# Regulation of strawberry growth and development

**Timo Hytönen**

Department of Applied Biology
Horticulture
Faculty of Agriculture and Forestry
and
Viikki Graduate School in Biosciences
University of Helsinki
Finland

Academic dissertation

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Supervisors: Professor Paula Elomaa
Department of Applied Biology
University of Helsinki, Finland

Professor Olavi Junttila
Department of Biology
University of Tromsø, Norway

Reviewers: Professor Yrjö Helariutta
Department of Biological and Environmental Sciences
University of Helsinki, Finland

Dr. Päivi Rinne
Department of Plant & Environmental Sciences
Norwegian University of Life Sciences, Norway

Opponent: Dr. Béatrice Denoyes-Rothan
Plant Breeding and Genetics Division
I'institut de la National Recherché Agronomique, France

Custos Professor Paula Elomaa
Department of Applied Biology
University of Helsinki, Finland

Cover: Everbearing and seasonal flowering genotypes of woodland strawberry.

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*"Oma maa mansikka, muu maa mustikka"*

*-Finnish proverb-*

**TABLE OF CONTENTS**

## LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following publications, which will be referred in the text in Roman numerals (I-IV). The published papers are reprinted with the permission from the publishers.

I **Hytönen T, Palonen P, Mouhu K, Junttila O.** 2004 Crown branching and cropping potential in strawberry (*Fragaria* x *ananassa*, Duch.) can be enhanced by daylength treatments. Journal of Horticultural Science & Biotechnology 79: 466-471.

II **Hytönen T, Moritz T, Elomaa P, Junttila O.** 2008. Gibberellin mediates daylength-regulated differentiation of vegetative meristems in strawberry (*Fragaria* x *ananassa* Duch.). BMC Plant Biology, in press.

III **Hytönen T, Mouhu K, Koivu I, Elomaa P, Junttila O.** 2008. Prohexadione-calcium enhances the cropping potential and yield of strawberry (*Fragaria* x *ananassa* Duch.). European Journal of Horticultural Science 73: 210-215.

IV **Mouhu K, Hytönen T, Folta K, Rantanen M, Paulin L, Auvinen P, Elomaa P.** 2008. Characterization of flowering pathways in strawberry -a perennial SD plant. Submitted.

## AUTHORSHIP STATEMENT

Publication I

Timo Hytönen was responsible for the planning and design of the experimental work together with Pauliina Palonen and Olavi Junttila. He did the experimental work together with Katriina Mouhu. He wrote the first draft of the manuscript and he was responsible for writing the final version in collaboration with the other authors.

Publication II

Timo Hytönen was responsible for the planning and design of the experimental work together with Olavi Junttila and Paula Elomaa. He did all the experimental work himself, except the quantification of GAs by GC-MS and cDNA sequencing. He had the main responsibility in the writing of the paper.

Publication III

Timo Hytönen was responsible for the planning and design of the experimental work. He carried out the experiments together with Ilpo Koivu and Katriina Mouhu. In addition, he was responsible for arranging the field experiments in commercial strawberry farms. He contributed to the writing of the paper equally with Katriina Mouhu.

Publication IV

Timo Hytönen was responsible for the planning and design of the experimental work together with Paula Elomaa and Katriina Mouhu. He established the collaboration with Kevin Folta. He did the real-time RT-PCR analyses and searched flowering time gene homologs included in the paper from Rosaceae EST sequence databases. He contributed to the writing of the paper equally with Katriina Mouhu.

**ABBREVIATIONS USED**

| | |
|---|---|
| 2ODD | 2-oxo-glutarate dependent dioxygenase |
| AMO-1618 | 2-isopropyl-5-methyl-4-piperidylcarbonyloxy trimethylammonium chloride |
| bHLH | basic helix-loop-helix |
| cDNA | complementary DNA |
| cM | centimorgan |
| DN | day-neutral |
| EB | everbearing |
| EST | expressed sequence tag |
| FR | far red (light) |
| GA | gibberellin |
| GC-MS | gas chromatography – mass spectrometry |
| GDR | Genome Database for Rosaceae |
| GGDP | geranylgeranyl diphosphate |
| H2A.Z | histone variant 2A.Z |
| H3K4me3 | histone 3 lysine 4 trimethylation |
| H3K9me2 | histone 3 lysine 9 dimethylation |
| H3K27me3 | histone 3 lysine 27 trimethylation |
| H3K36me2 | histone 3 lysine 36 dimethylation |
| H3K36me3 | histone 3 lysine 36 trimethylation |
| H4R3sme2 | histone 4 arginine 3 symmetric dimethylation |
| HMT | histone methyl transferase |
| HPLC | high pressure liquid chromatography |
| HPS | high pressure sodium |
| IAA | indoleacetic acid |
| ISSR | inter simple sequence repeat |
| LD | long day |
| miR | micro-RNA |
| mRNA | messenger RNA |
| NCBI | National Center for Biotechnology Information |
| NCGR | National Clonal Germplasm Repository |
| PAF1 | RNA polymerase 2 associated factor 1 |
| PCR | polymerase chain reaction |
| PP333 | paclobutrazol |
| PRC2 | Polycomb repressive complex 2 |
| ProCa | prohexadione-calcium |
| QTL | quantitative trait locus |
| R | red (light) |
| RACE | rapid amplification of cDNA ends |
| RNAi | RNA interference |
| RT-PCR | reverse transcription PCR |
| SCAR | sequence characterized amplified region |
| SCF | SKP/Cullin/F-box E3 ubiquitin ligase complex |
| SD | short day |
| SFL | seasonal flowering locus |
| SSH | suppressive subtractive hybridization |

**SUMMARY**

Strawberries (*Fragaria* spp.) are adapted to diverse environmental conditions from the tropics to about 70ºN, so different flowering responses to environmental conditions can be found. Most genotypes of garden strawberry (*F.* x *ananassa*) and woodland strawberry (*F. vesca*) are short-day (SD) plants that are induced to flowering by photoperiods under a critical limit, but also various photoperiod x temperature interactions can be found. In addition, continuously flowering everbearing (EB) genotypes are found in both species. In addition to flowering, axillary bud differentiation in strawberry is regulated by photoperiod, at least in SD genotypes. In SD conditions, axillary buds differentiate to rosette-like structures called "branch crowns", whereas in long-day conditions (LD) they form runners, branches with 2 long internodes followed by a daughter plant (leaf rosette). The number of crown branches determines the yield of the plant, since inflorescences are formed from the apical meristems of the crown. Although axillary bud differentiation is an important developmental process in strawberries, its environmental and hormonal regulation has not been characterized in detail. Moreover, the genetic mechanisms underlying axillary bud differentiation and regulation of flowering time in these species are almost completely unresolved. These topics have been studied in this thesis in order to enhance strawberry research, cultivation and breeding.

The results showed that axillary bud differentiation in garden strawberry cv. Korona can be strictly controlled by photoperiod. Runner initiation is suppressed by 8-12 SD cycles with the concomitant induction of crown branching, and 3 weeks of SD is sufficient for flowering induction in the main crown. Furthermore, a second SD treatment given a few weeks after the first SD period can be used to induce flowering in the primary branch crowns and to induce the formation of secondary branches. Thus, artificial SD treatments effectively stimulate crown branching, providing one means for the increase of cropping (yield) potential in strawberry. It was also found that gibberellin (GA) is one of the key signals involved in the photoperiod control of shoot differentiation. This idea is supported by the findings that (1) the inhibitor of GA biosynthesis, prohexadione-calcium (ProCa) prevented runner initiation with a concomitant enhancement of crown branch formation in LD, and this effect of ProCa was completely reversed by GA application; (2) the inhibition of runner formation correlated with a similar decline in $GA_1$ level in both ProCa and SD treatments, and (3) several GA biosynthetic, signalling and target genes were similarly affected by ProCa and SD (GA signalling homeostasis), correlating with shoot differentiation and with $GA_1$ levels. It was further showed that chemical control of GA biosynthesis by ProCa can be utilized to prevent excessive runner formation and induce crown branching in strawberry fields in northern LD conditions. Moreover, ProCa increased berry yield up to 50%, showing that it is an easier and more applicable alternative to artificial SD treatments for controlling strawberry crown development and yield. Finally, flowering gene pathways in *Fragaria* were explored by searching for homologs of 118 *Arabidopsis thaliana* flowering-time genes. In total, 66 gene

homologs were identified, and they distributed to all known flowering pathways, suggesting the presence of these pathways also in strawberry. Expression analysis of selected genes revealed that the mRNA of putative floral identity gene *APETALA1* (*AP1*) began to accumulate in the shoot apex of the EB genotype after the one leaf stage in LD, correlating with floral development. However, *AP1* was absent in vegetative SD genotype, indicating the usefulness of this gene product as the marker of floral initiation. The present data enables the further exploration of strawberry flowering pathways with genetic transformation, gene mapping and transcriptomics methods.

# 1 INTRODUCTION

Garden strawberry (*Fragaria* x *ananassa* Duch.) is one of the most popular soft fruit species because of its unique aroma and healthy composition. The importance of this species has inspired several researchers to develop new cultivation practices for strawberry. These studies include detailed exploration of environmental regulation of growth and development in garden strawberry, as well as its ancestor species woodland strawberry (*F. vesca* L.) (e.g. Heide 1977, Konsin et al. 2001, Heide and Sønsteby 2007). Several studies have revealed an obvious antagonism between generative and vegetative development, e.g. runner formation and induction of flowering seem to be almost mutually exclusive processes. Regardless of the obvious linkage between these two processes, they are genetically separate in two woodland strawberry genotypes (Brown and Wareign 1965, Battey et al. 1998), whereas branch crown formation is controlled by the same gene loci as runner formation in woodland strawberry (Brown and Wareign 1965). Obviously, the regulation of flowering in strawberry is a complex process that is intertwined with the regulation of vegetative growth including axillary bud differentiation, as well as other developmental processes occurring during the annual life cycle. Thus, detailed molecular as well as metabolic analyses are needed to clarify how these processes are controlled separately and how they interact. This endeavour is of utmost importance, and it will strongly enhance the development of new cultivars and cultivation techniques for strawberries as well as other species of the Rosaceae family.

## 1.1 Strawberry species, morphology and growth habit

The strawberry genus *Fragaria* belongs to the rose family (Rosaceae) and consists of 22 known species, including 13 diploids, four tetraploids, one hexaploid, and four octoploids (Folta and Davis 2006). The garden strawberry is an octoploid hybrid species (2n=8x=56), originating from interspecific hybridization of *F. chiloensis* L. and *F. virginiana* Mill. (Darrow 1966). Strawberry is one of the most important berry crops worldwide, with a global production of over 3.5 million tons and a production area of about 200 000 ha in 2006 (http://www.fao.org). The three biggest strawberry producers, USA, China and Spain, produced about half of the total annual harvest in 2006. In Finland, the garden strawberry is the most important berry, with a cultivation area of approximately 3300 ha and total production of 9700 tons in 2007 (http://www.hedelmatmarjat.fi).

Strawberry is a typical perennial rosette plant with very short internodes in its stem (Figure 1). The stem is called "a crown", and it consists of both aerial and underground parts. In the vegetative stage, the apical meristem of the crown produces new internodes and one trifoliate leaf with a long petiole in each node. One axillary bud is also formed in each node. Further development of these buds can be inhibited, but typically they initiate branch crowns or runners, depending on the growing

conditions. Runners consist of two long internodes followed by a terminal daughter plant. After formation of the daughter plant, and in favourable growing conditions the second axillary bud of the runner continues runner elongation. The first axillary bud may also produce a new runner, but it may also remain quiescent.

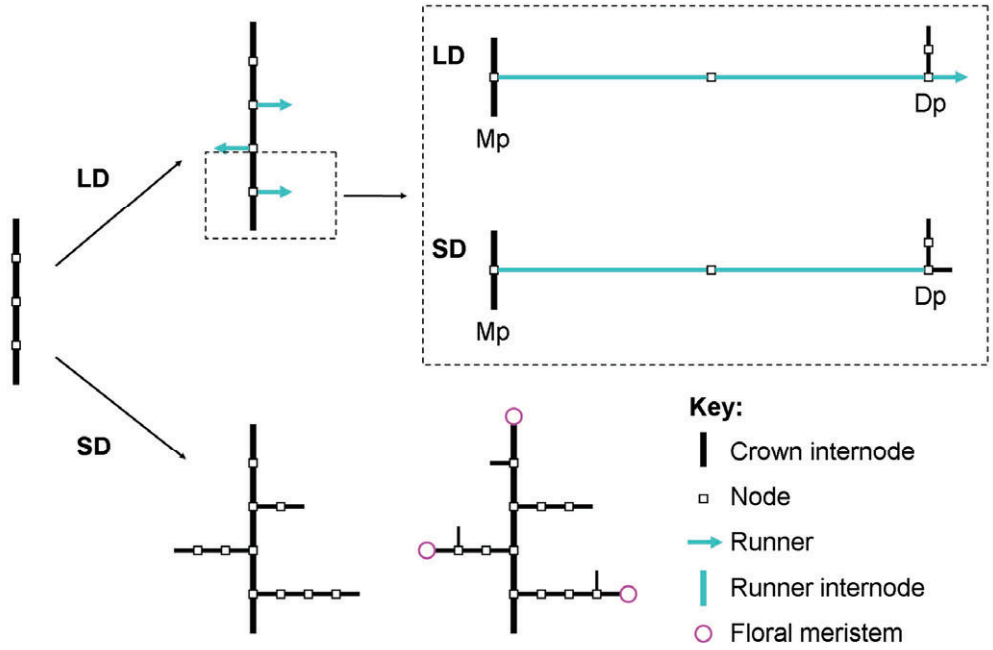


Figure 1. Schematic representation of the strawberry development. Strawberry crown (left) consists of short internodes. One leaf and axillary bud is formed in each node. In LD conditions, axillary buds are differentiated to runners, whereas in SD branch crowns are formed. After a certain number of SD cycles, apical meristems of the main crown and branch crowns are induced to flower. At this stage, axillary buds below the floral meristem continue the crown development. Runner development is shown in the inset. Runners consist of two long internodes followed by a terminal daughter plant (Dp). In LD conditions, the axillary bud in the second node of the runner continues runner growth, whereas in SD, this bud differentiates to branch crown and runner elongation ceases. Mp = mother plant.

Strawberry inflorescences are formed terminally, and first morphological sign of the flowering induction in the apex is the raising and flattening of the apical meristem followed by the formation of bracts (Jahn and Dana 1970, Taylor et al. 1997). The development of the primary flower begins in centripetal order, and the formation of higher orders of flowers follows sympodially from the bracts of the previous inflorescence axis. After termination of vegetative growth of the crown by the inflorescence, the uppermost axillary buds continue the vegetative extension growth

of the crown on its secondary axis producing new leaf rosettes (Guttridge 1985). Under inductive conditions, terminal inflorescences are formed also in the secondary axis of the crown, leading to further crown branching. Because of the terminal flowering habit, the number of inflorescences is dependent on the number of apical meristems in the crown.

### 1.2 Distinct flowering types in strawberry

Strawberries are widely distributed in the Northern hemisphere. Different species can be found from the tropics to latitudes of about 70ºN (Darrow 1966). This wide distribution is evidence of good ability to adapt to different growing conditions. A good example of the adaptability of different strawberry species and genotypes is their ability to grow and flower in very diverse environments (Darrow 1966). Different researchers have tried to group strawberry genotypes according to their flowering behaviour, but none of the proposed models has been generally accepted.

According to their flowering and cropping characters, strawberries can be classified into Junebearing and everbearing types. Junebearing genotypes form a clearly separate group that is induced to flower in photoperiods under a certain critical limit, and are therefore classified as short-day (SD) plants (Guttridge 1985). In contrast, the classification of everbearing cultivars has been a topic of debate for several decades, with different publications, referring to everbearing genotypes as either day-neutral (DN) or long-day (LD) plants (Durner et al. 1984, Guttridge 1985 and references therein). In early experiments, everbearing cultivars of European origin were classified as LD plants, because of apparent flowering promotion by long photoperiod (Darrow and Waldo 1934, Downs and Piringer 1955). Later, American everbearing cultivars were considered to be day-neutrals, meaning that flowering induction occurs independently of the photoperiod (Smeets 1980). Moreover, Nicoll and Galletta (1987) suggested that different cultivars form a continuum from SD plants through DNs to LD plants. One reason for this inconsistency between different studies is that everbearing cultivars are induced to flower at a very early stage, so it is likely that plants were already generative at the beginning of the experiments in most studies (Sønsteby and Heide 2007a). Recently, Sønsteby and Heide (2007b) stated that in all critical studies, LD has been shown to promote flowering of everbearing cultivars regardless of their origin, and hence they should all be classified as LD plants.

### 1.3 Environmental regulation of growth

Environmental regulation of strawberry growth and development has been extensively studied for several decades (reviewed by Taylor 2000). In Junebearing strawberry genotypes, vegetative and generative development are oppositely regulated by photoperiod and temperature. This antagonism between vegetative and generative

development is a widespread phenomenon in the perennial life cycle, and strawberry provides a good model species to study these aspects (Battey et al. 1998). In this section, the effects of photoperiod and temperature on strawberry vegetative and generative development are discussed.

*1.3.1 Vegetative growth*

Reduced petiole elongation is the first sign of reduction of the vegetative growth caused by shortening photoperiod. The effect of SD on petiole elongation can be measured as early as two days after the beginning of the first SD cycle (Wiseman and Turnbull 1999a). It is caused by reduced cell elongation (Gosselink and Smith 1967), but later, after about 2 weeks in SD, cell division also becomes reduced (Wiseman and Turnbull 1999a). After prolonged SD exposure, plants attain a semi-dormant state, in which emerging leaves remain small and petioles short, and the rate of leaf production decreases (Jonkers 1965, Sønsteby and Heide 2006).

Photoperiod also regulates axillary bud differentiation to either runners or branch crowns (Figure 1). Runnering is clearly promoted by long photoperiod (>14 – 16 h) and high temperature (>17 – 20°C), in SD cultivars of strawberry (Darrow 1936, Heide 1977, Durner et al. 1984, Guttridge 1985, Le Mière et al. 1996). Similarly, high temperature enhances runner formation also in everbearing cultivars of the garden strawberry, while the effect of photoperiod is not consistent in different reports (Durner et al. 1984, Manakasem and Goodwin 2001, Sønsteby and Heide 2007a, 2007b). In general, everbearing cultivars form fewer runners than Junebearing cultivars, possibly because of continuous flowering (Sønsteby and Heide 2007a) and consequent enhancement of crown branching. In *F. vesca*, runnering shows a clear photoperiod x temperature interaction (Battey et al. 1998, Heide and Sønsteby 2007). High temperature (21°C) promotes runnering in both SD and LD, at 15°C LD enhances runner initiation, and at 9 – 10°C runner formation ceases (Heide and Sønsteby 2007). Most everbearing *F. vesca* genotypes show an extreme phenotype without runners.

The development of the strawberry crown is regulated by environmental factors as well as by the endogenous developmental program. Konsin et al. (2001) showed that in cv. Korona a 15 h photoperiod initiates the formation of branch crowns from the axillary buds of the main crown. A shorter photoperiod (12 h) was even more effective, whereas in LD (18 h), no branch crowns were formed. The extension of SD treatment increased the number of branch crowns, providing more meristems for floral development. Light intensity also correlated positively with crown branching in everbearing cv. Everest (Wagstaffe and Battey 2004). The authors further suggested that inadequate crown branching may be the limiting factor for cropping potential in shade conditions. On the other hand, excessive branching may also have negative effects on berry yield (Chercuitte et al. 1991).

*1.3.2 Generative growth*

Many garden strawberry cultivars are classified as facultative SD plants, because of their conditional SD requirement. In general, SD is a primary factor for flowering induction of these cultivars, but there is a strong interaction between photoperiod and temperature (Heide 1977, Guttridge 1985 and references therein, Sønsteby and Nes 1998). At temperatures over 15ºC, most of these cultivars have an obligatory SD requirement, but at lower temperatures the role of photoperiod as an inductive signal is reduced (Ito and Saito 1962, Guttridge 1985). The significance of photoperiod and temperature for floral induction is cultivar-dependent (Heide 1977). For example, Sønsteby and Heide (2006) did not find photoperiod x temperature interaction in cv. Korona and cv. Elsanta, but these cultivars were induced by SD only between 9 and 21ºC, and flowering was inhibited at high (27ºC) or low (<9ºC) temperatures as also found in other studies (Ito and Saito 1962, Zhang et al. 2000, Verheul et al. 2006). The number of SD cycles needed for flowering induction varies between 7 and 28, depending on cultivar, temperature and photoperiod (Guttridge 1985, Sønsteby and Nes 1998). After the induction of flowering, SD promotes flower initiation, but delays differentiation of flower organs in strawberry (Durner and Poling 1987). Thus, during autumn, flower initiation continues until growth ceases, and the initiation rate is positively correlated with temperature (Le Mière et al. 1996).

Exploration of flowering physiology in everbearing cultivars is challenging, because they are induced to flower at a very early developmental stage. Sønsteby and Heide (2007a), however, were able to critically analyse the photoperiodic effect on flowering in everbearing hybrid cv. Elan, which is propagated by seeds. In their experiments, the movement of SD-grown seedlings to various photoperiod x temperature combinations uncovered a clear LD promotion of flowering at temperature range of 15 - 27ºC and a critical photoperiod of 15 h at 18ºC. Later, they also showed photoperiod x temperature interactions in five other cultivars having different origins of the everbearing character (Sønsteby and Heide 2007b). Sønsteby and Heide (2007b) stated that everbearing cultivars, in general, are qualitative LD plants at high temperature (27ºC), quantitative LD plants at intermediate temperatures and day-neutrals below 10 ºC.

In woodland strawberries, the significance of temperature in floral transition appears to dominate over that of photoperiod. Plants collected from different locations in Norway had an obligatory SD requirement at 15-18°C, whereas at 9°C flowering was independent of daylength and at 21°C flowering was inhibited (Heide and Sønsteby 2007). Interestingly, the authors did not find a correlation between environmental conditions and the induction of flowering in latitudinal and altitudinal sequences of populations ranging from 60 to 70°N and 5 – 1080 m. Thus, more research is needed to clarify how this species is induced to flower at correct time in diverse environments.

*1.3.3 Suspension of growth – semi-dormancy*

Strawberry plants do not have a true dormancy, defined as the lack of meristematic activity (Rinne et al. 2001), since they continue growth in SD. However, after a long period under SD, their growth becomes strongly reduced so that they enter a so-called semi-dormant state, in which emerging leaves are small with short petioles, runnering has ceased, and the growth habit is dwarfed (Guttridge 1985). For example, in cvs. Korona and Elsanta ten weeks under SD at 15ºC is enough to induce the semi-dormant state (Konsin et al. 2001, Sønsteby and Heide 2006). In this state, plants cannot resume normal growth when shifted to LD conditions (Jonkers 1965, Sønsteby and Heide 2006). Similarly, as the removal of true dormancy of perennial plants (e.g. Rinne et al. 2001), normal spring growth in strawberry is released by adequate exposure to chilling at temperatures between -2 and 8ºC (Porlingis and Boynton 1961, Jahn and Dana 1966, Avigdori-Avidov et al. 1977, Guttridge 1985). The release of growth is proportional to the length of the chilling treatment. Depending on the cultivar, 4 – 8 weeks of chilling is enough for full restoration of vegetative growth, (Guttridge 1958, Guttridge 1985, Tehranifar et al. 1998). Plants are not competent to the induction of flowering for 1 – 2 months after the release from semi-dormant state (Guttridge 1985, Battey 1998).

## 1.4 Mobile signals in flowering and runner formation

Defoliation studies and experiments using mother - daughter plant pairs suggest that runnering and flowering induction in strawberry are regulated by mobile signals originating from leaves (e.g. Hartmann 1947, Thompson and Guttridge 1960). Mobile signals that either induce or inhibit flowering have been proposed (reviewed by Durner and Poling 1988). In general, these signals seem to have opposite but inseparable effects on flowering and runnering, and therefore, they are handled together in this section.

Hartmann (1947) showed that flowering was induced and runnering was decreased if only some leaves were exposed to SD, and this effect was proportional to the number of leaves in SD. He also found that SD-grown mother plants induced flowering in attached daughter plants, suggesting that the quantitative flowering-inducing signal mediates this response. In contrast, the findings by Guttridge (1959) and Thompson and Guttridge (1960) supported the model in which vegetative growth-promoting but flowering-inhibiting hormone was produced in LD-grown leaves and moved to the growing regions. This signal was shown to move acropetally along the concentration gradient from LD-grown mother plants to daughter plants attached by a runner (Guttridge 1959, Leshem and Koller 1964). In addition to photoperiod, light quality and timing of the light treatment also affected the production of the proposed inhibitor. Red (R) light prevented flowering in the second half and far-red (FR) during the first half of the 16 h night, whereas the 1:1 combination of R and FR was inhibitory at both times (Vince-Prue and Guttridge 1973). Given that the induction of

flowering is regulated by an inhibitor gene in the perennial life cycle of strawberry, this inhibitor should undergo cyclic inactivation/activation steps (Battey et al. 1998).

## 1.5 Hormonal regulation of vegetative and generative growth

Most studies on the role of plant hormones in the regulation of strawberry development have concentrated on the effects of exogenous GA on both vegetative and generative development (e.g. Thompson and Guttridge 1959). The effects of other hormones have been less studied. According to a few reports, cytokinin may affect axillary bud differentiation, and IAA seems to promote flowering (Waithaka et al. 1980, Braun and Kender 1985, Hou and Huang 2005). The effects of GA on strawberry growth and development are described below.

### *1.5.1 GA biosynthesis and signal transduction*

GAs are diterpenes that are known to regulate several aspects of plant development including seed germination, stem elongation, leaf expansion, and flower and seed development. They are synthesized through a well known branch of the terpenoid pathway starting from geranylgeranyl diphosphate (GGDP) (reviewed by Sun and Kamiya 1997, Hedden and Phillips 2000, Sponsel and Hedden 2004, Yamaguchi 2008). The first GA in this pathway, $GA_{12}$, is produced from GGDP in sequential reactions catalysed by several enzymes (Figure 2). Following the formation of $GA_{12}$, the GA biosynthetic pathway is divided into two branches, the 13-hydroxylated and non-13-hydroxylated pathways. In the non-13-hydroxylated pathway, active $GA_4$ is synthesized from $GA_{12}$ through a few intermediates by 2-oxo-glutarate dependent dioxygenases (2ODD), GA 20-oxidase and GA 3-oxidase (GA20ox and GA3ox). In the 13-hydroxylated pathway, $GA_{12}$ is first oxidized by an unknown GA 13-oxidase to produce $GA_{53}$, which is further converted to bioactive $GA_1$ by GA20ox and GA3ox (Lange et al. 1994, Hedden and Kamiya 1997). Some GA3ox enzymes are also able to produce biologically active $GA_3$, $GA_5$ and $GA_6$ from $GA_{20}$ (Kwak et al. 1988, Fujioka et al. 1990). GA 2-oxidases (GA2ox), which are also 2ODDs, provide a major deactivation mechanism in the GA pathway. These enzymes are able to deactivate bioactive $GA_1$ and $GA_4$, and also several intermediates of the pathway reducing the pool of GA precursors (Thomas et al. 1999, Schomburg et al. 2003). In *Arabidopsis* and rice, all 2ODDs are encoded by small gene families (e.g., Hedden and Phillips 2000, Sakamoto et al. 2004, Yamaguchi 2008).

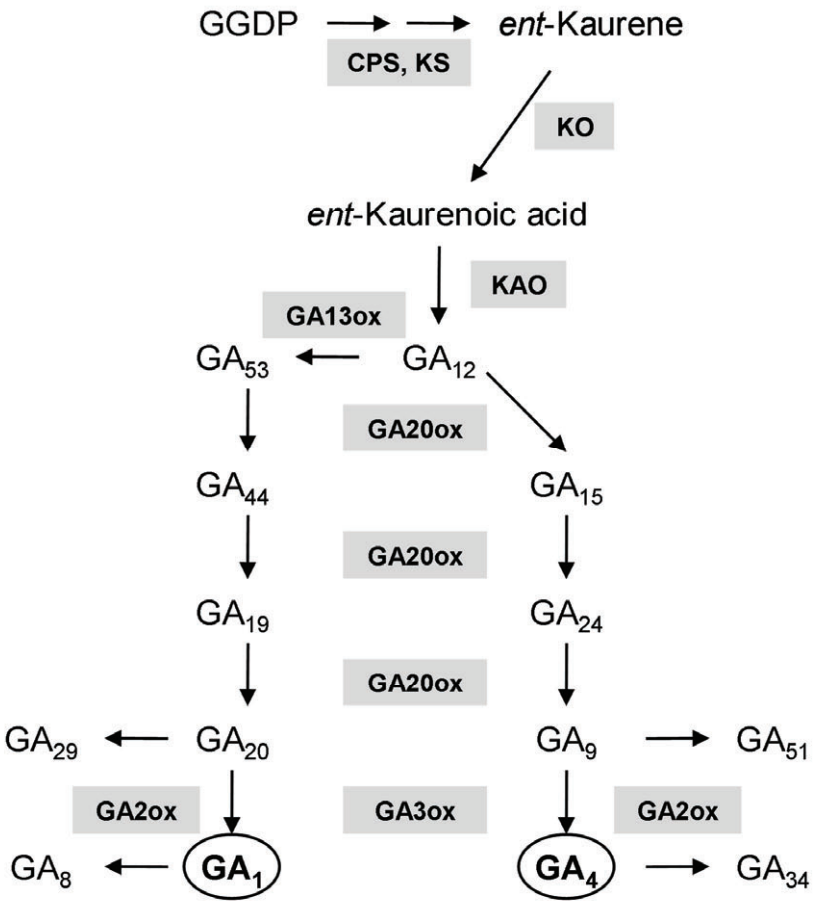


Figure 2. Simplified representation of the GA biosynthetic pathway in higher plants. Enzymes catalysing different steps of the pathway are shown, and bioactive GAs synthesized by 13-hydroxylation (left) and non-13-hydroxylation pathways (right); $GA_1$ and $GA_4$, respectively, are highlighted. GGDP = geranylgeranyl diphosphate, CPS = *ent*-copalyl diphosphate synthase, KS = *ent*-kaurene synthase, KO = *ent*-kaurene oxidase, KAO = *ent*-kaurenoic acid oxidase, GA13ox, GA20ox, GA3ox, GA2ox = GA-oxidases.

The recent identification of GA receptors has shed more light on the GA signalling pathway. (reviewed by Sun and Gubler 2004, Jiang and Fu 2007, Schwechheimer 2008). GA promotes plant growth by repressing DELLA proteins, the central components of GA signalling pathway that suppress GA-mediated growth responses (Silverstone et al. 2001). DELLA proteins, encoded by five genes in *Arabidopsis*, *GAI, RGA, RGL1, RGL2* and *RGL3* (Peng et al. 1997, Silverstone et al. 1997, 1998, Tyler et al. 2004), are transcriptional repressors that may directly bind to the promoters of several GA-regulated genes (Zentella et al. 2007). The first step in GA signalling is the binding of active GA to the soluble GA receptor GID1 (Figure 3), which is encoded by one and three genes (*GID1a*, *GID1b* and *GID1c*) in rice and *Arabidopsis*, respectively (Uecuchi-Tanaka et al. 2005, Nakajima et al. 2006). GA

binding activates the receptor and leads to a physical interaction between GID1 and the DELLA domain of GAI (GA INSENSITIVE) (Willige et al. 2007, reviewed by Hirano et al. 2008). Receptor-bound DELLA protein is next recruited by SLY1 F-box protein, which is a component of the E3 ubiquitin ligase SCF complex (SKP/Cullin/F-box). This complex links the polyubiquitin chain to the DELLA protein, and targets it to degradation in the 26S proteasome, releasing plant growth from the DELLA-mediated restraint (Dill et al. 2004). Also SPY (SPINDLY) is involved in the GA pathway as a negative regulator of GA signalling (Silverstone et al. 2007). It is an O-linked N-acetylglucosamine transferase that may activate DELLA protein function.

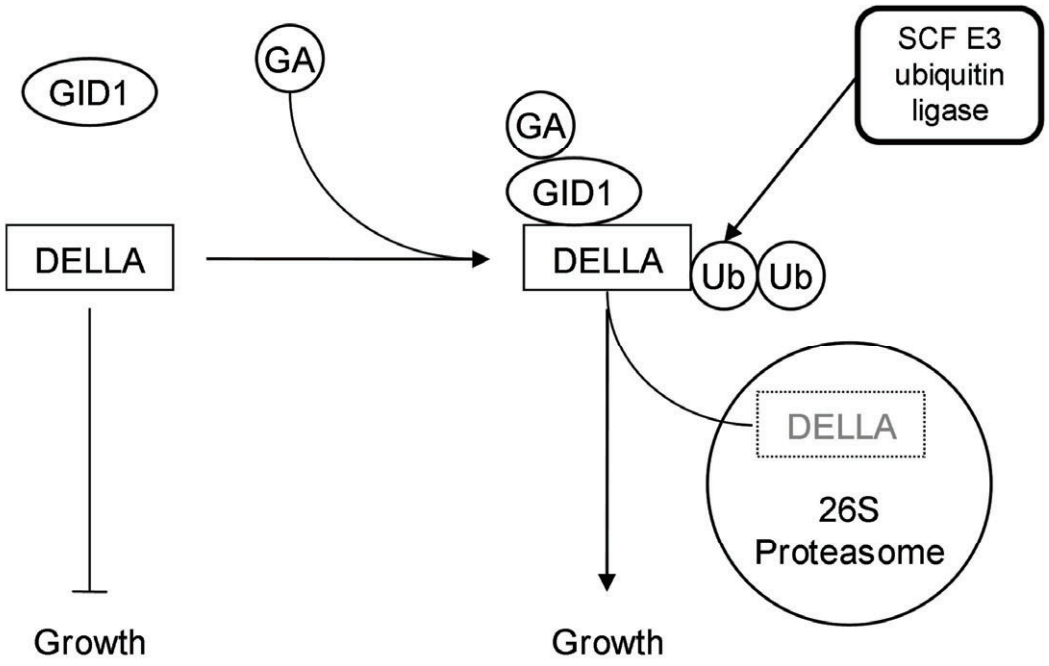


Figure 3. GA signalling pathway in higher plants. In the absence of bioactive GA, DELLA proteins repress the expression of genes needed for GA-mediated growth responses (left). Binding of GA to its receptor GID1 leads to direct interaction between GID1 and DELLA and to the ubiquitinylation of GID1-bound DELLA by the SCF E3 ubiquitin ligase complex. Subsequent degradation of DELLA by the 26S proteasome releases plant growth from DELLA-mediated restraint.

*1.5.2 Regulation of the GA pathway*

Both biosynthetic and signalling pathways of GA are regulated by various external and internal factors, including growing conditions, developmental stage and feedback mechanisms. GA is an important link between plant growth responses and the perception of environmental signals, including light (reviewed by García-Martinez and Gil 2002, Zhao et al. 2007a), temperature (Stavang et al. 2005, Stavang et al. 2007) and abiotic stress (Achard 2006). The effect of light, its quality, intensity and

photoperiod, has been most intensively studied, and it has been shown to control the GA pathway differently at different stages of plant development (e.g. Ait-Ali et al. 1999, Reid et al. 2002, García-Martinez and Gil 2002, Oh et al. 2007, Zhao et al. 2007a, 2007b, Archard et al. 2007). In adult plants, for example in spinach, LD promotes GA biosynthesis by activating *GA20ox1*, while *GA3ox1* is not clearly affected (Lee and Zeevaart 2002). In addition to photoperiodic effects, also diurnal changes in GA levels and in the expression of biosynthetic genes are found, and they may have a role in plant development (Carrera et al. 1999). The mechanisms of light regulation of the GA pathway have been studied in detail during seed germination and in de-etiolation responses. In *Arabidopsis* seeds, Phy activated by red light causes rapid degradation of a bHLH transcription factor, PIL5 (PHYTOCHROME INTERACTING FACTOR 3-LIKE 5) that controls both GA biosynthesis and signalling (Oh et al. 2006, 2007). Moreover, in seedlings, two other Phy-interacting factors, PIF3 and PIF4, are involved in GA-light interactions (Feng et al. 2008, de Lucas et al. 2008). It is not known whether PIFs are involved in GA x light interactions in adult plants.

Different genes of the GA biosynthetic pathway are spatially and temporally regulated during plant development in several plant species, including spinach (Lee and Zeevaart 2002), *Arabidopsis* (Mitchum et al. 2006), pea (Ross et al. 2003, Weston et al. 2008), and rice (Itoh et al. 2001). In general, biologically active GAs are thought to be synthesized at the site of GA action. For example, two GA biosynthetic genes, *GA20ox* and *GA3ox*, are expressed in rapidly elongating and dividing cells in rice internodes (Kaneko et al. 2003), tobacco rib meristems (Itoh et al. 1999) and hybrid poplar xylem (Israelsson et al. 2005). However, in some plant species the expression of different GA biosynthetic genes is spatially separated, suggesting the movement of certain intermediates (Israelsson et al. 2005, Mitchum et al. 2006). In fact, the precursors of bioactive $GA_1$, $GA_{19}$ and probably $GA_{20}$, are able to move within the plant (Proebsting et al. 1992). Thus, the local GA pools may be determined by a complex equation including distant and local biosynthesis as well as inactivation of GAs.

The GA pathway is also effectively regulated by GA itself, maintaining GA (signalling) homeostasis (Ross et al. 1999, Yamaguchi 2008, Schwechheimer 2008). Some of the *GA20ox* and *GA3ox* genes are under negative feedback control by GA, whereas certain *GA2ox* genes are oppositely regulated (Thomas et al. 1999, Hedden and Phillips 2000). Different GA metabolism genes have different sensitivity to changes in GA level, providing more flexibility to the system (Gallego-Giraldo et al. 2008). In addition to GA biosynthetic genes, some positive regulators of GA signalling (*GID1*, *SLY1*) are repressed, and negative signalling components (some *DELLA* genes) are activated by GA (Willige et al. 2007). DELLA proteins may directly regulate some of these genes, but other feedback responses are indirect (Ishida et al. 2004, Matsushita 2007, Dai et al. 2007, Willige et al. 2007). GA

signalling homeostasis functions also at the protein level, since a reduced level of a certain DELLA protein can be compensated by another DELLA (Willige et al. 2007).

*1.5.3 The role of GA in strawberry*

Exogenous GA, applied under SD, has been shown to inhibit flowering and promote runnering and petiole elongation in strawberry SD genotypes (Thompson and Guttridge 1959, Porlingis and Boynton 1961, Avigdori-Avidov et al. 1977). Similar effects of $GA_3$ have been shown also in *F. virginiana*, everbearing *F.* x *ananassa*, and Junebearing and everbearing types of *F. vesca*, but the size of the response varies among genotypes and species (Guttridge and Thompson 1963, Tafazoli and Vince-Prue 1978, Chroma and Himelrick 1984). Interestingly, $GA_3$ induced runnering even in non-runnering everbearing genotypes of *F. vesca* and *F.* x *ananassa*. Furthermore, $GA_3$ applied to the stump of a cut petiole was able to substitute the flowering inhibitor produced in leaf blades under LD, indicating that GA itself may be the inhibitor (Guttridge and Thompson 1963).

Several studies on the forms of GAs in strawberry clearly show that the 13-hydroxylation pathway (Figure 2) is predominant in strawberry tissues (Taylor et al. 1994, Wiseman and Turnbull 1999b, Taylor et al. 2000a, 2000b), and some novel GAs have been found (Blake et al. 2000). The results of Taylor et al. (1994) indicate possible modulation of the GA pathway by photoperiod. They found that $GA_5$ was present in SD-grown petioles, but not in LD. This finding is relevant also since $GA_5$ has been shown to act as a potent florigenic signal in *Lolium temulentum* (King et al. 2001), although in earlier studies, $GA_5$ inhibited flowering in *F. vesca* (Guttridge and Thompson 1963).

Different inhibitors of GA biosynthesis decrease vegetative growth in strawberry, but their effect on flowering time has not been reported. Both paclobutrazol (PP333) and prohexadione-calcium (ProCa) have been shown to reduce petiole elongation (Wiseman and Turnbull 1999a, Reekie and Hicklenton 2002). However, SD has an additive effect on petiole elongation, showing that rapid reduction in petiole growth in SD is at least partially mediated by factors other than GA biosynthesis. In fact, clear changes in GA levels of petioles were not found until eight days in SD (Wiseman and Turnbull 1999b). This shows clearly that early reduction of petiole elongation in SD is not caused by reduced GA biosynthesis, but a possible photoperiodic effect on GA responsiveness in petioles should be tested. Inhibitors of GA biosynthesis, PP333, AMO-1618 and ProCa, reduce runner formation and increase the number of branch crowns (Avigdori-Avidov et al. 1977, Nishizawa 1993, Reekie and Hicklenton 2002, Black 2004), and therefore, they may have a positive effect on flowering. The molecular mechanisms underlying the regulation of axillary bud differentiation by GA in strawberry are not known. Therefore, GA x light interactions in other systems resembling the differentiation of strawberry axillary buds are discussed in the following section.

*1.5.4 Initiation and cessation of elongation growth in other plants*

The differentiation of a strawberry axillary bud into a runner or a branch crown is obviously dependent on the activation/repression of elongation growth in the bud internodes, and hence it resembles, at least superficially, photoperiodic bolting of LD rosette plants, growth cessation in trees and tuberization in potato stolons. Thus, similar molecular mechanisms may be involved in these developmental processes and GA has a role in all these systems. In spinach and *Arabidopsis*, LD increases the expression of *GA20ox1* in the shoot apex, which is followed by the accumulation of bioactive GA and, consequently, elongation of the inflorescence stem (bolting) (Wu et al. 1996, Xu et al. 1997, Lee and Zeevaart 2002). Similarly, in trees, a certain level of bioactive GA seems to be needed to maintain elongation growth (reviewed by Junttila 2007). For example, in *Salix pentandra* and in poplar, growth cessation correlates with the reduced level of active $GA_1$ in shoots (Olsen et al. 1995, Olsen et al. 1997, Hansen et al. 1999). Moreover, SD represses *GA20ox* and activates two DELLA genes in hybrid poplar, indicating that both GA biosynthesis and signalling are diminished by SD (Eriksson and Moritz 2002, Ruttink et al. 2007). Also in potato stolon tips, SD down-regulates GA biosynthesis by repressing *GA20ox* and *GA3ox*, and activating *GA2ox* expression, which leads to the cessation of elongation growth and to tuber formation (Xu et al. 1998, Carrera et al. 2000, Rodríguez-Falcón et al. 2006, Kloosterman et al. 2007). The regulation of *GA20ox* has been studied in detail, and a Knotted1-type transcription factor, POTH1, has been shown to repress its expression in the stolon tips (Rosin et al. 2003). POTH1 function is also dependent on an interacting partner BEL5 (BEL1-like transcription factor), which may be a mobile signal moving from leaves to stolon tips via the phloem stream, preferentially in SD. (Chen et al. 2003, 2004, Banarjee et al. 2006).

Interestingly, the well known flowering-time genes *CO*, *FT* and *TFL1* have been linked to the photoperiodic regulation of growth cessation. In hybrid poplar, the CO/FT regulatory module controls SD-induced growth cessation and bud set, the timing of *CO* expression having a clear latitudinal gradient correlating with the critical photoperiod for growth cessation (Böhlenius et al. 2006). Furthermore, growth cessation is associated with the down-regulation of *FT* in both transgenic (RNAi) and WT plants. Later, Böhlenius (2007) suggested that, similarly to the *Arabidopsis* flowering pathway, the *Populus GI* homolog may connect the circadian clock and the *CO/FT* regulon. Another member of the hybrid poplar *FT/TFL* family, *CENL1* (*CENTRORADIALIS-LIKE 1*), as well as apple *TFL1* and Norway spruce *FT4*, may have an opposite role in growth cessation (Kotoda et al. 2006, Gyllenstrand et al. 2007, Ruonala et al. 2008). Interestingly, the *CO/FT* module may also control photoperiodic tuberization in potato (Martinez-Garcia et al. 2002, Rodríguez-Falcón et al. 2006). The probable connection between the photoperiodic *CO/FT* pathway and the GA pathway in the regulation of growth in different systems is an interesting open question.

## 1.6 Genetic regulation of growth and development in strawberry

Genetic regulation of strawberry runner formation, crown development and flowering has been studied by segregation analysis and QTL mapping. In octoploid *Fragaria* species, inheritance of the everbearing flowering character is complex. The everbearing character in most American cultivars originates from *F. virginiana* ssp. *glauca* (Staudt), in which this character was originally proposed to be regulated by a single dominant gene (Ahmadi et al. 1990). However, Weebadde et al. (2008) found eight QTLs linked with the everbearing character in garden strawberry, indicating that the determination of flowering type is a polygenic trait, and this is also supported by other studies (Shaw 2003, Hancock et al. 2004, Serce and Hancock 2005a). Garden strawberry has an octoploid genome with a proposed constitution of AAA'A'BBB'B' (Bringhurst 1990), indicating that it originates from four different diploid species. Thus, possible dosage effects of different alleles and variation in meiotic configurations makes the inheritance of the strawberry genome complex, resulting in variation in segregation ratios (Serce and Hancock 2005a). Therefore, more simple *Fragaria* species, like diploid *F.vesca*, are valuable tools to analyse the significance of different candidate genes affecting flowering time and other important traits.

In *F. vesca*, the difference between seasonal flowering and everbearing types has been shown to be determined by a single gene, *SEASONAL FLOWERING LOCUS* (*SFL*), in crosses between SD *F. vesca* and two everbearing genotypes (Baron Solemacher and Bush White), with seasonal flowering being a dominant (Brown and Wareign 1965, Battey et al. 1998). ISSR and SCAR markers have been developed around *SFL* and the closest marker is inseparable from the phenotype, but the gene has not been found (Cekic et al. 2001, Albani et al. 2004).

Like seasonal flowering, runnering may be regulated by a single dominant gene (*RUNNERING LOCUS, R*) in *F. vesca* (Brown and Wareign 1965). Moreover, flowering (everbearing/seasonal) and runnering (runnering/non-runnering) characters segregate independently, showing that at least in *F. vesca* they are regulated by different genes. Like *SFL*, the *R* locus has been mapped on the *F. vesca* x *F. nubicola* genetic map, and it is located in a 0.49 cM region in the middle of linkage group II (Sargent et. al. 2004). However, Sargent et al. (2004) reported that the segregation of this locus deviates significantly from the 3:1 ratio expected for a single dominant gene model, indicating that more than one gene is involved.

Brown and Wareign (1965) also studied the genetics of the bushy growth habit of everbearing, non-runnering *F.vesca* cv. Bushy White by crossing it with a seasonal flowering, runnering genotype. The bushy habit was tightly linked with a recessive *r* allele, suggesting regulation by the same gene or by two closely located genes. An *arborea* (*arb*) mutant having an opposite phenotype was found from the island of Madeira (Staudt 1959). This long-stemmed "strawberry tree" mutant has long internodes, it continuously produces runners, and no branch crowns are formed.

Guttridge (1973) characterized this mutant genetically by crossing it with everbearing non-runnering cv. Baron Solemacher, and found the arboreal phenotype to be a recessive character determined by one gene (*arb*). This gene is clearly epistatic to the *R* gene, because crossing progenies with two recessive *r* alleles still produced runners. The phenotype of the *arb* mutant closely resembles GA-treated plants, suggesting a mutation in some negative regulator in GA pathway. In contrast to the inhibitory effect of GA on flowering, everbearing segregants with long internodes were found in cv. Baron Solemacher x *arb* progenies (Guttridge 1973).

*F. vesca* has been recognized as one of the model species for the Rosaceae family (Shulaev et al. 2008). It provides a good experimental system for molecular studies because it has a small diploid genome (about 200 Mb), a short generation time of only 3 - 4 months and an effective transformation method is available for it (Akiyama et al. 2001, Oosumi et al. 2004, Folta and Davis 2006). Furthermore, Sargent et al. (2006, 2008) have produced a genetic linkage map with almost 200 markers, most of which are transferable between different ploidy levels and even between different genera in the Rosaceae (Monfort et al. 2006, Sargent et al. 2007). Furthermore, about 50000 EST sequences are currently available in public databases (http://www.bioinfo.wsu.edu/gdr/, http://www.ncbi.nlm.nih.gov/). In spite of the availability of EST resources and different molecular methods for strawberry, molecular information on the regulation of flowering and runnering is almost completely lacking (Folta and Davis 2006). Only a few flowering time genes have been reported, including a putative *CO* (*CONSTANS*), *SOC1* (*SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1*) and *VIN3* (*VERNALIZATION INSENSITIVE 3*) (Folta et al. 2005). Among these, the expression of only *CO* has been studied, and in contrast to other plants, it shows a peak in the morning (Yano et al. 2000, Suarez-Lopez et al. 2001, Stewart 2007). Understanding the regulation of flowering in strawberry requires detailed functional studies of these genes, as well as other potential regulators of flowering time. The fact that several genes of flowering pathways are conserved between different plant species may help in the understanding of the regulation of flowering in strawberry (Hetch et al. 2005, Ausin et al. 2005, Dennis and Peacock 2007). Therefore, *Arabidopsis* flowering pathways are described in the following sections.

### 1.7 Genetic pathways regulating flowering

In the model plant *Arabidopsis*, four major genetic pathways regulating flowering are known -photoperiod, vernalization, autonomous and gibberellin pathways (reviewed by Boss et al. 2004, Putterill et al. 2004, Parcy 2005, Ausín et al. 2005, Dennis and Peacock 2007, Zhou et al. 2007). In addition, light quality and ambient temperature pathways play roles in specific circumstances (Cerdán and Chory 2003, Samach and Wigge 2005). The photoperiod pathway dominates in annual rapid-cycling ecotypes of *Arabidopsis*, whereas in winter-annuals the vernalization pathway dominates

(Simpson and Dean 2002). Furthermore, the GA pathway is needed for flowering in SD, and the functional autonomous pathway responds to endogenous cues, including developmental stage and plant age, to promote flowering (Boss et al. 2004, Simpson 2004). Floral promoting or inhibiting signals from distinct pathways ultimately converge onto a subset of genes, including *FT* (*FLOWERING LOCUS T*), *SOC1* and *LFY* (*LEAFY*), known as floral integrators (Figure 4) (Boss et al. 2004, Parcy 2005). The floral integrators, in turn, activate the floral meristem identity genes *AP1* (*APETALA1*), *FUL* (*FRUITFULL*) and *CAL* (*CAULIFLOWER*), to initiate flowering (Wagner et al. 1999, Ferrandiz et al. 2000).

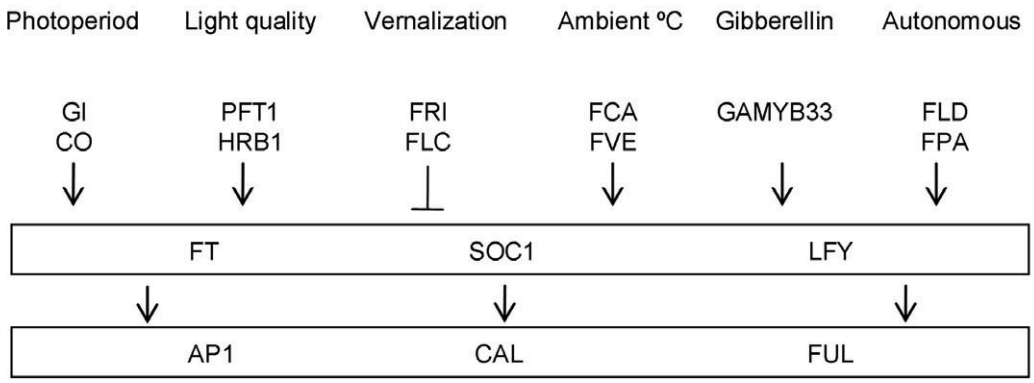


Figure 4. Organization of the genetic regulation of flowering in *Arabidopsis*. Different flowering pathways and their major genes are shown in the upper part of the figure. Activating and inhibiting signals from these pathways are integrated by floral integrator genes (middle part). Floral integrator genes in turn activate floral identitity genes (lower part) and consequently flowering initiation. Arrows indicate positive regulation and bars negative regulation.

*1.7.1 Photoperiod pathway*

Photoperiodic regulation of flowering is best explained according to the external coincidence model (Bünning 1936), according to which flowering occurs when the light or darkness coincides with the light-sensitive period of the endogenous rhythm in LD or SD plants, respectively. Molecular mechanisms underlying photoperiodic regulation of flowering in the LD plant *Arabidopsis* and the SD plant rice are consistent with this model. In both species, the circadian clock generates the expression rhythm of the key regulator, *CO*, with a peak in late afternoon. In *Arabidopsis*, flowering is activated if this peak occurs during the light period in LD, while in rice, the coincidence of CO peak with darkness (SD) is needed for flowering (Yano et al. 2000, Suarez-Lopez et al. 2001, Yanovsky and Kay 2002, Hayama et al. 2003). Three important questions have to be answered before the molecular mechanism underlying photoperiodic control of flowering can be understood. First, how is the *CO* expression rhythm generated; second, how is the coincidence of light

and *CO* expression peak detected; and finally, how does CO in the leaves induce floral development in distant meristems? These issues are discussed in following sections.

Plants use different light-absorbing photoreceptors (Jiao et al. 2007). In the case of photoperiodic regulation of flowering, PhyA and Cry2 are dominant photoreceptors, but other Phys and Cry1 also play a role (Guo et al. 1998, Lin 2000, Mockler et al. 2003, Halliday et al. 2003, Thomas et al. 2006). Dusk and dawn signals perceived by photoreceptors entrain the circadian clock, in which cyclical activation and repression steps maintain the rhythm (Figure 5) (McClung 2006). In the clock negative feedback loop, two partially redundant MYB transcription factor genes, *LHY* (*LONG HYPOCOTYL*) and *CCA1* (*CIRCADIAN CLOCK ASSOCIATED 1*), are expressed at dawn and repress the expression of *TOC1* (*TIMING OF THE CAB EXPRESSION 1*) during the day (Schaffer et al. 1998, Wang and Tobin 1998, Strayer et al. 2000). Later, in the evening, TOC1 peaks and indirectly activates *CCA1* and *LHY*, closing the loop (Alabadi et al. 2001). This clock mechanism generates several output rhythms that are needed for tight regulation of *CO* mRNA expression (Yakir et al. 2007, Niwa et al. 2007).

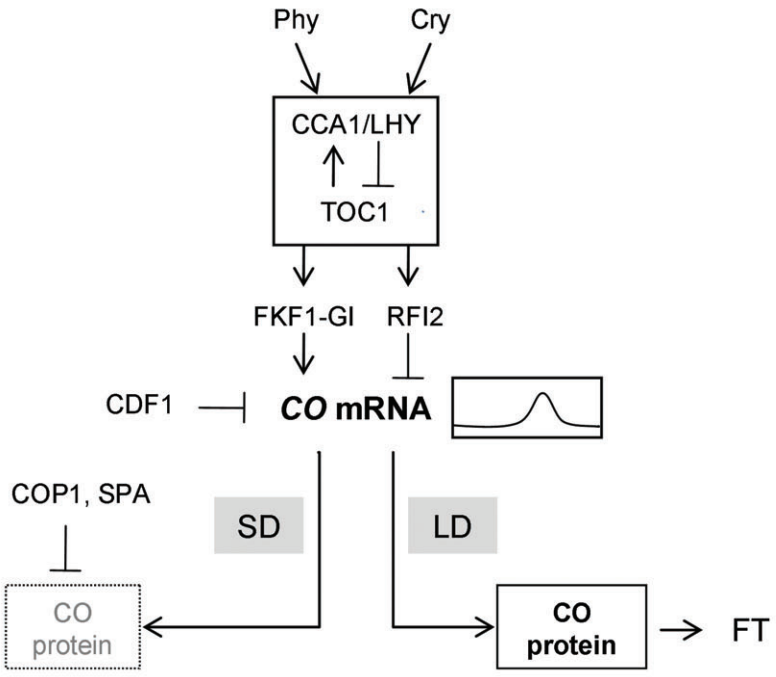


Figure 5. The photoperiod pathway regulating flowering in *Arabidopsis*. Light signals perceived by photoreceptors (Phy and Cry) entrain the circadian clock including CCA1/LHY and TOC1. CDF1 and the clock generate the rhythmic expression of *CO*, a major gene of the pathway. In SD (left), the *CO* expression peak appears at night when the CO protein is rapidly degraded. In LD, in contrast, light stabilizes the CO protein, which activates flowering through florigen FT. Arrows indicate positive regulation and bars negative regulation.

The function of CO as a seasonal time sensor is based on its lability in darkness. In SD, *CO* peaks during the night, when CO protein is rapidly degraded and flowering does not occur. In contrast, under LD, *CO* expression coincides with light, leading to accumulation of CO protein, which activates flowering induction (Suarez-Lopez et al. 2001, Yanovsky and Kay 2002). Accumulation of CO protein is also dependent on light quality, since red light perceived by phyB promotes CO degradation, whereas far-red and blue light stabilize it (Valverde et al 2004). The mechanism of CO degradation is emerging after recent findings, showing that COP1 ubiquitin ligase targets CO for degradation, and this process may need interaction with SPA proteins (Figure 5) (Ishikawa et al. 2006, Laubinger et al. 2006, Jang et al. 2008). CO is more stable in light, probably because Cry interaction with COP1 prevents CO degradation by COP1 (Liu et al. 2008). The Cry-COP1-CO interaction cascade is consistent with the finding that Cry2 regulates flowering in the vascular bundles, the tissue where also *CO* is expressed (An et al 2004, Endo et al. 2007). In contrast, regulation of CO by PhyB requires cell-to-cell signalling, since PhyB functions in the mesophyll (Endo et al. 2005). In sum, the clock-generated expression rhythm of *CO* determines the critical photoperiod for flowering, since CO protein can accumulate only if its mRNA is expressed during the photoperiod. Thus, any change in the circadian clock function that alters the timing of *CO* expression peak changes the critical photoperiod for flowering, providing a mechanism of adaptation to different growing conditions.

CO protein controls flowering by activating a graft-transmissible signal in the phloem companion cells, and this signal (florigen) induces flowering in the shoot apex (An et al. 2004, Ayre et al. 2004). Several lines of evidence support the role of FT as a florigen. FT is a potent floral activator, and CO activates its expression indirectly in the phloem (Kardailsky et al. 1999, Cai et al. 2007). As shown in rice, *Arabidopsis* and cucurbit, FT protein is able to move to the shoot apex and induce flowering (Lin et al. 2007, Corbesier et al. 2007, Tamaki et al. 2007). Moreover, *FT* has been shown to induce flowering in most plant species tested, showing evidence that FT is a major part of the florigen signal (Turck et al. 2008). In the meristem, FT needs an interacting transcription factor FD to induce flowering by activating *SOC1* and *AP1* (Abe et al. 2005, Yoo et al. 2005). Among additional members of *FT* gene family, *TSF1* is partially redundant with *FT*, whereas *TFL1* function as the repressor of floral transition in *Arabidopsis* (Hanzawa et al. 2005, Yamaguchi et al. 2005).

*1.7.2 Vernalization pathway*

Many plant species growing in temperate regions need vernalization, i.e., prolonged cold treatment during winter, to become competent to initiate flowers. In the vernalization pathway, the most important gene is *FLC* that prevents flowering by repressing *FT, FD* and *SOC1* (Michaels and Amasino 1999, Searle et al. 2006). In addition to *FLC*, there are five *FLC*-like genes in the *Arabidopsis* genome, and at least two of them function as floral repressors (Scortecci et al. 2001, Ratcliffe et al. 2003). Regulation of vernalization involves several histone modifications at the *FLC* locus, collectively called 'the histone code' (Table 1). The mechanisms controlling the

histone code have been extensively studied and similar protein complexes appear to be involved in its regulation both in plants and in other eucaryotes (Dennis and Peacock 2007). The protein complexes associated with active chromatin include PAF1 (RNA Polymerase II associated factor 1) and SWR1, whereas PRC2 (Polycomb repressive complex 2) has an opposite role (Figure 6) (He et al. 2004, Wood et al. 2006, Choi et al. 2007, Dennis and Peacock 2007). In addition, the plant-specific FRI complex is needed for the activation of *FLC* (Kim et al. 2006).

Table 1. Different histone marks associated with *FLC* chromatin, their effect on transcription, and proteins and/or protein complexes (in bold) involved in inserting/removing the marks.

| Histone mark | Effect on chromatin | Acquisition | Removal |
|---|---|---|---|
| H3K4me3 | Activation | **PAF1**, ATX1 | LDL1/2, FLD |
| H3K36me3 | Activation | EFS/SDG8 | **VRN2-PRC2**, VIN3 |
| H3K36me2 | Activation | EFS/SDG8 | **VRN2-PRC2**, VIN3 |
| H3K27me3 | Repression | **VRN2-PRC2**, VRN5 | at meiosis |
| H3K9me2 | Repression | **VRN2-PRC2**, VIN3 | at meiosis |
| H4R3sme2 | Repression | SKB1, PRMT5 | at meiosis |
| Acetyl group | Activation | **SWR1**, ARP6 | **VRN2-PRC2**, VIN3, FLD, FVE, CZS |

FRI is a major activator of *FLC* transcription in *Arabidopsis*, as shown by the finding that differences in flowering time between rapid-cycling and winter-annual ecotypes can be explained by allelic variation at the *FRI* locus in most cases (Johanson et al 2000). The activation of *FLC* by the FRI complex is poorly understood, but recent characterization of SUF4 provided one possible model to explain the function of the FRI complex (Michaels et al. 2004, Schmitz et al. 2005, Kim and Michaels 2006, Kim et al. 2006). SUF4 is needed for insertion of transcription-activating H3K4me3 (histone 3 lysine 4 trimethylation) marks into *FLC* chromatin. Because the methylation of H4K3 needs a functional PAF1 complex and histone methyltransferase (HMT), SUF4 has been suggested to recruit these regulators to the *FLC* chromatin (He et al. 2004, Kim and Michaels 2006, Pien et al. 2008). In addition to the H4K3 mark, di- and tri-methylation of H3K36 of the *FLC* locus also marks active chromatin (Xu et al. 2008).

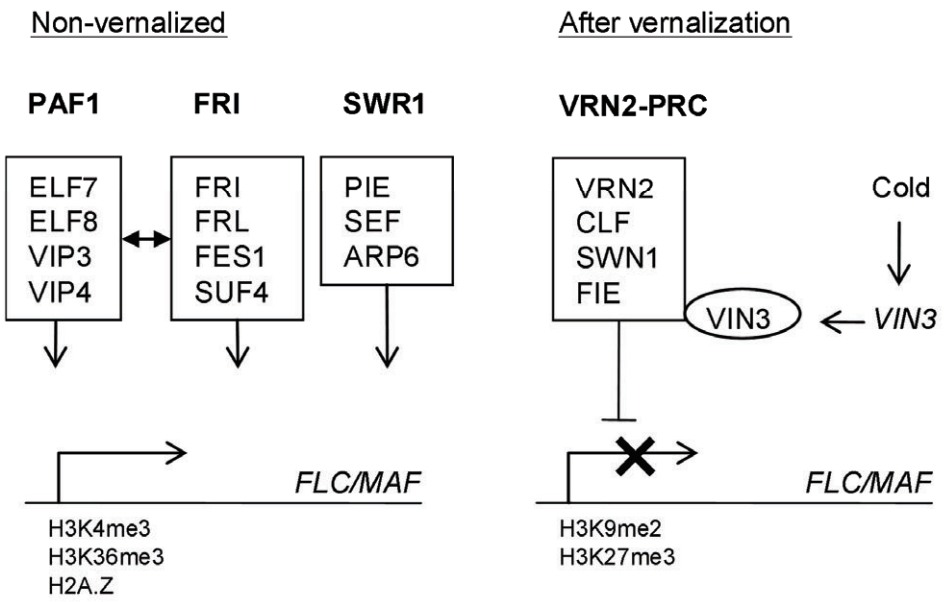


Figure 6. Activation and repression of the major flowering inhibitor *FLC* and homologous *MAF* genes in the *Arabidopsis* vernalization pathway. In non-vernalized plants (left), PAF1 and FRI complexes together activate the transcription of *FLC/MAF* genes by inserting indicated histone marks, and the role of SWR1 is to insert specific histone variant H2A.Z needed for the transcriptional activation of *FLC/MAF*. After vernalization (right), VRN2-PRC2 complex recruits cold-activated VIN3 and silences *FLC/MAF* by removing activating histone marks and by inserting repressive marks, allowing plants to flower.

In addition to histone marks, the activation of *FLC* transcription requires the insertion of histone variant H2A.Z into *FLC* chromatin (Deal et al. 2007). This is probably done by SWR1, a putative chromatin remodelling complex, since mutations in any gene of SWR1 prevent the establishment of H2A.Z on the chromatin (Noh and Amasino 2003, Deal et al. 2005, March-Diaz et al. 2007, Deal et al. 2007). According to the current model, SWR1 adds H2A.Z mainly to the promoter and 3'-region of H3K4 tri-methylated *FLC* chromatin, and this modification may facilitate the access to the chromatin by transcriptional machinery (Santos-Rosa et al. 2003, March-Diaz et al. 2007).

Prolonged cold treatment makes plants competent to flower by repressing *FLC* expression. This repression is stable during mitotic cell divisions, suggesting that vernalization may cause heterochromatin formation at *FLC* locus (Dennis and Peacock 2007). The central gene in *FLC* down-regulation is *VIN3*, the transcript and protein levels of which increase during vernalization (Sung and Amasino 2004). After vernalization, the VRN2-PRC2 complex may bind *FLC* chromatin and recruit VIN3 (Gendall et al. 2001, Wood et al. 2006). This complex removes activating histone marks and inserts repressive marks (Table 1), which are commonly found in stably

silenced heterochromatin (Alexandre and Hennig 2007, Greb et al. 2007). After the extensive chromatin modifications, LHP1 together with VRN1 may bind to H3K27me3 and maintain the silenced stage of the *FLC* until meiosis (Levy et al. 2002, Sung et al. 2006). A different mechanism is needed in vernalization-requiring perennial plants, since the vernalization pathway has to be cyclically activated and repressed from year to year.

*1.7.3 Autonomous pathway*

Vernalization is not needed for flowering in rapid-cycling ecotypes of *Arabidopsis*, because the autonomous pathway, responding to endogenous cues from the developmental program of the plant, is able to repress low expression level of *FLC* (Simpson 2004). Thus, all genes of the autonomous pathway promote flowering, and most of them express their function by modifying *FLC* chromatin or mRNA. Chromatin modifications regulated by the autonomous pathway include both insertion of different repressive histone marks as well as removal of transcription-activating marks (He et al. 2003, Ausín et al. 2004, Jiang et al. 2007, Krichevsky et al. 2007, Wang et al. 2007, Lu et al. 2008, Bäurle et al. 2008). In addition to different chromatin modifiers, RNA-binding proteins FPA, FLK and FCA function in parallel pathways to repress *FLC* (MacKnight 1997, Schomburg et al. 2001, Lim et al. 2004, Quesada et al. 2005). Among these, FCA and FPA may repress transcription of several target loci by siRNA-directed DNA methylation (Bäurle et al. 2008).

*1.7.4 Gibberellin pathway*

In *Arabidopsis*, $GA_4$ promotes flowering induction in LD and is needed for flowering in non-inductive SD (Boss et al. 2004, Eriksson et al. 2006). Promotion of flowering by GA occurs through activation of *LFY* and *SOC1* (Blázquez et al. 1998, Moon et al. 2003). This effect of GA may be mediated by the GA inducible MYB transcription factor GAMYB33 that accumulates in the apical meristem simultaneously with *LFY* up-regulation and is able to bind *LFY* promoter (Gogal et al. 2001). GA regulation of flowering may also involve miR159, which has been shown to control *GAMYB33* in GA-dependent manner (Achard et al. 2004). In contrast to *Arabidopsis*, GA inhibits flowering for example in grape, where a gain-of-function mutation in the DELLA protein, *VvGAI1*, caused dwarfism and the formation of inflorescences instead of tendrils (Boss and Thomas 2002).

*1.7.5 Ambient temperature pathway*

Ambient temperature makes an important input to flowering time, but little is known about the mechanisms of temperature sensing (Samach and Wigge 2005, Penfield 2008). In Arabidopsis, flowering is significantly delayed if plants are moved from 23ºC to 16ºC. This delay is completely absent in mutants lacking a functional autonomous pathway gene, *FCA* or *FVE* (Blázquez et al. 2003). Lee et al. (2007) showed that also MADS-box protein SVP (Hartmann et al. 2000) functions downstream of *FCA* and *FVE* and suppresses *FT* expression by direct binding to *FT* promoter. Thus, *FVE, FCA* and *SVP* provide a thermosensory pathway delaying

*Arabidopsis* flowering at low temperature (Lee et al. 2007). Recently, SVP was shown to bind FLC, and together they down-regulate *SOC1* (Li et al. 2008). SVP-FLC complex also plays a more general role in the regulation of flowering time, since it integrates signals also from the gibberellin and autonomous pathways.

## 2 AIMS OF THE STUDY

Environmental regulation of runnering and flowering induction in strawberry are quite well documented, and several studies suggest that vegetative and generative development in strawberry are antagonistic processes. However, molecular and metabolic regulation of vegetative of generative growth in strawberry is almost completely unknown. The objectives of this study, therefore, were to analyse the regulation of flowering induction and axillary bud differentiation to runners and branch crowns in strawberry, and to find ways to control these developmental processes in strawberry cultivation. Moreover, hormonal and genetic regulation of these processes were characterized. This information can be used to accelerate strawberry breeding and research in this area. The specific objectives addressed in the papers were:

1) To study the effect of artificial SD treatments on strawberry crown branching and cropping potential, as well as linkage between these processes;
2) To examine the role of daylength and GA in the regulation of axillary bud differentiation in strawberry;
3) To examine the chemical control of axillary bud differentiation by GA biosynthesis inhibitor ProCa in strawberry production; and
4) To explore genetic pathways regulating flowering in strawberry.

## 3 MATERIALS AND METHODS

Materials and methods are summarized in Table 2, and strawberry germplasm used in this study are listed in Table 3. Detailed descriptions of materials and methods can be found in the original publications I - IV.

Table 2. The methods used in this study. Publications in parenthesis indicates that work has been carried out only by co-authors

| Method | Publication |
|---|---|
| Daylength treatments | I, II |
| Flowering time measurement | IV |
| GA treatments | II |
| Pro-Ca treatments | II, III |
| GA analysis | II |
| Suppressive subtractive hybridization | (IV) |
| EST sequencing | (IV) |
| Real-time RT-PCR | II, IV |
| cDNA synthesis | II, IV |
| Bioinformatics analyses | IV |
| cDNA cloning | II |
| RACE | II |
| RNA extraction | II, IV |
| mRNA extraction | IV |

Table 3. The list of germplasm used in this study.

| Species | Genotype | Publication |
|---|---|---|
| *Fragaria* x *ananassa* | 'Korona' | I, II |
| *Fragaria* x *ananassa* | 'Polka' | III |
| *Fragaria* x *ananassa* | 'Honeoye' | III |
| *Fragaria vesca* | NCGR accession 551792 | IV |
| *Fragaria vesca* | 'Baron Solemacher' | IV |
| *Fragaria vesca* | 'Hawaii-4' | IV |
| *Fragaria vesca* | 'Yellow wonder' | Thesis |
| *Fragaria vesca* | 'Alexandria' | Thesis |
| *Fragaria vesca* | white unknown (Piikkiö) | Thesis |

# 4 RESULTS AND DISCUSSION

## 4.1 Environmental regulation of growth in strawberry

*4.1.1 Flowering and runnering in everbearing woodland strawberry (IV)*

Environmental regulation of flowering in SD genotypes of woodland strawberry and garden strawberry is well documented. In general, flowering induction occurs in SD, but high temperature inhibits flowering. Moreover, some genotypes flower independently of photoperiod at low temperature (Heide 1977, Konsin et al. 2001, Sønsteby and Heide 2006, Heide and Sønsteby 2007). In everbearing genotypes, the regulation of flowering is not so clear, and they have been classified as day-neutrals in most reports (e.g. Darrow 1966, Guttridge 1985). The effect of photoperiod and temperature on flowering time in everbearing genotypes Baron Solemacher and Hawaii-4 were analysed by counting the number of leaves in the main crown before the development of the terminal inflorescence (developmental stage-based method). These genotypes, as well as three other everbearing genotypes, flowered very early in LD at 18°C (IV, Figure 7), but when plants of these same two genotypes were given 5 weeks SD treatment after germination, the induction of flowering was delayed by 4 – 6 leaves. Because plants formed 4 – 5 leaves during the SD treatment, flowering was probably induced immediately after the movement of plants to LD. Low-temperature (11°C) treatment of 5 weeks delayed flowering by about 3 leaves, which is equal to the number of leaves formed during the treatment (IV). These data confirm the results from a recent study showing that LD and high temperature accelerate flowering in two everbearing woodland strawberry genotypes, Rugen and Baron Solemacher (Sønsteby and Heide 2008). However, flowering time was measured as days to flowering, a method that does not take into account the effect of environmental conditions on general growth rate, in contrast to our developmental stage-based method. Taken together, SD and everbearing genotypes of woodland strawberry show opposite flowering responses to photoperiod and temperature.

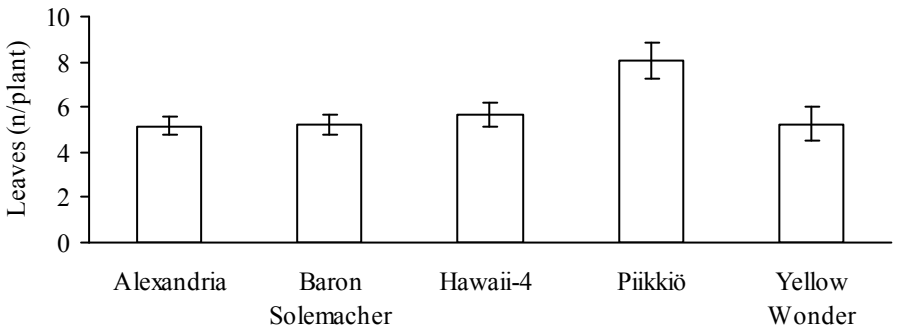


Figure 7. Flowering time of different everbearing genotypes of *F. vesca* in LD at 18ºC indicated as the number of leaves in the main crown before the terminal inflorescence. Values are averages of 10 plants ± standard deviation.

Runner formation in runner-producing, everbearing genotype Hawaii-4 began four weeks earlier in SD-grown plants, and at the end of the experiment, they had three times more runners than LD-grown plants (Figure 8). This is in contrast to SD genotypes, where runnering is suppressed by SD (Heide and Sønsteby 2007). Thus, in both SD genotypes of woodland strawberry and everbearing genotype Hawaii-4, runnering and flowering induction are clearly antagonistic processes. This is also supported by the finding that everbearing cv. Baron Solemacher, considered as a non-runnering genotype, produces runners in SD at high temperature, conditions that inhibit flowering (Sønsteby and Heide 2008). Moreover, in SD garden strawberry cv. Korona, runnering is inhibited in photoperiods ≤ 14 – 15 h, and the same photoperiod is close or equal to the critical photoperiod for flowering induction (II, Konsin et al. 2001). In conclusion, these data indicate that the regulation of runnering and flowering are connected in strawberries, and GA has been proposed to be a link between these processes (reviewed by Guttridge 1985). However, crossing experiments in woodland strawberry show that runnering and flowering are genetically separate processes, since functional alleles of separate genes are the basis of seasonal flowering or runner production, and mutation in one of these genes causes everbearing flowering habit and non-functional alleles of the other gene prevent runner formation (Brown and Wareign 1965, Battey et al. 1998). In the light of these findings, the connection between flowering and runnering in strawberries is indirect. The simplest explanation for reduced runnering after the induction of flowering is that crown branching occurring after the formation of terminal inflorescence has consumed axillary buds that would have differentiated to runners in vegetative plants.

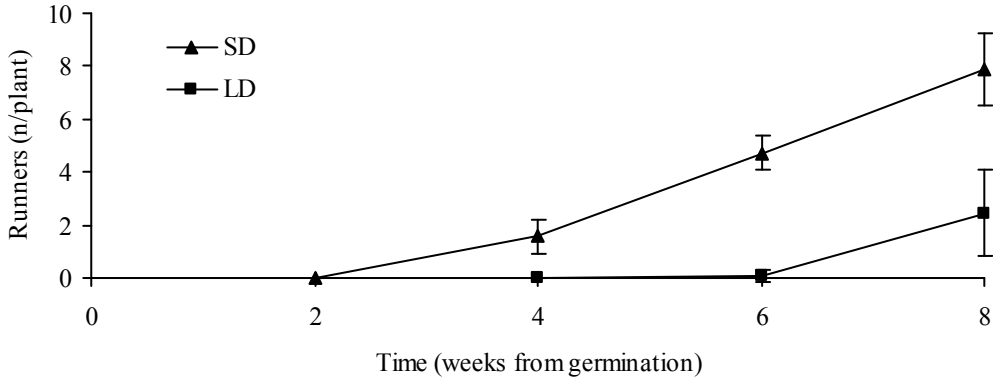


Figure 8. The effect of photoperiod on the cumulative number of runners in Hawaii-4 genotype of *F. vesca*. Plants were grown for five weeks in photoperiods of 12 h (SD) or 18 h (LD) at 18°C, after which SD grown plants were moved to LD. Values are averages of 13 plants ± standard deviation.

*4.1.2 Crown development and flower initiation (I)*

Strawberry flowering is dependent on the number of crown branches, since flowers are initiated terminally from the apical meristems of the main crown and branch crowns. In SD cultivars, both flowering induction (Darrow 1936, Heide 1977, Guttridge 1985) and crown branching (I, II, Konsin et al. 2001) are induced by SD, and the number of SD cycles determines the size of the response. SD treatment of 12 h photoperiod for three weeks initiated the formation of several primary branch crowns from the axillary buds of the main crown in garden strawberry cv. Korona, but secondary branches were found in only a few plants (I). This treatment also induced flowering in the apical meristem of the main crown. In contrast, most branch crown initials remained vegetative, probably because they had not reached competence to initiate flowering, which is attained after formation of at least 2 - 4 leaf initials (Arney 1953). However, when plants were exposed to a second 3 week SD treatment starting four weeks later, primary branch crowns were induced to flower and the number of secondary branch crowns started to increase rapidly. Again, most of the secondary crown branches induced by the second SD treatment did not reach competence to initiate flowers during SD and remained vegetative. Furthermore, under continuous SD of 15 weeks, a steady increase in the number of branch crowns was found, and meristems initiated flowers when they had reached competence. In conclusion, artificial SD treatments increase the number of meristems capable of initiating flowers, and therefore, these treatments can be used to enhance the cropping potential of strawberry plants.

According to these data, it could be concluded that flowering induction in the apical meristem is a primary factor initiating crown branching from axillary meristems. However, in a 12 h photoperiod, the first branch crowns were initiated after 8 - 12 SD cycles in cv. Korona, whereas flowering induction required more than 14 SD cycles (II, Hytönen et al. 2003). These data support the view that photoperiod is a primary factor affecting axillary bud differentiation in cv. Korona, and the differentiation of apical meristem after flowering induction is a secondary factor. However, possible involvement of early processes of incomplete flowering induction cannot be ruled out. Thus, careful analysis using marker genes for flowering induction should be done in order to find out if photoperiod controls axillary bud differentiation directly or indirectly through floral transition. As discussed below, the strawberry homolog of *AP1* can be used as a marker of floral transition in woodland strawberry (IV), and it should be tested also in garden strawberry.

*4.1.3 Runner axillary bud #2 as a model for long/short shoot differentiation (II)*

Strawberry runners and branch crowns are analogous to long and short shoots of several species of the Rosaceae, including apple, pear and cherry. In all these species, axillary buds are able to form both shoot types, and flowers are typically found on short shoots (Tukey 1964, Westwood 1993, Dennis 2003). Because the differentiation of strawberry axillary buds to runners and branch crowns can be strictly regulated by photoperiod (I, II, Konsin et al. 2001), strawberry could provide a good model system

to study this process. The photoperiodic responses of axillary buds on runners (axillary bud #2) and crowns were compared in cv. Korona, in order to use the axillary bud #2 as a model system for bud differentiation studies. These buds are more accessible and, therefore, more suitable for molecular and analytical studies. Runner growth ceased after 2 - 3 weeks in photoperiods of 10 or 14 h, because axillary bud #2 differentiated into a branch crown, whereas in LD (18 h), continuous runner growth was found (II). Axillary buds of the crown showed similar photoperiod regulation, supporting the use of runner axillary bud #2 as a model.

## 4.2 Hormonal regulation of axillary bud differentiation in strawberry

### *4.2.1 GA is needed for runner outgrowth (II)*

Both exogenously applied GA and the inhibitors of GA biosynthesis have been shown to affect runner formation in strawberry, indicating that GA plays a role in axillary bud differentiation (Thompson and Guttridge 1963, Avigdori-Avidov et al. 1977, Nishizawa 1993, Black 2004). The effect of prohexadione-calcium (ProCa), the inhibitor of GA3-oxidase (Rademacher 2000), was tested on GA levels and axillary bud diffentiation in cv. Korona (II). ProCa clearly blocked GA3-oxidase in strawberry, since the amount of $GA_1$ dropped by almost 50% two days after the ProCa treatment, whereas the immediate precursors $GA_{19}$ and $GA_{20}$ accumulated to high levels. ProCa also blocked runner formation in about two days, with a concomitant induction of crown branching, indicating that 50% reduction in the level of bioactive GA is sufficient to change the fate of the axillary buds (II). These effects of ProCa were completely reversed by GA application, confirming the causality of the reduced level of bioactive GA and the cessation of runner initiation in ProCa-treated plants.

Interestingly, in this runner bud model system, the reduction in $GA_1$ level in SD-grown buds compared with LD-grown buds was similar to that in ProCa-treated plants compared with non-treated plants, and these changes correlated with bud fate (II). The observed responses after a moderate drop in $GA_1$ level may be biologically relevant, because similar changes have been shown to precede growth cessation in subapical tissues of *Salix pentandra* (Olsen et al. 1995). Taken together, results from growth regulator experiments and GA analyses suggest that a certain level of $GA_1$ is needed for runner initiation in strawberry, and the reduction of $GA_1$ concentration below this level leads to branch crown formation. However, SD-grown axillary buds also showed reduced responsiveness to applied GA (II), indicating that photoperiod affects the GA signalling pathway in strawberry. Therefore, detailed studies are needed to uncover the relative role of reduced GA level and signalling in photoperiodic shoot differentiation in strawberry. This is challenging, since mechanisms of photoperiod x GA interaction are mostly unresolved (Schwechheimer 2008). However, recent findings indicate that the interaction of PIF3, PIF4 and PIF1/PIL5 with DELLA proteins could provide some possible answers about the mechanisms underlying light x GA interaction (Oh et al. 2006, Feng et al. 2008, de Lucas et al. 2008). Moreover,

an example of a photoperiod-regulated signalling cascade affecting GA biosynthesis is found in potato. In this cascade, SD-induced *BEL5* generates a long-distance signal that interacts with a Knotted1-type transcription factor POTH1 in the stolon tip, and together they down-regulate GA biosynthesis and consequently induce tuber formation (Chen et al. 2004, Banarjee et al. 2006).

*4.2.2 Feedback mechanisms reveal changes in GA signal of axillary buds (II)*

GA biosynthesis and signalling homeostasis is a well documented phenomenon in plants and reflects the tight control of the whole GA pathway (Schwechheimer 2008). The GA homeostasis is maintained by DELLA proteins that affect the expression of several genes in GA biosynthetic and signalling pathways directly or indirectly (Zentella et al. 2007). This part of the study aimed to find GA-regulated genes in strawberry, in order to use them as markers of the activity of GA signal. Therefore, the expression of ten putative GA biosynthetic, signalling and target genes was studied in cv. Korona by real-time RT-PCR in parallel with GA analyses. Many of these transcripts showed a clear response to reduced $GA_1$ levels in ProCa-treated plants (II), providing evidence for similar feedback-regulation of the GA pathway in strawberry as found in *Arabidopsis* (Zentella et al 2007, Willige et al. 2007). In general, positive components of the GA pathway (*GA3ox, GID1b, SLY*) were up-regulated and genes encoding negative regulators (*GA2ox, GAI, RGA*) were repressed by ProCa. In addition, the homolog of GA-repressed *XERICO* (Zentella et al. 2007) was upregulated by ProCa, as expected.

Similar changes in gene expression levels were expected in SD-grown differentiated axillary bud #2 as in ProCa-treated plants, because of the comparable drop in $GA_1$ level. This was indeed the case with all of these genes except *GA3ox* (II). These data indicate that GA regulation of these genes is functional in SD-grown axillary buds and provides molecular evidence for GA regulation of axillary bud fate. The finding that *GA3ox* was downregulated in SD-grown buds compared to LD, instead of upregulation by feedback mechanism as in ProCa-treated plants, provides one possible means to reduce the level of $GA_1$. However, GA-oxidases are encoded by small gene families in other plant species (Hedden and Phillips 2000, Sakamoto et al. 2004), so the presence and the expression of redundant genes in strawberry should be explored.

*4.2.3 ProCa, the inhibitor of GA biosynthesis, enhances cropping potential (III)*

GA is one of the signals determining the differentiation of strawberry axillary buds to runners and branch crowns (II), and therefore the manipulation of GA biosynthesis should affect the cropping potential in strawberry. Several growth regulators are available to control GA biosynthesis, but most of them have a long half-life. In contrast, ProCa is a non-toxic growth regulator with a short half-life (Rademacher 2000). Therefore, its effect on vegetative and generative development of strawberry cvs. Polka and Honeoye was tested in northern LD conditions. ProCa treatment during the planting year reduced runner growth and the formation of new runners in five

experiments out of six, with a concomitant increase of crown branching in three experiments. These changes in axillary bud differentiation were associated at best with about 50% increase in the number of inflorescences and berry yield during the following year. Our findings on strawberry vegetative development are in line with previous studies (Reekie and Hicklenton 2002, Black 2004). However, in Black's study, ProCa did not affect flowering and yield. These differences may be due to different climatic and daylength conditions, since Black (2004) conducted his experiments at more southern latitudes in the USA. Reekie et al. (2003, 2005), however, reported that ProCa treatment given in a nursery field increased the berry yield, probably because of better establishment of the plants in the production field. In conclusion, planting year ProCa treatment can be used in strawberry fields to control axillary bud diffenrentiation and enhance cropping potential in northern LD conditions.

## 4.3 Genetic regulation of flowering in strawberry

*4.3.1 Involvement of multiple flowering pathways (IV)*

Environmental regulation of flowering in strawberry has been characterized in detail (Taylor 2000, Sønsteby and Heide 2006, 2007a, 2007b, 2008, Heide and Sønsteby 2007), but almost nothing is known about the genes and regulatory pathways underlying the induction of flowering. In the model plant *Arabidopsis*, four major genetic pathways, photoperiod, vernalization, autonomous and gibberellin pathways, are known, and more than one hundred genes are involved in these pathways (Simpson 2004, Boss et al. 2004, Yakir et al. 2007, Dennis and Peacock 2007, Turck 2008). Therefore, putative flowering time genes of strawberry were sought by EST sequencing and bioinformatics analysis. Two subtracted (flowering gene enriched) cDNA libraries from the apical buds of SD and EB woodland strawberry were constructed by the SSH-method (Diatchenko et al. 1996), and about 2700 ESTs were sequenced (IV). Among these were 14 gene homologs of *Arabidopsis* flowering time genes from all major flowering pathways (Table 4). Gene homologs of the photoperiod and thermosensory/autonomous pathways were found only in the SD library, whereas putative members of the vernalization pathway were identified only in the EB library, suggesting differences in the function of these pathways between EB and SD genotypes. However, expression analysis of selected genes in parallel samples did not reveal differential expression between the genotypes. According to these data, the SSH did not enrich differentially expressed flowering time genes. However, given that only a little overlap was found between the cDNA libraries, they were effectively normalized by SSH, and therefore a high number of putative flowering time genes was identified.

For a more comprehensive analysis of strawberry flowering pathways, homologous sequences for 118 *Arabidopsis* flowering time genes were sought in the *Fragaria* EST and EST contig collections available at the Genome database for Rosaceae (GDR). In

these searches, we found 52 additional gene homologs among 51000 sequences. Thus, in total, we found strawberry homologs for 66 flowering time regulators of *Arabidopsis*, of which few were reported earlier (Folta et al. 2005, Stewart 2007). Moreover, gene homologs absent in *Fragaria* sequence resources were further searched from GDR Rosaceae EST database containing 410000 sequences. In this search, 22 additional gene homologs were identified.

Table 4. Putative flowering time gene homologs identified from subtracted cDNA libraries. Sequences corresponding to *Arabidopsis* genes of different flowering pathways are grouped. The cut-off value in BLASTx searches was 1E-10.

| Gene | Biological function | SD/EB | Reference | E-value |
|---|---|---|---|---|
| *Photoperiodic pathway* | | | | |
| PhyA | Red light photoreceptor | SD | Lin 2000 | 5E-33 |
| FYPP3 | Ser/Thr-specific protein phosph. 2A | SD/EB | Kim et al. 2002 | 1E-56 |
| LHY | Myb domain TF | SD | Schaffer et al. 1998 | 9E-19 |
| PRR7 | Pseudo-response regulator | SD | Nakamichi et al. 2007 | 5E-52 |
| ELF6 | Jumonji/zinc finger-class TF | SD | Noh et al. 2004 | 1E-45 |
| AP2 | AP2 TF | SD | Aukermann et al. 2003 | 9E-16 |
| *Vernalization pathway* | | | | |
| SUF4 | putative zinc finger containing TF | EB | Kim et al. 2006 | 5E-46 |
| ELF8 | RNA pol. 2 associated factor -like | EB | He et al. 2007 | 3E-42 |
| MSI1 | WD40 protein | EB | Bouveret et al. 2006 | 4E-62 |
| *Autonomous and thermosensory pathways* | | | | |
| SVP | MADS-box TF | SD | Hartmann et al. 2000 | 5E-22 |
| FVE | retinoblastoma associated | SD | Ausin et al. 2004 | 3E-76 |
| *Gibberellin pathway* | | | | |
| SPY | O-linked N-acetylglucosamine transf. | SD/EB | Tseng et al. 2004 | 2E-93 |
| *Light quality pathway* | | | | |
| PFT1 | vWF-A domain protein | EB | Cerdán et al. 2003 | 1E-17 |
| HRB1 | ZZ type zinc finger protein | SD | Kang et al. 2007 | 7E-22 |

The role of the photoperiodic flowering pathway is to control flowering time according to daylength signals. The major regulator in this pathway is CO, which performs seasonal time measurement in plants (Yanovsky and Kay 2002). Putative strawberry *CO* has been cloned earlier (Stewart 2007), but its regulatory pathways have not been characterized. In the present study, several putative photoreceptor sequences encoding phy, cry and ZTL-like photoreceptors were found (IV) and several candidate genes were identified, corresponding to the circadian clock genes known in *Arabidopsis*, including *LHY1* (Schaffer et al. 1999) and *TOC1* (Strayer et al. 2000) belonging to the core feedback loop. However, *CCA1*, a gene encoding MYB domain transcription factor redundant with LHY1 (Wang and Tobin 1998) was found neither in *Fragaria* nor in Rosaceae EST sequence collections, indicating that this gene may be absent from the Rosaceae, as it is from the Fabaceae (Hetch et al. 2005).

Among the regulators of *CO* transcription and protein stability, putative COP1 (Jang et al. 2008) and two sequences encoding putative SPA proteins (Laubinger et al. 2006) were found in the present work. In contrast, ESTs for two important regulators, GI and CDF1 (Fowler et al. 1999, Imaizumi et al. 2005, Sawa et al. 2007) were not identified in *Fragaria*, but they were present in Rosaceae EST database.

The central flowering repressor in the vernalization pathway, FLC, prevents flowering by suppressing *FT* and *SOC1*, thereby overriding the promotive effect of other flowering pathways (Michaels and Amasino 1999). The activation of *FLC* involves complex histone modifications generated by FRI, PAF1 and SWR1 protein complexes and vernalization is needed to overcome FLC function (He et al. 2004, Kim et al. 2006, Choi et al. 2007) Neither *FLC*-like sequences nor its major activator FRI were found in *Fragaria* or Rosaceae EST databases, but *Fragaria* genes homologous with the components of FRI complex were identified, including two putative *FRL* genes and *SUF4* (Michaels et al. 2004, Kim et al. 2006). Homologs of all known genes of SWR1 complex (*PIE, SEF1, ARP6*) and four genes belonging to PAF1 (*ELF8, VIP3, VIP4, ATX1*) were also present in *Fragaria* EST collections. In addition to the activators of *FLC*, sequences were found corresponding to *SWN1*, *FIE* and *VIN3* that are involved in the silencing of *FLC* during vernalization as the components of VRN2-PRC2 complex (Chanvivattana et al. 2004, Sung and Amasino 2004, Wood et al. 2006). Taken together, the presence of this complex machinery needed to control *FLC*, suggests that *FLC*-like sequence(s) exists in strawberry. This is also supported by the finding that *FLC*-like sequences are present in several eudicot lineages (Reeves et al. 2007).

Many genes have been identified as associated with the autonomous flowering pathway in *Arabidopsis*. The function of these genes is to downregulate *FLC* and *FLC*-like genes according to the developmental program of the plant (Simpson 2004). At least 14 genes are thought to belong to this pathway, and homologs for 8 and 12 of these genes are currently found in *Fragaria* and Rosaceae, respectively (IV). Sequences found in *Fragaria* correspond to *Arabidopsis* RNA processing factors *FLK, FY*, *LD* (Quesada et al. 2005), and regulators of histone methylation including *LDL1*, *LDL2* and *REF6* (Noh et al. 2004, Jiang et al. 2007). Moreover, ESTs homologous to the recently characterized ambient temperature pathway, *SVP* and *FVE* (Hartmann 2000, Blázquez et al. 2003), were identified from the present cDNA libraries (Table 4).

$GA_4$ promotes flowering in Arabidopsis (Eriksson et al. 2006), so all changes affecting the activity of GA signal should affect flowering time. Several putative GA biosynthetic and signalling genes were cloned or identified from strawberry (II). In addition, flowering time genes associated to the GA pathway were sought and homologs of *GAMYB33*, *FPF1* and *DDF1* were found in the strawberry EST database. Among these regulators, GAMYB33 may be a central regulator in the GA pathway, since it binds *LFY* promoter and is activated by GA (Gogal et al. 2001).

Floral inductive signals coming from different pathways are integrated by a few genes including *FT, LFY* and *SOC1*, and therefore these are called flowering integrator genes (Parcy 2005). These genes in turn trigger floral initiation by activating floral identity gene *AP1* (Wagner et al. 1999, Fernandiz et al. 2000, Abe et al. 2005). These genes were not found in the present EST libraries. The full length cDNA sequence of putative *SOC1* and partial *LFY* cDNA were cloned from woodland strawberry (IV), but *FT* was not found despite the number of attempts, although *FT* is present in Rosaceae, since both *Malus* and *Prunus FT* sequences were found at NCBI Genbank. Moreover, putative strawberry *AP1* was identified from sequence collections at GDR.

*4.3.2 SFL is a major inhibitor of flowering (IV)*

*Fragaria* genes were identified representing all flowering pathways known in *Arabidopsis*, but the functions of corresponding genes and pathways may differ between species. The major flowering repressor in woodland strawberry, SFL, is an example of a novel regulatory mechanism, and this gene provides a key for understanding the regulation of seasonal (SD) flowering induction. Dominant alleles of this gene have been shown to be the root of SD/low temperature flowering response in SD genotypes of woodland strawberry, whereas recessive alleles cause continuous (EB) flowering habit in cv. Baron Solemacher (Brown and Wareign 1965, Battey et al. 1998). Furthermore, in cv. Baron Solemacher, flowering induction is promoted by LD and high temperature (IV). Thus, SFL makes a difference between SD/low temperature and LD/high temperature flowering responses. The expression analysis of selected genes corresponding to *Arabidopsis* genes from different flowering pathways did not reveal the location of *SFL* in strawberry flowering pathways (IV).

Since seasonal and everbearing woodland strawberries show different photoperiodic responses, *SFL* may lie in the photoperiodic flowering pathway. Different photoperiodic responses of the SD plant rice and the LD plant *Arabidopsis* can be explained by different function of CO. In *Arabidopsis,* coincidence of CO and light period promotes flowering in LD (Suarez-Lopez et al. 2001), whereas the rice CO homolog, Hd1, is inhibitory during photoperiod, but promotes flowering in darkness (Yano et al. 2000). This raises the question whether CO has a similar inhibitory role in strawberry, in which case *CO*, or some activator of its transcription or gene participating in its post-transcriptional modification, could be *SFL*. Strawberry *CO* has been mapped to the *Fragaria* reference map, and its location does not support its role as *SFL* (Sargent et al. 2006, Stewart 2007). However, a photoperiod pathway operating through *CO* cannot be ignored as the site of floral inhibitor *SFL*, because transcriptional or post-transcriptional regulation of CO is still an option. Another possibility is that CO is a floral activator (Stewart et al. 2007), in which case seasonal flowering could be explained by a dominant flowering inhibitor overriding the promotive function of CO. In fact, this model is the easiest way to explain the opposite environmental regulation of flowering in SD and EB genotypes. In this case,

FT would be a logical candidate for the mobile signal promoting flowering in strawberry (Hartmann 1947, Corbesier et al. 2007).

Vernalization pathways in *Arabidopsis* and cereals provide examples of inhibitory pathways overriding the promotive effect of the photoperiod (Trevaskis et al. 2007, Ausín et al. 2005). Thus, the homolog of *Arabidopsis* flowering repressor, FLC (Michaels and Amasino 1999), is an attractive alternative for SFL function, as also suggested by Battey (2000). There is, however, discrepancy in the hypothesis that *SFL* could be the *FLC*-like gene, since flowering induction in strawberry and vernalization in *Arabidopsis* occur between different temperature limits. In strawberry, flowering is induced by temperatures above the vernalizing range, whereas vernalizing temperature (6°C) inhibits flowering (Ito and Saito 1962, Sønsteby and Heide 2006, Heide and Sønsteby 2007). Moreover, winter chilling under vernalizing temperatures promotes vegetative growth and prevents further flowering induction in the spring, indicating that SFL is activated again (Battey 2000). Thus, the regulation of the *FLC*-like gene should be mechanistically different in strawberry from that in *Arabidopsis*. If the *FLC*-like gene is a key floral repressor in strawberry, its activators should also be considered as potential candidates for *SFL*.

GA has also been suggested to be a flowering inhibitor in strawberry, because exogenously applied GA acts in that way (Thompson and Guttridge 1959, Guttridge and Thompson 1963). However, the present gene expression data does not support this idea (IV). In the present work, *GA3ox* was strongly down-regulated in EB apices at three- and four-leaf stages compared to the two-leaf stage, and *GA2ox* showed a similar trend, but these changes in the GA pathway did not coincide with the timing of flowering induction that occurs before the two-leaf stage. Given that *GA2ox* is strongly upregulated by GA in strawberry (II), these findings indicate that GA activity was not modified in the EB apex before flowering initiation started (IV). Moreover, the expression levels of these genes were no higher in the shoot apex of SD genotype compared to EB at one- and two-leaf stages, suggesting that GA activity was also no higher there then. Taken together, these data suggests that endogenous GA is not a major flowering inhibitor in strawberry, but they do not exclude the possibility that some flowering-time gene(s) are differentially regulated by GA in SD and EB genotypes. Thus, the GA pathway as well as its target genes in flowering pathways should be carefully characterized in order to unravel the role of GA in the regulation of flowering in strawberry.

*4.3.3 Co-regulation of AP1, LFY and SOC1 during floral transition (IV)*

The developmental regulation of putative *AP1, LFY* and *SOC1* was studied in the shoot apex samples of EB and SD genotypes. PCR analysis revealed that the putative floral identity gene *AP1* accumulated rapidly after the one-leaf stage in the EB genotype, whereas it was absent in the SD genotype (IV). *LFY* showed a similar trend in the EB genotype, starting to accumulate at the two-leaf stage. *SOC1*, instead, peaked at the two-leaf stage and was slightly down-regulated during later stages.

These expression profiles are in agreement with the data available in *Arabidopsis* (Wagner et al. 1999, Abe et al. 2005, Liu et al. 2007), and they indicate that flowering induction occurs before the two-leaf stage in EB cv. Baron Solemacher in LD. Moreover, these data show that *AP1* can be used as the marker in order to strictly determine the timing of floral induction in woodland strawberry.

## 5 CONCLUSIONS AND PROSPECTS

In this thesis, photoperiodic, hormonal and genetic regulation of flowering as well as axillary bud differentiation in strawberry were studied. Flowering induction in woodland strawberry *sfl* mutant cv. Baron Solemacher was promoted by LD and high temperature, opposite to SD genotypes, indicating that the unknown *SFL* gene makes the difference between these opposite flowering responses. Moreover, 66 putative flowering time genes were identified from strawberry EST collections, representing all major pathways known to regulate flowering in *Arabidopsis*. One of the identified genes, *AP1*, was found to be specifically expressed in the shoot apices of the EB genotype after flowering induction, showing that it can be used as the marker of floral transition in woodland strawberry. Several central regulators were not found, including *FT* and *FLC*, and therefore they are among the most important targets of future research. Because *SFL* is a putative repressor gene linked to both photoperiod and temperature responses, it will most likely lie on the photoperiodic or vernalization flowering pathway. Therefore, functional analysis of central genes from these pathways, including *CO, FT, FLC* and *VIN3*, should be carried out. Moreover, the function of *SOC1, LFY* and *AP1* as floral integrator and identity genes needs to be confirmed. The identification of putative flowering regulators as well as the *AP1* marker gene will strongly enhance the exploration of strawberry flowering pathways by genetic transformation, QTL mapping and transcriptomics analysis.

Strawberry axillary bud differentiation was shown to be strictly regulated by photoperiod and GA was one of the key signals mediating this differentiation. Confirming this finding, the inhibitor of GA biosynthesis, prohexadione-calcium, can be used to control axillary bud differentiation with a concomitant increase in the berry yield in northern LD conditions. In future studies, the molecular mechanisms of GA x photoperiod interaction should be studied, in order to understand the regulation of axillary bud differentiation in strawberry, and more broadly, short/long shoot differentiation in the Rosaceae. Interestingly, also runnering vs. non-runnering phenotypes in woodland strawberry are controlled by a single gene, *R* locus, and therefore, the identification of the *R* gene is an important goal.

In conclusion, detailed understanding of strawberry flowering pathways and molecular factors controlling axillary bud differentiation as well as their interaction will ultimately enhance the production of strawberry and other species of the Rosaceae family through improved cultivars produced with the aid of molecular markers and transgenes. Obviously *SFL* and *R* genes provide keys for the understanding of these central characters in strawberry growth and development.

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