MOLECULAR CONTROL OF THE YEARLY GROWTH CYCLE IN WILD STRAWBERRY

DOCTORAL THESIS IN HORTICULTURE

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ACADEMIC DISSERTATION

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III **Katriina Mouhu**, Takeshi Kurokura, Elli A. Koskela, Victor A. Albert, Paula Elomaa and Timo Hytönen. 2013. *Fragaria vesca* homolog of *SUPPRESSOR OF OVER-EXPRESSION OF CONSTANS1* represses flowering and promotes vegetative growth. Plant Cell 25: 3296–3310.

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ABSTRACT

Strawberries (*Fragaria* sp.) are found throughout the Northern Hemisphere, growing in a wide variety of climatic conditions. The economically most important species is the garden strawberry (*Fragaria* x *ananassa* Duch.). Strawberries are perennial rosette plants with distinct developmental phases regulated by day length during the growing season. During long days (LDs) in spring and summer, strawberries grow actively with axillary buds developing into stolons called runners. During short days (SDs) in autumn, runner formation is replaced by branch crown formation and an inflorescence is initiated in the shoot meristem of a rosette crown. After winter rest, the inflorescence formed in the previous autumn flowers and vegetative growth is again activated by LDs.

The wild strawberry (*F. vesca* L.) has been used as a model plant in strawberry research for several years. The wild strawberry is a seasonally flowering SD plant, but several perpetually flowering strawberry genotypes have been found. These types differ by a single recessive locus, the *SEASONAL FLOWERING LOCUS* (*SFL*), but the regulatory gene behind this trait has not been identified. This thesis aimed to identify the genes related to flowering and vegetative development in the wild strawberry.

Expressed sequence tag (EST) sequencing of SD *F. vesca* and a perpetually flowering genotype were combined with data mining in published Fragaria and Rosaceae EST databases using known *Arabidopsis thaliana (L.) Heynh.* flowering-related genes as a reference. The results revealed that most genes in the *Arabidopsis* flowering pathways could be identified among strawberry ESTs, indicating putative conservation in flowering pathway genes between these species.

Fragaria vesca TERMINAL FLOWER 1 (*FvTFL1*), a homologue of the A*rabidopsis thaliana TFL1,* was confirmed to be the *SFL*, encoding the flowering repressor in wild strawberry. *Fragaria vesca* SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (FvSOC1), a homologue of *Arabidopsis* SOC1, represses flowering via *FvTFL1* activation in shoot apices. Both *FvSOC1* and *FvTFL1* expression are under photoperiodic regulation, controlled at least in part via *F. vesca* FLOWERING LOCUS T 1 (FvFT1), encoding a putative homologue of the *Arabidopsis* mobile flowering signal FT, and expressed specifically under LDs. It was concluded that FvTFL1 functions as the flowering repressor and *FvTFL1* activation under LDs occurs by FvFT1 via FvSOC1 in wild strawberry.

FvSOC1 regulates vegetative growth independently of FvTFL1. FvSOC1 enhanced runner formation from the axillary buds, which involves changes in the levels of gibberellin biosynthesis genes. These results were used to construct a model of the yearly growth cycle in wild strawberry.

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ABBREVIATIONS

13-OH	13-hydroxylation
AFLP	amplified fragment length polymorphism
cDNA	complementary DNA
COMPASS	Complex Proteins Associated with Set1 complex
DN	day-neutral
EB	ever-bearing
EST	expressed sequence tag
F. vesca	*Fragaria vesca*
FAC	florigen activation complex
FM	floral meristem
FRIc	FRIGIDA complex
GA	gibberellin
GAox	GAoxidase
GA20ox	GA20oxidase
GA3ox	GA3oxidase
GDR	Genome Database for the Rosaceae
'H4'	*Fragaria vesca* ssp. *semperflorens* 'Hawaii-4'
IM	inflorescence meristem
ISSR-PCR	inter-simple sequence repeat PCR
H2A	histone 2A
H2Aub1	histone 2A monoubiquitination
H2A.Z	histone 2A variant
H2Bub1	histone 2B monoubiquitination
H3K4me2/3	histone 3 lysine 4 di- or trimethylation
H3K27me3	histone 3 lysine 27 trimethylation
H3K36me2/3	histone 3 lysine 36 di- or trimethylation
HDac	histone deacetylation
HUB-UBC	HUB-UBC complex
LD	long day
miR156	micro-RNA 156
miR172	micro-RNA 172
non-13-OH	non-13-hydroxylation
PAF1c	RNA Polymerase II-Associated Factor 1 complex
qPCR	quantitative real time polymerase chain reaction
QTL	quantitative trait locus
PCR	polymerase chain reaction
PHD	plant homeodomain
PRC1	Polycomb repressive complex 1
PRC2	Polycomb repressive complex 2
RAPD	random amplified polymorphic DNA
SD	short day
SFL	*SEASONAL FLOWERING LOCUS*

SNP	single nucleotide polymorphism
SSR	simple sequence repeat
STS	sequence-characterized sequence-tagged site
SWR1c	SWR1 chromatin remodelling complex
R	*RUNNERING LOCUS*
TF	transcription factor

Keywords: Strawberry, *Fragaria*, *Fragaria vesca*, day length, flowering, runnering, vegetative growth, gibberellin, EST sequencing, FvTFL1, FvSOC1.

1 INTRODUCTION

1.1 STRAWBERRY

Strawberries (*Fragaria* L. sp) belong to the large and economically important Rosaceae family, which includes a variety of species from fruit crops, such as apple, pear, peach, plum and cherry, to ornamental trees and shrubs, such as rose, cinquefoil, rowan and hawthorn (Potter et al. 2007). Strawberry species are found throughout the Northern Hemisphere (Darrow 1966, Hummer et al. 2011). The most cultivated and hence the most important strawberry species is the garden strawberry, *Fragaria* x *ananassa* Duch. In 2011, garden strawberry production was 4.6 million t on 244 000 ha, with an increasing trend during the last five years (FAOSTAT 2013). Therefore, the market for new cultivars is large. With the development of genetics and genomics in plant science, new tools have also been used in strawberry research to provide more detailed understanding of the regulation of growth. This rapidly expanding knowledge of molecular regulation is a significant tool for breeding new cultivars and decreasing the time between breeding and a marketable cultivar.

1.1.1 Strawberry genomics

The genus *Fragaria* includes 25 species that are found at various ploidy levels ranging from diploid to decaploid (Darrow 1966, Hummer et al. 2011). The most widely spread species is the diploid wild strawberry *F. vesca* L., which is native to North America and Europe, westward of Lake Baikal. Nine diploid species are native to regions in Asia, such as *F. mandshurica* Staudt in North China and *F. iinumae* Makino in Japan. Five tetraploids are found in Southeast and East Asia, the single hexaploid *F. moschata* Weston in Euro-Siberia, wild octoploids in North America, Hawaii and Chile, and the decaploid *F. iturupensis* Staudt in the Kurile Islands. The garden strawberry is an octoploid hybrid species between two octoploids, *F. chiloensis* (L.) Miller and *F. virginiana* Miller. The current genome model for the octoploids is Y'Y'Y''Y''ZZZZ (or YYYYZZZZ) and the diploid origin of the octoploid species was suggested to be *F. vesca* ($Y^{(')}$ in the model), *F. mandshurica* ($Y^{(")}$ in the model) and *F. iinumae* (Z in the model; Rousseau-Gueutin et al. 2009).

The octoploid (2n = 8x = 56) genome is complicated and therefore genetic and genomic studies have been largely performed in wild strawberry, *F. vesca* (2n = 2x = 14). The first linkage map containing seven linkage groups in wild strawberry was developed in 1997, using random amplified polymorphic DNA (RAPD), isozyme and morphological

markers analysed in an F_2 crossing population between *F. vesca* clone WC6 and an alpine (continuously flowering) variety 'Baron Solemacher' (Davis and Yu 1997). Later linkage maps in wild strawberry were based on inter-simple sequence repeat polymerase chain reaction (ISSR-PCR), simple sequence repeats (SSRs) and ISSR-PCR-derived, locus-specific, sequence-characterized amplified region markers (Cekic et al. 2001, James et al. 2003, Albani et al. 2004, Sargent et al. 2004). The most comprehensive genetic map used for *Fragaria* species has been developed for the reference mapping population between *F. vesca* '815' and *F. bucharica* '601' Losinsk., and it contains at least 600 sequence-characterized sequence-tagged site (STS) markers spanning over seven linkage groups (Sargent et al. 2004, 2006, 2008, 2011, Ruiz-Rojas et al. 2010). Furthermore, 90% of the published genome of the *F. vesca* (L.) var. *semperflorens* (Duch.) Staudt 'Hawaii-4' ('H4') is anchored in the reference map using STS markers and 97.6% using conventional and bin-mapping strategies (Ruiz-Rojas et al. 2010, Sargent et al. 2011, Shulaev et al. 2011).

The first linkage map for the garden strawberry was based on amplified fragment length polymorphism (AFLP) markers (Lerceteau-Köhler et al. 2003). AFLP markers, like RAPD and ISSR-PCR markers, are difficult to transfer between species, so later linkage maps for the garden strawberry were based on SSR markers, especially on *F. vesca* SSR and STS markers (Hadonou et al. 2004, Sargent et al. 2004, 2006, 2009, Monfort et al. 2006, Rousseau-Gueutin et al. 2008, Isobe et al. 2013).

The linkage maps are used for marker-assisted selection in strawberry breeding programmes. The markers used for selection are associated with the gene and/or quantitative trait locus (QTL) of interest, due to genetic linkage, and are useful in selection for traits that are difficult to measure with conventional methods. Additionally, linkage maps are valuable tools in map-based cloning of genes and also in researching genome evolution between species.

1.1.2 Strawberry plant

Strawberries are small, perennial rosette herbs. The rosette stem is called a crown and consists of short internodes with a single trifoliate leaf and an axillary bud in the base of the petiole (Darrow 1966, Guttridge 1985). Strawberries reproduce both sexually and clonally. Clonal reproduction occurs via development of aboveground stolons called runners that comprise two long internodes followed by a daughter rosette plant from the axillary buds in the crown. Runner formation, also called runnering, continues from the axillary buds of the daughter plants, resulting in a strongly expanding net of daughter plants around the mother plant. Instead of a runner, an axillary bud can also form a new rosette stem called a branch crown, which is structurally similar to a runner, but without the first

long internodes. The inflorescence forms terminally in the apical meristem of a crown, after which the vegetative growth is continued by branch crowns from the axillary meristems below the apical meristem. The inflorescence is basically a dichasial cyme, but the inflorescence structure can vary widely between cultivars and depending on the environmental conditions (Anderson and Guttridge 1982).

As a perennial species, the strawberry undergoes repeated cycles of vegetative and generative phases throughout the years (Figure 1). When spring arrives, strawberry plants enter an active growth phase that can be observed as an increase in leaf petiole growth and growth of inflorescences. The plants begin to flower in June and the cropping season is during high summer, peaking in July. During summer, runners begin to form from the axillary buds. Towards autumn, runner formation ceases and axillary bud development shifts from runner formation to branch crown formation. In autumn, the petiole growth is suppressed and the strawberry enters the generative growth phase, in which the apical meristem of the shoot forms an inflorescence initial. Towards winter, the strawberry growth rate decreases, and the winter is passed by under rest.

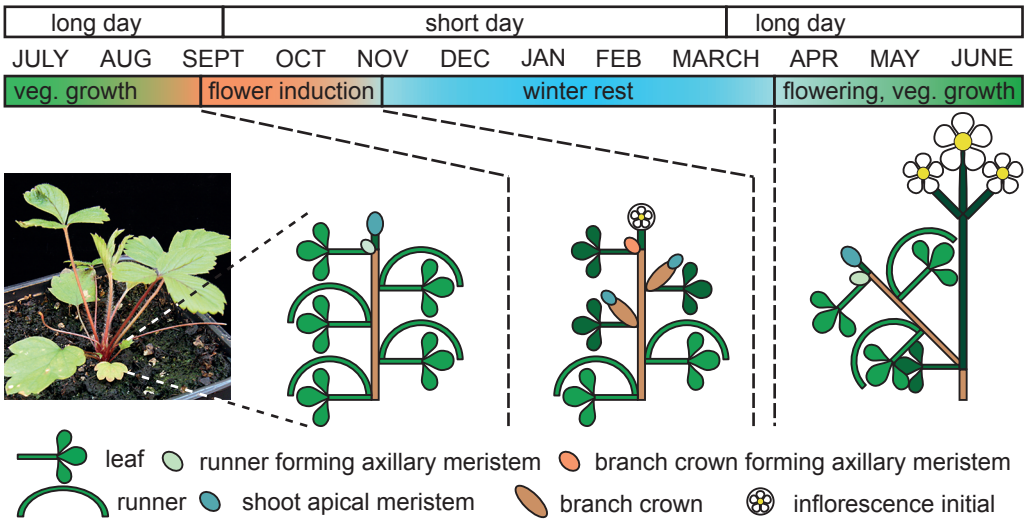

Figure 1 Structural changes in a strawberry main crown under different photoperiods during the year. Under long days in high summer, axillary meristems develop into runners. As the day length decreases towards autumn, axillary meristems begin to form branch crowns and petiole growth is suppressed. The shoot meristem of the main crown forms a terminal inflorescence initial and the vegetative growth is continued in the shoot apical meristems of the branch crowns formed. Under long days during next spring/summer after winter rest, the inflorescence of the main crown flowers and the branch crowns continue plant growth, each behaving as the main crown of the previous growth season.

1.1.3 Strawberry physiology

Photoperiodic responses

Photoperiodism in vegetative and generative plant growth was described as early as in the 1920s (Garner and Allard 1920). Plants can be classified in three main categories, based on their flowering response to the photoperiod: short day (SD) plants that flower under a relatively short day length, when the day length is under the critical value for the species, long day (LD) plants that flower under a relatively long day, when the day length is longer than the critical day length for the species, and day-neutral (DN) plants that flower regardless of the day length. Most strawberry species are believed to exhibit the SD-flowering habit naturally (Darrow 1966). Garden strawberry cultivars are mostly SD plants, often also called June-bearing cultivars, in which flower induction occurs under a shortening photoperiod in autumn followed by a winter rest and flowering the following spring (Guttridge 1985). The critical day length is strongly dependent on the cultivar, but in general is between 11 and 16 h, the optimal being between 8 and 11 h (Heide 1977, Durner et al. 1984, Guttridge 1985, Konsin et al. 2001, Verheul et al. 2007). Cultivars vary in their sensitivity to flower-inductive conditions and the number of inductive days needed for flower induction ranges from 7 to 23, on average, depending on the day length (Guttridge 1985, Konsin et al. 2001, Sønsteby and Heide 2008b).

Some octoploid species exhibit a perpetually flowering trait (Darrow 1966). In garden strawberry, this trait may have been derived from three main sources. The first source, in European everbearing (EB) cultivars, may have been derived from 'Gloede's Seedling', introduced in France in 1866 (Richardson 1913, Ahmadi et al. 1990). The next EB trait source was developed in North America, where 'Pan American' was introduced in 1890 (Darrow 1966). The third source, in North America as well, is reported to be *F. virgianiana* ssp. *glauca*, the origin of many modern DN varieties (Ahmadi et al. 1990). The genetic regulation of this trait has been under speculation. Ahmadi et al. (1990) suggested that in varieties derived from *F. virgianiana* ssp. *glauca*, day-neutrality is conferred by a single dominant gene, but later it was suggested that most likely the DN trait is polygenic, possibly still with a single major dominant locus (Serçe and Hancock 2005, Shaw and Famula 2005, Weebadde et al. 2008). Recently, Gaston et al. (2013) showed that a single major QTL, *FaPFRU*, does control both perpetual flowering habit (positive effect) and runnering (negative effect) in a dominant manner.

In addition to flowering response, the photoperiod also affects vegetative growth. Interestingly, this effect constrasts with that on generative growth: both runner formation and vegetative growth, often measured in parameters such as petiole and pedicle length, are enhanced under LDs and suppressed under SDs (Heide 1977, Durner et al. 1984, Sønsteby and Nes 1998, Konsin et al. 2001, Hytönen et al. 2009).

Temperature

Temperature strongly affects flowering response in strawberries. Most SD cultivars are facultative SD plants: when the temperature falls below a critical value, flowering is induced photoperiod-independently (Guttridge 1985). In general, the critical temperatures for flower induction are 14-18 ˚C (Durner et al. 1984, Guttridge 1985, Manakasem and Goodwin 2001, Heide and Sønsteby 2007). The temperatures at which flower induction is photoperiod-sensitive, in general 14-20 °C, are called intermediate temperatures. In turn, higher temperatures, generally > 20 ˚C, suppress flower induction, regardless of the photoperiod (Durner et al. 1984, Guttridge 1985, Manakasem and Goodwin 2001). These temperature limits for photoperiod-insensitive, photoperiod-sensitive and temperature-suppressed flower induction are strongly cultivar-dependent. The same temperature can be under the critical temperature for one cultivar, intermediate for another and even over the flowering-suppressing temperature for a third cultivar.

In several cultivars, the critical day length for the induction increases when the temperature decreases within these intermediate temperatures (Heide 1977, Guttridge 1985, Sønsteby and Heide 2008b). Additionally, the critical number of inductive days needed for flower induction exhibits a day length x temperature interaction as well (Guttridge 1985). These effects are particularly important for strawberry cultivation in northern latitudes, where the LDs during autumn would otherwise delay flower induction.

There is considerable variation between cultivars in both day length and temperature requirements, and day length x temperature interaction for flower induction, which also enables strawberry cultivation under a wide range of climatic conditions and latitudes (Heide 1977, Sønsteby and Nes 1998, Manakasem and Goodwin 2001, Sønsteby and Heide 2006, Verheul et al. 2007). This strong day length x temperature interaction characteristic, however, can also lead to confusion in defining the flowering response of a cultivar,since it can behave as DN under one temperature condition and as an SD plant under another (Durner et al. 1984, Manakasem and Goodwin 2001, Sønsteby and Heide 2007). As a result, it has been proposed that perpetually flowering cultivars previously classified as EB or DN should instead be classified as qualitative LD plants at high temperatures (over 27 ˚C), quantitative LD plants at intermediate temperatures and DN at low temperatures (under 10 ˚C; Durner et al. 1984, Sønsteby and Heide 2007, Bradford et al. 2010).

In addition to flowering response, temperature also affects the fate of axillary buds. Warm temperatures enhance runner formation in both SD and LD cultivars (Heide 1977, Durner et al. 1984, Konsin et al. 2001, Sønsteby and Heide 2007, Hytönen et al. 2009, Bradford et al. 2010). Warm temperatures also advance inflorescence growth and flowering (Verheul et al. 2006, 2007).

Gibberellin

The plant hormone group gibberellins (GAs) promote cell division and cell elongation and are involved in the normal growth of plant organs (Mutasa-Göttgens and Hedden 2009). Active GA is formed from *trans*-geranylgeranyl diphosphate in 12 steps that are catalysed by six enzymes. The major sites of regulation in this pathway are the last steps, where the 2-oxoglutarate-dependent dioxygenases, GA20oxidase (GA20ox) and GA3oxidase (GA3ox), catalyse the formation of active GA (Figure 2). GA20ox catalyses three steps in the GA biosynthesis pathway: from GA_{12} to the immediate precursors of active GA. GA3ox catalyses 3β-hydroxylation of these precursors, GA_9 and GA_{20}, to GA_4 and GA_1, respectively. Active GAs are inactivated by 2β-hydroxylation by GA2oxidase (GA2ox). Both the 13-hydroxylation (13-OH) and non-13-hydroxylation (non-13-OH) pathways seem to be present in strawberry: endogenous GAs in the 13-OH pathway have been found in leaves, stems, axillary buds, receptacles and berries, and endogenous GAs in the non-13-OH pathway in petioles (Guttridge and Thompson 1964, Taylor et al. 1994, 2000, Wiseman and Turnbull 1999b, Hytönen et al. 2009, Symons et al. 2012). Additionally, Guttridge and Thompson (1964) showed that GA_4 treatment enhanced petiole growth more than did GA_1, and GA_1 enhanced stem growth and runner formation more than did GA_4 in *F. vesca*. This suggests that strawberries may use both pathways to synthesize bioactive GA in different tissues. Recent studies with rice (*Oryza sativa* L.) indicate that plants may fine-tune growth responses to GA by regulating the levels of strongly bioactive and less bioactive GAs in different tissues via distinct use of these two GA biosynthesis pathways (Sun 2011, Magome et al. 2013).

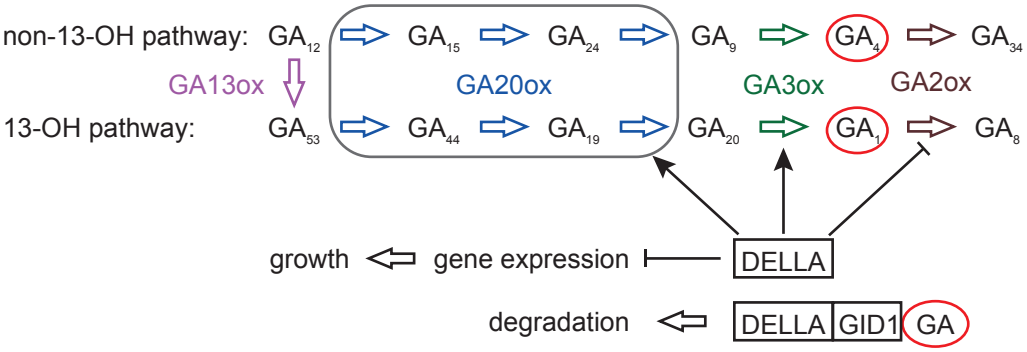

Figure 2 Gibberellin (GA) biosynthesis and signalling. The last steps in the GA biosynthesis in the non-13-hydroxylation (non-13-OH) pathway and 13-hydroxylation (13-OH) pathway begin with GA_{12}. GA oxidase (GAox) responsible for the conversion is next to the corresponding step (block arrow in corresponding colour). Active GA (circled with a red ellipse) binds to the GA receptor GIBBERELLIN INSENSITIVE DWARF 1 (GID1), which binds to DELLA protein, directing it to degradation. DELLA regulates its own turnover by regulating GA biosynthetic genes. The black arrows denote transcriptional activation and the bars transcriptional repression.

GA promotes growth by directing growth-suppressing DELLA proteins to degrade (Figure 2; Mutasa-Göttgens and Hedden 2009, Sun 2011). Active GA binds to the GA receptor GIBBERELLIN INSENSITIVE DWARF 1, which then interacts with DELLA protein (Sun 2011). DELLA protein is polyubiquitinated by the Skp1–Cullin–F-box protein E3 ligase and subsequently degraded by the 26S proteasome, removing DELLA repression of the target genes. In strawberries, the few reports on GA receptors and DELLA genes have focused mainly on fruit development, indicating the receptacle as the main site for GA receptor and DELLA gene expression (Csukasi et al. 2011, Kang et al. 2013).

GA strongly affects strawberry growth and flowering, specifically enhancing vegetative growth. Exogenously applied GA enhances petiole elongation and runner formation and represses flower induction in plants grown under growth-suppressing and flower-inductive SDs (Thompson and Guttridge 1959, Guttridge and Thompson 1964, Braun and Kender 1985, Braun and Garth 1986). Applied after flower induction, GA enhances the emergence and length of inflorescences along with petiole length and leaf area (Tafazoli and Vince-Prue 1978, Paroussi et al. 2002). Applications of GA biosynthesis inhibitors suppress petiole elongation and induce branch crown formation instead of runner formation from the axillary buds (Wiseman and Turnbull 1999a, Black 2004, Hytönen et al. 2008, 2009).

Short photoperiods reduce active GA levels in petioles and axillary buds (Wiseman and Turnbull 1999b, Hytönen et al. 2009). However, the reduction in petiole length occurs earlier than the decrease in GA levels, indicating that SDs suppress petiole growth not only via GA synthesis but with an additional mechanism (Wiseman and Turnbull 1999b). Recently, Hytönen et al. (2009) showed that GA application under SDs and transfer from SDs to LDs induced runner formation at a similar level, whereas transfer to LDs combined with GA treatment strongly enhanced runner formation from axillary buds. These data indicate that not only GA levels but also sensitivity to GA determines growth rate, and that LDs increase this sensitivity to GA, thereby enhancing vegetative growth and runner formation.

1.1.4 *Fragaria vesca* – the model plant

The garden strawberry is not well suited for molecular research purposes, due to its octoploid genome, size and relatively long growth cycle. Instead, the diploid wild strawberry has become the model plant in strawberry research. The wild strawberry has several advantages over the garden strawberry: it has a short generation time of approximately 3–4 months, it is small in size and it is easy to propagate vegetatively via runner formation. In addition, since strawberries are readily self-pollinating, the production of highly inbred lines is easy (Slovin et al. 2009). Robust and efficient *in vitro* regeneration and transformation methods have been

developed for wild strawberry, facilitating the use of genetic tools for structural and functional analyses (El Mansouri et al. 1996, Haymes and Davis 1998, Alsheikh et al. 2002, Oosumi et al. 2006). Furthermore, the recently published genome of *F. vesca* accession 'H4' and the comprehensive and detailed reference map provide a powerful basis for genomic studies both in wild strawberry and other *Fragaria* species (Shulaev et al. 2011, Sargent et al. 2011).

Wild strawberry, like garden strawberry, is a facultative SD plant: flower induction is inhibited at temperatures over 20 °C, a short photoperiod is required at intermediate temperatures and cool temperatures around 10 °C induce flowering, regardless of the photoperiod (Heide and Sønsteby 2007). In addition to SD accessions, several perpetually flowering forms of wild strawberry (*F. vesca* ssp. *semperflorens*) have been found and taken into limited cultivation (Brown and Wareing 1965, Sønsteby and Heide 2008a, Slovin et al. 2009). These accessions may have originated from the European Alps and are often called alpine strawberries (Darrow 1966, Ahmadi et al. 1990).

Classical crossing experiments between the SD type and perpetually flowering accessions conducted by Brown and Wareing in 1965 showed that the photoperiodic flowering response in *F. vesca* is regulated by a single gene, later named the *SEASONAL FLOWERING LOCUS* (*SFL*): the dominant allele confers seasonal flowering and the recessive allele perpetual flowering (Brown and Wareing 1965, Albani et al. 2004). Although often called perpetually flowering or DN, these cultivars with recessive *sfl* alleles appear to be LD plants, as has also been suggested for garden strawberry cultivars, with strong interaction between day length requirements and temperature (Sønsteby and Heide 2007, 2008a). As in seasonal flowering, the runnering trait is also controlled by a single dominant gene, *RUNNERING LOCUS* (*R*; Brown and Wareing 1965). *SFL* and *R* segregate separately, and *SFL* has been mapped to linkage group VI and *R* to linkage group II in the *F. vesca* reference map (Brown and Wareing 1965, Sargent et al. 2006, Iwata et al. 2012).

1.2 MOLECULAR CONTROL OF FLOWERING

Regulation of flowering has been most extensively studied using the model plants thale cress *Arabidopsis thaliana* (L.) Heynh. (a facultative LD plant) and rice (an SD plant). These studies have revealed that both environmental and endogenous cues regulate the transition to flowering (Mutasa-Göttgens and Hedden 2009, Huijser and Schmid 2011, Jarillo and Piñeiro 2011, Andres and Coupland 2012). External cues include light and temperature effects, and endogenous cues include hormonal effects and age-related changes. The regulative effect of these cues at the molecular level has been assigned to genetic flowering pathways, which form a regulatory network that in the end activates floral meristem (FM) identity

genes, resulting in flowering. Based on *Arabidopsis* research, the major flowering pathways are often divided into photoperiod, light quality, vernalization, autonomous, ambient temperature and GA pathways (Figure 3).

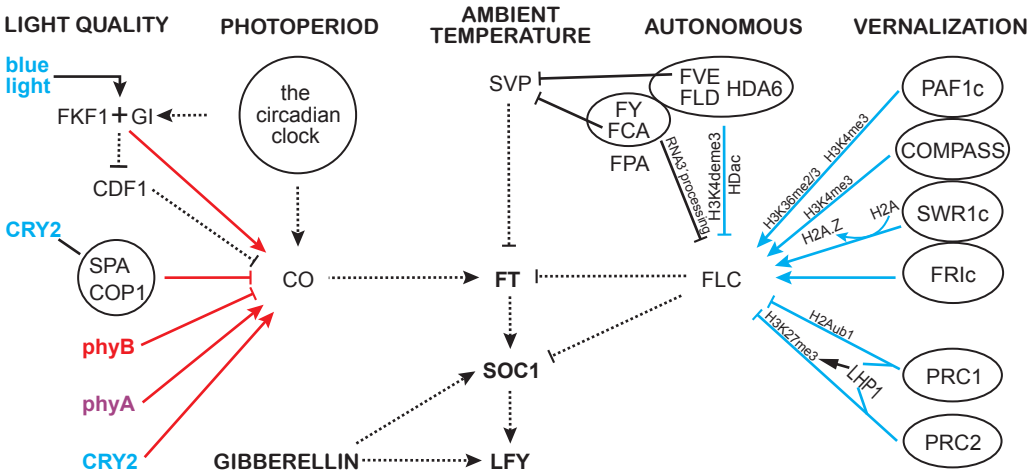

Figure 3 A simplified model of the *Arabidopsis* main flowering pathways and their activational/repressional effect on the floral integrator genes FT, SOC1 and LFY. Black arrows and bars, activation and repression, respectivel; dotted arrows and bars, transcriptional activation and repression, respectively; red arrows and bars, posttranscriptional activation and repression, respectively; blue arrows and bars, chromatin modificational activation and repression, respectively. See text below for detailed explanation.

Genes in the flowering pathways function in different ways to regulate the onset of flowering. Many genes in the flowering pathways encode transcription factors (TFs) that bind to target gene DNA to activate or repress transcription. TFs can act alone, with other TFs as cobinders and/or as the DNA-binding parts of larger protein complexes. DNA binding of TFs is affected by chromatin modifications, and several genes in the flowering pathways are associated with these modification processes. Common chromatin modifications that are linked with gene transcription rates include histone acetylation, histone methylation and histone monoubiquitination (Berr et al. 2011, He 2012, Zentner and Henikoff 2013).

The flowering pathways converge in a set of genes, *FLOWERING LOCUS T* (*FT*), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) and *LEAFY* (*LFY*), which are often called floral integrator genes (Blázquez and Weigel 2000, Lee et al. 2000, Samach et al. 2000, Moon et al. 2003, 2005, An et al. 2004). These genes receive both promoting and repressing signals from the flowering pathways and also activate each other in a cascade: FT activates *SOC1* and SOC1 activates *LFY* to induce floral transition (Moon et al. 2005).

When flowering is initiated, the vegetative shoot meristem transforms into the inflorescence meristem (IM), from which the FMs arise (Liu et al. 2009). In *Arabidopsis*, the central IM regulator is TERMINAL FLOWER 1

(TFL1), which functions in maintaining IM indeterminacy (Ratcliffe et al. 1998, 1999, Liu et al. 2009). FMs are formed on the flanks of the IM, and FM initiation is induced by APETALA 1 (AP1) and LFY (Liu et al. 2009). AP1 is a central regulator of FM identity, involved in repressing flowering time genes and regulation of flower organ formation (Liu et al. 2009, Kaufmann et al. 2010). TFL1, AP1 and LFY are involved in defining IM and FM boundaries; TFL1 suppresses *AP1* and *LFY* in the IM and both AP1 and LFY down-regulate *TFL1* in the FM (Ratcliffe et al. 1998, 1999, Liljegren et al. 1999). *TFL1* is weakly expressed in the vegetative shoot apical meristem and more strongly later in development in the centre of the IM (Ratcliffe et al. 1999). *AP1* and *LFY*, in turn, are activated by FT and SOC1, respectively, during floral transition (Liu et al. 2009, Andres and Coupland 2012). These genes regulating floral transition and IM/FM formation have been identified in several plant species, both in monocots and dicots (Liu et al. 2009, Moyroud et al. 2010, Andres and Coupland 2012, Mimida et al. 2013), suggesting a conserved regulatory pathway for inflorescence formation in plants.

1.2.1 Flowering pathways

Photoperiod and light quality pathway

The photoperiodic pathway activates the floral integrator gene *FT* (Suárez-López et al. 2001, Yanovsky and Kay 2002, An et al. 2004). In *Arabidopsis*, CONSTANS (CO) is required for the promotion of flowering under LDs (Putterill et al. 1995, Suárez-López et al. 2001). Flower induction is dependent on both the *CO* expression and CO protein levels during the day, and both the transcriptional and posttranscriptional levels of CO are tightly regulated to ensure the proper timing of flowering (Andres and Coupland 2012).

CO transcription is repressed by CYCLING DOF FACTORs (CDFs; Imaizumi et al. 2005, Fornara et al. 2009). During the day, CDFs are degraded by FLAVIN-BINDING, KELCH REPEAT, F-BOX 1 (FKF1) and GIGANTEA (GI), which form a blue light-activated complex, allowing CO transcripts to accumulate towards evening (Imaizumi et al. 2005, Sawa et al. 2007, Song et al. 2012). FKF1-GI complex formation requires that *FKF1* and *GI* expressions coincide, which occurs during the afternoon under LDs, but not under SDs.

CO protein degradation is promoted by a ubiquitin ligase complex containing CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) and SUPPRESSOR OF PHYA-105 (SPA; Laubinger et al. 2006, Jang et al. 2008, Andres and Coupland 2012). During the day, blue light activates CRYPTOCHROME 2 (CRY2), and enhances CRY2-COP1 interaction, which in turn inhibits the COP1-SPA-mediated CO degradation (Liu et al. 2008b, Zuo et al. 2011). Additionally, the blue light-induced FKF1-GI complex also stabilizes CO protein in the afternoon (Sawa et al. 2007,

Song et al. 2012). Red light promotes CO protein degradation (through phytochrome B) in the early part of the light period and this effect is counteracted by CO protein stabilization by far-red light (through phytochrome A), possibly via interaction with COP1-SPA in the latter part of the light period (Yanovsky and Kay 2002, Valverde et al. 2004, Andres and Coupland 2012). Thus, under LDs both *CO* transcription and CO protein accumulation increase towards the evening, whereas under SDs *CO* expression peaks during the night, when CO protein is efficiently degraded.

CO accumulates under LDs and up-regulates *FT* in the phloem (Samach et al. 2000, An et al. 2004). FT protein moves from the phloem to the shoot apex (Corbesier et al. 2007, Jaeger and Wigge 2007, Mathieu et al. 2007). In the shoot apex, FT interacts with FD to activate *AP1*, a key regulatory gene in flower development (Abe et al. 2005, Wigge et al. 2005, Kaufmann et al. 2010). This system, in which FT accumulates in leaves and translocates to the shoot apex to induce flowering, agrees with the concept of florigen, a graft-transmissible flowering signal functioning in both SD and LD plants (Andres and Coupland 2012, Taoka et al. 2013). Research on rice suggests that a florigen activation complex (FAC) formed in the shoot apex consists of the FT homologue Hd3a and the FD homologue OsFD1, which are connected via a 14-3-3 protein (Taoka et al. 2013). The components and interaction partners of FAC have been identified in several plant species (Taoka et al. 2013), indicating that FAC formation and function are conserved in plants.

Vernalization pathway

Some plant species need to experience a period of cold before flowering can be initiated (He 2012, Andres and Coupland 2012). This vernalization process ensures that the plant does not flower before winter, but instead during the next spring. In plants that need vernalization, flowering is blocked until cold temperatures gradually lift this block.

In *Arabidopsis*, the winter-annual ecotypes require vernalization for flowering. In these ecotypes, the vernalization requirement is conferred by two genes, *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC;* Michaels and Amasino 1999, Johanson et al. 2000, Michaels et al. 2004). FRI functions primarily as an *FLC* activator (Johanson et al. 2000), while FLC functions as a flowering repressor by suppressing the floral integrator genes *FT* and *SOC1* (Hepworth et al. 2002, Helliwell et al. 2006, Searle et al. 2006). In winter-annual ecotypes, vernalization gradually overrides *FLC* activation by FRI, inducing stable repression of *FLC* expression (He 2012).

FLC regulation is mediated to a great extent by chromatin modifications (Berr et al. 2011, He 2012). Chromatin modifications that are associated with active *FLC* transcription include di- and trimethylation of lysine 4 in histone 3 (H3K4me2/3), of lysine 36 in histone 3 (H3K36me2/3) and histone 2B monoubiquitination (H2Bub1). Several chromatin-modifying complexes mediating these modifications have been associated with FRI-

dependent *FLC* activation (He 2012). FRI acts as a scaffold protein to form the FRIGIDA complex (FRIc), which associates with the *FLC* locus via interaction with SUPPRESSOR OF FRIGIDA 4 and recruits these complexes into *FLC* chromatin to activate *FLC* transcription (Jiang et al. 2007, Cao et al. 2008, March-Díaz et al. 2008, Pien et al. 2008, Choi et al. 2011, He 2012, Yun et al. 2012). FRIc associates with EARLY FLOWERING IN SHORT DAYS (EFS), which mediates H3K4me2/3 and H3K36me2/3 at the *FLC* locus, and with the Complex Proteins Associated with Set1 complex (COMPASS) and the RNA Polymerase II-Associated Factor 1 complex (PAF1c) to further deposit H3K4me2/3 and H3K36me3 into the *FLC* chromatin. PAF1c recruits the HUB-UBC complex, which consists of two E3 ubiquitin ligases HISTONE MONOUBIQUITINATION 1 (HUB1) and HUB2 and an ubiquitin-conjugating enzyme UBIQUITIN CARRIER PROTEIN 1 (UBC1) or UBC2, to induce H2Bub1 at the FLC locus. Additionally, the SWR1 chromatin remodelling complex (SWR1c) is involved in the substitution of histone 2A (H2A) by the histone variant H2A.Z, which promotes gene transcription.

Vernalization-induced chromatin modifications that repress *FLC* expression include histone 3 lysine 27 trimethylation (H3K27me3) and H2A monoubiquitination (H2Aub1; Berr et al. 2011, He 2012). Vernalization is triggered by cold-activated transcription of *COLD ASSISTED INTRONIC NONCODING RNA* (*COLDAIR*), which recruits the Polycomb repressive complex 2 (PRC2) to the *FLC* chromatin (Heo and Sung 2011). PRC2 associates with plant homeodomain (PHD) proteins and deposits the H3K27me3 mark across the *FLC* locus (He 2012). This H3K27me3 mark is recognized by LIKE HETEROCHROMATIN PROTEIN 1 (LHP1), which links not only PRC2 but also H2Aub1 mediating Polycomb repressive complex 1 (PRC1) to *FLC* chromatin for stable silencing of *FLC* (Mylne et al. 2006, Derkacheva et al. 2013, Molitor and Shen 2013).

Autonomous pathway

In summer-annual *Arabidopsis* ecotypes lacking FRI, *FLC* is repressed by autonomous or constitutive FLC repressors (He 2012). Several of these repressors mediate chromatin modifications associated with transcriptional suppression, such as histone demethylation and histone deacetylation (HDac). For example, *Arabidopsis* relatives of human Lysine-Specific Demethylase 1 (LSD1) FLOWERING LOCUS D (FLD), LSD1-LIKE1 and LSD1-LIKE 2 demethylate H3K4 at the *FLC* locus (Jiang et al. 2007). HISTONE DEACETYLASE 6 (HDA6) and FVE, which deacetylate histones H3 and H4, respectively, are components of an HDac complex that requires interaction with FLD to repress *FLC* (Jiang et al. 2007, Yu et al. 2011, Jeon and Kim 2011, He 2012). Several genes in the autonomous pathway are also involved in RNA processing (He 2012). For example, the RNA-binding proteins FCA and FPA may recognize *FLC* RNA and interact with RNA 3'-end processing factors, and also with FLD-HDA6-FVE complex to silence *FLC* (Bäurle and Dean 2008).

These complexes involved in chromatin modification and RNA processing of *FLC* are common mechanisms in gene transcription regulation during plant development (Berr et al. 2011, He 2012, Holec and Berger 2012, Molitor and Shen 2013, Zentner and Henikoff 2013). For example, the floral integrator gene *FT* chromatin is modified by PRC1 and PRC2, and the repressive H3K27me3 mark is bound by LHP1 (Adrian et al. 2010, Derkacheva et al. 2013, Lee et al. 2013). However, unlike during the stable silencing of the *FLC* locus *FT* chromatin is simultaneously marked with both H3K4me3 and H3K27me3 (Jiang et al. 2008, He 2012), indicating that the *FT* transcription rate is also controlled by the relative levels of these activating and repressing marks.

Ambient temperature pathway

In addition to cold vernalization temperatures, *Arabidopsis* flowering is also regulated by ambient temperatures: cool temperatures delaying and warm temperatures advancing flowering (Jarillo and Piñeiro 2011). This thermosensory flowering is mediated at least by CO, FT and SOC1 (Balasubramanian et al. 2006, Lee et al. 2007, Jung et al. 2012c). The CO protein level is regulated by the E3 ubiquitin ligase HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE 1, which targets CO for protein degradation in response to brief cold temperatures (Jung et al. 2012c, Lazaro et al. 2012). *FT* is directly regulated by PHYTOCHROME INTERACTING FACTOR 4 (PIF4), which binds to *FT* promoter to activate transcription in response to warmer temperatures (Kumar et al. 2012). This PIF4 binding is modulated by chromatin modification: at higher temperatures the histone variant H2A.Z levels at *FT* chromatin decrease, allowing PIF4 to bind more strongly to the *FT* promoter (Kumar et al. 2012).

The RNA-binding protein FCA is involved in *FT* activator micro-RNA 172 (miR172) processing, increasing miR172 levels, and consequently *FT* levels, at higher temperatures (Jung et al. 2012b). SHORT VEGETATIVE PHASE (SVP) directly represses *FT* in leaves and *SOC1* in the shoot apex downstream of *FCA* and *FVE* in the ambient temperature pathway (Lee et al. 2007, Li et al. 2008). SVP also binds directly to miR172 (Tao et al. 2012), but recent reports on SVP repressing miR172 in response to low temperature have been conflicting (Cho et al. 2012, Jung et al. 2012b).

Gibberellin pathway

Growth in plants is regulated by the plant hormone GA (Mutasa-Göttgens and Hedden 2009). The role of GA in floral induction has been shown mainly in LD and biennial plants, in which GA is required for bolting before floral induction can occur. GA is also involved in floral transition: in *Arabidopsis*, GA activates flowering both under LDs and SDs (Wilson et al. 1992, Moon et al. 2003, Porri et al. 2012). The LD enhancement of flowering occurs in the leaves, where GA up-regulates *FT* transcription (Porri et al. 2012). Under SDs, GA up-regulates *LFY* in the shoot meristem

(Blázquez et al. 1997, 1998, Moon et al. 2003, Eriksson et al. 2006). GA activation of flowering under LDs has been believed to occur via up-regulation of *SOC1* transcription (Moon et al. 2005, Eriksson et al. 2006), but recently Porri et al. (2012) showed that GA is not involved in *SOC1* regulation, but instead activates *SQUAMOSA PROMOTER-BINDING PROTEIN LIKE 3* (*SPL3*), *SPL4*, *SPL5* and *SPL9*, which act downstream of *SOC1* (Porri et al. 2012, Jung et al. 2012a).

Age

Plants generally undergo developmental transitions or phases. After germination plants grow vegetatively (juvenile vegetative phase) until they reach reproductive competence (adult vegetative phase) and begin to flower (adult reproductive phase; Huijser and Schmid 2011). The juvenile-to-adult vegetative phase is often marked by changes in morphological traits, such as leaf shape and size, which are often more prominant in perennials than in annuals.

In *Arabidopsis*, the phase change from juvenile to adult vegetative and adult generative phases is largely regulated by two micro-RNAs, miR156 and miR172 (Huijser and Schmid 2011). The function of miR156 is to maintain the juvenile phase and its expression decreases as the plant ages (Wu and Poethig 2006, Wu et al. 2009). In contrast, miR172 expression increases after germination (Aukerman and Sakai 2003). miR156 represses *SPL* genes that promote the adult phase (Huijser and Schmid 2011). Among the targets of miR156 are *SPL3*, *SPL4* and *SPL5*, which control floral transition by activating *SOC1* and *FRUITFULL* (*FUL*), which activates flowering redundantly with *AP1* (Ferrándiz et al. 2000, Wu and Poethig 2006). miR172 represses AP2 domain genes, which suppress *FT* (Aukerman and Sakai 2003). This miR156/miR172 developmental regulation system is conserved in angiosperms, in both annuals and perennials (Huijser and Schmid 2011).

2 OBJECTIVES OF THE STUDY

Strawberries are an economically important species that have been cultivated for several hundred years. The physiology of strawberries is well known, but the molecular regulation of growth in strawberries, however, is relatively unknown. With the development of precise tools in genetic and genomic research, and especially the publishing of the wild strawberry genome, research at the molecular level has been initiated during recent years. The general aim of this study was to identify the genes that regulate the vegetative and generative growth of wild strawberry. Since the wild strawberry is used as the model plant for the garden strawberry, this knowledge would be ultimately useful for research and breeding purposes in strawberry and even other Rosaceae species.

For initial gene identification, an expressed sequence tag (EST) collection, using the suppression subtractive hybridization method, was constructed and candidate genes in existing databases were searched. Potential flowering regulating genes were then selected for expression analysis (I) and two genes were functionally analysed to build up a model of strawberry-flowering regulation and the yearly growth cycle (II, III).

3 MATERIAL AND METHODS

Methods used in this thesis work are summarized in Table 1 and described in detail in publications I to III. Methods performed by co-authors are denoted by publication number in parenthesis.

Table 1 The methods used in this thesis.

Method	**Publication**
Bioinformatic analysis	I
cDNA cloning	I
cDNA synthesis	I, II, III
Crossing population	II
Day length treatments	(I), II, III
EST sequencing	(I)
Flowering time measurements	(I), (II), III
Gibberellin treatments	(III)
GATEWAY plasmid construction	(II), III
In situ -hybridization	(II)
Genetic mapping	(II)
Phylogenetic analysis	(III)
Prohexadione-Calsium treatments	(III)
Quantitative RT-PCR	(I), II, III
RNA extraction	I, II, III
SNP marker identification	(II)
Suppression subtractive hybridization	I
Genetic transformation	II, III

4 RESULTS AND DISCUSSION

4.1 IDENTIFICATION OF VEGETATIVE GROWTH AND FLOWERING-RELATED GENES IN WILD STRAWBERRY (I)

In strawberries, the photoperiod regulates vegetative and generative growth: LDs enhance vegetative growth and SDs induce flowering. The effect of the photoperiod on vegetative growth is most noticeable in axillary bud development: LDs activate runner formation and SDs branch crown formation. Branch crown formation is also essential for flowering, since the inflorescence is formed terminally in the shoot apex; i.e. the potential number of inflorescences in a plant is dependent on the number of branch crowns developed. For commercial cultivation, balancing between vegetative and generative growth is vital for optimal cropping. Therefore, identification of the genes controlling both vegetative and generative phases would increase our understanding of the regulation of growth processes, and ultimately advance cultivar breeding. In addition, identification of the flowering repressor *SFL* would be a major breakthrough for breeding cultivars with extended cropping season.

To identify putative flowering regulators in wild strawberry, both data mining and EST sequencing were combined. Since the wild strawberry exhibits two different flowering types, seasonally flowering and perpetually flowering genotypes, the suppression subtractive hybridization method was employed to separate gene transcripts between the two genotypes, aiming to identify genes that were related specifically to flowering or vegetative growth processes. In total, 1172 ESTs were sequenced from the library containing SD genotype complementary DNA (cDNA) and 1344 ESTs from the library containing EB genotype cDNA (I, Table 1). Searching these ESTs against *Arabidopsis*, Swissprot and nonredundant protein sequence databases revealed that more that 70% of the ESTs in both libraries resulted in a Blastx hit in one or all databases (I, Table 1). Furthermore, comparison of the EST sequences from both libraries against the Genome Database for the Rosaceae (GDR) database revealed that 38% of the sequences encoded novel *Fragaria* transcripts. In all, 14 putative flowering-related genes were found in the two EST libraries: eight in the SD library and four in the EB library (I, Figure 2). These genes were placed in all major flowering pathways in *Arabidopsis*, suggesting initially that these pathways also exist in strawberries (I, Figure 2).

In addition to these 14 ESTs, 118 *Arabidopsis* flowering genes were searched against public *Fragaria* and Rosaceae EST databases in the GDR, and an additional 52 and 88 ESTs, respectively, were identified. One of the central flowering-regulating genes in *Arabidopsis* is *FLC*, but no *FLC* or *FLC*-like genes were found in the EST sequences, or in the *Fragaria*

and Rosaceae databases. This was expected, since functional *FLC* and *FLC*-like genes have been, so far, found only in the Brassicaceae (Jarillo and Piñeiro 2011).

Putative homologues for most genes in the photoperiodic pathway were found in the EST collection or the *Fragaria* database (I, Table 2). The most notable exceptions were *GI*, *FT* and *TFL1*. However, a putative homologue for *GI* was found in the Rosaceae database. Putative homologues were identified for most genes in the chromatin-modelling complexes in the vernalization pathway (I, Table 3). In addition to regulating *FLC* expression, these complexes also regulate other flowering-related genes; e.g. the *FT*, *LFY* and FM identity genes *AGAMOUS* and *PISTILLATA* are known target loci for PRC1 and PRC2 complexes (Adrian et al. 2010, Molitor and Shen 2013, Holec and Berger 2012). As with the photoperiod and vernalization pathway genes, putative homologues were found for most genes in the autonomous pathway and GA signalling pathway (I, Table 4). These results and, later, the analysis of the 'H4' genome published in 2011 suggest that the flowering pathway genes are mostly conserved between *Arabidopsis* and the wild strawberry (Shulaev et al. 2011).

No putative homologues for the floral integrator genes *FT*, *SOC1* or *LFY* were identified, nor for the FM gene *AP1*, either in the EST sequences or in the Fragaria and Rosaceae databases. Therefore, a full-length *Fragaria vesca SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*FvSOC1*) and a fragment of *Fragaria vesca LEAFY* (*FvLFY*) were cloned from EB *F. vesca.* Despite several attempts, *Fragaria vesca FLOWERING LOCUS T* (*FvFT*) was not successfully cloned in either SD or EB *F. vesca*, and thus datamining was applied to find a putative *FT* in the *Prunus* and *Malus* protein databases at the National Centre for Biotechnology Information, and a putative *AP1/ FUL* in the *Fragaria* EST collection at the GDR for expression analysis. The expression of these and a selection of the putative flowering-related genes identified (I, Table 5) was examined in SD *F. vesca* and the EB genotype 'Baron Solemacher'. However, no clear differences in the expression levels were found between the genotypes, except that a putative *FvAP1/FUL* was expressed only in the shoot apices of 'Baron Solemacher', correlating with the perpetually flowering trait (I, Table 6). Both the *FvAP1/FUL* and *FvLFY* transcription levels increased after the two-leaf stage in young seedlings of the EB genotype compared with the SD genotype (I, Figure 4 A and B). In *Arabidopsis*, *AP1*, *LFY* and *FUL* are up-regulated during floral initiation, marking the floral transition (Hempel et al. 1997). The very early increase in *FvAP1/FUL* and *FvLFY* transcription in the EB genotype indicates that these genes, especially *FvAP1/FUL*, are also useful in determining the stage of floral transition in the shoot apex in wild strawberry.

4.2 MOLECULAR CONTROL OF FLOWERING IN WILD STRAWBERRY (II, III)

4.2.1 Floral integrator genes in wild strawberry

The floral integrator genes *FT*, *SOC1* and *LFY* are central to floral transition. In several plant species, the *FT* and *FT* homologues participate in flowering activation (Jarillo and Piñeiro 2011). Based on *Arabidopsis* and rice, *FT*/*Hd3a* acts as a florigen, a mobile flowering signal that moves from the leaf to the shoot apex to activate floral transition (Corbesier et al. 2007, Tamaki et al. 2007). Florigen *FT* and *FT*-like genes are expressed in the leaf and are under photoperiodic regulation, leading to transcriptional diurnal oscillation (Yanovsky and Kay 2002, Hayama et al. 2003, 2007, Andres and Coupland 2012). In wild strawberry, three *FT*-like genes are found in the 'H4' genome. *FvFT1* was expressed mainly in the leaves and *FvFT2* in the flower buds in SD *F. vesca* (II, Figure 6 A and B). *FvFT3* transcripts were not detected in the plant parts tested (data not shown). *FvFT1* was photoperiodically regulated and also showed dawn/dusk expression peaks under LDs (II, Figure 6 C and D), suggesting that *FvFT1* may be a functional homologue of *FT*.

In *Arabidopsis*, FT activates *SOC1* transcription under LDs in the shoot apex but not in the leaves (Borner et al. 2000, Moon et al. 2005, Wigge et al. 2005). In contrast, the SOC1 homologue OsMADS50 in rice activates the *FT* homologues *Hd3a* and *RFT1* in leaves under LDs (Lee et al. 2004, Ryu et al. 2009). In SD *F. vesca*, the *SOC1* homologue *FvSOC1* (III, Supplemental Figure 1–4) was down-regulated both in the leaves and shoot apices under SDs (III, Figure 2 B–D), in parallel with *FvFT1* expression in leaves (II, Figure 6 C and E). This SD down-regulation of *FvSOC1* expression was also released after transfer to LD conditions (III, Figure 2 D). In the EB genotype 'H4', three independent *FvFT1* silencing lines showed decreased *FvSOC1* expression in the shoot apices (III, Figure 2 E), indicating that FvFT1 regulates *FvSOC1*, as in *Arabidopsis*, and not *vice versa*, as in rice. However, this result should also be confirmed in the SD *F. vesca* background. Furthermore, *FvFT1* expression was not changed in either *FvSOC1* overexpression or silencing lines compared with untransformed SD *F. vesca* plants (Figure 4), indicating that *FvFT1* is not downstream of *FvSOC1*. These data suggest that FvFT1 functions as an activator of *FvSOC1* in shoot meristem in wild strawberry.

In *Arabidopsis*, FT activates *SOC1* directly and *LFY* via *SOC1* activation (Wigge et al. 2005, Moon et al. 2005, Lee et al. 2008). However, overexpression or silencing of *FvSOC1* in the SD *F. vesca* did not affect *FvLFY* expression, either under LD or SD conditions (III, Figure 4 D, Supplemental Figure 10 B). Since SOC1 interacts with AGAMOUS-LIKE 24 (AGL24) to activate *LFY* and there is a positive feedback loop between *SOC1* and *AGL24* during floral transition (Liu et al. 2008a, Lee et al. 2008),

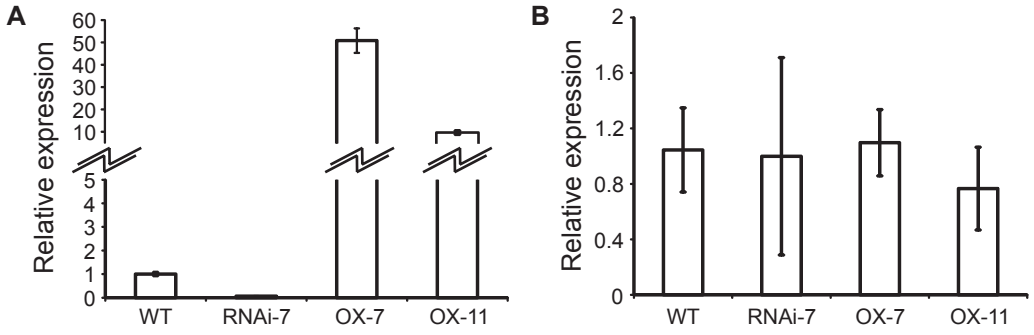

Figure 4 Expression of *FvSOC1* (A) and *FvFT1* (B) in leaves of SD *F. vesca* (WT) and transgenic lines silencing (RNAi) or overexpressing (OX) *FvSOC1*. Rooted runner plants were grown under SDs (day length 12 h, temperature 19 °C) for four weeks. Expression levels were normalized with *FvMSI1* and relative expression calculated to WT. qPCR reactions were performed, as described (III).

the *FvAGL24* expression levels were examined in the *FvSOC1* overexpression or silencing lines. The *FvAGL24* levels were slightly up-regulated in the *FvSOC1* overexpression lines, but in the *FvSOC1*-silencing lines the *FvAGL24* levels were relatively unchanged (III, Figure 4 E, Supplemental Figure 10 A). Therefore, it seems unlikely that FvSOC1 regulates either *FvAGL24* or *FvLFY* during floral transition. In rice, the FLO/LFY homologue RFL also functions differently from *Arabidopsis*: RFL activates *RFT1* and also *OsMADS50* in leaves (Rao et al. 2008). However, the expression of *FvLFY* did increase in the shoot apices of the EB genotype 'Baron Solemacher' under LDs (I, Figure B). Since *FvLFY* up-regulation occurred approximately 1 week later than *FvAP1* expression (I, Figure 4 A), *FvLFY* may play a role in FM regulation. Since LFY in *Arabidopsis* is involved in FM development and floral organ patterning (Liu et al. 2009), it would be interesting to elucidate the possible role of *FvLFY* in FM development in wild strawberry.

4.2.2 TFL1 homologue FvTFL1 represses flowering in wild strawberry

In *Arabidopsis*, FT is counteracted in the shoot apex by the flowering repressor TFL1 (Kobayashi et al. 1999, Hanano and Goto 2011). Iwata et al. (2012) detected a 2-base pair deletion in *Fragaria vesca TERMINAL FLOWER 1* (*FvTFL1*) sequence, leading to a putatively truncated and therefore nonfunctional protein, and associated *FvTFL1* with the *SFL* in an 11-Mb window in linkage group VI (Sargent et al. 2006, Iwata et al. 2012). This window was further narrowed down to 248 kb (II, Supplemental Figures S1 and S2). Using the EB genotype 'H4' it was shown that under LDs (flower-inducing conditions for 'H4') the primary transgenic plants overexpressing *FvTFL1* without this deletion remained vegetative, whereas overexpression of *FvTFL1* with the deletion did not change the flowering phenotype (II, Figure 1 A–D, Supplemental Figure S3). To further test the

hypothesis of FvTFL1 functioning as a flower repressor, *FvTFL1* was overexpressed and silenced in SD *F. vesca*. Both under continuous LDs (flower-repressing conditions for SD *F. vesca*) and after strong flower-inducing treatment, the primary transgenic plants overexpressing functional *FvTFL1* remained vegetative, as did untransformed plants under LDs (II, Figure 2 A and B). In contrast, *FvTFL1* silencing plants began flowering under both SDs and LDs at the same time as the untransformed control plants after flower-inducing SD treatment (II, Figure 2 A and B), showing that FvTFL1 represses flowering in wild strawberry. This functional analysis confirmed that *FvTFL1* is the wild strawberry *SFL* (Brown and Wareing 1965, Albani et al. 2004, Iwata et al. 2012). The flower-repressing function of FvTFL1 seems to be conserved in the Rosaceae; silencing *TFL1* homologues leads to perpetual flowering in apple, pear and rose (Kotoda and Wada 2005, Kotoda et al. 2006, Freiman et al. 2012, Iwata et al. 2012).

In *Arabidopsis*, TFL1 regulates IM identity in an antagonist manner to the FM-regulating genes *LFY*, *AP1*, *FUL* and *CAULIFLOWER* (*CAL;* Liljegren et al. 1999, Ratcliffe et al. 1999, Ferrándiz et al. 2000). *TFL1* is expressed in the centre of the shoot meristem, keeping the meristem indeterminate (Ratcliffe et al. 1999). In contrast, *FvTFL1* was expressed throughout the apical meristem under LDs (II, Figure 4 B and C), and furthermore was strongly down-regulated in shoot apices under SDs (II, Figure 5 A). In apple, down-regulation of the *TFL1* homologue *MdTFL1* occurs during floral transition with a concomitant up-regulation of *MdAP1* in the shoot apex (Mimida et al. 2011). Both strawberry and apple form a terminal inflorescence, in which lateral flower meristems arise from the flanks of the terminal flower meristem (Jahn and Dana 1970, Foster et al. 2003). In consequence, the meristems are determinate and thus *TFL1* behaves differently.

The TFL1 and AP1/CAL/FUL genes repress each other in *Arabidopsis* IM (Ratcliffe et al. 1999, Liljegren et al. 1999, Ferrándiz et al. 2000). When functional *FvTFL1* was overexpressed in ‘H4’, the putative *FvAP1*/*FUL* homologues were down-regulated (II, Figure 1 E–H), but when functional *FvTFL1* was silenced in SD *F. vesca*, *FvAP1* was up-regulated in the shoot apices (II, Figure 2 C and D). Furthermore, under flower-inducing conditions *FvTFL1* expression was repressed with concomitant up-regulation of *FvAP1*/*FUL*-like FM identity genes and flowering in SD *F. vesca* (II, Figure 5 A–D). After transfer to flower noninducing conditions *FvTFL1* expression was up-regulated and *FvAP1*/*FUL* expression down-regulated in the shoot apices of the branch crowns formed under these noninductive conditions (II, Figure 5 A–D). This gave further confirmation that FvTFL1 represses flowering by regulating FM identity genes. It would also be very worthwhile determining whether FvAP1/FUL and FvLFY regulate *FvTFL1* in the apical meristem of wild strawberry.

4.2.3 SOC1 homologue FvSOC1 represses flowering in wild strawberry

SOC1 and *SOC1* homologues encode flowering activators in several plant species, both in monocots and dicots (Borner et al. 2000, Watson and Brill 2004, Sreekantan and Thomas 2006, Shitsukawa et al. 2007, Tan and Swain 2007, Ryu et al. 2009, Nakano et al. 2011, Ding et al. 2013). However, in SD *F. vesca* background silencing and not overexpression of *FvSOC1* induced flowering (III, Figure 3 A and B, Table 1). Therefore, flowering of selected strong *FvSOC1*-silencing lines was followed under continuous LDs and after flower-inducing SD treatment. The *FvSOC1*-silencing lines formed inflorescences continuously, regardless of the photoperiod, while under LDs wild-type plants did not form inflorescences at all or after SD treatment ceased to form inflorescences after a few weeks (III, Figure 3 C and D). Furthermore, SD-treated *FvSOC1* overexpression lines failed to flower (III, Table 1). These data show that FvSOC1 has a flower-repressing function in wild strawberry.

Since *FvTFL1* is the flowering trait-controlling gene in wild strawberry (II, Figure 1 A–C, Figure 2 A and B, Supplemental Figures S1 and S2; Brown and Wareing 1965, Albani et al. 2004, Iwata et al. 2012) and the expression of *FvTFL1* was under photoperiodic control correlating with the flowering phenotype (II, Figure 4 D–F, Figure 5 A and D), the expression of *FvTFL1* in *FvSOC1* silencing and overexpression plants was examined in the SD *F. vesca* background. Silencing *FvSOC1* led to down-regulation of *FvTFL1* in the shoot apices under the LDs (III, Figure 3 E–G) and after SD treatment (III, Figure 3 E and G). In contrast, overexpression of *FvSOC1* led to up-regulation of *FvTFL1* under both LDs and SDs in the shoot apices (III, Figure 4 A and B). Furthermore, *FvSOC1* expression was relatively unchanged in the shoot apices of *FvTFL1* silencing or overexpression lines (III, Supplemental Figure 7), indicating that *FvSOC1* is not a downstream gene of FvTFL1. These data suggest that FvSOC1 mediates the repressor function in flowering via *FvTFL1* activation. However, the down-regulation of *FvTFL1* expression under SDs in the shoot apices of a strong *FvSOC1*-silencing line was stronger than in wild-type SD *F. vesca* (III, Figure 3 F and G) and *FvTFL1* up-regulation in *FvSOC1* overexpression lines was not as pronounced under SDs as under LDs (III, Figure 3 F and G, Figure 4 B), suggesting that an additional *FvTFL1*-regulating pathway functions in wild strawberry.

SOC1 or SOC1 homologues do not regulate *TFL1* directly. However, FvSOC1 belongs to MADS box TFs, which bind to certain sequence motifs called CArG boxes in promoter regions of their target genes. FvSOC1 may be able to bind to *FvTFL1* promoter to repress *FvTFL1* transcription. This hypothesis is supported by the presence of a CArG box motif that is almost identical to a SOC1-binding site in the *AGL24* promoter in *Arabidopsis*, with an adjacent AAA triplet that may be essential for MADS box protein-binding action, in the *FvTFL1* promoter region (III; Liu et al. 2008b, Deng et

al. 2011, Tao et al. 2012). This potential binding of FvSOC1 to *FvTFL1* promoter should be investigated to verify any possible direct regulation.

4.3 MOLECULAR CONTROL OF VEGETATIVE GROWTH IN WILD STRAWBERRY (II, III)

Runner formation was strongly affected in *FvSOC1* transgenic plants; *FvSOC1* overexpression induced continuous runner formation and *FvSOC1* silencing suppressed runner formation, regardless of the photoperiod in the SD *F. vesca* background (III, Figure 6). This FvSOC1 effect on runner formation is also independent of FvTFL1, because neither runner formation nor branch crown formation was altered in *FvTFL1* transgenic plants under either LD or SD conditions (II, Figure 3 A–D). *FvSOC1* overexpression was linked to long petioles, whereas *FvSOC1*-silencing plants had short petioles and produced branch crowns from the axillary buds (III, Supplemental Figures 8 and 13). Since strawberry growth and axillary bud development are known to be regulated by GA (Thompson and Guttridge 1959, Guttridge and Thompson 1964, Braun and Garth 1986, Wiseman and Turnbull 1999a, Black 2004, Hytönen et al. 2008), this vegetative phenotype of *FvSOC1* transgenic plants indicated that FvSOC1 may be involved in regulation of GA levels. Treatment with prohexadione-Calcium, which inhibits the last steps in the GA biosynthesis pathway, arrested runner formation in *FvSOC1* overexpressing plants (III, Figure 7 A) and, correspondingly, GA treatment induced runner production in *FvSOC*-silencing plants (III, Figure 7 B). The recovery of the SD *F. vesca* vegetative phenotype, after treatments with either the GA biosynthesis inhibitor or GA, confirmed that FvSOC1 is involved in GA regulation.

The last steps in the GA biosynthesis are regulated by GA-oxidases (GAox; Mutasa-Göttgens and Hedden 2009). The expression of *GAox* genes was altered in *FvSOC1* transgenic plants; several *FvGA20ox* and *FvGA3ox* genes were up-regulated in leaves in the *FvSOC1* overexpression line under LDs (III, Figure 7 C and D), while in the *FvSOC1*-silencing line the *GA20ox* and *GA3ox* genes were down-regulated (III, Figure 7 E and F). This indicates that FvSOC1 controls vegetative growth via regulating biosynthesis of active GA. In *Arabidopsis*, SOC1 promotes flowering not upstream but downstream of GA (Borner et al. 2000, Moon et al. 2003). However, Dorca-Fornell et al. (2011) showed that SOC1 could be involved in regulating *GA20ox1* levels. SOC1 may regulate GA biosynthesis indirectly, possibly via the *TEMPRANILLO* genes that repress the GA biosynthesis genes (Tao et al. 2012, Osnato et al. 2012). This putative GA regulation pathway should be investigated in wild strawberry.

The runnering trait is controlled by *R* in wild strawberry (Brown and Wareing 1965). *R* has been located in linkage group II (Sargent et al.

2004), whereas *FvSOC1* is located in linkage group VII in the diploid map (Shulaev et al. 2011). This indicates that *FvSOC1* is not *R* in wild strawberry. Recently, Gaston et al (2013) showed that both seasonal flowering and runnering in garden strawberry is controlled by a single gene, *FaPFRU*. However, *FaPFRU* is located in linkage group IVb-f within homologous group IV, corresponding to diploid linkage group IV (Rousseau-Gueutin et al. 2008, Gaston et al. 2013), indicating that the runnering trait is controlled by different genes in wild and garden strawberry. Nonetheless, the identification of *R* would be important for understanding the regulation of axillary bud development in wild strawberry.

4.4 CONCLUDING REMARKS: MOLECULAR CONTROL OF THE YEARLY GROWTH CYCLE IN WILD STRAWBERRY

Based on the results in this thesis, the wild strawberry yearly growth model can be extended with a molecular regulation level, summarized in Figure 5. In spring, when strawberry growth resumes after resting over winter, the increasing day length induces *FvSOC1* expression in the apical and axillary meristems (III, Figure 2 C and D), via activation by FvFT1 (II, Figure 6 C–E; III, Figure 2 E). FvSOC1 up-regulates *FvTFL1* expression in the apical meristem, leading to down-regulation of *FvAP1* (II, Figure 2 C and D, Figure 4 D, Figure 5 A and B; III, Figure 3 E–H) and repression of flowering. High *FvSOC1* expression during spring and summer induces expression of GA biosynthesis genes (III, Figure 7 C–F), which likely leads to accumulation of active GA in the plant. Active GA enhances vegetative growth and induces runner formation from the axillary buds (III, Figure 7 A and B; Hytönen et al. 2009). In autumn, the shortening day represses *FvSOC1*, leading to down-regulation of GA biosynthesis genes (III, Figure 2 B, Figure 7 C–F). The decreasing amount of active GA induces branch crown formation instead of runners from the axillary buds and suppresses vegetative growth (Wiseman and Turnbull 1999b, Hytönen et al. 2009). At the same time, *FvSOC1* expression decreases with concomitant down-regulation of *FvTFL1* expression in the apical meristem, and *FvAP1* repression by FvTFL1 is relieved (II, Figure 2 C and D, Figure 4 D, Figure 5 A and B; III, Figure 2 C and D, Figure 3 E–H). Consequently, the apical meristem is induced to form an inflorescence initial. The plant overwinters and next spring the increasing day length activates flowering and a new growth cycle.

In the perpetually flowering genotypes, the 2-base pair deletion in the first exon of *FvTFL1* causes a putative truncated and therefore nonfunctional FvTFL1 protein translation (II, Supplemental Figure S2; III, Supplemental Figure 12; Iwata et al. 2012). These *SFL* mutant genotypes flower continuously and photoperiod-independently, after initial flower induction has occurred under LDs (I, Figure 1; II, Figure 5 D).

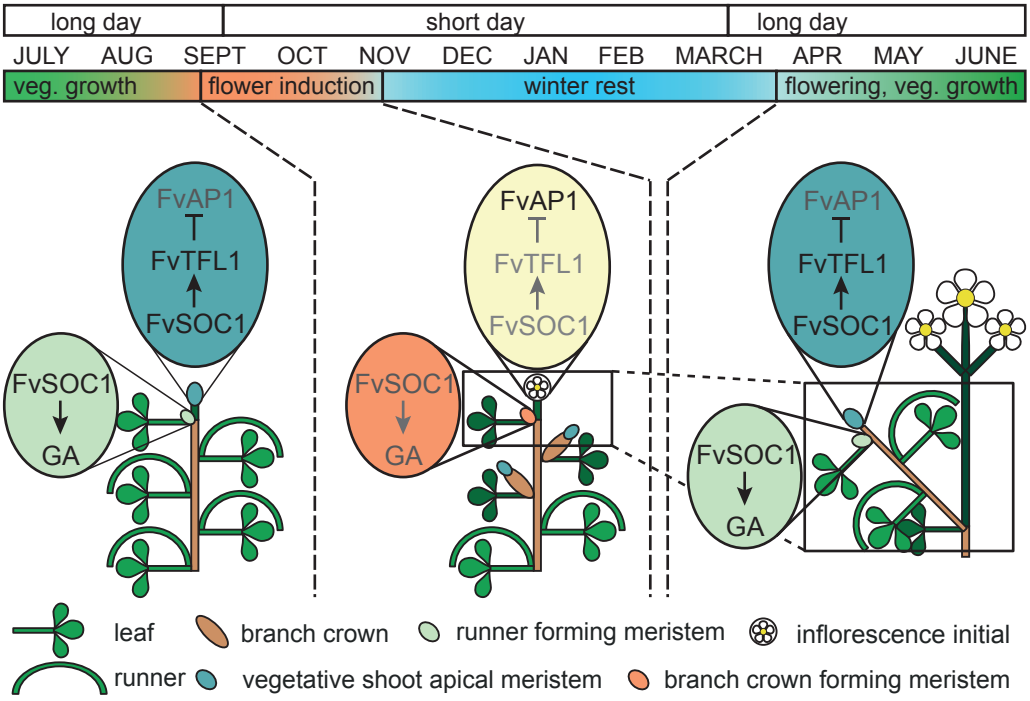

Figure 5 Model of the yearly growth cycle at the molecular regulation level in wild strawberry. FvSOC1, *F. vesca SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1*; FvTFL1, *F. vesca TERMINAL FLOWER 1*; FvAP1, *F. vesca APETALA 1,* GA, gibberellin. The arrows denote transcriptional activation and the bars transcriptional repression. See Figure 1 and text above for detailed explanation.

The results obtained here in this thesis improve our understanding of the regulation of the strawberry yearly growth cycle at the molecular level. The two, apparently separate *FvSOC1*-GA and *FvSOC1-FvTFL1* pathways controlling vegetative growth and flowering, respectively, present interesting prospects for research in strawberries, especially considering the recent discovery of a major locus, *FaPFRU,* controlling both seasonal flowering and runner formation in garden strawberry (Gaston et al. 2013). The discovery of the central role of FvSOC1 in the photoperiodic regulation of both vegetative and generative growth in wild strawberry also raises the possibility that similar regulation may be present in other Rosaceae species.

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