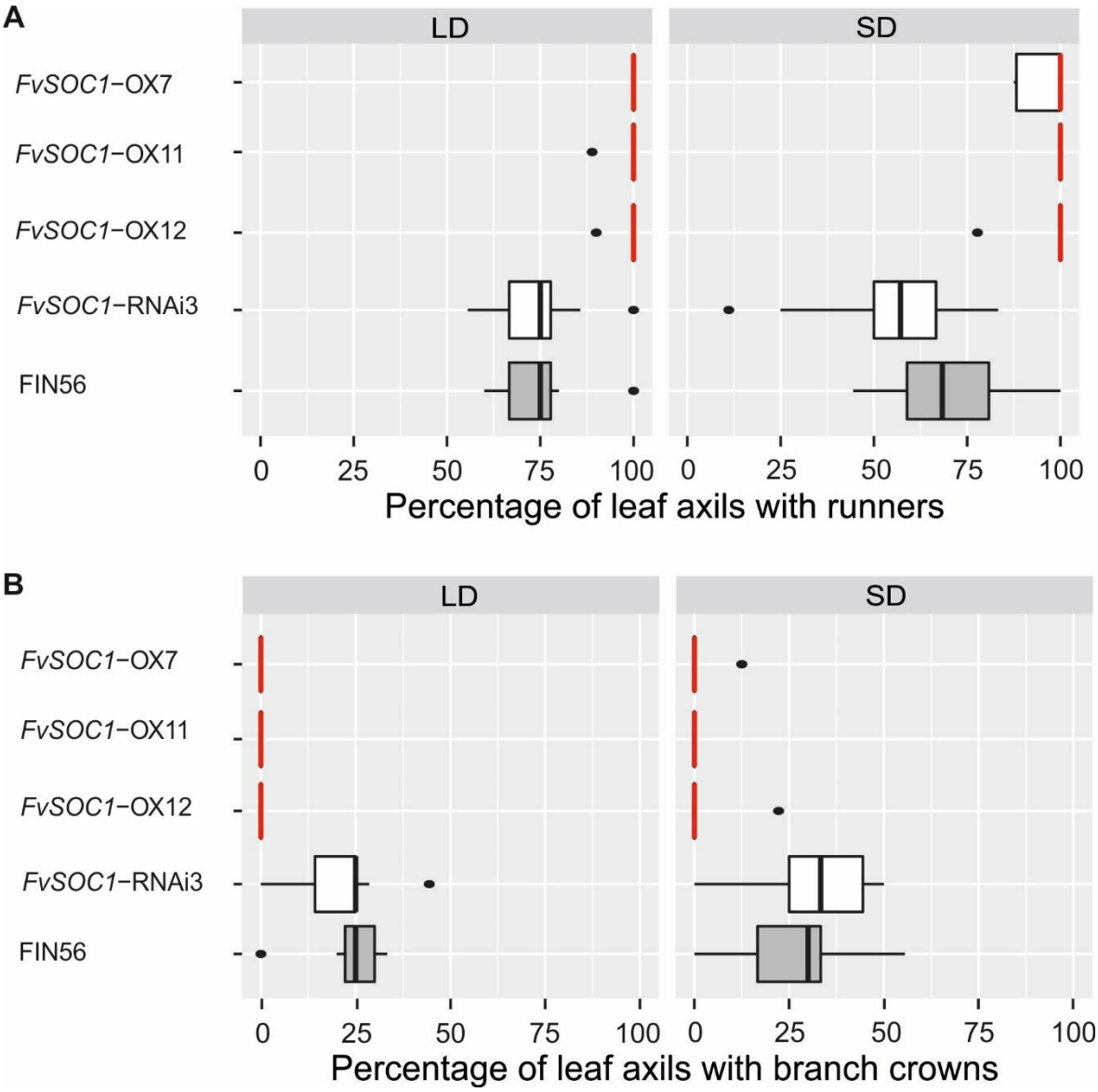
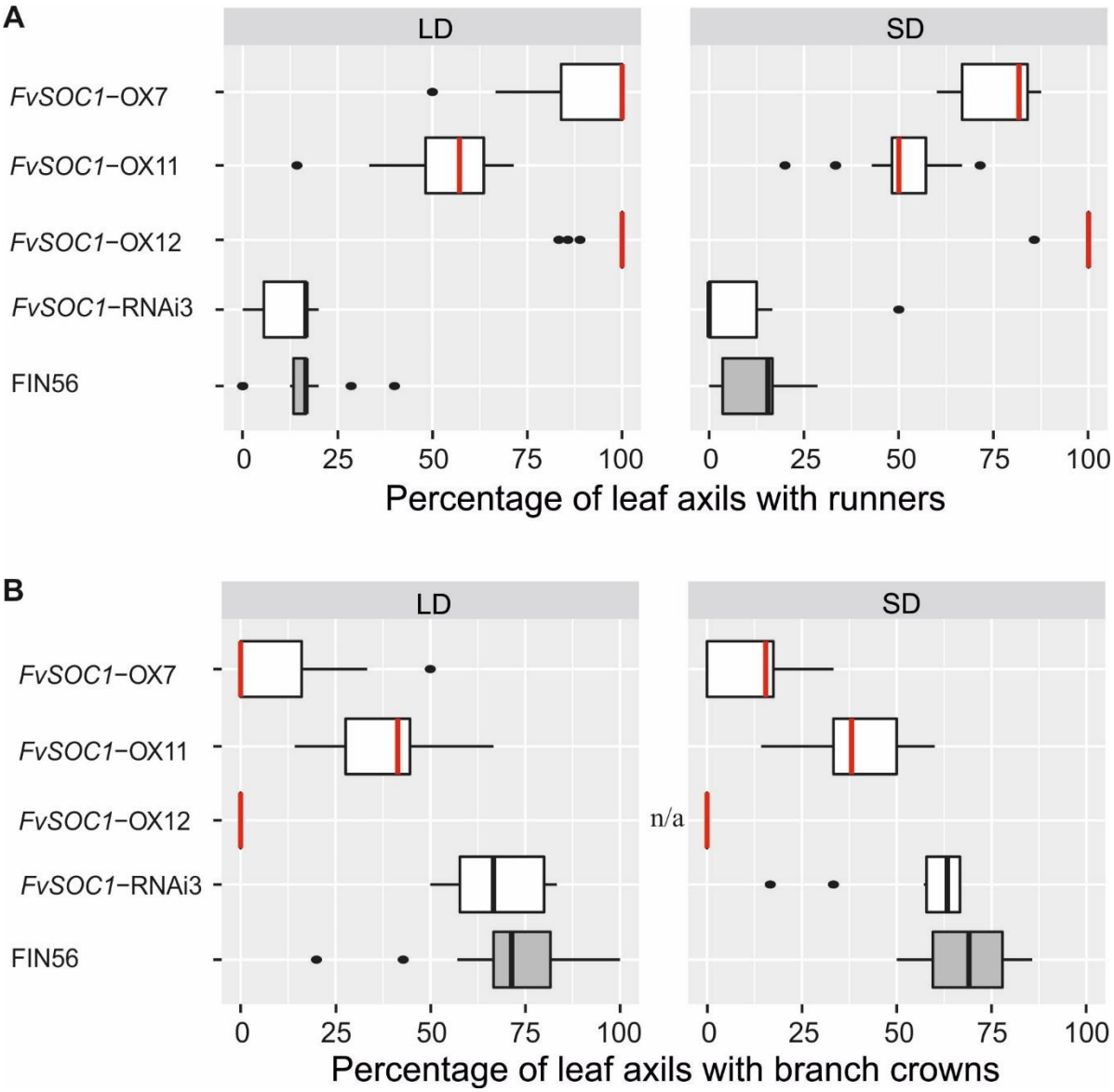


Figure 5 Percentage of axillary meristems producing either runners (A) or branch crowns (B) per genotype grown at warm temperature (daytime: 22ºC and night: 20ºC), where LD and SD indicate 16-h long days and 12-h short days, respectively. The ends of the box represent the lower and upper quartiles, so that the box spans the interquartile range with the median marked with a solid vertical line. The whiskers represent the highest and lowest values observed in each category while the dots represent outliers. Medians marked in red denote genotypes which differed significantly ($p < 0.05$) from FIN56 under a given photoperiod ($n = 9–16$).



485

486 Figure 6 Percentage of axillary meristems producing either runners (A) or branch crowns (B) per
487 genotype grown at cool temperature (daytime: 11ºC and night: 10ºC), where LD and SD indicate
488 16-h long days and 12-h short days, respectively. The ends of the box represent the lower and
489 upper quartiles, so that the box spans the interquartile range with the median marked with a solid
490 vertical line. The whiskers represent the highest and lowest values observed in each category while
491 the dots represent outliers. Medians marked in red denote genotypes which differed significantly
492 ($p < 0.05$) from FIN56 under a given photoperiod ($n = 11–16$).

Supplement information

Supplemental table S*1 Transgenic lines used in the study.*

Supplemental table S*2 Number of plants per treatment.*

Supplemental table S3. *Binomial regression model for the effects of temperature, photoperiod and genotype on the proportion of AXMs producing branch crowns.*

Supplemental table S4. *Binomial regression model for the effects of temperature, photoperiod and genotype on the proportion of AXMs producing runners.*

Supplemental table S5. *Pairwise proportions test for the proportion of AXMs producing runners at intermediate temperature in FIN56 and genotypes with higher-than-normal FvTFL1 expression.*

Supplemental table S6. *Pairwise proportions test for the proportion of AXMs producing branch crowns at intermediate temperature in FIN56 and genotypes with higher-than-normal FvTFL1 expression.*

Supplemental table S7. *Pairwise proportions test for the proportion of AXMs producing runners at cool temperature in FIN56 and genotypes with higher-than-normal FvTFL1 expression.*

Supplemental table S8. *Pairwise proportions test for the proportion of AXMs producing branch crowns at cool temperature in FIN56 and genotypes with higher-than-normal FvTFL1 expression.*

Supplemental table S9. *Pairwise proportions test for the proportion of AXMs producing runners at warm temperature in FIN56 and genotypes with higher-than-normal FvTFL1 expression.*

Supplemental table S10. *Pairwise proportions test for the proportion of AXMs producing branch crowns at warm temperature in FIN56 and genotypes with higher-than-normal FvTFL1 expression.*

Supplemental table S11. *Pairwise proportions test for the proportion of AXMs producing runners at intermediate temperature in FIN56 and FvSOC1 overexpressing genotypes.*

Supplemental table S12. *Pairwise proportions test for the proportion of AXMs producing branch crowns at intermediate temperature in FIN56 and FvSOC1 overexpressing genotypes.*

Supplemental table S13. *Pairwise proportions test for the proportion of AXMs producing runners at cool temperature in FIN56 and FvSOC1 overexpressing genotypes.*

Supplemental table S14. *Pairwise proportions test for the proportion of AXMs producing branch crowns at cool temperature in FIN56 and FvSOC1 overexpressing genotypes.*

Supplemental table S15. *Pairwise proportions test for the proportion of AXMs producing runners at warm temperature in FIN56 and FvSOC1 overexpressing genotypes.*

Supplemental table S16. *Pairwise proportions test for the proportion of AXMs producing branch crowns at warm temperature in FIN56 and FvSOC1 overexpressing genotypes.*

Supplement Figure S 1 *Average no. of leaves at flowering developed after the beginning of the treatments with standard error (N = 11- 19).*

Supplement Figure S 2 *(A) Percentages of flowering plants by treatment. Note, that SOC1-RNAi3 line was not included at the intermediate temperature. (B) Pairwise proportions test for the percentage of flowering plants. Warm temperature was not included in the statistical analysis. Values in orange font indicate significant differences at α = 0.05. Correction for multiple*

comparisons was done by false discovery rate procedure. In (A), the error bars denote 95 % confidence intervals around the mean. For both (A) and (B), n = 9–19.

Supplement data

Supplement Table S 1 *Transgenic lines used in the study.*

Genotype	Transgenic line	Publication
SOC1-OX	*FvSOC1*-OX7	Mouhu, et al. (2013)
	FvSOC1-OX11	Mouhu, et al. (2013)
	FvSOC1-OX12	Mouhu, et al. (2013)
SOC1-RNAi	*FvSOC1*-RNAi3	Mouhu, et al. (2013)
TFL1-OX	*FvTFL1*-OX2	Unpublished data
	FvTFL1-OX3	Unpublished data
FIN56	*WT*	PI551792
NOR1	*WT*	(Heide and Sønsteby, 2007)

Supplement Table S 2 *Number of plants per treatment.*

Total number	Cool		Intermediate		Warm
	SD	LD	SD	LD	SD
FIN56	14	15	15	15	16
FvSOC1-OX11	16	16	18	19	16
FvSOC1-OX12	14	15	18	16	15
FvSOC1-OX7	16	14	19	18	11
FvSOC1-RNAi3	11	11	-	-	9
FvTFL1-OX2	15	16	14	14	16
FvTFL1-OX3	16	16	18	18	15
NOR1	16	12	14	13	13

Supplement Table S 3 *Binomial regression model for the effects of temperature, photoperiod and genotype on the proportion of AXMs producing branch crowns. The estimate is the estimated amount by which the log odds of the proportion of branch crowns (out of total number of AXMs) changes as compared to LDs, warm temperature or FIN56.*

Coefficients	Estimate	Standard error	Pr (>|z|)	Significance
(intercept)	-1.121e+00	2.102e-01	9.82e-08	***
Photoperiod SD	-4.689e-03	2.828e-01	0.986772	
Temperature Cool	1.943e+00	3.063e-01	2.26e-10	***
NOR1	-4.038e+00	1.025e+00	8.11e-05	***
FvTFL1-OX2	-1.997e+01	2.340e+03	0.993190	
FvTFL1-OX3	-1.687e+00	4.424e-01	0.000137	***
FvTFL1-OX4	-1.195e+00	3.427e-01	0.000487	***
FvSOC1-RNAi3	-1.322e-01	3.529e-01	0.708034	
FvSOC1-OX7	-1.984e+01	2.071e+03	0.992354	
FvSOC1-OX11	-1.988e+01	1.924e+03	0.991755	
FvSOC1-OX12	-2.000e+01	1.973e+03	0.991912	

Supplement Table S 4 *Binomial regression model for the effects of temperature, photoperiod and genotype on the proportion of AXMs producing runners*. The estimate is the estimated amount by which the log odds of the proportion of branch crowns (out of total number of AXMs) changes as compared to LDs, warm temperature or FIN56.

Coefficients	Estimate	Standard error	Pr (>|z|)	Significance
(intercept)	1.07687	0.20796	2.24e-07	***

Photoperiod SD	-0.12319	0.27610	0.655457	
Temperature Cool	-2.83226	0.35640	1.91e-15	***
NOR1	2.97201	0.61838	1.54e-06	***
FvTFL1-OX2	19.01346	1419.14400	0.989310	
FvTFL1-OX3	0.82337	0.33925	0.015224	*
FvTFL1-OX4	1.10055	0.32975	0.000845	***
FvSOC1-RNAi3	-0.05102	0.33874	0.880280	
FvSOC1-OX7	18.88568	1255.89198	0.988002	
FvSOC1-OX11	3.79066	1.02515	0.000218	***
FvSOC1-OX12	3.86477	1.02489	0.000163	***

548

549 Supplement Table S 5 *Pairwise proportions test for the proportion of AXMs producing runners*
550 *at intermediate temperature in FIN56 and genotypes with higher-than-normal FvTFL1*
551 *expression. P values were adjusted for multiple testing by the Holm method.*

		FIN56		NOR1		*FvTFL1*-OX2	
		SD	LD	SD	LD	SD	LD
FIN56	LD	0.01048					
NOR1	SD	0.14370	1.1e-08				
	LD	2.7e-08	0.20925	<2e-16			
FvTFL1-	SD	0.90721	1.4e-06	1	6.5e-14		
OX2	LD	7.3e-10	0.03531	<2e-16	1	8.2e-16	
FvTFL1-	SD	0.50253	1.3e-07	1	1.6e-15	1	<2e-16
OX4	LD	0.00151	1	4.5e-10	0.62062	8.7e-08	0.14370

Supplement Table S 6 *Pairwise proportions test for the proportion of AXMs producing branch crowns at intermediate temperature in FIN56 and genotypes with higher-than-normal FvTFL1 expression. P values were adjusted for multiple testing by the Holm method.*

		FIN56		NOR1		*FvTFL1*-OX2	
		SD	LD	SD	LD	SD	LD
FIN56	LD	0.00232					
NOR1	SD	1	0.00279				
	LD	2.9e-09	0.18106	3.9e-09			
FvTFL1-	SD	0.31027	1	0.33961	0.00123		
OX2	LD	2.8e-15	7.2e-05	3.8e-15	0.52286	3.7e-08	
FvTFL1-	SD	1	0.01281	1	3.5e-08	0.78718	5.3e-14
OX4	LD	2.9e-09	0.98456	3.1e-07	1	0.02381	0.06822

Supplement Table S 7 *Pairwise proportions test for the proportion of AXMs producing runners at cool temperature in FIN56 and genotypes with higher-than-normal FvTFL1 expression. P values were adjusted for multiple testing by the Holm method.*

		FIN56		NOR1		*FvTFL1*-OX2		*FvTFL1*-OX4
		SD	LD	SD	LD	SD	LD	SD
FIN56	LD	1	-	-	-	-	-	-
NOR1	SD	0.00133	0.00432	-	-	-	-	-
	LD	0.00026	0.00087	1	-	-	-	-
FvTFL1-	SD	0.76308	1	1	1	-	-	-
OX2	LD	0.01331	0.04047	1	1	1	-	-

	SD			0.0944				
FvTFL1-OX4		1	1	0	0.02198	1	0.61626	-
	LD	1	1	1	1	1	1	1

558

559 Supplement Table S 8 *Pairwise proportions test for the proportion of AXMs producing branch*
560 *crowns at cool temperature in FIN56 and genotypes with higher-than-normal FvTFL1*
561 *expression. P values were adjusted for multiple testing by the Holm method.*

		FIN56		NOR1		*FvTFL1*-OX2	
		SD	LD	SD	LD	SD	LD
FIN56	LD	1	-	-	-	-	-
NOR1	SD	0.00128	0.00031	-	-	-	-
	LD	0.64155	0.29784	1	-	-	-
FvTFL1-OX2	SD	0.66874	0.31590	1	1	-	-
	LD	0.90518	0.45283	1	1	1	-
FvTFL1-OX4	SD	1	1	1	1	1	1
	LD	0.17235	0.06896	1	1	1	1

562

563 *Supplement Table S 9 Pairwise proportions test for the proportion of AXMs producing runners at*
564 *warm temperature in FIN56 and genotypes with higher-than-normal FvTFL1 expression. P*
565 *values were adjusted for multiple testing by the Holm method.*

		FIN56		NOR1		*FvTFL1*-OX2	
		SD	LD	SD	LD	SD	LD
FIN56	LD	1	-	-	-	-	-

NOR1	SD	0.00051	0.00619	-	-	-	-
	LD	8.5e-09	2.5e-07	1	-	-	-
FvTFL1-OX2	SD	0.14734	0.88176	1	0.04929	-	-
	LD	8.3e-06	5.6e-05	1	1	0.13246	-
FvTFL1-OX4	SD	1	1	0.02244	1.7e-06	1	0.00019
	LD	0.01834	0.16631	1	0.28212	1	0.40075

Supplement Table S 10 *Pairwise proportions test for the proportion of AXMs producing branch crowns at warm temperature in FIN56 and genotypes with higher-than-normal FvTFL1 expression. P values were adjusted for multiple testing by the Holm method.*

		FIN56		NOR1		*FvTFL1*-OX2	
		SD	LD	SD	LD	SD	LD
FIN56	LD	1	-	-	-	-	-
NOR1	SD	0.00788	0.01288	-	-	-	-
	LD	2.0e-08	3.2e-08	1	-	-	-
FvTFL1-OX2	SD	0.16154	0.24039	1	0.05494	-	-
	LD	8.7e-05	0.00011	1	1	0.54174	-
FvTFL1-OX4	SD	1	1	0.04395	2.2e-07	0.66874	0.00035
	LD	0.06597	0.10589	1	0.11411	1	0.82326

Supplement Table S 11 *Pairwise proportions test for the proportion of AXMs producing runners at intermediate temperature in FIN56 and FvSOC1 overexpressing genotypes. P values were adjusted for multiple testing by the Holm method.*

		FIN56		*FvSOC1*-OX7		*FvSOC1*-OX11	
		SD	LD	SD	LD	SD	LD
FIN56	LD	0.01048					
FvSOC1-	SD	<2e-16	2.4e-13				
OX7	LD	<2e-16	5.0e-11	1			
FvSOC1-	SD	<2e-16	3.6e-07	0.91271	1		
OX11	LD	<2e-16	1.5e-11	1	1	1	
FvSOC1-	SD	<2e-16	3.3e-16	1	0.53851	0.01256	0.91271
OX12	LD	<2e-16	2.7e-12	1	1	0.62062	1

574

575 Supplement Table S 12 *Pairwise proportions test for the proportion of AXMs producing branch*
576 *crowns at intermediate temperature in FIN56 and FvSOC1 overexpressing genotypes.P values*
577 *were adjusted for multiple testing by the Holm method.*

		FIN56		*FvSOC1*-OX7		*FvSOC1*-OX11	
		SD	LD	SD	LD	SD	LD
FIN56	LD	0.00232					
FvSOC1-OX7	SD	<2e-16	1.7e-13				
	LD	<2e-16	1.0e-11	1			
FvSOC1-	SD	<2e-16	2.8e-05	0.01403	0.07197		
OX11	LD	<2e-16	9.9e-11	1	1	0.22813	
FvSOC1-	SD	<2e-16	9.1e-12	-	1	0.03277	1
OX12	LD	<2e-16	4.1e-11	-	1	0.04411	1

578

Supplement Table S 13 *Pairwise proportions test for the proportion of AXMs producing runners at cool temperature in FIN56 and FvSOC1 overexpressing genotypes. P values were adjusted for multiple testing by the Holm method.*

		FIN56		*FvSOC1*-RNAi3		*FvSOC1*-OX7		*FvSOC1*-OX11	
		SD	LD	SD	LD	SD	LD	SD	LD
FIN56	LD	1.00000							
FvSOC1-	SD	1.00000	1.00000						
RNAi3	LD	1.00000	1.00000	1.00000					
FvSOC1-	SD	9.1e-16	2.8e-15	6.7e-14	1.1e-12				
OX7	LD	<2e-16	<2e-16	<2e-16	<2e-16	1			
FvSOC1-	SD	1.3e-05	4.3e-05	3.6e-05	0.00037	0.0326 1	2.0e-06		
OX11	LD	2.0e-06	6.7e-06	7.9e-06	8.3e-05	0.0874 8	7.7e-06	1	
FvSOC1- OX12	SD	<2e-16	<2e-16	<2e-16	<2e-16	0.0018 5	1	9.0e-12	4.9e-11
	LD	<2e-16	<2e-16	<2e-16	<2e-16	0.0086 9	1	2.0e-11	1.2e-10

Supplement Table S 14 *Pairwise proportions test for the proportion of AXMs producing branch crowns at cool temperature in FIN56 and FvSOC1 overexpressing genotypes. P values were adjusted for multiple testing by the Holm method.*

		FIN56		*FvSOC1*-RNAi3		*FvSOC1*-OX7		*FvSOC1*-OX11	*FvSOC1*-OX12
		SD	LD	SD	LD	SD	LD	SD	LD
FIN56	LD	1	-	-	-				
FvSOC1-RNAi3	SD	1	1	-	-				
	LD	1	1	1	-				
FvSOC1-OX7	SD	7.3e-11	8.5e-12	2.5e-05	1.0e-08				
	LD	1.1e-12	1.3e-13	5.4e-07	1.6e-10	1			
FvSOC1-OX11	SD	0.02480	0.00827	1	0.18527	0.03442	0.00527		
	LD	0.00422	0.00121	1	0.04884	0.11936	0.99553	1	
FvSOC1-OX12	SD	<2e-16	<2e-16	1.7e-13	<2e-16	0.06168	0.70279	6.0e-09	4.2e-08
	LD	<2e-16	<2e-16	1.3e-14	<2e-16	0.03367	1	9.4e-10	7.4e-09

587

588 Supplement Table S 15 *Pairwise proportions test for the proportion of AXMs producing runners*
589 *at warm temperature in FIN56 and FvSOC1 overexpressing genotypes. P values were adjusted*
590 *for multiple testing by the Holm method.*

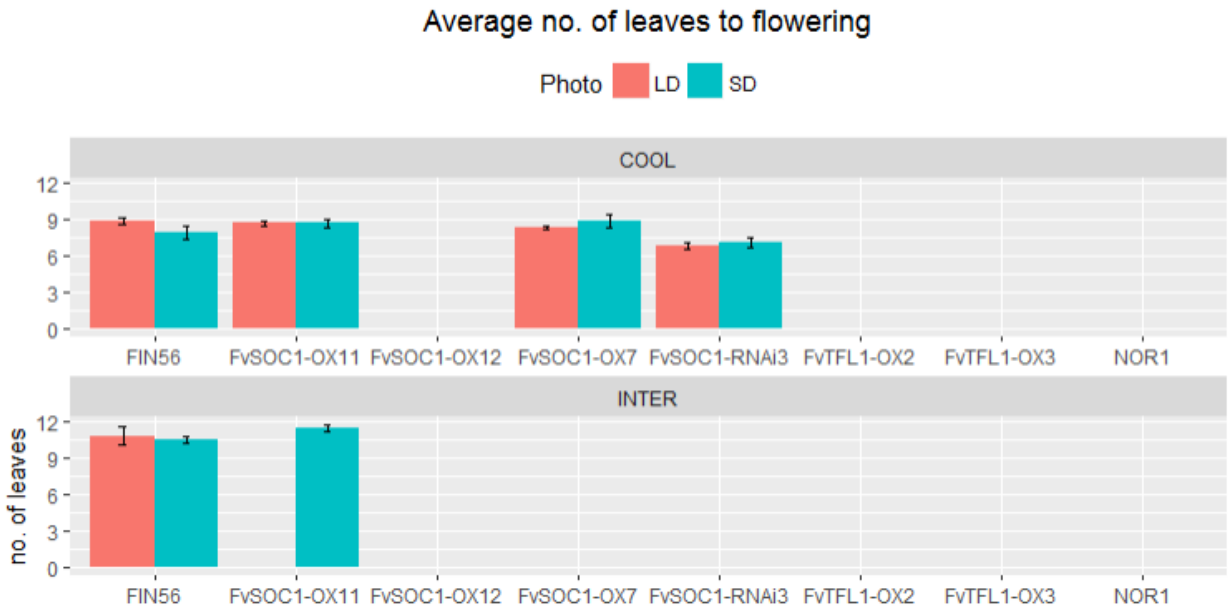
		FIN56		*FvSOC1*-RNAi3		*FvSOC1-OX7*		*FvSOC1-OX11*	
		SD	LD	SD	LD	SD	LD	SD	LD
FIN56	LD	1							
FvSOC1-	SD	0.95468	0.49689	-					
RNAi3									
	LD	1	1	1					
FvSOC1-	SD	0.00400	0.02207	2.5e-07	0.03574				
OX7									
	LD	1.4e-06	1.2e-05	3.2e-12	1.5e-05	1			
FvSOC1-	SD	5.2e-08	6.7e-07	1.6e-14	8.6e-07	1	-		
OX11	LD	2.4e-07	3.1e-06	1.1e-13	5.1e-06	1	1	1	
FvSOC1-	SD	1.2e-07	2.0e-06	2.4e-14	4.3e-06	1	1	1	1
OX12	LD	5.5e-08	8.7e-07	1.1e-14	1.5e-06	1	1	1	1

591

592 Supplement Table S 16 *Pairwise proportions test for the proportion of AXMs producing branch*
593 *crowns at warm temperature in FIN56 and FvSOC1 overexpressing genotypes. P values were*
594 *adjusted for multiple testing by the Holm method.*

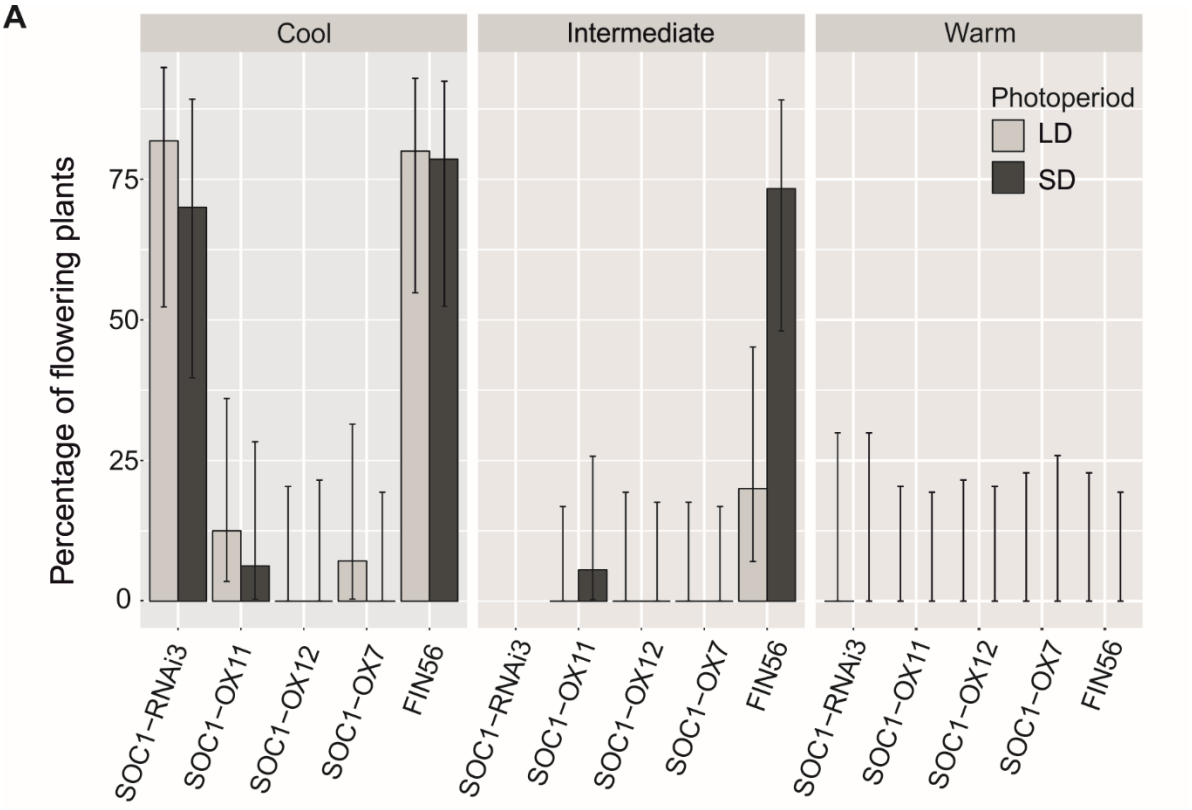
		FIN56		*FvSOC1*-RNAi3		*FvSOC1*-OX7		*FvSOC1*-OX11	
		SD	LD	SD	LD	SD	LD	SD	LD
FIN56	LD	1							
FvSOC1-	SD	1	1						
RNAi3	LD	1	1	1					

FvSOC1-OX7	SD	0.00342	0.00438	0.00027	0.03679					
	LD	1.8e-05	2.3e-05	5.5e-07	0.00026	1				
FvSOC1-OX11	SD	1.1e-06	1.5e-06	1.7e-08	2.4e-05	1	-			
	LD	1.1e-06	1.5e-06	1.7e-08	2.4e-05	1	-	1		
FvSOC1-OX12	SD	2.9e-06	4.6e-06	6.9e-08	0.00015	1	1	1	1	
	LD	3.0e-07	4.2e-07	3.7e-09	8.2e-06	1	-	-	-	



Supplement Figure S 1 *Average no. of leaves at flowering developed after the beginning of the treatments with standard error (N = 11- 19).*

A



B

			COOL						INTERMEDIATE		
			FIN56		SOC1-RNAi3		SOC1-OX11		FIN56		SOC1-OX11
			SD	LD	SD	LD	SD	LD	SD	LD	SD
COOL	FIN56	LD	1								
	SOC1-RNAi3	SD	1	1							
		LD	1	1	1						
	SOC1-OX11	SD	0,00103	0,00085	0,00661	0,00124					
		LD	0,00318	0,00207	0,01953	0,00382	1				
INTERMEDIATE	FIN56	SD	1	1	1	1	0,00185	0,00542			
		LD	0,01156	0,00785	0,06700	0,01312	0,84576	1	0,01953		
	SOC1-OX11	SD	0,00084	0,00057	0,00389	0,00085	1	1	0,00103	0,74761	
		LD	0,00026	0,00026	0,00096	0,00026	1	0,65314	0,00034	0,26301	1

Supplement Figure S 2 *(A) Percentages of flowering plants by treatment. Note, that SOC1-RNAi3 line was not included at the intermediate temperature. (B) Pairwise proportions test for the percentage of flowering plants. Warm temperature was not included in the statistical analysis. Values in orange font indicate significant differences at α = 0.05. Correction for multiple comparisons was done by false discovery rate procedure. In (A), the error bars denote 95 % confidence intervals around the mean. For both (A) and (B), n = 9–19.*

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ARTICLE

Additive QTLs on three chromosomes control flowering time in woodland strawberry (*Fragaria vesca* L.)

Samia Samad[1,2,7], Takeshi Kurokura[3,7], Elli Koskela[1], Tuomas Toivainen[1], Vipul Patel[4], Katriina Mouhu[1], Daniel James Sargent[2,5] and Timo Hytönen[1,6]

Flowering time is an important trait that affects survival, reproduction and yield in both wild and cultivated plants. Therefore, many studies have focused on the identification of flowering time quantitative trait locus (QTLs) in different crops, and molecular control of this trait has been extensively investigated in model species. Here we report the mapping of QTLs for flowering time and vegetative traits in a large woodland strawberry mapping population that was phenotyped both under field conditions and in a greenhouse after flower induction in the field. The greenhouse experiment revealed additive QTLs in three linkage groups (LG), two on both LG4 and LG7, and one on LG6 that explain about half of the flowering time variance in the population. Three of the QTLs were newly identified in this study, and one co-localized with the previously characterized *FvTFL1* gene. An additional strong QTL corresponding to previously mapped *PFRU* was detected in both field and greenhouse experiments indicating that gene(s) in this locus can control the timing of flowering in different environments in addition to the duration of flowering and axillary bud differentiation to runners and branch crowns. Several putative flowering time genes were identified in these QTL regions that await functional validation. Our results indicate that a few major QTLs may control flowering time and axillary bud differentiation in strawberries. We suggest that the identification of causal genes in the diploid strawberry may enable fine tuning of flowering time and vegetative growth in the closely related octoploid cultivated strawberry.

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INTRODUCTION

Synchronizing floral development with local climatic conditions is important for crop and wild plants because of its significant impact on reproduction, yield and ultimately survival. The genetic control of flowering time is thus of immense significance. As a result, numerous mapping studies have focused on the identification of flowering time QTLs in different crops, and a detailed molecular understanding of flowering time regulation in model plants has facilitated the identification of causal genes.[1–4] In the cultivated strawberry, one of the most economically important berry crops throughout the world, flowering time is a major breeding target because of year-round demand for fresh berries. To extend the cropping season, breeders are developing early and late flowering cultivars as well as everbearing (EB; also called day-neutral) cultivars, which display a continuous flowering habit.[5–8]

In seasonal flowering 'Junebearing' strawberries, flower induction occurs in autumn, and the timing of the induction correlates with flowering time in the following season.[9] Both short days (SD) and cool temperatures can induce flowering. Typically, flower induction occurs independently of day-length at temperatures below ~13 °C, whereas SDs are required at intermediate temperatures, and at temperatures above 20–24 °C floral induction is inhibited.[10–13] There is however significant variation in critical temperature limits between cultivars,[10,14] and some cultivars possess an obligatory SD requirement for flower induction even under cool temperatures.[15] In contrast to seasonal flowering strawberries, EB cultivars flower earlier under long days (LD) than SDs.[16,17] Both seasonal flowering and EB habits are also found in the woodland strawberry.[18]

As the cultivated strawberry is a complex allo-octoploid ($2n = 8x = 56$), the woodland strawberry (*F. vesca*; $2n = 2x = 14$) and other diploid species have been used as a genetically facile surrogate system for genetic investigation. Several genetic linkage maps have been developed for diploid strawberry species. Initially saturated maps were developed using large numbers of microsatellite (SSR) and other PCR-based markers,[19–25] and the *Fragaria* SSR-based reference linkage map was used to anchor the *F. vesca* 'Hawaii-4' genome sequence.[26] More recently, high-throughput genotyping has been employed using various platforms to produce dense SNP-based linkage maps that have enabled more precise sequence scaffold anchoring and orientation,[27] whereas the most comprehensive linkage map for diploid *Fragaria* to date is that of *F. iinumae* produced using the *Fragaria* Axiom SNP genotyping array (Axiom IStraw90, Affymetrix, Santa Clara, CA, USA), supplemented with SNPs scored using genotyping-by-sequencing (GBS).[28,29] The linkage maps have been used to map a number of genetic loci in diploid strawberry species, including fruit color, runnering, leaf color, and seasonal flowering,[20,30–32] and represent a powerful tool for the genetic dissection of traits of economic importance in the cultivated strawberry.

Previous genetic mapping studies have shown that the gene causing seasonal flowering habit in woodland strawberry is

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located on linkage group 6 (LG6).[21,31] This gene has been recently shown to encode a strong repressor of flowering, TERMINAL FLOWER 1 (FvTFL1), and a 2-bp deletion in the first exon of *FvTFL1* causes expression of the EB phenotype in woodland strawberry.[32] Furthermore, homologs of FvTFL1 have been found to repress flowering in several rosaceous species suggesting the conservation of the genetic mechanism of flowering time regulation.[33–36] Only a few other flowering time genes have been functionally characterized in woodland strawberry. Using genetic transformation, recent studies have revealed how homologs of *FLOWERING LOCUS T* (*FvFT1*) and *SUPPRESSOR OF THE OVEREXPRESSION OF CONSTANS1* (*FvSOC1*) mediate photoperiod and temperature signals and how they are integrated to control flower induction.[32,37–39] In the seasonal flowering woodland strawberry, cool temperatures below 13 °C cause photoperiod-independent downregulation of *FvTFL1* by an unknown mechanism leading to flower induction, whereas at higher temperatures, photoperiod controls flowering through *FvTFL1*. Under LD, leaf-expressed FvFT1 activates *FvSOC1* in the shoot apex leading to the upregulation of *FvTFL1* and the maintenance of a vegetative stage, whereas under SD, this *FvFT1-FvSOC1-FvTFL1* pathway is silenced and flower induction occurs. However, at temperatures above 20 °C *FvTFL1* is highly upregulated by an unknown activator that functions independently of FvSOC1, and plants remain vegetative.[37,39] This model contrasts with the mechanism in *Arabidopsis* in which both FT and SOC1 function as floral activators.[1] However, FvFT1 is a strong floral activator in EB woodland strawberries that are lacking functional *FvTFL1*.[32,38]

The genetic control of the EB habit has also been studied in the cultivated strawberry. Weebadde *et al.*[5] suggested the presence of several QTLs with significant genotype × environment interaction,[5] whereas other studies indicated that a single dominant locus causes continuous flowering.[40,41] The presence of a single QTL was supported by recent genetic mapping studies by two groups,[6,8] who found a major dominant QTL on LG4 in two independent crossing populations.[6,8] Moreover, Honjo *et al.*[42] demonstrated that the EB trait is controlled by the same gene in the EB parents of these populations.[42] In addition to the EB trait, this locus called *PFRU* also controls the production of runners, which are long shoots that enable the efficient clonal reproduction of the species.[6] Although studies indicate that different genes cause the EB phenotype in the woodland and cultivated strawberries, a recent study has shown that *TFL1* homologs encode major floral repressors in both species, and that the silencing of this gene also causes the EB habit in cultivated strawberry.[43] Moreover, the regulation of *FaTFL1* correlates with flower induction in different seasonal flowering cultivars.[43,44]

As strawberries form terminal inflorescences from the apical meristems of the crowns, the number of inflorescences, and consequently the yield potential, depends on the number of side shoots called branch crowns that are produced.[45–48] SD conditions promote the differentiation of strawberry axillary buds to branch crowns, whereas under LDs axillary buds typically differentiate to runners.[10,14,45] Changes in gibberellin biosynthesis and signaling are known to mediate the photoperiodic differentiation of axillary buds.[49] This differentiation is not understood at the molecular level, although runnerless mutants are known in woodland strawberry.[18]

Here, we aimed to identify novel genetic loci controlling flowering time and axillary bud differentiation using the large woodland strawberry F2 mapping population produced by Koskela *et al.*[32] We demonstrate that five QTLs with additive effects explain about half of the flowering time variance found in the population. One of the QTLs co-localized with the previously identified *FvTFL1* and another with *PFRU*, whereas the others were newly identified in this study. In addition, we report the mapping of two QTLs that affect the number of branch crowns and runners in our mapping population.

MATERIALS AND METHODS

Mapping population and phenotyping

Selected lines from a F2 population derived from the cross *F. vesca f. semperflorens* 'Hawaii-4' (H4) × *F. vesca* subsp. *vesca* (FV), (denoted H4 × FV),[32] were used in this study. The population consisted of 735 seasonal flowering plants that were planted in an experimental field at the University of Helsinki at the beginning of August 2009. In the spring 2010, flowering time was observed in the field. On the basis of these observations and previously scored genotypes for the *FvTFL1* gene,[32] 335 seasonal flowering lines containing one or two functional *FvTFL1* alleles were selected for flowering time observations. At the beginning of August 2010, four clonal daughter plants per line were potted into 6 cm square plastic pots filled with peat moss (Kekkilä, Finland). Plants were kept under natural environmental conditions and periodically supplemented with fertilizer (NPK 17-4-25; Kekkilä) applications until 13 December 2010. Then, the plants were moved into a greenhouse and repotted into 8 × 8 cm plastic pots and arranged according to completely randomized design. The greenhouse temperature was set to 18 °C and plants were kept under an 18 h photoperiod illuminated with high pressure sodium lamps (Airam 400 W, Kerava, Finland) providing a light intensity of 100 μmol m^{-2} s^{-1}. Plants were automatically watered with the fertilizer solution. The date of first flower of all genotypes was recorded and flowering time was calculated as days after the anthesis of the earliest flowering F2 line. Runners were removed and counted regularly in the greenhouse, and the total number of runners produced per plant before 8 March 2011 was calculated. In addition, the number of branch crowns was observed on the same date. On the basis of the greenhouse phenotypic data, 32 F2 lines with extreme flowering time values (16 early and 16 late flowering lines) were selected for growth chamber experiment. Four clones of each F2 line were rooted in 8 × 8 cm plastic pots and plants were subjected to 12 h short-day treatment (AP67 LED lamps; 200 μmol m^{-2} s^{-1}; Valoya, Finland) at 11 °C for 6 weeks followed by flowering time observations in the greenhouse as described above. In the greenhouse and growth chamber experiments, mean phenotypic values of four replicates were used for QTL mapping.

DNA extraction and quantification

DNA was extracted from young leaves of the parents 'H4' and 'FV', along with the F1 and 335 F2 seedlings of the progeny, using a modified version of the CTAB method.[31] The DNA was quantified using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and was diluted 1:100 (5–10 ng μl^{-1}) for use in PCR. For genome resequencing an additional purification step was carried out. NaCl was added at a final concentration of 0.2 M, and DNA was precipitated by adding 2/3 volumes of cold isopropanol. After centrifugation, the pellet was washed with 100 μl of 70% ethanol and re-suspended in 50 μl of TE.

Amplification and scoring of genetic markers

A total of 73 published SSR primer pairs (Supplementary File 1) labeled on the forward primer with either 6-FAM or HEX fluorescent dyes[19–22,25,50–52] were screened for polymorphism in the parental and F1 lines using the 'Type-it' PCR mastermix (Qiagen, Hilden, Germany), following the PCR protocol reported in Sargent *et al.*[53] In addition, 12 SSR primer pairs were tested using the fluorescent labeling method described by Schuelke[24] (Supplementary File 1). Samples were diluted 1:75, separated by capillary electrophoresis (Foster City, CA, USA) on a genetic analyzer (ABI 3100, ABI 3130 XL or ABI 3700, Applied Biosystems), and the resultant data were collected and analyzed using GeneMapper or Peak Scanner 2 (Applied Biosystems).

SNPs were identified in the parental genome sequences and the temperature switch PCR (TSP) protocol[23] was used to develop assays for 49 SNP markers within flowering time QTL regions identified in this investigation. In brief, two sets of primers, a locus specific (LS) set and a nested-locus specific (NLS) set were designed per SNP using Primer3 v.0.40 (ref. 54) and NetPrimer (http://www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html), respectively. The LS primers were designed to have a Tm between 60–65 °C (optimal 63 °C) and a product size >400 bp, whereas the NLS primers were designed to have a T_m of 43–47 °C (optimal 45 °C) and were extended using a non-complementary 5′ tail region that increased the overall primer T_m to 52–55 °C (optimal 53 °C).[23] PCR was performed as described previously.[23] The primer concentrations and the numbers of PCR cycles were adjusted for each primer individually to optimize marker amplification for ease of scoring. Products were visualized

over UV light following electrophoresis through a 1.5% (w/v) TAE agarose gel containing gel red (Applied Bioprobes, Rockville, MD, USA) at a constant voltage of 100 V for 2–3 h, depending on the sizes of the fragments produced. The TSP loci were named UH, followed by the chromosome number and finally a unique primer pair reference number (Supplementary File 1).

Genotyping-by-sequencing

DNA samples (30–100 ng μl^{-1}) of 188 seedlings selected to represent the full seedling population genotypically (based on the SSR and TSP data) and phenotypically, were sent to the Cornell University where genotyping-by-sequencing (GBS) was carried out according to Elshire *et al.*[55] GBS libraries were sequenced using the Illumina HiSeq2500 platform (San Diego, CA, USA). GBS tags with a minimum number of three reads and unique alignment to the reference genome (https://www.rosaceae.org/species/fragaria_vesca/genome_v2.0.a1)[27] were identified, and from them SNPs were called by Genomic Diversity Facility in Cornell using the GBS-pipeline[56] implemented in the TASSEL 3.0 software application.[57] To remove genotyping errors, several filtering steps were performed for each polymorphic site using vcftools v. 0.1.12b.[58] A site was accepted if genotype calling rate was over 90% per site, the minor allele frequency was >0.1, and the proportion of heterozygote genotypes fit to the Hardy–Weinberg expectations (2pq, $P < 0.01$). Missing data was imputed with BEAGLE4 (ref. 59) and the TASSEL5 was used to produce ABH file with 4672 SNPs. Following an initial round of linkage mapping (described below), a second filtering and manual imputation step was performed using the rationale previously reported by Ward *et al.*[60] This second imputation step removed suspected false homozygous calls that created multiple double-recombination events, inflating mapping distances and confounding local marker ordering.

Heritability, linkage mapping and QTL analysis

The broad sense heritability for flowering time and numbers of runners and branch crowns were calculated in a population of 188 individuals according the formula $H^2 = \sigma^2 g/(\sigma^2 g + \sigma^2 e)$, where $\sigma^2 g = (\text{MS(genotype)} - \text{MS(environment)})/r$ and $\sigma^2 e = \text{MS(environment)}$. MS(genotype) and MS(environment) were obtained from analysis of variance for completely randomized design as the mean sums of squares for genotype and residual error, respectively, and r was the effective number of replicates (three to four per genotype). Estimation of these variance components were performed by REML as implemented in PROC VARCOMP procedure in SAS/STAT software, version 9.4 (Copyright 2013, SAS Institute, Cary, NC, USA). Linkage mapping was performed using JOINMAP 4.1 (Kyazma, Wageningen, The Netherlands)[61] using maximum likelihood and the default mapping criteria. The maps presented were illustrated using MapChart 2.2.[62] The significance of association between individual markers on the H4 × FV linkage map and the phenotypes was calculated with linear regression using MapQTL 6.0 (Kyazma).[63] QTLs were identified employing interval mapping with a step size of 1 cM, considering a maximum of five neighboring markers. The genome-wide log of odds (LOD) threshold was determined over 10 000 permutations and the most significant markers were then used as co-factors for restricted multiple QTL mapping (rMQM) with a step size of 1 cM. Linkage mapping and QTL analyses were performed on 335 individuals using SSR and TSP markers and on 186 individuals using all marked data.

Genome resequencing and the identification of single-nucleotide polymorphisms between the 'H4' and 'FV' genomes

The genome of the 'FV' parent was re-sequenced at DNA Sequencing and Genomics Laboratory, Institute of Biotechnology, University of Helsinki, Finland. Briefly, DNA was sheared using a Bioruptor NGS sonicator (Diagenode, Denville, NJ, USA) and the obtained fragments were end-repaired and A-tailed, and truncated Illumina Y-adapters were ligated. In a PCR step (20 cycles), full-length P5 and indexed P7 adapters were introduced using KAPA Hifi DNA Polymerase (KAPA Biosystems, Wilmington, MA, USA). The obtained libraries were purified and size selected using AMPure XP beads (Beckman Coulter, Brea, CA, USA). The obtained final libraries were paired-end (300 bp+300 bp) sequenced on a MiSeq Sequencer (Illumina, San Diego, CA, USA). Adapter sequences were removed from paired-end reads with cutadapt 1.8.1.,[64] and reads were aligned against the strawberry reference genome v2.0.a1 with bwa-mem[65] using default settings. After the alignment, samtools 1.3 (refs 66,67) was used for sorting, indexing, filtering and removing PCR duplicates. Reads with mapping quality of <25 were discarded. After filtering, average coverage was 14.8, and 91.5% of the reference genome was covered. Genetic variants including SNPs and indels were called with bcftools after calculating genotype likelihoods with samtools mpileup function. To ensure high quality variants, additional filtering was conducted with vcftools,[58] and only sites having at least 10-fold coverage and <50-fold coverage were accepted for further analysis. Genomic regions spanning flowering time QTL were identified from the genomic positions of flanking SSR markers mapped in the full mapping progeny, within which polymorphic SNPs were identified for subsequent marker development.

Identification of candidate genes in the QTL regions

To identify candidate genes for the QTLs, predicted genes located between the markers flanking the most significant markers were BLAST searched against the RefSeq or UniRef90 protein database using the blastx algorithm in Blast2GO[68] or blast+ (2.4.0). Query sequences longer than 8000 bp were restricted to the first 8000 bp for Blast2GO as this is the maximum length accepted by the software. Homologs of known flowering time genes were selected as candidate genes. Parental genetic variation in the coding regions of candidate genes were retrieved in the Illumina whole genome resequencing data using vcftools,[58] and translated amino acid sequences were compared to reveal non-synonymous variation in the coding regions.

RESULTS

Segregation of flowering time and vegetative traits in H4 × FV mapping population

Initial field observations revealed over one month variation in flowering time among the 735 seasonal flowering F2 lines of H4 × FV mapping population in Helsinki in summer 2010 (Supplementary Figure 1). The first plants started to flower at week 20, and almost 70% of the plants flowered by week 22. However, many plants flowered significantly later, and about 5% of plants flowered at week 25 or later. In autumn 2010, 335 F2 selected lines were subjected to natural autumn conditions followed by growth observations in a greenhouse. Again, significant variation in flowering time was observed (Figure 1a). On average, these plants started to flower 39 days after the transfer to the greenhouse, and an 18-day variation in flowering time was observed. The broad sense heritability for flowering time was $H^2 = 0.61$. Greenhouse flowering data correlated positively, but weakly with field data ($r = 0.39$). Flowering time of the 16 earliest and the 16 latest F2 lines were further tested in a growth chamber. In this experiment, over two week variation in flowering time and clear positive correlation with greenhouse flowering data ($r = 0.71$) was observed (Supplementary Figure 2).

Variation was also observed in the vegetative development of the F2 lines. By the end of the experiment, most F2 lines produced 2–4 branch crowns per plant. However, a significant number of genotypes produced an average of less than two, or more than four branch crowns (Figure 1b). The total number of runners produced per genotype varied between 5 and 22 (Figure 1c). The broad sense heritability (H^2) was 0.48 and 0.41 for branch crowns and runners, respectively. The number of runners was positively correlated with the number of branch crowns ($r = 0.785$; Figure 1d). However, no correlation was found between flowering time and the number of branch crowns or runners.

Marker development and linkage map construction

Of the 73 reported SSR and 49 TSP markers screened (Supplementary File 1), 47 and 13 markers, respectively, were polymorphic between the 'H4' and 'FV' genotypes and segregated in the progeny. Following linkage analysis on 335 F2 lines, these 60 markers coalesced into seven discrete linkage groups spanning the majority of the *Fragaria* genome (Supplementary Figure 3). To increase mapping resolution, GBS was carried out in 188 F2 seedlings. Out of 271 473 572 barcoded Illumina reads, 2 391 856 GBS tags with a minimum coverage of three were found, and

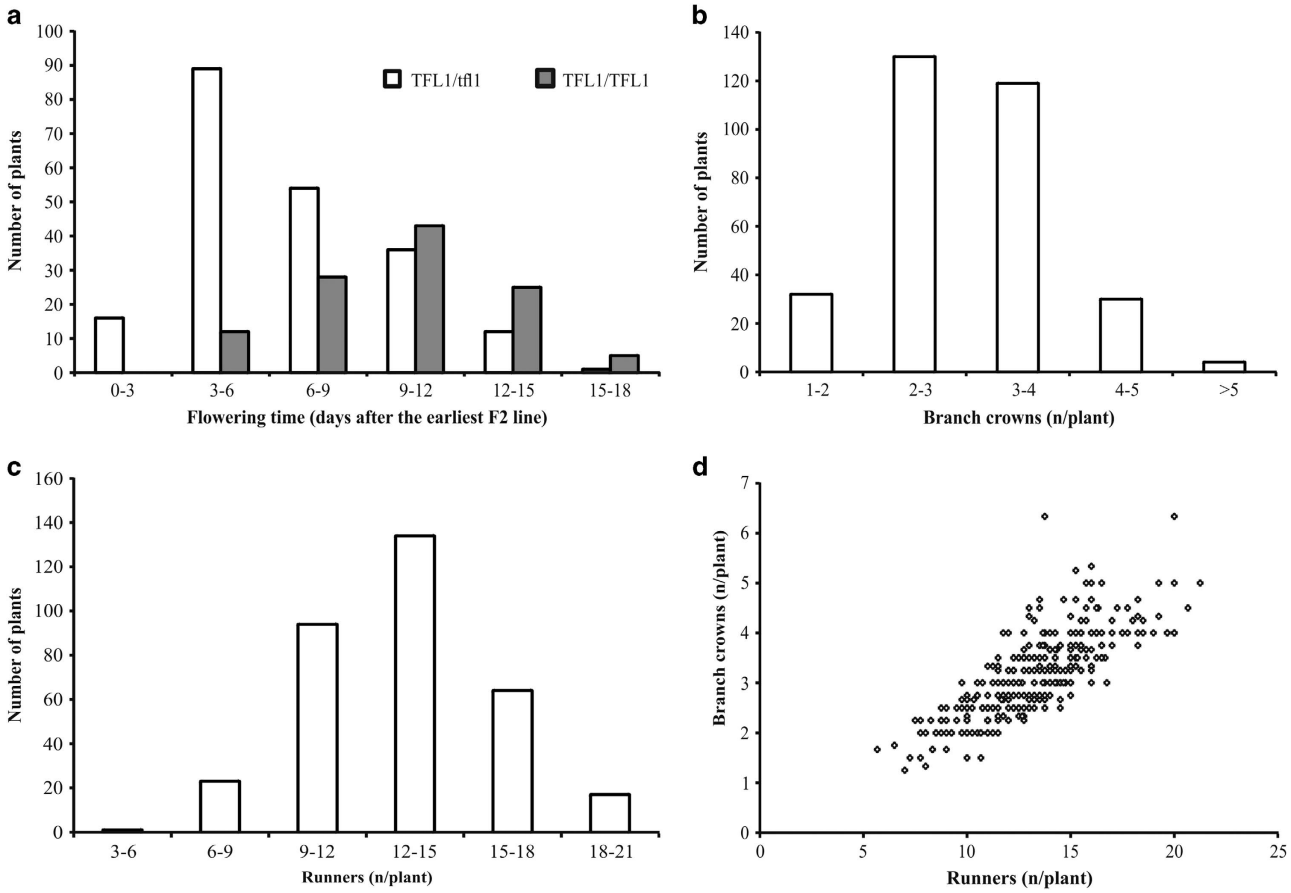

Figure 1. Segregation of flowering time and vegetative traits in H4 × FV mapping population. Segregation of flowering time (**a**), the number of branch crowns (**b**) and runners (**c**), and correlation between the number of branch crowns and runners (**d**) are shown. Flowering time data is shown separately for plants homozygote or heterozygote for functional *FvTFL1* alleles (FV alleles). Four clones of each of 335 F2 plants were phenotyped and mean values were used for analyses. Standard deviations of clones of each F2 line varied between 0.0 and 4.0 for flowering time, 0.0 and 2.1 for branch crowns, and 0.5 and 7.6 for runners.

1 755 780 tags (73.4%) were uniquely aligned on the H4 reference genome. Initial SNP calling revealed 133 930 SNPs, but after Hardy–Weinberg filtering and removal of SNPs that had more than 10% missing data and a minor allele frequency below 0.1, an ABH file of 4672 SNPs was retained. Following initial mapping, imputation and filtering, 2395 loci in 186 F2 lines, comprising 53 SSR/TSP markers and 2342 GBS-derived SNP markers coalesced into a linkage map of the seven expected linkage groups that covered a total genetic distance of 558 cM (Figure 2). Plotting genetic distances against the physical positions of the markers on the *Fvb* genome sequence assembly demonstrated that the genetic map was predominantly co-linear with the genome sequence (Figure 2) and thus marker placement on the map could be considered generally reliable.

Flowering time QTLs in greenhouse and field experiments

QTL analysis was first carried out using greenhouse flowering time data on the progeny of 335 seedlings and segregation data for 60 genetic loci. This analysis revealed four significant QTLs, one on both LG6 and LG7, and two on LG4 (Supplementary Figure 4). QTL analysis was also performed in growth chamber flowering time data using 32 F2 lines that were selected based on their extreme flowering time phenotypes in the greenhouse experiment. Using these lines, the same four QTLs with a LOD value above 3 were detected in both greenhouse and growth chamber flowering time data (Table 1). To increase the mapping resolution, we performed QTL analysis using the additional GBS based markers on selected 186 seedlings including individuals that exhibited informative recombination close to the QTL regions mapped in a larger progeny. This high-resolution mapping identified one additional QTL peak on LG7 and narrowed down the other QTL peaks (Figure 3). The peak of the LOD of the QTL on LG6 corresponded to the position of the *FvTFL1* gene previously identified by Koskela *et al.*[32] (Figure 3).[32] This QTL explained 17% of the observed variance; plants homozygous for 'FV' alleles of *FvTFL1* flowered later than heterozygous lines (Table 2). The peaks of the LOD for two significant loci on LG4 were associated with markers SFvb4_7853414 and bx083, both explaining about 15% of the observed variance. In both cases, the allele from the 'FV' parent promoted flowering. The peaks of the LOD of the significant QTLs on LG7 were associated with markers SFvb7_16204472 and SFvb7_21710529 that explained over 12% of the observed variance each, with the allele of the 'FV' parent promoting earlier flowering. The analysis of different haplotype combinations indicated that QTLs on all three LGs had additive effects on flowering time (Figure 4; Table 2). Plants with 'FV' markers in both LG4 and LG7 flowered first, whereas the latest plants typically had one or two 'H4' alleles in both LGs. In addition, lines containing two functional *FvTFL1* alleles tended to flower later than heterozygous lines, regardless of LG4 and LG7 marker genotypes.

The analysis of field phenotypes for flowering time revealed highly significant QTL only on LG4 (Figure 5). The peak of the LOD of this QTL was associated with marker bx083 as in the greenhouse dataset, but there was also another equally high

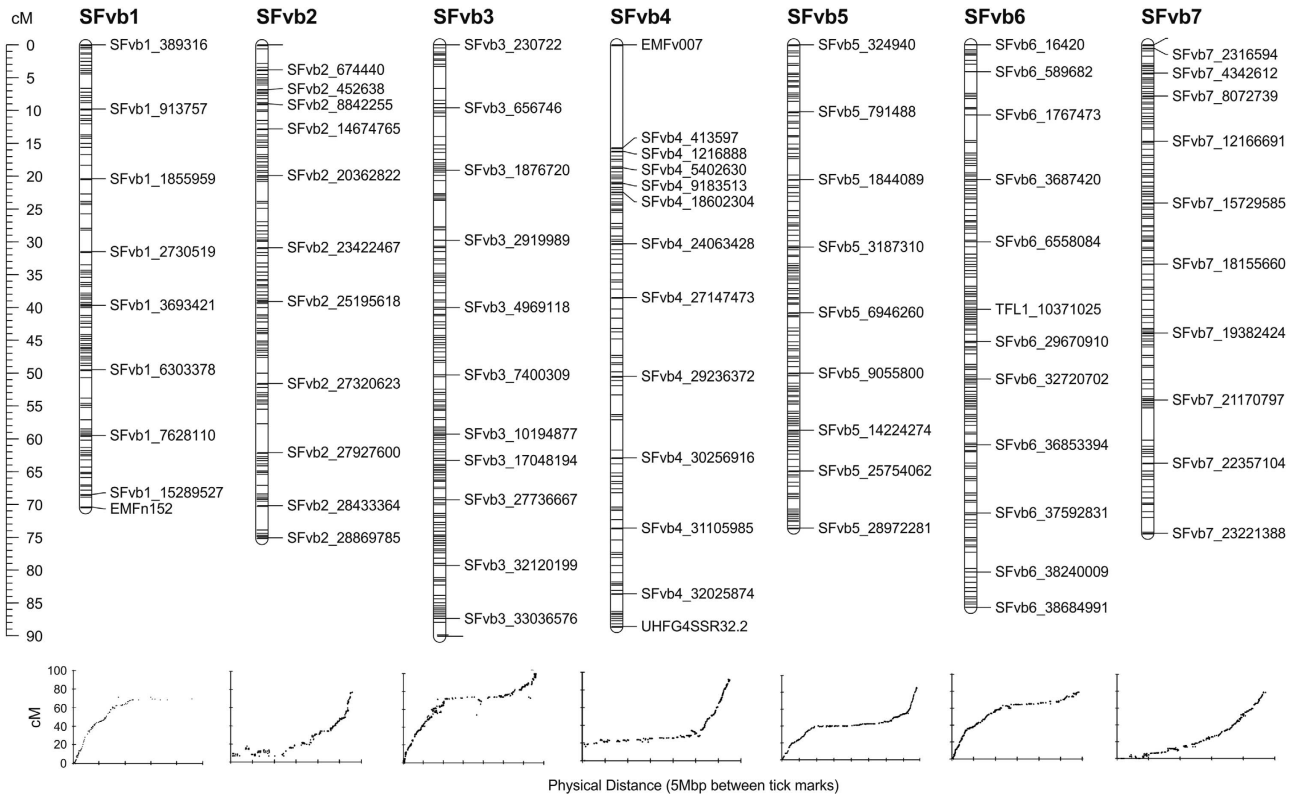

Figure 2. Linkage map of 186 F2 progeny of the H4 × FV cross comprising of 2395 molecular markers. The numbers at the top denote the chromosome number and the genetic distances are in centiMorgan (cM, scale in the left). In the small panels, all markers of each chromosome are plotted according to their position in the genome v.2.0 (*x*-axis) and in the genetic map (*y*-axis).

Table 1. Flowering time QTLs mapped in 32 F2 lines with extreme phenotypes

Marker	*Linkage group*	*Position (cM)*	*LOD value, growth chamber*	*LOD value, greenhouse*
UHFG4M19[a]	LG4	18.91	3.8	6.42
bx083	LG4	37.70	4.1	7.42
TFL1	LG6	17.84	5.01	13.59
BFaCT44	LG7	40.59	3.57	5.59

[a]Markers at 15.91–20.17 cM showed the same LOD value. The most significant markers for each QTL, their positions and LOD values in growth chamber and greenhouse experiments are shown.

peak ~ 1 Mb upstream of bx083. This QTL explained almost 25% of the phenotypic variance. Another minor QTL just above the genome-wide significance threshold of 3 was detected on LG2 with a peak of the LOD associated with the marker CFVCT020 at 11.94 cM. However, no QTLs were found on LG6 or LG7 in the field using phenotypic data of 186 F2 lines.

Runnering and branch crown evaluation

QTL were revealed on LG4 and LG5 associated with branch crown formation and runnering. The peak of the LOD of the significant QTL for the number of branch crowns was associated with the marker SFvb4_29399865 at 53.184 cM on LG4 and with the marker SFvb_25913832 at 65.718 cM on LG5 (Figures 6a and b), and QTLs for the number of runners were located in the same regions (Supplementary Figure 5). Both QTLs explained over 20% of the variance in the number of branch crowns, but their effect on runnering was less pronounced (Table 2). Increased numbers of runners and branch crowns were associated with 'FV' alleles on both LG4 and LG5.

Candidate genes for QTLs

To identify candidate genes around the QTLs, BLAST searches were carried out on the genomic regions between the flanking markers. In most cases, no obvious candidate genes were detected inside the narrow QTL peaks, but promising candidates were found very close to the highest LOD values. *FvFT2* (mrna04680.1-v1.0-hybrid) that was also identified in another QTL mapping study,[69] was found within the ~ 400 kb QTL region in the end of the LG4. However, no candidate genes were searched for the other QTL on the LG4 because the physical position of the QTL was uncertain. On LG7, a homolog of *EARLY FLOWERING 6* (*ELF6*, gene23255-v1.0-hybrid)[70] was located about 600 kb upstream of the first QTL peak, and a gene encoding a homolog of floral repressor TFL1 (*FvCENTRORADIALIS1*, *FvCEN1*; gene13304-v1.0-hybrid)[32] was found ~ 650 kb upstream of the second QTL peak.

Candidate genes were also identified within the QTL intervals controlling branch crown and runner formation. On LG4, a TCP transcription factor (FvTCP7, gene04759-v1.0-hybrid)[71] was found 150 kb upstream of the QTL peak. Furthermore, genes encoding two closely related MADS transcription factors that are homologous to DORMANCY ASSOCIATED MADS BOX (DAM, mrna12119.1-v1.0-hybrid, mrna12120.1-v1.0-hybrid),[2,72,73] were found inside the QTL region on the LG5. No non-synonymous variation was found in the coding sequence of any candidate gene between the parental genome sequences (data not shown).

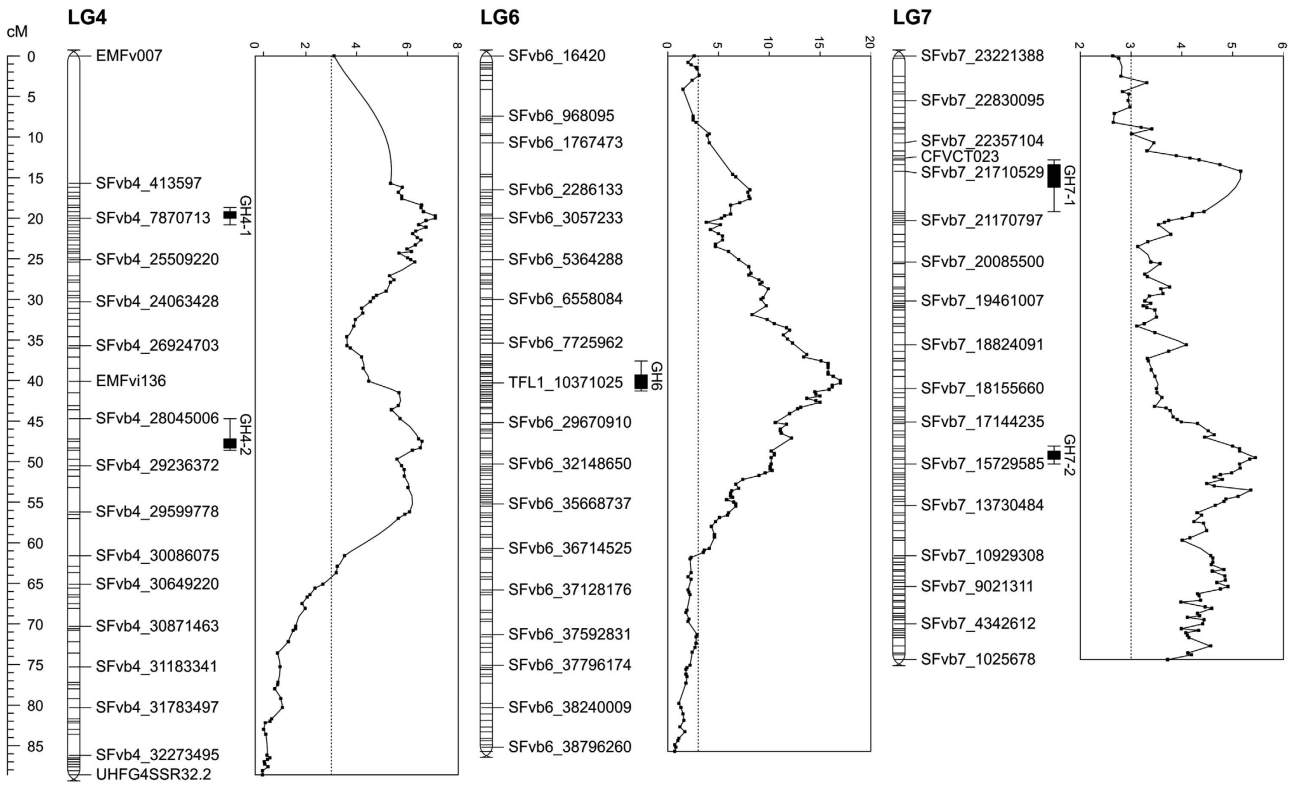

Figure 3. Flowering time QTLs in a greenhouse experiment. QTLs for flowering time were detected on chromosomes 4, 6, and 7. The genetic map (left) and LOD scores (right) are shown for each chromosome. Bars denote areas with the highest LOD scores (QTL regions). Field-grown plants were moved to the greenhouse in the middle of December 2011 for flowering time observations. Four clones of each of 186 F2 plants were phenotyped and mean values were used for the QTL analysis.

Table 2. QTLs mapped in the greenhouse experiment

QTL/ phenotype	*Marker*	*Position (bp)*	*Position (cM)*	*LOD value*	*Explained variance (%)*	*Dominance*	*Additive*
FT	SFvb4_7870713	Fvb4: 7870713	20.012	7.1	16.1	−0.137	1.807
FT	Bx083/ SFvb4_28667943	Fvb4: 28667943	47.485	6.58	15	0.427	1.784
FT	TFL1	Fvb6: 10371025	40.306	7.59	17	−4.380	1.623
FT	SFvb7_16204472	Fvb7: 16204472	24.895	5.45	12.6	0.232	1.775
FT	SFvb7_21710529	Fvb7: 21710529	14.215	5.16	12	−0.212	1.617
BC	SFvb4_29399865	Fvb4: 29399865	53.184	8.8	20.5	−0.053	−0.552
BC	SFvb5_25913832	Fvb5: 25913832	65.718	9.78	22.5	0.151	−0.582
RU	SFvb4_29399865	Fvb4: 29399865	53.184	6.56	15	0.536	−1.382
RU	SFvb5_25814268	Fvb5: 25814268	64.908	6.13	14.1	0.899	−1.271

Abbreviations: BC, branch crowns; FT, flowering time; RU, runners The most significant markers for each QTL, their positions and different genetic parameters are shown. Negative or positive value for dominance indicate that the allele of 'H4' or 'FV' parent has larger effect on the phenotype, respectively.

DISCUSSION

Extension of the strawberry production season in the open field is of significant economic importance, and two different breeding strategies have been implemented to maximize the length of the season. Several breeding programs are focusing on EB cultivars that can produce berries for several months during a single season,[6,8,42] whereas the breeding of early and late ripening cultivars is an alternative approach to extend the ripening season.[7] Previous QTL mapping studies in strawberry have focused on the perpetual flowering habit,[5,6,8,42] and no QTLs have been reported for earliness. Here, we report the identification of additive QTLs on three LGs that explain over 50% of the observed 18-day variance in flowering time in woodland strawberry mapping population previously reported by Koskela *et al.*[32] Moreover, we have found two QTLs that affect branch crown formation, the trait that has a direct effect on strawberry yield potential.[45,47,48]

Additive QTLs on three linkage groups control flowering time in woodland strawberry

QTL mapping using replicated greenhouse flowering time data in our H4 × FV F2 population revealed one new QTL on the LG4, two on the LG7, and two additional QTLs that co-localized with previously mapped QTLs on the LG4 and LG6. Four of these QTLs were also detected in a replicated growth chamber experiment using 32 F2 selected lines that exhibited extreme flowering time phenotypes in a greenhouse experiment, indicating that these QTLs are robust at least in controlled climate. In the non-replicated

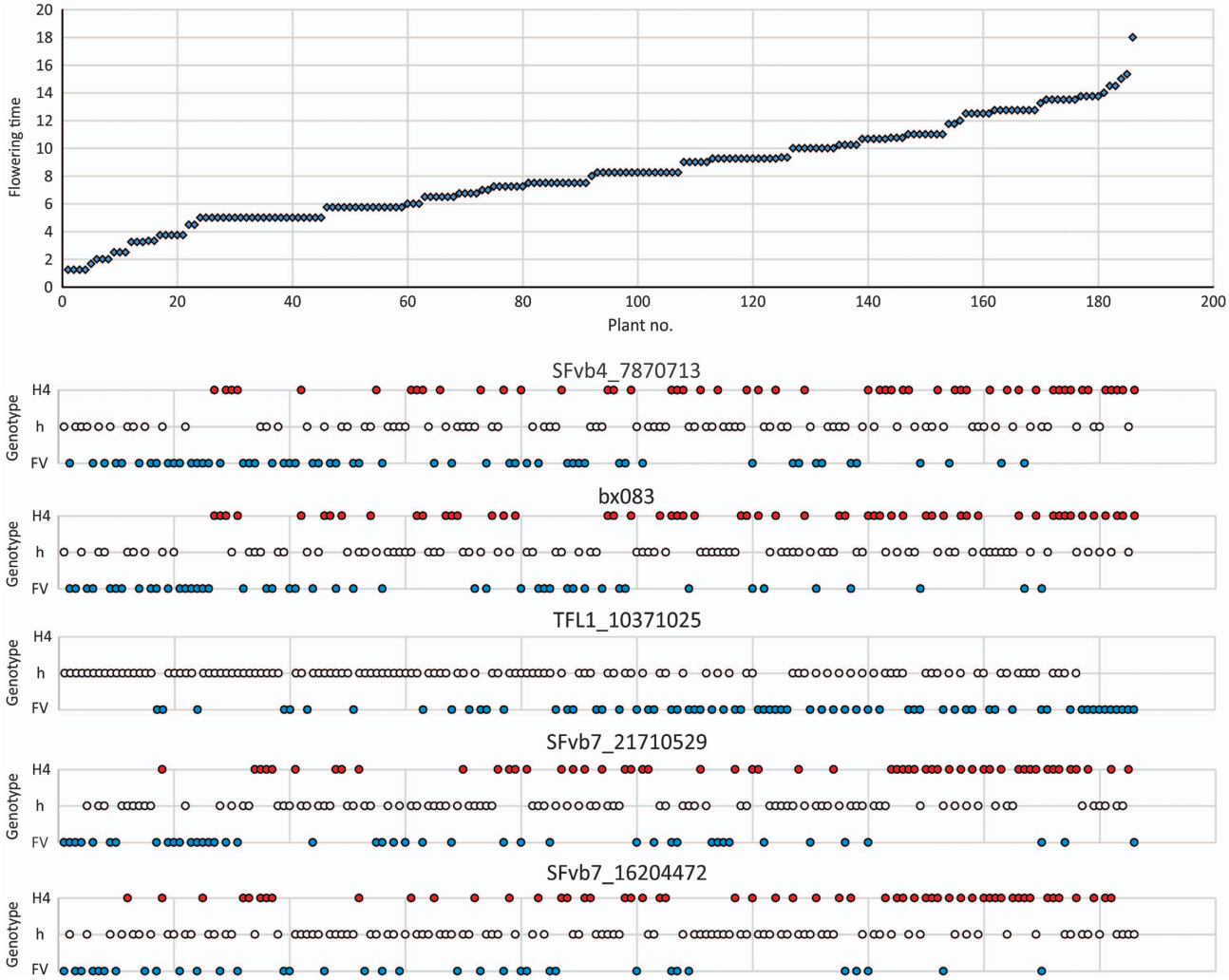

Figure 4. The effect of different haplotype combinations on flowering time. Flowering time of each 186 F2 line and their haplotypes on five QTL regions are shown. Haplotypes were inferred from the marker with the highest LOD score for each QTL region. H4 and FV denote parental genotypes (H4 = *F. vesca f. semperflorens* Hawaii-4, FV = *F. vesca* spp. *vesca*). h = heterozygote. Flowering time is shown as days after the earliest flowering line.

field experiment, the analysis using GBS data on 186 lines revealed major QTL only on LG4. However, QTL mapping on a larger population of 335 lines using SSR markers showed LOD values close to 4.0 in both LG6 and LG7 (data not shown), but a replicated field experiment is needed to confirm these QTLs in the field.

On the LG6, the QTL mapped on the same region than a previously characterized *FvTFL1* gene that encodes a repressor of flowering.[32] In cultivated strawberry, a major QTL for perpetual flowering and runnering (*PFRU*) was previously identified on the LG4.[6,8,42] A recent fine-mapping study placed this QTL close to marker bx083,[69] and the same marker was located in the QTL peak also in our greenhouse and field experiments. Therefore, our data suggest a new role for *PFRU* in the control of earliness, in addition to its role in the flowering period, at least in woodland strawberry. The effect of *PFRU* on flowering time seems to be robust in different environments. However, the other QTL, that is located upstream of *PFRU* on LG4, was only detected in controlled climate experiments indicating that it may function only in specific environments.

We found other QTLs on LG7 where no flowering related QTLs have been previously detected in strawberries. These QTLs showed high LOD values in the greenhouse experiment with the F2 population of 186 lines (GBS data), but not in the field indicating that they were also influenced by the environment. One of these QTLs matched also with the QTL region detected in our growth chamber experiment (Table 1). This QTL was associated with the marker BFaCT044 at 22 166 857 bp on LG7 in a set of 32 early and late flowering lines in both growth chamber and greenhouse, and about 400 kb upstream (the most significant marker SFvb7_21710529) in the GBS dataset of 186 lines.

The analysis of allele combinations on QTL positions demonstrated their additive effects on flowering time (Figure 4; Table 2). Clearly, 'H4' specific markers on LG4 and LG7 QTL regions, in both heterozygous and homozygous condition delayed flowering. Furthermore, homozygous *FvTFL1* alleles from 'FV' parent caused additional delay compared to heterozygous plants. Plants homozygous for 'H4' alleles of *FvTFL1* were excluded from this study because they exhibit perpetual flowering habit.[32]

Candidate genes for flowering time QTLs

We searched for candidate flowering time genes in the genomic regions containing the markers with the highest LOD if the physical and genetic maps on the region were mostly co-linear

(Figure 2). This was not the case for the newly identified QTL on the LG4, and therefore, a better genome assembly is needed to enable reliable candidate gene search on this region.

Candidate genes for *PFRU*, that co-localize with the QTL identified in our greenhouse and field studies on the LG4, has been recently reported.[69] These genes include *FvFT2* and *FvCDF2* that are homologs of known regulators of photoperiodic flowering. In *Arabidopsis*, CDFs are floral repressors that contribute to photoperiodic flowering by down-regulating both *CO* and *FT* mRNA expression especially in the morning.[74] Furthermore, specific alleles of potato *CDF* gene were selected during domestication allowing the cultivation of this SD crop in LD conditions in northern latitudes.[75] Tissue specific gene expression analyses in woodland strawberry showed that *FvFT2* is mainly expressed in flowers and fruits.[32,76] However, both FvFT2 and FvCDF2 may also have important roles in the control of photoperiodic development in strawberries and are thus good candidates for future functional studies.

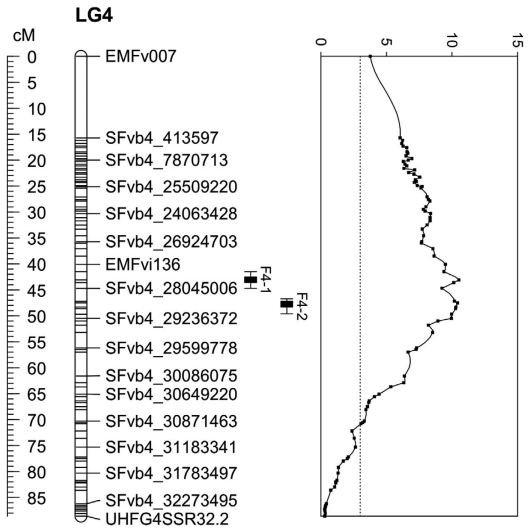

Figure 5. Flowering time QTL on the chromosome 4 in the field experiment. The genetic map (left) and LOD scores (right) are shown. Bars denote areas with the highest LOD scores (QTL regions). H4 × FV F2 lines ($n = 186$) were grown in the field and flowering time was observed in summer 2011.

FvELF6 was identified as a candidate gene for the QTL on LG7. ELF6 was originally identified as a repressor of photoperiodic flowering in *Arabidopsis*.[70] It is a histone demethylase that control gene expression through chromatin regulation.[77,78] As histone methylation is a common mechanism in the control of flowering time,[79] and a homologous gene to *ELF6* co-localizes with a QTL for spring heat requirement for flowering on almond LG2,[2] thus, *ELF6* is an interesting candidate for future studies.

The homolog of *TFL1/CEN* is located in the QTL region for flowering time in peach [72,80] and gene functional studies showed that *TFL1* homologs encode major floral repressors in several rosaceous species.[32–35] Interestingly, *TFL1/CEN* homologs were identified as candidate genes for two QTL regions in this study. A QTL on the LG6 co-localize with *FvTFL1* that encodes a major floral repressor,[32] and closely related *FvCEN1* is located near the second QTL peak on LG7 and awaits for functional validation. Our finding that plants containing only one functional *FvTFL1* copy flowered

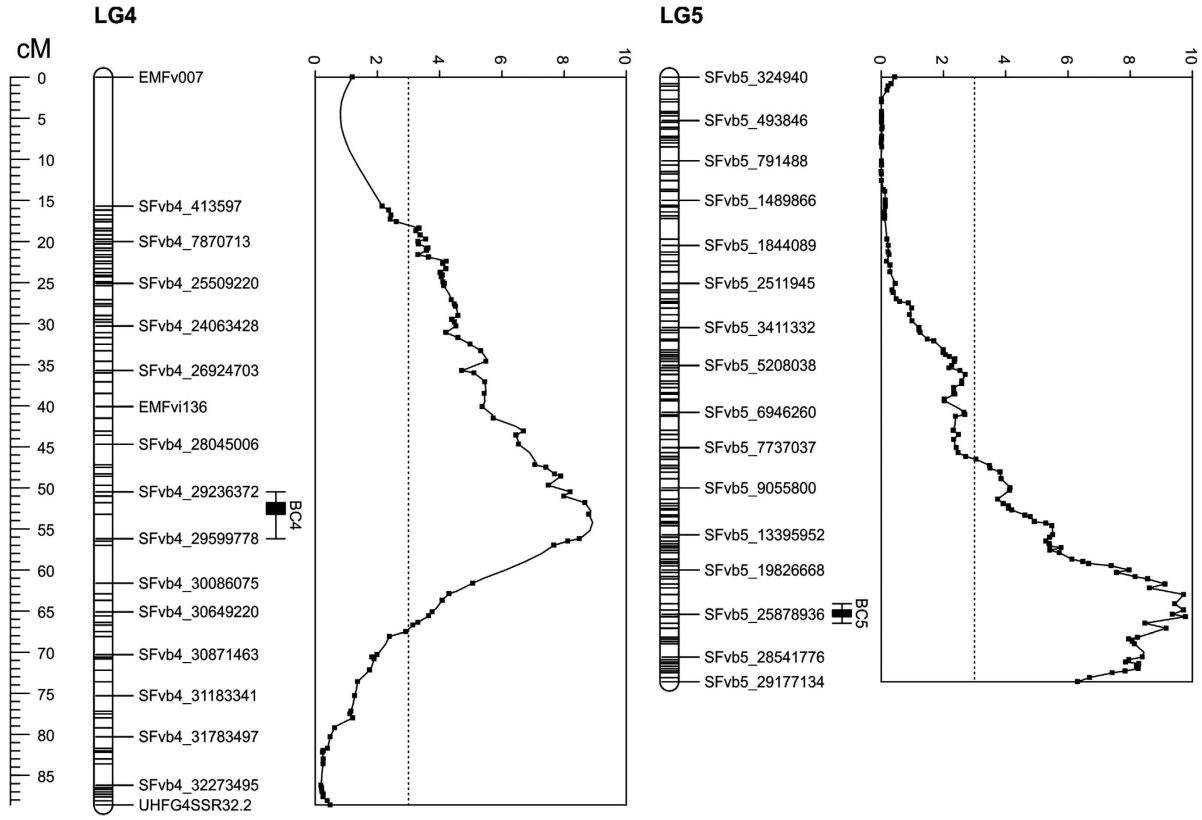

Figure 6. QTLs for the number of branch crowns in the greenhouse experiment. Two QTLs for the number of branch crowns (BC4 and BC5) were detected in chromosomes 4 and 5. The genetic map (left) and LOD scores (right) are shown for each chromosome. Bars denote areas with the highest LOD scores (QTL regions). Field-grown plants were taken into the greenhouse in the middle of December 2011 for growth observations. Four clones of each of 186 F2 plants were phenotyped and mean values were used for the QTL analysis.

earlier than plants homozygous for functional 'FV' alleles provides interesting opportunities to control flowering time, particularly in polyploid crops such as the octoploid cultivated strawberry. In fact, a recent report showed that FaTFL1 is a major floral repressor also in cultivated strawberry, and that altered regulation of *FaTFL1* mRNA expression is associated with different flowering responses in this species.[43] To enable more efficient breeding of flowering time, further studies are needed to uncover whether the variation in *FaTFL1* expression between cultivars is caused by allelic variation of *FaTFL1* itself or by upstream regulators. Among these regulators, the expression of *FT1* and *SOC1* homologs correlate with *TFL1* mRNA levels only in specific conditions in woodland and cultivated strawberries suggesting that *TFL1* alleles or its other regulators should be major targets of future investigations.[39,43] Whether some of the candidate genes identified in this study control flowering time through *FvTFL1* is an interesting open question.

Identification of QTLs controlling crown branching and runner formation

Although we mainly focused on flowering time, we also identified two QTLs on the LG4 and LG5 controlling the number of branch crowns and runners in woodland strawberry. These traits showed a strong positive correlation because the production of branch crowns increases the number of axillary buds that are able to form runners under LD conditions. Therefore, these QTL primarily control the branching of the leaf rosette in woodland strawberry. On the LG4, the QTL co-localized with previously mapped *PFRU* locus.[6,69] However, the peak of the LOD was located about 700 kb downstream of the flowering time QTL mapped in this study. This indicates that two closely located QTLs may control flowering and axillary bud differentiation to runners and branch crowns in our population, but additional studies are needed to confirm this hypothesis.

We identified a gene encoding TCP transcription factor close to the most significant marker on the LG4. This TCP, recently named as FvTCP7,[71] shows the highest similarity with *Arabidopsis* TCP14 and TCP15, which control cell proliferation and internode elongation.[81] As runners are long shoots with elongated internodes this is a promising candidate gene for the strawberry axillary bud differentiation. Initial characterization of *FvTCP7* indicated that it is highly expressed in vegetative tissues including runners, but also in flower buds, and the protein is localized to nucleus.[71] However, the function of FvTCP7 in strawberry is unknown. Further studies are needed to elucidate what is the relationship between the branching QTL detected here and *PFRU* that controls runner formation, in addition to perpetual flowering, in cultivated strawberry.[6,8]

Also the newly identified QTL region on the LG5 contained interesting candidate genes for future studies including two genes homologous to *DAM* and *SHORT VEGETATIVE PHASE* (*SVP*) that encode major regulators of dormancy in Rosaceae and floral repressor in *Arabidopsis*, respectively.[2,72,73,82] Although strawberries do not have true dormancy, their growth vigor is strongly reduced under SDs in autumn including the cessation of runner formation, and a long period of chilling is needed to resume normal vegetative development.[83] Functional studies would reveal whether identified FvDAM transcription factors control dormancy and what is their role in the control of axillary bud differentiation. Furthermore, the possible interaction of the branching/runnering QTLs identified in this study with the FvSOC1 and gibberellin pathway, that were previously shown to control runnering in woodland strawberry,[37,49] should be elucidated.

CONCLUSIONS

Here, we have reported the mapping of three new flowering time QTLs in woodland strawberry, which co-localize with homologs of known flowering time regulators that have not been functionally characterized in this species. In addition, we have shown that *PFRU*, another QTL that was previously reported to control the duration of flowering and the number of runners in cultivated strawberry,[6,69] affects flowering time, crown branching and runner formation in woodland strawberry. Although we were able to detect four out of five flowering time QTLs in two independent experiments in controlled climate, only one of these showed a LOD value above 3 in the non-replicated field experiment. Therefore, further replicated experiments are needed to reveal the role of these QTLs in different environments. On the basis of the evidence shown here, identified QTLs control flowering time additively along with a previously identified floral repressor FvTFL1 that has quantitative effect on flowering time. As *TFL1* homologs likely integrate both light and temperature signals to control flowering time in woodland and cultivated strawberries,[39,43] we suggest that candidate genes on the LG4 and LG7 QTL regions may control flowering time through *TFL1*. However, further studies are needed to clarify causal genes and to reveal their roles in the control of flowering time and vegetative traits. As cultivated strawberry is an octoploid species with at least two subgenome donors,[27,84] we hypothesize that it may contain significant genetic variation in these regulators that may enable fine tuning of flowering time and shoot architecture in different environments through marker assisted selection or genomic selection strategies that have recently been developed in this species.[85]

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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RESEARCH PAPER

Fragaria vesca CONSTANS controls photoperiodic flowering and vegetative development

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Abstract

According to the external coincidence model, photoperiodic flowering occurs when *CONSTANS* (*CO*) mRNA expression coincides with light in the afternoon of long days (LDs), leading to the activation of *FLOWERING LOCUS T* (*FT*). CO has evolved in Brassicaceae from other Group Ia CO-like (COL) proteins which do not control photoperiodic flowering in Arabidopsis. COLs in other species have evolved different functions as floral activators or even as repressors. To understand photoperiodic development in the perennial rosaceous model species woodland strawberry, we functionally characterized FvCO, the only Group Ia COL in its genome. We demonstrate that FvCO has a major role in the photoperiodic control of flowering and vegetative reproduction through runners. FvCO is needed to generate a bimodal rhythm of *FvFT1* which encodes a floral activator in the LD accession Hawaii-4: a sharp *FvCO* expression peak at dawn is followed by the *FvFT1* morning peak in LDs indicating possible direct regulation, but additional factors that may include FvGI and FvFKF1 are probably needed to schedule the second *FvFT1* peak around dusk. These results demonstrate that although FvCO and FvFT1 play major roles in photoperiodic development, the *CO*-based external coincidence around dusk is not fully applicable to the woodland strawberry.

Key words: CONSTANS, FLOWERING LOCUS T, *Fragaria*, photoperiod, reproduction, runner, strawberry.

Introduction

Plants use various environmental cues, such as light and temperature, to synchronize their life cycles according to local climate (Yanovsky and Kay, 2003). The external coincidence model indicates how environment is linked to flowering in Arabidopsis (Salomé and McClung, 2004; Nozue *et al.*, 2007). According to this model, flower induction takes place when external stimuli such as photoperiod meet with the active phase of an internal oscillator (Sawa *et al.*, 2008). A small transcription factor CONSTANS (CO) is at the heart of the external coincidence model (Suárez-López *et al.*, 2001; Valverde *et al.*, 2004): photoperiodic flowering occurs when the *CO* mRNA expression peaks at the end of the light period in long days (LDs) and CO activates the expression of *FLOWERING LOCUS T* (*FT*) that encodes a mobile flowering-inducing signal (Corbesier *et al.*, 2007; Tamaki *et al.*, 2007).

The circadian clock indirectly generates the rhythmic expression of *CO*. The basic mechanism of this clock involves

feedback loops of genes that are expressed in different phases of the daily cycle (McClung, 2009). The core feedback loop, formed by *TIMING OF CAB EXPRESSION* (*TOC1*) and *LATE ELONGATED HYPOCOTYL/CIRCADIAN CLOCK ASSOCIATED1* (*LHY/CCA1*) (Yanovsky and Kay, 2003; Más, 2008; McClung, 2008), generates the rhythmic expression of several flowering time regulators including FLAVIN-BINDING, KELCH REPEAT, AND F-BOX 1 (FKF1), GIGANTEA (GI), and CYCLING DOF FACTOR1 (CDF1) (Huq *et al.*, 2000; Mizoguchi *et al.*, 2005; Fornara *et al.*, 2009). In the morning, CDF1 directly binds to the promoter of *CO* to suppress the transcription of the gene (Imaizumi *et al.*, 2005). CDF is degraded by the GI–FKF1 protein complex during the day, leading to a peak in *CO* expression in the afternoon (Sawa *et al.*, 2007).

Along with transcriptional regulation, CO protein concentration is also strictly regulated by light. CO is unstable in darkness and in the morning, when E3 ubiquitin ligase HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES1 (HOS1) and phytochrome B (PHYB) activated by red light together destabilize CO (Valverde *et al.*, 2004; Lazaro *et al.*, 2015). In the afternoon, however, phytochrome A and cryptochrome 2 (PHYA and CRY2), which respond to far-red and blue light, respectively, stabilize CO. Therefore, CO protein only accumulates under LD conditions when *CO* mRNA expression peaks during the light period. This results in the activation of *FT* and thus flowering. On the other hand, under short-day (SD) conditions, *CO* mRNA expression peaks in the middle of the night, when CO protein cannot accumulate sufficiently to activate *FT* (Blázquez and Weigel, 1999; Suárez-López *et al.*, 2001; Izawa *et al.*, 2002; Valverde *et al.*, 2004; Endo *et al.*, 2005).

CO has two B-box type zinc finger domains which have been proposed to function in protein–protein interaction (Putterill *et al.*, 1995; Robson *et al.*, 2001). It also has one CCT (CO, CO-like, TOC1) domain on its C-terminus which mediates protein–protein interaction and nuclear localization (Robson *et al.*, 2001; Ben-Naim, 2006; Wenkel *et al.*, 2006). A total of 16 *CO* homologous genes, all of them with at least one B-box domain and one CCT domain, have been isolated from Arabidopsis and designated as *CO*-like (*COL*) *1–16* (Robson *et al.*, 2001; Griffiths *et al.*, 2003). These genes were allocated to Groups I–III, according to the degree of conservation and number of B-box domains (Robson *et al.*, 2001). Group I, which includes the *CO* gene, was subdivided into Ia–Id according to the extent of conservation of four highly conserved regions in the middle (Griffiths *et al.*, 2003). A recent study has provided evidence that *COL1* and *COL2*, that do not encode floral promoters, are ancestral Group Ia *COL* genes; the floral promoter CO evolved within the Brassicaceae after the family split from the Cleomaceae (Simon *et al.*, 2015). In addition to distinct functions, these *COL* genes show the highest expression at dawn, in contrast to *CO* which peaks in the afternoon (Ledger *et al.*, 2001; Simon *et al.*, 2015).

CO homologues have been isolated from other plants including woody plants, monocotyledons, and even single-celled *Chlamydomonas* (Song *et al.*, 1998; Lagercrantz and Axelsson, 2000; Yuceer *et al.*, 2002; Griffiths *et al.*, 2003; Nemoto *et al.*, 2003; Chia *et al.*, 2008; Holefors *et al.*, 2009; Serrano *et al.*, 2009). In the SD plant rice (*Oryza sativa*), the *CO* homologue *Heading date1* (*Hd1*) promotes expression of the *FT* homologue *Hd3a* under inductive SDs (Izawa *et al.*, 2002; Ishikawa *et al.*, 2005). Other *CO* homologues, *OsCO3* (*OsB*) and *OsCOL10*, have a negative effect on the expression of *Hd3a* under these conditions (Kim *et al.*, 2008; Tan *et al.*, 2016). Several *CO*-like genes have also been identified in *Chrysanthemum* spp., and one of these was found to promote *FT* expression and flowering (Fu *et al.*, 2015). However, studies in *Pharbitis nil* and *Medicago truncatula* indicated that their *COL* genes are not involved in the control of *FT* expression and flowering (Hayama *et al.*, 2007; Wong *et al.*, 2014).

FT has been shown to function as a floral activator in SD, LD, and day-neutral plants, while another member of the same gene family, TERMINAL FLOWER1 (TFL1), is a floral repressor (Wickland and Hanzawa, 2015). However, there are several independent examples about the evolution of FT homologues into floral repressors including BvFT1 in sugar beet, three FT homologues in tobacco, and a specific splicing variant of *Brachypodium* FT (Pin *et al.*, 2010; Harig *et al.*, 2012; Qin *et al.*, 2017). In seasonal flowering commercial strawberry (*Fragaria×ananassa* Duch.) and the diploid model woodland strawberry (*Fragaria vesca* L.), which are both SD plants (Ito and Saito, 1962; Battey *et al.*, 1998; Battey, 2000), TFL1 homologues are strong floral repressors. *FvTFL1* and *FaTFL1* are highly expressed under LDs, and their repression under SDs and low temperature conditions enables flower induction to take place (Koskela *et al.*, 2012; Nakano *et al.*, 2015; Rantanen *et al.*, 2015; Koskela *et al.*, 2016). Interestingly, *FT* homologues, *FvFT1* and *FaFT1*, are expressed specifically under LDs and correlate negatively with flower induction, indicating that they may also repress flowering in SD strawberries (Koskela *et al.*, 2012; Nakano *et al.*, 2015). A natural mutant of woodland strawberry (*F. vesca semperflorens*) lacks functional FvTFL1 and is an LD plant which flowers perpetually after flower induction (Koskela *et al.*, 2012). In this mutant, *FvFT1* is also expressed under LDs and functions as a promoter of flowering (Koskela *et al.*, 2012; Rantanen *et al.*, 2014). *FvFT1* is normally expressed diurnally with peaks 4 h and 16 h after dawn; its expression is most effectively induced artificially by FR daylength extension in the mutant. (Koskela *et al.*, 2012; Rantanen *et al.*, 2014).

A close *CO* homologue (*FvCO*) has been previously identified in woodland strawberry (Shulaev *et al.*, 2011), but its function has not been tested. Here, using transgenic overexpression and RNAi lines of woodland strawberry, we demonstrate that *FvCO* has a major role in the photoperiodic development of this species. We show that, although the gene expression rhythms of *FvCO* and *FvFT1* do not coincide, *FvCO* is needed to activate *FvFT1* that controls reproductive and vegetative development in response to photoperiodic signals.

Materials and methods

Plant material

Experiments were mostly performed with the LD-flowering accession 'Hawaii-4' (H4; National Clonal Germplasm Repository

accession number PI551572). Gene expression analyses were also carried out in a Finnish SD accession FIN56 (PI551792). Seedlings or plants clonally propagated from runner cuttings were used for the experiments as indicated in the text and figure legends.

Growth conditions and phenotypic observations

Plants were raised in a growth chamber or a greenhouse under a non-flower-inductive photoperiod at 20–22 °C, under SDs (12/12 h light/dark) for H4 and LDs (16/8 h light/dark) for FIN56. Fluorescent tubes (Warm white 30W/32-930, Osram, Germany) or light-emitting diodes (LEDs; AP67, Valoya, Finland) were used as the white light source at a photosynthetic photon flux density (PPFD) of 200 µmol m^{-2} s^{-1} in growth chambers. High pressure sodium (HPS) lamps (Airam 400W, Kerava, Finland) at a PPFD of 120 µmol m^{-2} s^{-1} were used to supplement the natural light in the greenhouse. Seedlings were transplanted to 8 × 8 cm pots at the cotyledon stage, while runner cuttings were directly rooted in these pots. Fertilized peat (Kekkilä, Finland) supplemented with 25% (v/v) vermiculite (Ø2 mm) was used as a growing medium. Plants were fertilized with liquid fertilizer (Kekkilä; N-P-K: 17-4-25) biweekly.

Both flowering time and vegetative development were studied in the experiments. To observe flowering time differences between H4 and transgenic lines, either the number of leaves in the primary leaf rosette before the terminal inflorescence or the number of days before the first open flower was recorded. In addition, the differentiation of axillary buds into either axillary leaf rosettes called branch crowns or runners (stolons) was observed.

Gene expression analysis

Leaf and shoot apex samples were frozen in liquid nitrogen and stored at –80 °C before total RNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) method as described in Koskela *et al.* (2012). cDNAs were synthesized from 1 µg of total RNA using Superscript III reverse transcriptase (Invitrogen). SYBR Green I master mix (Roche) was used for real-time PCRs which were performed in the Light Cycler 480 instrument (Roche) as described previously (Mouhu *et al.*, 2009). Real-time PCR reactions were performed with three technical replicates and two or three biological replicates as mentioned in the figure legends. Relative expression levels were calculated by the ΔΔCt (cycle threshold) method, with *FvMSI1* as the normalization gene as described previously (Mouhu *et al.*, 2013). Primers used in the real-time PCR are listed in Supplementary Table S1 at *JXB* online. Primer efficiencies were almost equal for all primer pairs (Rantanen *et al.*, 2014).

Plasmid constructs

Plasmid constructs for overexpression and RNAi silencing lines were created according to Gateway technology with Clonase II (Invitrogen). For *FvCO* overexpression and RNAi constructs, cDNA from *F. vesca* H4 was amplified with primer pairs 5Y76J-(attB1)-TGAGAGTGAGGAGGAAACAACA-3' and 5'-(attB2)-TTGCTGCAAAAGGTTGAACT-3', and 5'-(attB1)-ACAATCCGGTATGCCTCAAG-3' and 5-(attB2)-AGGAACAATGCCGTATCCAG-3', respectively. The destination vectors were pK7WG2D.1 for overexpression and pK7GWIWG2D(II) for RNAi silencing (Karimi *et al.*, 2002). Both vectors contain green fluorescent protein as a positive selection marker.

Transformation

Vectors carrying overexpression and RNAi constructs were electroporated into *Agrobacterium tumefaciens* strain GV3101 and transformed into H4 as described previously (Oosumi *et al.*, 2006). Several transgenic lines were generated for both constructs. Transgenic lines were selected for the experiments based on their phenotypes and *FvCO* expression levels.

Sequence alignment and phylogenetic analysis

Amino acid sequence alignment was conducted using the ClustalW program with the BLOSUM62 matrix. MrBayes 3.2.2 was used to construct a Bayesian estimation of a phylogeny of CO-like proteins. Two independent runs were performed, the averaged. WAG (Whelan and Goldman) matrix was used as a substitution model, and gamma distribution was set for among-site rate variation with the rate category of 4. The Markov chain Monte Carlo algorithm was run with chain length of 1 000 000 with four heated chains (heated chain temperature=0.2). Subsampling was performed every 200 generations and burn-in length was set to 10%. CrCO from *Chlamydomonas reinhardtii* was used as the outgroup.

Statistical analyses

ANOVA was conducted on the averages using the general linear model, and differences between means were analysed by Tukey–Kramer test. All statistical analyses were conducted using the R package (ver. 3.3.2).

Accession numbers

Sequence data from this article can be found in the GenBank/National Center for Biotechnology Information data library under the following accession numbers: *FvSOC1* (FJ531999) and *FvFT1* (JN172098). Predicted gene models (Hybrid V2) can be found in the Genome Database for Rosaceae (http://www.rosaceae.org): *FvCO* (gene04172), *FvMSI1* (gene03001), gene02008, gene03742, gene14015, gene14981, gene15552, gene24941, gene25039, gene25171, and gene27383. Accession numbers of the protein sequences used in the phylogenetic analysis are listed in Supplementary Table S2.

Results

Isolation, structure, and phylogenetic analysis of Fragaria CO

One woodland strawberry homologue of *CO*, *FvCO* (gene04172), was previously annotated in the *F. vesca* whole-genome v1.1 assembly (Shulaev *et al.*, 2011). To explore the strawberry *CO*-like gene family, a BLASTx database search was performed using the full-length sequence of *FvCO* against the whole-genome assembly. In total, nine additional putative CO-like protein sequences longer than 200 amino acids were identified. These protein sequences were subjected to a phylogenetic analysis to identify putative regulators of flowering time.

A phylogenetic tree of COL proteins was constructed using CrCO from *C. reinhardtii* as the outgroup. FvCO was placed in the same clade with CO homologues of eastern cottonwood, morning glory, and tomato, and with Arabidopsis Group Ia proteins CO, COL1, and COL2 (Fig. 1; Supplementary Fig. S1). The predicted protein for gene14981 was placed in the clade comprised of *Malus domestica* CO-like proteins, BvCOL2 of sugar beet, and Arabidopsis COL3 and COL4, which are categorized as CO Group Ib proteins (Griffiths *et al.*, 2003; Chia *et al.*, 2008); the predicted protein for gene27383 was close to COL5 (Fig. 1; Supplementary Fig. S1). Other predicted proteins clustered in Group II (gene03742 and gene25171) or Group III (gene14015, gene15552, and gene24941); gene02008 and gene25039 made up an isolated clade of their own (see Supplementary Fig. S1).

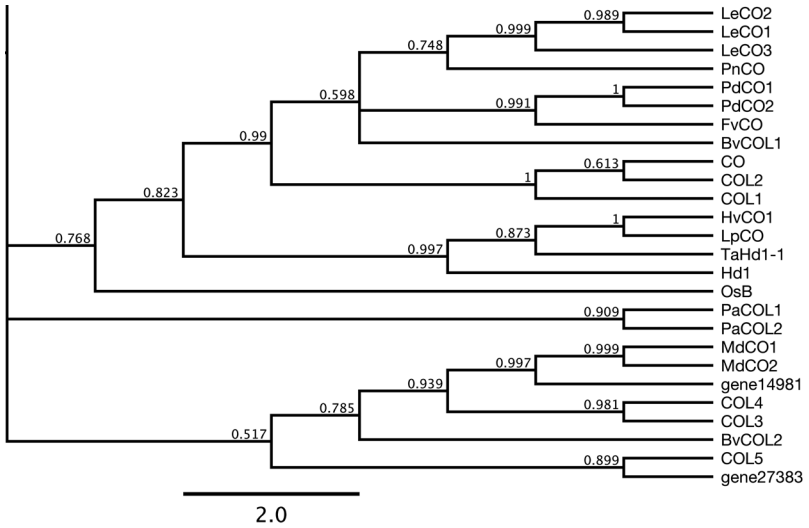

Fig. 1. A phylogenetic tree of COL proteins from woodland strawberry and other species. A part of the phylogenetic tree containing Group I genes is shown. The full tree structure is available as Supplementary Fig. S1. The list of species and protein accessions is available in Supplementary Table S2. Numbers on each node indicate posterior probabilities.

As the phylogenetic tree indicated that *FvCO*, gene14981, and gene27383 belong to Group I, conserved domains of the corresponding protein sequences were subjected to further analysis (Supplementary Fig. S2a). Predicted protein sequences of these genes were aligned with other CO-like proteins by ClustalW. The alignment showed that two B-box domains (Griffiths *et al.*, 2003) and the CCT domain (Wenkel *et al.*, 2006) were highly conserved in these three *Fragaria CO*-like sequences (Supplementary Fig. S2b, c). FvCO showed the highest level of conservation in the M1–M4 conserved regions. Gene27383 had glutamate to aspartate, tryptophan to leucine, and leucine to isoleucine substitutions in the M1 region found in Group Ia–Ic (Glu-X_2-Ser-Trp-Leu-Leu), while the other two have a conserved sequence (Supplementary Fig. S2d). The M2 region (Leu-Val-Asp/Gly-Tyr) of FvCO had an aspartate/glycine to glutamate substitution similarly to PnCO (Group Ia), and the other two were lacking valine similarly to COL3 and COL4 (Group Ic) (Supplementary Fig. S2e). The M3 region (Gly-X-Asp/Glu-X-Ile/Val-Val-Pro) of gene14981 had a substitution of the first glycine residue to alanine (Supplementary Fig. S2f), and the M4 region (Ser-X-Glu/Asp-X_3-Val-Pro) of gene14981 had a substitution of the first serine to proline (Supplementary Fig. S2g). As the phylogenetic tree and further analyses on conserved domains indicated that FvCO was the only Group Ia COL protein encoded by the accessible woodland strawberry genome, functional analysis was mainly focused on FvCO.

FvCO *expression peaks at dawn*

The diurnal expression patterns of *FvCO* and *FvFT1* were investigated in woodland strawberry accessions with contrasting photoperiodic responses. In the perpetual flowering LD accession H4and the seasonal flowering SD accession FIN56, *FvCO* exhibited a single mRNA expression peak at dawn under both LD and SD conditions, and its expression stayed low during the day regardless of the accessions examined (Fig. 2A; Supplementary Fig. S3). In H4 under LDs, the expression of *FvFT1* peaked 4–8 h after dawn and again in the evening (ZT16–ZT20), the second peak being slightly higher than the first (Fig. 2B). A similar diurnal rhythm of *FvFT1* mRNA expression was observed in FIN56 under LDs, but the morning peak was higher than the evening peak (Supplementary Fig. S3). The morning peak of *FvFT1* followed that of *FvCO*, but the evening peak of *FvFT1* did not coincide with high *FvCO* mRNA levels. In H4 under SDs, the *FvFT1* mRNA level was very low or undetectable throughout the 24 h cycle (Fig. 2B). In addition to *FvCO*, we explored the rhythmic expression of two *COL* genes showing the highest homology with *FvCO*. The gene27383 exhibited rhythmic expression, peaking at the same time as *FvCO*, whereas the expression of gene14981 did not show a clear rhythm (Supplementary Fig. S4).

Our data indicated that *FvCO* expression peaked at dawn under different photoperiods, so we tested whether the dawn signal was critical for the timing of its expression. LD-grown plants were transferred to darkness (DD) and *FvCO* mRNA levels measured. Under DD conditions, in contrast to the LD control, *FvCO* expression continued to rise after the subjective dawn (the beginning of the light period in the LD control) and stayed high during the next 8 h (Fig. 2C). These results suggest that the up-regulation of *FvCO* takes place in darkness and the dawn signal is needed for its down-regulation.

FvCO controls vegetative and generative development

To test the role of *FvCO* in the photoperiodic control of vegetative and reproductive development, we generated transgenic plants of the H4 accession with *FvCO* overexpressed [driven by the *Cauliflower mosaic virus* (CaMV) *35S* promoter] or RNAi silenced. The expression levels of *FvCO* mRNA were clearly altered in these transgenic lines (Fig. 3A, B). In the overexpression lines, strong up-regulation of *FvCO* was observed especially in the evening (ZT16) when its expression

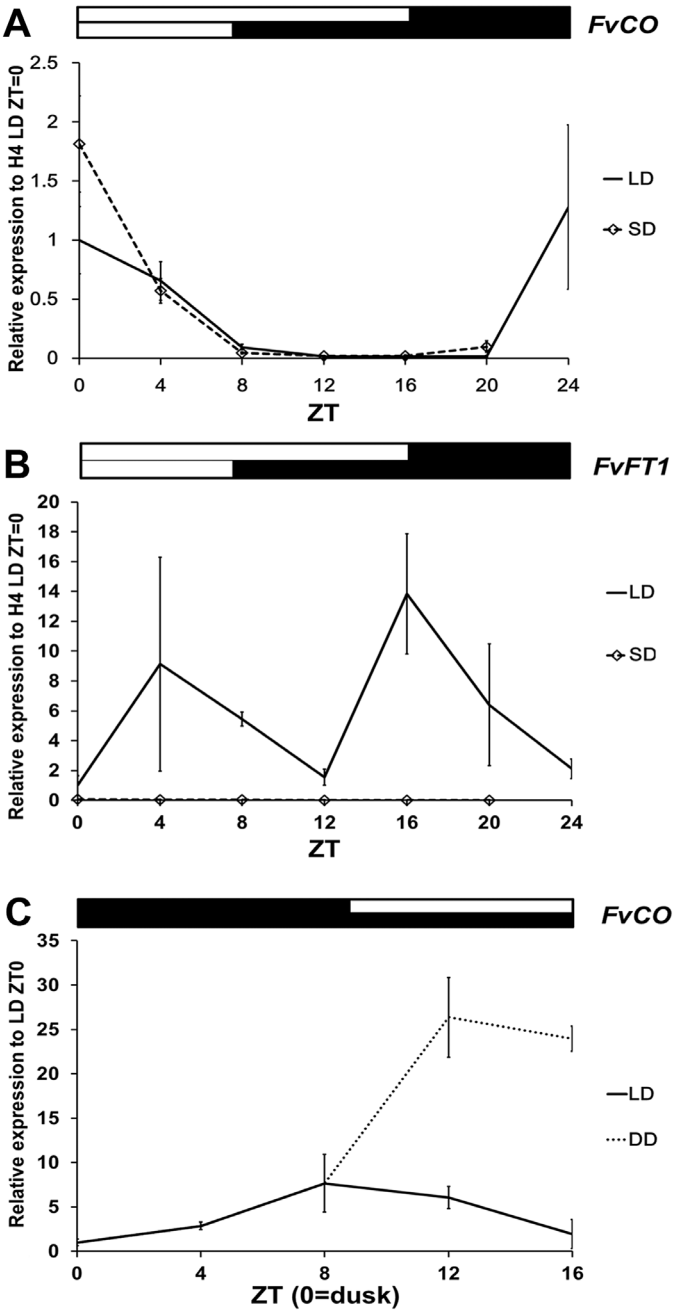

Fig. 2. Expression patterns of *FvCO* and *FvFT1*. mRNA expression patterns of *FvCO* (A) and *FvFT1* (B) were analysed in the leaf samples of short day- (SD) and long day- (LD) grown H4 plants. FvCO mRNA expression was also analysed in plants moved from LDs to darkness (DD) (C). White and black bars above the panels indicate light and dark periods, respectively. The average expression level of three biological replicates is shown for each time point, all normalized to the expression level of *FvMSI1*. Error bars indicate the SD.

level in the wild-type H4 is low. In RNAi lines, in contrast, clear down-regulation of *FvCO* was observed, but no silencing of two other Group I *COL* genes was detected, confirming the specificity of our RNAi construct (Supplementary Fig. S4).

We recorded the number of leaves in the primary leaf rosette before the terminal inflorescence in plants that had been subjected to LDs or SDs. Overexpression lines produced slightly fewer leaves before the terminal inflorescence compared with wild-type plants under LDs, whereas a strong promotion of flowering was observed in overexpression lines under SDs (Figs 3C, D, 4A; Supplementary Table S3). In *FvCO* RNAi lines, in contrast, flowering was significantly delayed compared with non-transgenic control plants under LDs, while under SDs, both H4 and *FvCO* RNAi lines remained vegetative or flowered very late, depending on the experiment (Figs 3E, F, 4A; Supplementary Table S3). An additional experiment revealed that *FvCO* overexpression plants flowered within 4 weeks and wild-type H4 after 5 weeks in LDs, whereas *FvCO* RNAi lines flowered ~1 month later (Supplementary Fig. S5). Comparison of *FvCO* RNAi lines with the previously published *FvFT1* RNAi lines (Koskela *et al.*, 2012) showed that both constructs had a similar effect on flowering time in H4 (Fig. 4B; Supplementary Table S3).

Flower-inducing conditions promote the differentiation of axillary buds to axillary leaf rosettes called branch crowns, while in non-inductive conditions vegetative reproduction through runners takes place. To gain insight into the effect of FvCO and FvFT1 on vegetative development, we studied the differentiation of axillary buds of the primary leaf rosette. In H4, most axillary buds differentiated to runners in SD conditions and only a few branch crowns were observed, whereas the effect of LDs was opposite (Fig. 4C–F). A clear photoperiodic response was also observed in *FvCO* overexpression lines, although they tended to produce fewer runners and more branch crowns than the wild type. In both *FvCO* and *FvFT1* RNAi lines, in contrast, axillary buds did not show a clear photoperiodic response (Fig. 4C–F; Supplementary Fig. S5). In all RNAi lines, roughly two-thirds of axillary buds differentiated to runners and only very few buds produced branch crowns in both photoperiods. Moreover, in H4 and all transgenic lines, ~20–30% of axillary buds remained dormant (data not shown).

To explore further the effect of FvCO on the balance between generative and vegetative development, we observed the cumulative number of inflorescences and runners in generative plant materials. *FvCO* overexpression plants produced slightly more new inflorescences than the wild type (Fig. 5A). In *FvCO* and *FvFT1* RNAi plants, however, inflorescence production was reduced so that by the end of the experiment they had almost 50% fewer inflorescences than the H4 accession. In contrast to the intense flowering, runner production was strongly suppressed in overexpression and wild-type plants, whereas all RNAi lines continuously produced new runners at the rate of approximately one runner per week (Fig. 5B).

FvCO *up-regulates* FvFT1 *in light*

Next, we examined the expression of flowering time genes in *FvCO* transgenic lines. First, leaf samples were collected 4 h or 16 h after dawn (ZT=4 or 16) under LD conditions, as the *FvFT1* mRNA level peaks at these times in wild-type plants (Fig. 2B). The up-regulation of *FvFT1* was observed at both time points in *FvCO* overexpression lines (Supplementary Fig. S6). In RNAi lines, however, *FvFT1* mRNA expression

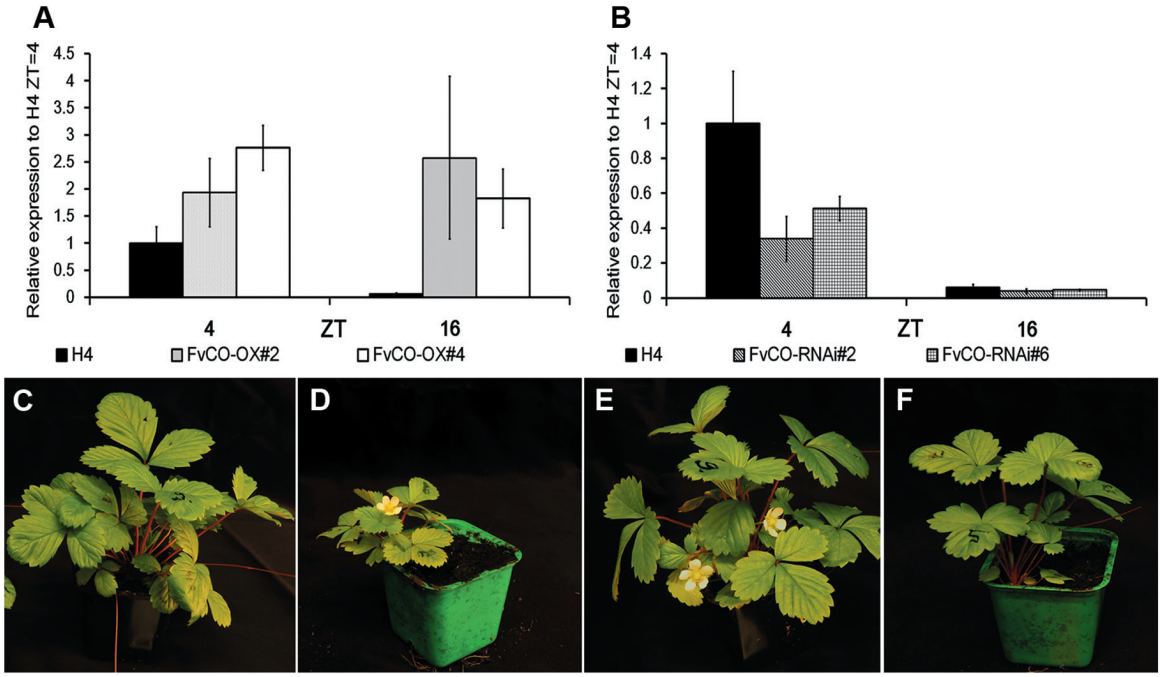

Fig. 3. Flowering phenotypes of *FvCO* transgenic plants. mRNA expression levels of *FvCO* were analysed in the leaf samples of overexpression (A) and RNAi lines (B) after plants were transferred to LD conditions for 2 weeks. Samples were taken at ZT4 and ZT16. The average expression level of three biological replicates is shown for each time point, all normalized to the expression level of *FvMSI1*. Error bars indicate the SD. (C–F) Flowering phenotypes of wild-type H4 (C, E), the overexpression line #2 (D), and the RNAi line #2 (F) after plants were placed under SD (C, D) or LD (E, F) conditions for 6 weeks.

was not detected. To understand the regulation of *FvFT1* by FvCO in more detail, we explored diurnal expression patterns in H4 and *FvCO* transgenic lines grown under LD and SD conditions. Overexpression of *FvCO* induced expression of *FvFT1* under both LD and SD conditions, but the normal diurnal expression cycle was lost (Fig. 6). Under LDs, up-regulation of *FvFT1* was observed during the light period from ZT0 to ZT16 in overexpression plants (Fig. 6A, B), while under SDs a strong up-regulation was observed only at ZT4 and another minor peak was present 4 h after dusk at ZT12 (Fig. 6C, D). In *FvCO* RNAi plants, in contrast, *FvFT1* mRNA levels remained extremely low or undetectable during the whole diurnal cycle under SD and LD conditions (Fig. 6B, D), even under continuous light which strongly increased *FvFT1* mRNA levels (Supplementary Fig. S7). These data indicated that FvCO affected both morning and evening peaks in *FvFT1* expression, even though *FvCO* expression was high only around dawn. Moreover, *FvFT1* expression is dependent on the light/dark cycle also in *FvCO* overexpression lines that highly express *FvCO* mRNA throughout the day.

To explore further the downstream flowering gene pathway, we studied the expression of *FvSOC1*, that is activated by FvFT1 in shoot apices in LDs (Mouhu *et al.*, 2013), and the expression of the floral meristem identity gene *FvAP1*. *FvSOC1* was strongly activated in *FvCO* overexpression lines compared with H4 especially under SD conditions (Fig. 7A). In RNAi lines, however, the *FvSOC1* mRNA level was reduced in LD conditions and, in contrast to wild-type H4, no clear photoperiodic regulation of the gene was observed. Consistent with the observed differences in flowering time, *FvAP1* was down-regulated in RNAi lines and highly activated in the stronger SD-grown *FvCO* overexpression line compared with H4 at 3 weeks after the beginning of the treatment (Fig. 7B). However, an equally high *FvAP1* expression level was detected in wild-type and overexpression lines in LDs at this time point, but in another experiment, at a 1 week earlier time point, an elevated *FvAP1* expression level was detected in overexpression lines compared with H4 in LDs (Supplementary Fig. S8).

FvGI *and* FvFKF1 *expression peaks precede the up-regulation of* FvFT1 *towards evening in LDs*

Although *FvCO* is clearly required for the activation of *FvFT1* mRNA expression, additional factors are probably needed to schedule its diurnal cycle, especially towards evening (Fig. 2). Therefore, we studied the diurnal expression patterns of strawberry homologues of *GI* and *FKF1*, genes which encode regulators of *FT* expression in Arabidopsis (Sawa *et al.*, 2007; Sawa and Kay, 2011). In the H4 accession under 12 h SDs, the expression of *FvGI* increased rapidly in the morning and stayed high until ZT12, after which time there was a rapid drop in expression (Fig. 8A). Slightly slower up-regulation was observed under 16 h LD condidions, and *FvGI* expression remained high until dusk at ZT16; a similar expression pattern was also observed in FIN56 (Supplementary Fig. S9A). The expression of *FvFKF1* began to increase in the morning and peaked 8–12 after dawn (Fig. 8B; Supplementary Fig. S9B). The up-regulation was slower under LDs, where the strong activation took place between ZT4 and ZT8. The peak of expression of both *FvGI* and *FvFKF1* therefore preceded

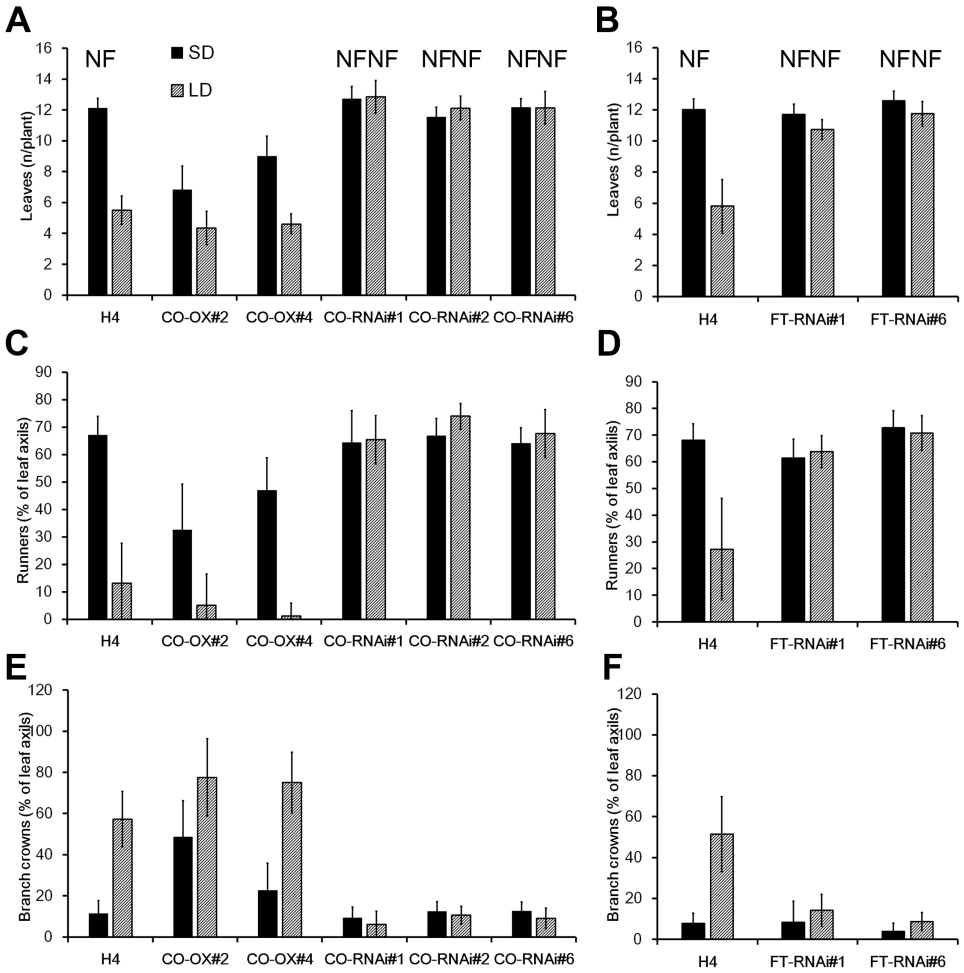

Fig. 4. Vegetative and generative development in transgenic lines. Number of leaves emerged before the terminal inflorescence (A, B) and the percentage of axillary buds of the primary shoot differentiated to runners (C, D) or branch crowns (E, F) in *FvCO* transgenic lines (A, C, E) or in *FvFT* RNAi lines (B, D, F) Plants were grown under SD or LD conditions for up to 10 weeks (n=10). NF, no flowering. Axillary buds that did not differentiate to runners or branch crowns remained dormant.

the up-regulation of *FvFT1* that takes place after ZT12, in the evening.

Discussion

Plants typically contain a large *COL* gene family; for example Arabidopsis and rice have 17 and 16 genes, respectively, while 26 genes have been identified in soybean (Griffiths *et al.*, 2003; Wu *et al.*, 2014). A few of these genes encode floral activators, but also repressors as well as regulators, with no effect on flowering (Putterill *et al.*, 1995; Wong *et al.*, 2014; Cao *et al.*, 2015; Mulki and von Korff, 2016; Tan *et al.*, 2016). Here, we have identified 10 *COL* genes in woodland strawberry and shown that, based on phylogenetic analysis (Fig. 1; Supplementary Fig. S1), the previously identified *FvCO* is the only Group Ia *COL* gene in the *F. vesca* genome (Shulaev *et al.*, 2011). We have also shown that it plays a major role in the photoperiodic control of reproductive and vegetative development in this species. Although *FvCO* mRNA is expressed at different phases during the day compared with Arabidopsis *CO*, it is nevertheless required to generate the evening expression peak of *FvFT1* (a feature similar to the expression pattern of Arabidopsis *FT*; see Fig. 6; Suárez-López *et al.*, 2001), as well as an additional peak in the morning.

FvCO controls photoperiodic flowering in strawberry

Previous studies suggested that the LD-activated *FvFT1–FvSOC1–FvTFL1* pathway represses flowering in woodland strawberry, and flower induction occurs after the silencing of this pathway by SDs and cool temperature in autumn. However, the characterization of the LD-flowering mutant H4, that is lacking the functional floral repressor FvTFL1, revealed a relic function of FvFT1 and FvSOC1 as floral activators in this accession (Koskela *et al.*, 2012; Mouhu *et al.*, 2013; Rantanen *et al.*, 2014, 2015). We show here that, similarly to the RNA silencing of *FvFT1*, the silencing of *FvCO* delays flowering in H4, while *FvCO* overexpression has an opposite effect (Figs 3, 4; Koskela *et al.*, 2012; Rantanen *et al.*, 2014). In agreement with these phenotypic observations, the silencing of *FvCO* strongly reduces *FvFT1* mRNA level in leaves, whereas *FvCO* overexpression leads to the activation of *FvFT1*. As previously observed in *FvFT1* RNAi lines (Mouhu *et al.*, 2013), in our *FvCO* transgenic

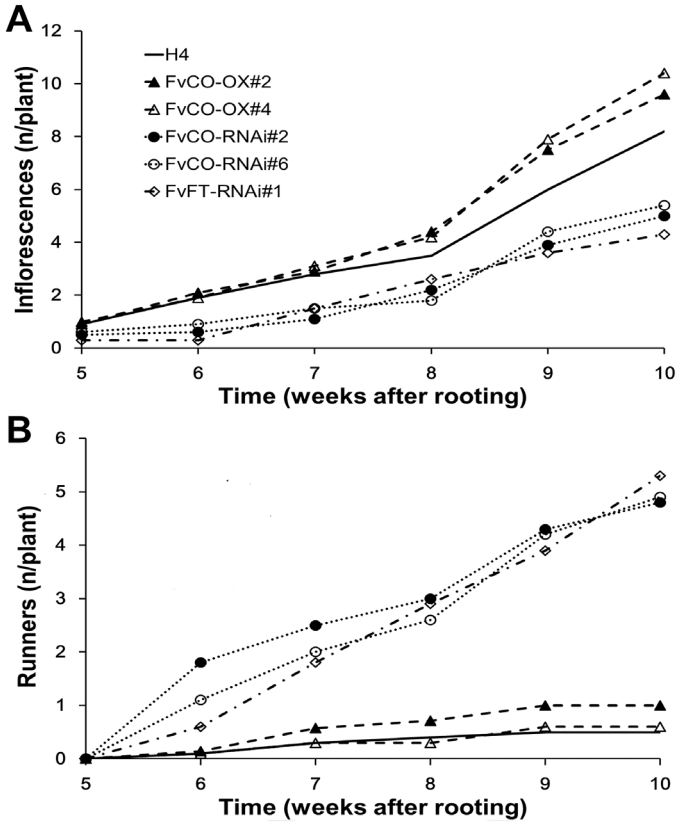

Fig. 5. FvCO controls the balance between vegetative and generative development. Cumulative number of inflorescences (A) and runners (B) in clonally propagated plants of H4 and the indicated *FvCO* and *FvFT1* transgenic lines grown under LD conditions (n=10). To obtain generative plant materials in both wild-type H4 and transgenic lines, runner cuttings of flowering plants were rooted.

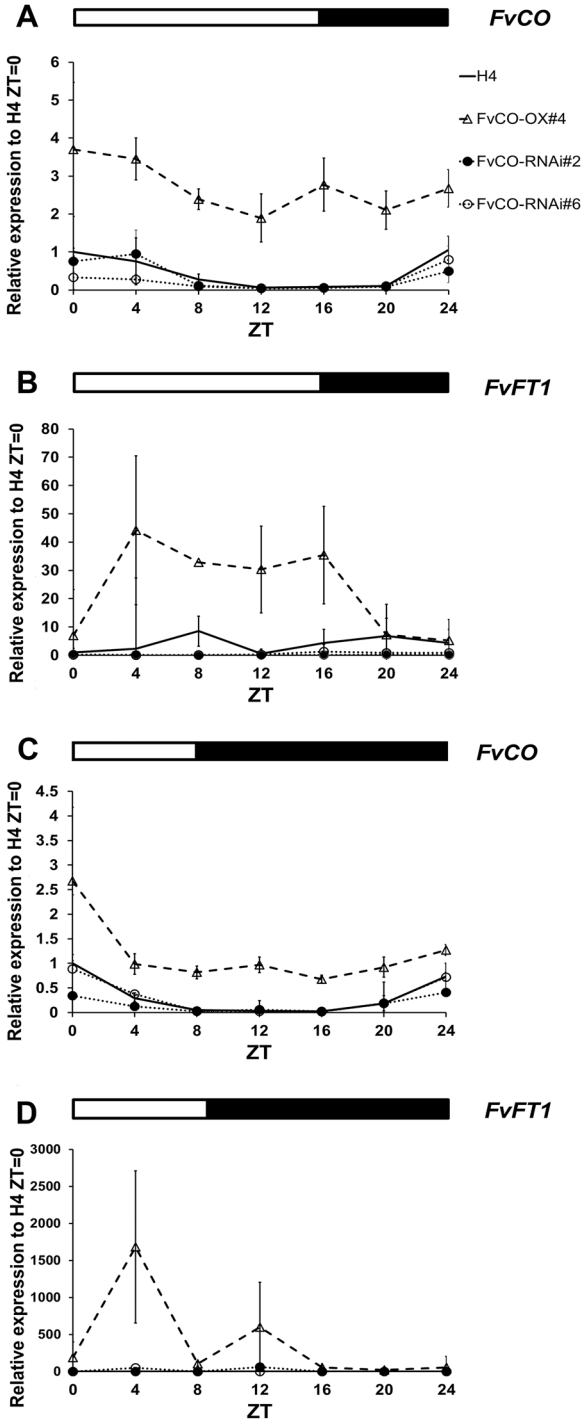

Fig. 6. FvCO activates *FvFT1* in light. Diurnal expression of *FvCO* (A, C) and *FvFT1* (B, D) in the leaves of H4 and the indicated *FvCO* transgenic lines grown under LD (A, B) or SD (C, D) conditions. White and black bars above the panels indicate light and dark periods, respectively. The average expression level of three biological replicates is shown for each time point, all normalized to the expression level of *FvMSI1*, and the average of H4 ZT=0 is set as 1. Error bars indicate the SA. ZT, time (h) after dawn.

lines, *FvSOC1* and *FvAP1* mRNA levels in shoot apices correlated positively with *FvFT1* expression in leaves. This indicates that in H4, *FvCO* has a major role in regulating *FvFT1* and *FvSOC1* expression to advance flowering under LD conditions. Also SD genotypes of woodland strawberry and cultivated strawberry may contain the relic flowering-promoting *FvCO–FvFT1–FvSOC1* pathway, but the activation of *FvTFL1* by FvSOC1 probably reverses the developmental outcome, namely the photoperiodic flowering response (Mouhu *et al.*, 2013; Koskela *et al.*, 2016). Direct functional analyses of *FvCO* and *FvFT1* in an SD genotype, however, are needed to confirm this model.

A recent study has suggested that another *FT*, *FaFT3*, is activated before *FaAP1* and may induce flowering in cultivated strawberry under SDs (Nakano *et al.*, 2015). A similar SD-specific activator may also function in the LD accession H4 which will eventually flower under SD conditions, when *FvFT1* expression is undetectable (Fig. 6D; Rantanen *et al.*, 2014). However, we found very low *FvFT3* expression in both H4 and *FvCO* transgenic lines in both SDs and LDs (data not shown). Thus, our results do not support the role of *FvFT3* in flower induction in H4.

Phylogenetic analysis grouped FvCO with other Group Ia COL proteins including Arabidopsis COL1 and COL2 that have no effect on flowering time (Ledger *et al.*, 2001; Kim *et al.*, 2013) and the major floral activator CO that has evolved from *COL1* or *COL2* by gene duplication in the Brassicaceae (Simon *et al.*, 2015). Unlike FvCO, studies on Group Ia COLs of the SD plant *P. nil* and the LD plant *M. truncatula* suggested that they do not promote flowering (Hayama *et al.*, 2007; Wong *et al.*, 2014); in *Glycine max*, COL1 functions as a

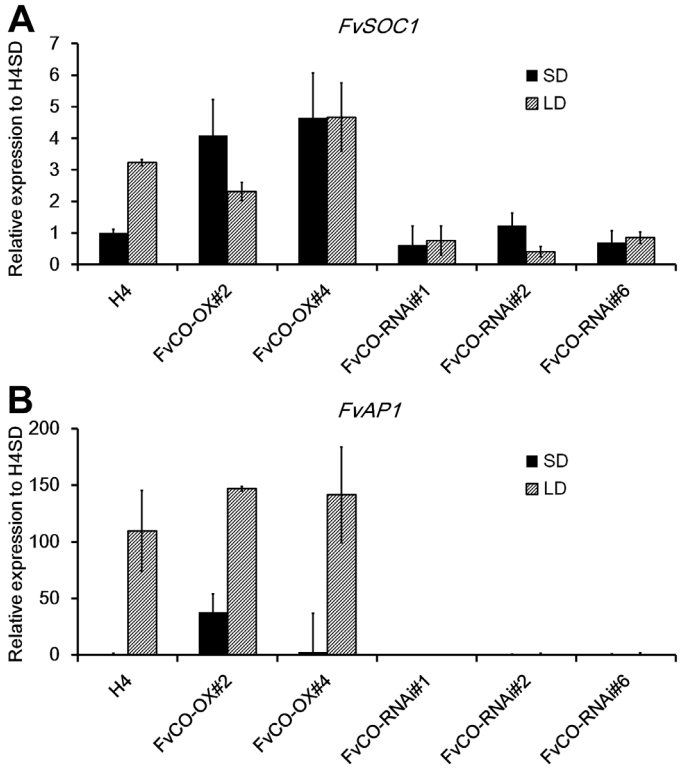

Fig. 7. FvCO activates *FvSOC1* and *FvAP1* in the shoot apex. The expression of *FvSOC1* (A) and the floral meristem identity gene *FvAP1* (B) in shoot apices of *FvCO* transgenic lines and H4 control plants grown under SD or LD conditions for 3 weeks. The average expression level of three biological replicates is shown, all normalized to the expression level of *FvMSI1*, and H4 SD is set as 1. Error bars indicate the SD.

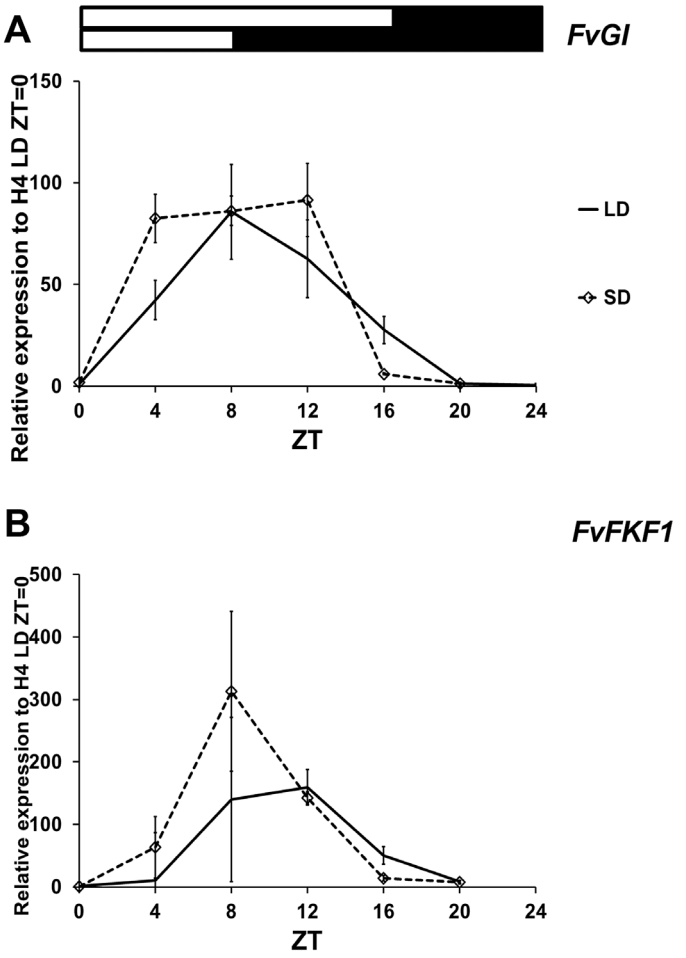

Fig. 8. Expression patterns of *FvGI* and *FvFKF1*. Diurnal expression of *FvGI* (A) and *FvFKF1* (B) in the leaves of H4 under LD or SD conditions. White and black bars above the panels indicate light and dark periods, respectively. The average expression level of three biological replicates is shown for each time point, all normalized to the expression level of *FvMSI1*, and the average of H4 ZT=0 is set as 1. Error bars indicate the SD. ZT, time (h) after dawn.

floral repressor under LDs (Cao *et al.*, 2015). In the monocots rice and spring barley, however, the closest CO homologues Hd1 and HvCO2, respectively, activate flowering (Izawa *et al.*, 2002; Mulki and von Korff, 2016). This indicates that the functions of Group Ia COLs are species specific. What causes these diverse functions of CO homologues in flowering time regulation is an interesting open question.

FvCO controls vegetative development in strawberry

Differentiation of strawberry axillary buds to runners and branch crowns is also regulated by photoperiod (Hytönen *et al.*, 2004). Our data demonstrate the major role of the FvCO/FvFT1-mediated photoperiodic pathway in this response as well as in controlling the balance between vegetative and floral development. H4 produced far more runners under SDs than under flower-inducing LDs, while the silencing of either *FvCO* or *FvFT1* caused continuous photoperiod-independent production of runners. *FvCO* overexpression plants, however, produced slightly fewer runners than H4 and, when these plants were moved from SDs to flower-inductive LD conditions, their runner production slowed down earlier than in H4. LD, in contrast, promoted the differentiation of axillary buds to branch crowns in H4 and *FvCO* overexpression lines, whereas RNAi lines did not show this response.

In contrast to runner production, generative *FvCO* RNAi plants produced fewer and overexpression lines slightly more inflorescences than H4 (Fig. 5). Such a balance between vegetative and generative growth is well documented in cultivated strawberries (e.g. Sønsteby and Heide, 2007), and it may be caused by competition for resources in clonal plants (Loehle, 1987). Furthermore, *FvCO* and its counterpart in cultivated strawberry can affect the expression/function of the gene at the *PFRU* locus that has been reported to control the balance between vegetative and generative growth (Gaston *et al.*, 2013; Samad *et al.*, 2017).

In the SD accession of woodland strawberry, FvSOC1 promotes runner formation in LDs (Mouhu *et al.*, 2013), and studies in non-flowering *FvTFL1* overexpression plants and in a non-transgenic SD cultivar of cultivated strawberry confirmed that direct photoperiodic regulation of axillary bud differentiation can occur (Hytönen *et al.*, 2009; Koskela *et al.*, 2012). In H4 and *FvCO* transgenic lines, however, we found a negative correlation between the *FvSOC1* expression level and the number of runners. Therefore, our data suggest that in this accession, which flowers perpetually after flower induction, axillary bud differentiation is primarily controlled

by flowering, and FvSOC1 may have a minor role. Runners are formed from axillary buds at the vegetative stage and, upon flower induction, the uppermost axillary buds differentiate into new branch crowns instead of runners, which leads to a reduction in runner formation and increases the number of meristems that can produce inflorescences (Hytönen *et al.*, 2004). Taken together with this information, our study indicates that the photoperiodic pathway affects the balance between vegetative and generative development in strawberries; further studies are needed to uncover how this balance is regulated in LD and SD genotypes.

The diurnal FvFT1 *expression is under control of FvCO*

In Arabidopsis, the *CO* mRNA level increases towards evening and, according to the external coincidence model, *FT* is activated under LDs when *CO* expression coincides with light (Suárez-López *et al.*, 2001). Similarly to Arabidopsis *FT*, *FT* homologues in woodland strawberry and cultivated strawberry (*FvFT1* and *FaFT1*, respectively), exhibited a major mRNA expression peak in the evening at ZT16–ZT20 (Fig. 6; Koskela *et al.*, 2012, 2016). However, an additional peak was observed between 4 h and 8 h after dawn; other work shows that the height of this peak depends on the light conditions (Rantanen *et al.*, 2014). *FvCO* is expressed in a different phase from Arabidopsis *CO* in both LD and SD accessions (Supplementary Fig. S2; Kurokura, 2009). It exhibits a sharp expression peak towards dawn, similar to *COL1*, *COL2*, and *COL5*, *BvCOL1* of *Beta vulgaris*, and *PnCO* of *P. nil* (Ledger *et al.*, 2001; Liu *et al.*, 2001; Chia *et al.*, 2008; Hassidim *et al.*, 2009). The dawn signal (dark to light) causes the down-regulation of the *COL* gene in the SD plant *Chenopodium rubrum* (Drabešová *et al.*, 2014), and this is also likely to be the case in woodland strawberry, because the transfer of plants to darkness caused accumulation of *FvCO* mRNA after subjective dawn (Fig. 2c).

Our studies on transgenic lines indicate that, although diurnal expression rhythms of *FvCO* and *FvFT1* do not match in woodland strawberry, functional FvCO is needed to activate *FvFT1* mRNA expression in both the morning and evening in LDs. *FvCO* RNAi lines exhibit very low *FvFT1* mRNA levels during the whole diurnal cycle compared with the wild type, whereas overexpression of *FvCO* results in the induction of *FvFT1* in a light-dependent manner with a broad peak during the light period under LDs. Under SDs, however, *FvFT1* is highly activated only in the morning in overexpression plants, with an additional minor peak after dusk (Fig. 6). Our results in *FvCO* overexpression plants suggest that the FvCO protein is regulated by light, as has been observed in Arabidopsis (Valverde *et al.*, 2004; Song *et al.*, 2012). Although the *FvCO* expression pattern is different from that of *CO* (Suárez-López *et al.*, 2001), light-regulated FvCO protein could form a part of the photoperiod measurement system that controls the gradual up-regulation of *FvFT1* under increasing photoperiods (Rantanen *et al.*, 2015). However, additional unknown factors are probably needed to schedule the evening peak of *FvFT1*. These factors may include CRYPTOCHROME-INTERACTING BASIC-HELIX-LOOP-HELIX and/or PHYTOCHROME-DEPENDENT LATE-FLOWERING proteins that activate *FT* specifically in the evening (Endo *et al.*, 2013; Liu *et al.*, 2013). Further studies on these regulators as well as on FvCO protein stability and activity are needed to understand the photoperiodic control of *FvFT1* mRNA expression.

In Arabidopsis, GI and FKF1 interact in a blue light-dependent manner to activate *CO* and *FT* mRNA expression by removing the repressor protein CDF1 in the afternoon (Imaizumi *et al.*, 2005; Sawa *et al.*, 2007; Fornara *et al.*, 2009; Song *et al.*, 2012). In addition, FKF1 and GI can directly activate the expression of *FT* (Sawa and Kay, 2011). Since *CO*-independent *FT* regulation has also been suggested in other species (Doi *et al.*, 2004; Hayama *et al.*, 2007; Ridge *et al.*, 2016), it is unlikely that *FvFT1* expression is regulated only by *FvCO* in woodland strawberry, even though *FvCO* seems to play a major role.

To gain insight into the function of these genes in woodland strawberry, we investigated their diurnal expression rhythms and observed that *FvGI* was highly expressed during the day in both SDs and LDs (Fig. 8A). *FvFKF1* exhibited a sharper expression peak in the afternoon, a few hours before the *FvFT1* evening peak (Fig. 8B). Therefore, FvFKF1 and FvGI may control the expression of *FvFT1* in the evening in LDs, but detailed gene functional studies are needed to confirm their roles in the photoperiodic flower induction of the woodland strawberry.

Conclusions

The *CO* homologue of the woodland strawberry, *FvCO*, has a diurnal expression rhythm with a sharp peak around dawn, regardless of photoperiodic conditions. FvCO plays a major role in the photoperiodic regulation of *FvFT1* and thus flowering time, as well as in vegetative reproduction (i.e. the production of runners). The expression of *FvCO* is promoted under darkness, and light is required to suppress its expression in the morning. Partial coincidence of the expression pattern of *FvCO* and *FvFT1* in the morning indicates that *FvCO1* regulates *FvFT1* expression in part, but other unknown factor(s) may be involved in the generation of the bimodal diurnal expression pattern of *FvFT1*. Woodland strawberry homologues of FvGI and FvFKF1 are good candidates for the factors that schedule *FvFT1* expression in the evening because, as in Arabidopsis, corresponding genes are expressed during the day before the *FvFT1* evening peak.

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Full structure of the phylogenetic tree of COL proteins.

Fig. S2. The analysis of conserved motifs of Group I COL proteins.

Fig. S3. Expression patterns of *FvCO* and *FvFT1* in FIN56.

Fig. S4. Expression patterns of *COL* genes 14981 and 27383 in H4 and *FvCO* RNAi lines.

Fig. S5. Vegetative and reproductive growth of *FvCO* transgenic lines under LD conditions.

Fig. S6. Expression of *FvFT1* in *FvCO* transgenic plants.

Fig. S7. *FvCO* and *FvFT1* expression under continuous light.

Fig. S8. *FvAP1* expression in *FvCO* transgenic plants.

Fig. S9. Expression patterns of *FvGI* and *FvFKF1* in SD accession FIN56.

Table S1. List of primers used in quantitative real-time PCR.

Table S2. List of protein accession numbers used in the phylogenetic tree.

Table S3. Flowering time of Hawaii-4 and *FvCO* transgenic lines.

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