FLOWER INDUCTION AND VEGETATIVE GROWTH CHARACTERISTICS IN *FRAGARIA X ANANASSA* CULTIVARS ‘CALYPSO’ AND ‘HAPIL’

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ABSTRACT

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Tiivistelmä ⁂ Referat ⁂ Abstract

Fragaria x ananassa is a widely appreciated berry with its production growing all around the world. Thus, there will be a huge demand for strawberry breeding in the future especially since the climate change is casting an extra shadow upon the growing conditions which is also why there is a need for better understanding of different cultivar types. There are everbearing and seasonally flowering cultivars of both *Fragaria x ananassa* and *Fragaria vesca.* The seasonally flowering types such as ‘Hapil’ flower once during the growing season whereas everbearing types such as ‘Calypso’ flower for a longer period. The gene behind the change in the flowering habit has been tracked to the photoperiodic pathway of *F. vesca* although the gene behind the trait in *F x ananassa* remains unknown.

The aim of this project was to compare flowering and vegetative responses of in vitro propagated everbearing *F x ananassa* cultivar ‘Calypso’ and seasonally flowering ‘Hapil’ in long and short day photoperiodic conditions in order to find out differences between everbearing and seasonally flowering cultivars. This was done by collecting data from phenotype observations linked to the vegetative and generative stages of the development of strawberries. The phenotype data was then combined with gene expression data of *FaSOC1*, *FaTFL1*, *FaGA20ox4* and *FaAP1* which are genes known to work on the photoperiodic pathway that regulates the switch between the vegetative and generative development of both *F x ananassa* and *F. vesca*. In addition, the expression of an everbearing phenotype associated gene *FaFT2* was analysed. This study was a part of a larger project aimed to find out the genetic basis for the everbearing habit of *F x ananassa.*

Part of the ‘Calypso’ plants were induced to flower already during the acclimatization period and the rest at the very beginning of the treatment period which then caused differential flowering times between the ‘Calypso’ groups. Short day grown ‘Hapil’ was induced to flower between weeks three and six whereas long day grown ‘Hapils’ remained vegetative. Phenotypic observations were also backed up by the expression of *FaTFL1* and *FaAP1*. Instead the *FaSOC1* expression was repressed in short day conditions more than in the long days regardless of the cultivar type.

‘Calypsos’ were capable of producing runners regardless of photoperiod or flower induction. Consequently the runner production seemed to be regulated by factors outside of the photoperiodic pathway. However, the expression of runnering associated *FaGA20ox4* was low and variable due to the sampling strategy. Interestingly most of the axillary meristems of short day grown ‘Hapils’ remained dormant for an unknown reason. Expression of *FaFT2* was low on the apical meristems and further support for the role of the gene in everbearing phenotype was not found.

Avainsanat – Nyckelord – Keywords

Fragaria x ananassa, Fragaria vesca, Flower induction*,* ‘Calypso’, ‘Hapil’, Gene expression, Plant breeding

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Tiivistelmä ⎄ Referat ⎄ Abstract

Fragaria x ananassa (puutarhamansikka) on laajalti arvostettu marja, jonka tuotanto lisääntyy jatkuvasti ympäri maailman. Näin ollen sen jalostukselle tulee olemaan valtavasti kysyntää varsinkin kun ilmastonmuutos luo oman varjonsa kasvuolojen ylle. Erilaisten lajiketyyppien vegetatiivisten ja generatiivisten vasteiden tuntemus on siten tärkeässä roolissa tulevaisuuden mansikantuotannon kannalta. Sekä puutarhamansikasta, että ahomansikasta (*Fragaria vesca*) on olemassa kahta lajiketyyppiä, jatkuvasatoisia sekä kausisatoisia. Jatkuvasatoiset lajikkeet, kuten ‘Calypso’ kukkivat ja tuottavat satoa läpi kasvukauden kun taas kausisatoiset lajikkeet, kuten ‘Hapil’ kukkivat lyhyemmän ajan, jolloin satokausikin on lyhyempi. Ahomansikan kukintatavan muutoksen taustalla vaikuttava geeni sijaitsee kukintaa säätelevällä päivänpituusreitillä, kun taas puutarhamansikalla ominaisuuden tausta on tuntematon.

Tutkimuksen tavotteena oli verrata kahden in vitro -kasvatetun puutarhamansikkalajikkeen ‘Calypson’ ja ‘Hapilin’ vegetatiivisia ja generatiivisia vasteita pitkässä ja lyhyessä päivässä ja löytää siten eroja jatkuva- ja kausisatoisten lajikkeiden välillä. Tämä tehtiin havainnoimalla mansikoiden vegetatiivisiin ja generatiivisiin kasvuvaiheisiin liittyviä fenotyppejä, joka yhdistettiin päivänpituusreitillä toimivien geenien *FaSOC1*, *FaTFL1*, *FaGA20ox4* ja *FaAP1* ekspressiodataan. Lisäksi analysoitiin puutarhamansikan jatkuvasatoisuuteen yhdistetyn geenin *FaFT2* ekspressio. Tämä tutkimus oli osa laajempaa projektia, jonka tavoitteena oli löytää puutarhamansikan jatkuvasatoisuuden taustalla vaikuttavat geenit.

Osa ‘Calypsoista’ indusoitui kukintaan jo sopeutumisjaksolla ennen valojaksokäsittelyiden aloittamista ja loput heti käsittelyiden alussa, mikä johti havaittuihin eroihin päivänpituuskäsittelyiden välillä. Lyhyen päivän olosuhteissa kasvatetut ‘Hapilit’ indusoituivat viikoilla 3–6, kun taas pitkän päivän olosuhteissa kasvatetut ‘Hapilit’ pysyivät vegetatiivisina. Geenien *FaTFL1* ja *FaAP1* ekspressio tuki havaittuja kukintafenotyyppejä, kun taas *FaSOC1* repressoitui enemmän lyhyen, kuin pitkän päivän olosuhteissa lajikeyypistä riippumatta.

‘Calypsot’ tuottivat rönsyjä valojaksosta tai kukintainduktiosta riippumatta, joten vaikuttaa siltä että ‘Calypsojen’ rönsyntuotanto oli päivänpituusreitistä riippumatonta. Toisaalta rönsyntuotantoon yhdistetyn geenin *FaGA20ox4* ekspressiotaso oli alhainen ja vaihteleva näytteenottostragegiasta johtuen. Valtaosa lyhyen päivän olosuhteissa kasvatettujen ‘Hapileiden’ hankasilmuista pysyi dormanttina toistaiseksi tuntemattomasta syystä. *FaFT2*:n ekspressiotaso puolestaan oli alhainen, eikä todisteita sen roolista puutarhamansikan jatkuvasatoisuuden taustalla löydetty.

Avainsanat – Nyckelord – Keywords

Fragaria x ananassa, Fragaria vesca, kukintainduktio*, ‘*Calypso’, ‘Hapil’, geeniekspressio, kasvinjalostus

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Table of contents

1. Abbreviations

AP1	*APETALA1*
BAP	6-benzylaminopurine benzyl adenine
CO	*CONSTANS*
FT1	*FLOWERING LOCUS T1*
FT2	*FLOWERING LOCUS T2*
FUL	*FRUITFULL*
GA	Gibberellic Acid
GA20ox4	*Gibberellin 20 oxidase 4*
GWAS	Genome-wide association study
IBA	Indole-3-Butyric Acid
MS	Murashige and Skoog medium
qPCR	Quantitative Polymerase Chain Reaction
SOC1	*SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1*
TFL1	*TERMINAL FLOWER1*

2. Introduction

Strawberry is a widely appreciated crop with its global production more than doubled during this millennium (FAOSTAT 2018). In addition to a delicious taste there is more behind the popularity and rising production rates of this jewel-like berry. For instance the cropping season has lengthened notably thanks to both, improved varieties and agronomic practices (Simpson 2018). Strawberries can also be cultivated in an area ranging from sub-tropics to temperate climate zones. Moreover, the shelf-life of the berries has also increased significantly enabling shipping for further distances (Simpson 2018).

The nutritional and economical value of strawberries is significant both domestically and internationally. Strawberries are cultivated in 76 countries (Simpson 2018), the largest producer being China with 71% of the global production in 2018 (FAOSTAT 2018). The popularity and cultivation is expected to grow even further with the consumption of fresh strawberries rising alongside the economical development of many countries (Simpson 2018). Strawberries are appreciated in Finland as well. The domestic production of berries in 2019 was 18 million kilograms of which strawberry made 85% making it the most popular domestically produced berry (LUKE 2019). Hence there is no doubt that in the future there will be a lot of demand for strawberry breeding and thus knowledge of strawberry genetics, especially now that climate change is affecting the growing conditions around the world.

There are everbearing and seasonally flowering cultivars of both *F. vesca* (woodland strawberry) and *F x ananassa* (garden strawberry) (Darrow 1977). The seasonal types flower only once during the growing season whereas everbearing types flower for a longer period. Vegetative growth including the runner formation has been characterized to be greater in seasonally flowering than in everbearing cultivars whereas the yields of the everbearers have been found to be bigger (Pérez de Camacaro et al. 2002). This is

because runnering is negatively correlated with flowering which is why the formation of runners has been found to be compromised in everbearing cultivars (Sønsteby & Heide 2007a). The bigger yield potential and earlier flower induction make everbearing strawberries ideal for cultivation. However, the limited capability for runner formation restricts the use of everbearing cultivars in professional cultivation since strawberries are usually propagated from runners (Battey et al. 1998).

Diploid *F. vesca* can be used as a model organism for the octoploid *F x ananassa*. The everbearing trait of *F. vesca* has been tracked to be caused by a mutation in the photoperiodic pathway gene *FvTFL1 (TERMINAL FLOWER1)* regulating the generative and vegetative development of *F. vesca* (Koskela et al. 2012) whereas on *F x ananassa* the genes behind the trait still remain a mystery regardless the intensive research around the subject. The photoperiodic pathway has been shown to function to some extent similarly between the two species with some of the known photoperiodic pathway genes such as *FT1* (*FLOWERING LOCUS T1*) and *SOC1* (*SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1*) responding to environmental cues similarly although the regulation of a major repressor *FaTFL1* on the pathway seem to be cultivar specific (Koskela et al. 2016). Moreover Koskela et. al (2016) found out in the same study that there are cultivar dependent differences in the response of *FaTFL1* to photoperiod and temperature.

The aim of the project was to compare flowering and vegetative responses of in vitro propagated everbearing *F x ananassa* cultivar ‘Calypso’ and seasonally flowering ‘Hapil’ in long and short day photoperiodic conditions in order to find out differences between everbearing and seasonally flowering cultivars. This was done by collecting data from phenotype observations linked to the vegetative and generative stages of the development of strawberries. The phenotype data was then combined with gene expression data of some of the known genes working on the photoperiodic pathway regulating the switch between the vegetative and generative development of both *F x ananassa* and *F. vesca*.

This project was a part of a larger project aimed to find out the genes behind the everbearing trait of octoploid *F x ananassa* by confirming a so-called three gene model. The model was proposed by Helen Cockerton and Richard Harrison from East Malling Research Station (EMR), as they found evidence in their unpublished GWA study that there could be three genes behind the everbearing trait. Thus they proposed a model in which at chromosome 4A there should be a dominant allele in order for the cultivar to be everbearing. In addition to the dominant allele in chromosome 4A there should also be one dominant allele in either chromosome 6A or 5C. The cultivar 'Calypso' was used specifically because it is heterozygous in each of the three loci. The results obtained from this project were planned to be an additional experiment bringing information about the function of the photoperiodic pathway of cultivar 'Calypso'.

3. Review

3.1. Phylogenetics and evolutionary history of *F x ananassa*

F x ananassa is octoploid and highly heterozygous (Hirakawa et al. 2014) hybrid between two octoploid strawberry species, *F. chiloensis* and *F. virginiana (Fragaria x ananassa* (Weston) Duchesne ex Rozier (1785)). The ploidy levels in the genus *Fragaria* are very variable and can vary from diploid to decaploid (Njunguna et al. 2013). In addition to the hermaphroditism of the polyploid species, the reproduction systems in the genus *Fragaria* include a vast array of different types such as gynodioecy, subdioecy, dioecy, heteroecy and both compatibility types (Njunguna et al. 2013, Staudt 2009).

The hybridization of *F x ananassa* happened in Europe partly by accident since only female plants of dioecious *F. virginiana* were imported to Europe at the early 1700's making the fertilization difficult. The plants were later crossed with *F. virginiana*, which also originates to the American continent. Crossing resulted in production of large

delicious berries in the hybrid progeny (Kingsbury 2009). The hybrid was given a name after pineapple since it was the most highly regarded and exotic fruit during the 1700's and was thought to portray this aromatic berry appropriately (Kingsbury 2009). This makes the garden strawberry one of the youngest domesticated species even though it's ancestors such as *F. chiloensis* have been cultivated for a millennia (Liston et al. 2014).

The North American origin can also be detected phylogenetically. Potter et al. (2000) suggested that the common octoploid ancestor of *F. virginiana* and *F. chiloensis* came through Bering Strait and then afterwards diverged into the eastern *F. virginiana* and more southern *F. chiloensis*. Similar results have been obtained by Staudt (2009) and Edger et al. (2019) as well. The rise of the octoploid clade of the *Fragaria* genus has been dated to 0.37–2.05 million years ago whereas the genus *Fragaria* itself is estimated to have diverged from *Potentilla* 12.1–38.8 million years ago (Njunguna et al. 2013). This makes the genus relatively young.

The previous hypothesis for the origin of F x *ananassa* suggested that the octoploid ancestor of the octoploids *F. chiloensis* and *F. virginiana* was a result of hybridization between *F. iinumae* and *F. vesca*-like diploids forming an allotetraploid species which then crossed with an unknown *F. iinumae* like allotetraploid (Tennessen et al. 2014). Even though the two known progenitor species are octoploids, the octoploidy (2n = 8x = 56) in *F x ananassa* has lately been analyzed to have arisen from four diploid progenitor species *F. iinumae*, *F. nipponica*, *F. viridis* and *F. vesca subsp. bracheata* of which *F. vesca subsp. bracheata* was probably the last progenitor (Edger et al. 2019). Two of these progenitors, *F. iinumae* and *F. vesca subsp. bracheata*, have been earlier identified by other groups as well (Hirakawa et al. 2014, Tennessen et al. 2014).

F x ananassa still has a remaining complete sets of chromosomes from each of the progenitor species and even though a part of *F. iinumae* subgenome has been replaced by the *F. vesca* genome (Edger et al. 2019) no evidence has been found for structural changes in the transition from diploid to octoploid (Hirakawa et al. 2014). Even though

chromosome losses, translocations, inversions, deletions and duplications can often happen during hybridization as a response to a genomic shock which polyploidization presents (McClintock 1984).

From these four progenitor species the *F. vesca subsp. bracheata* genome seems to be dominating the rest of the genomes. The *F. vesca subsp. bracheata* also encodes more dominantly expressed homoeologs than the other three submissive subgenomes combined with its power over the other subgenomes increasing over time (Edger et al. 2019). Tennessen et al. (2014) had similar results, they suggested that a *F. vesca* sequence per se could have higher fitness or then *F. vesca*-like genes might have been favored in the octoploid habitat. With such a big effect by the *F. vesca* subgenome, it is sensible to use *F. vesca* as a model organism when studying flowering responses to photoperiodic conditions in different cultivars of *F x ananassa*.

3.2. Plant structure and annual growth cycle of strawberries

3.2.1 Plant structure

The stem of a strawberry plant grows as a rosette with each leaf having a basal axillary bud. Each of these axillary buds can stay either dormant or produce a branch crown or a runner (Darrow 1966) whereas the apical meristem produces either new axils or an inflorescence (Figure 1) (Guttridge 1985). The inflorescence terminates the growth of the main crown whereas the continuation of the growth is continued by the uppermost axil (Darrow 1966). The conditions that are optimal for flower induction promote the formation of branch crowns whereas the conditions that promote vegetative growth turns the axillary buds into runners (Hytönen et al. 2004, Kurokura et al. 2017). These morphological changes can be taken advantage of when studying flower induction.

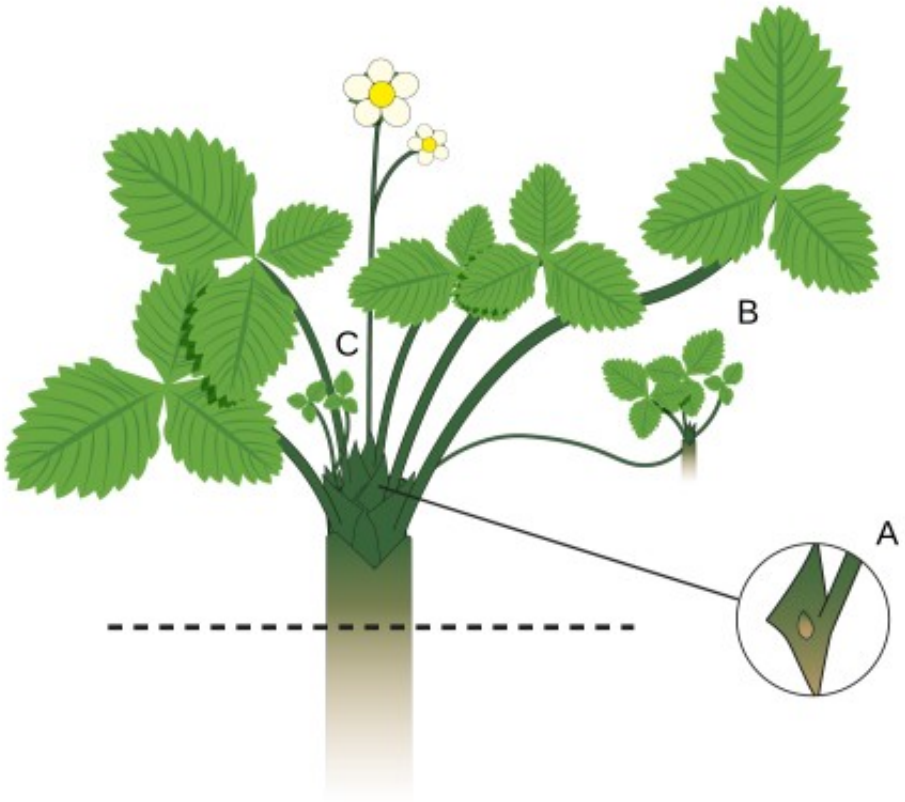

Figure 1. Structure of a strawberry plant, axillary buds can stay either dormant (A) or differentiate into runners from which a new daughter plant can grow (B) or differentiate into branch crowns (C).

3.2.2 Annual growth cycle of seasonally flowering strawberries

The seasonally flowering cultivars of both *F. vesca* and *F x ananassa* flower once during springtime after which the fruits are produced during a short period in summer (Figure 2). These seasonally flowering cultivars are short day plants, which means that the flowering is induced in short day photoperiod. In the case of strawberries, this means that the flower induction happens when the days shorten at the end of the summer (Figure 2). However, the flower induction of these short day accessions happens via photoperiodic pathway only when the temperature is between 13–20 °C (Rantanen et al. 2015). When the temperature is above this limit during the summertime, the flowering is inhibited by an unknown factor whereas at lower

temperatures the induction happens regardless of the photoperiod (Rantanen et al. 2015, Sønsteby 1997).

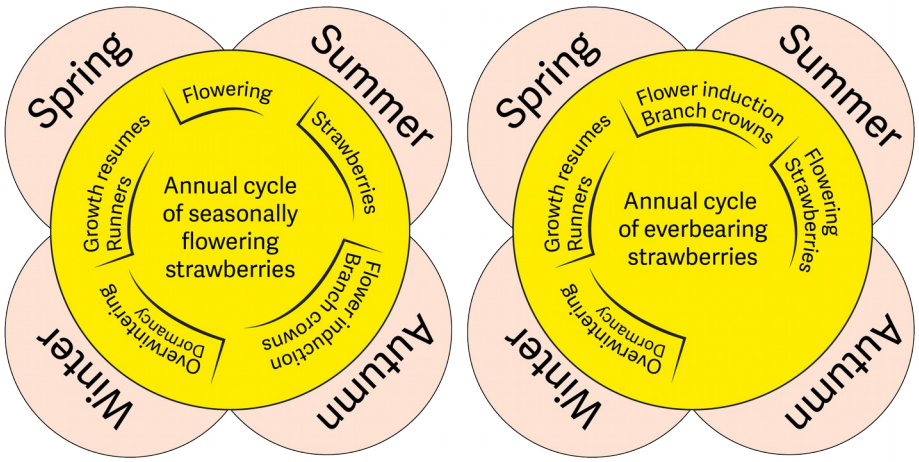

Figure 2. Main differences between the annual cycles of everbearing and seasonally flowering strawberries.

The flower induced meristem then overwinters as the plant enters dormancy after circa 4–6 weeks of short days (Guttridge 1985). However, the dormancy inducing photoperiod and temperature has been found to vary among different short day cultivars (Heide 1977, Konsin 2001). Then in the springtime, as the days get longer and temperatures higher, the plant resumes the vegetative growth and flowers subsequently (Figure 2) (Koskela & Hytönen 2018). The vegetative growth including runner formation then continues until the days shorten again and temperature decreases (Figure 2). In addition to flower induction the change in the growing conditions towards autumn causes the axillary meristems to develop branch crowns (Darrow 1966, Hytönen et al. 2004).

3.2.3 Annual growth cycle of everbearing strawberries

In addition to the seasonally flowering accessions, there are also accessions of both *F x ananassa* and *F. vesca* that display an everbearing flowering habit. Unlike the seasonally flowering cultivars, the everbearing cultivars flower more or less throughout the growing season (Figure 2). However, Perrotte et al. (2016a) have identified from three to four flowering phases that occur during the growing season of everbearing strawberries. Nonetheless the flowering season of the everbearing cultivars is substantially longer than for the seasonally flowering cultivars.

The key difference behind these distinct flowering habits of the everbearing and seasonally flowering cultivars is the flower induction. The photoperiod has been shown to have an opposite effect on flowering between the everbearing and seasonally flowering *F. vesca* accessions (Figure 2) (Mouhu et al. 2009). In addition, the flowering of the everbearing *F. vesca* cultivars is inhibited in low temperatures unlike in the seasonally flowering cultivars (Mouhu et al. 2009). Taken together the flower induction of the everbearing cultivars takes place in long day conditions thus these plants can be classified as long day plants.

The low temperature does not only inhibit the flowering but the temperature also interacts with the photoperiod regarding the everbearing *F x ananassa* cultivars. Everbearing strawberries have been shown to be obligatory long day plants at temperatures above 27 °C, quantitative long day plants in intermediate temperatures and day neutral at temperatures below 10 °C (Heide & Sønsteby 2007a). This means that at temperatures above 27 °C the flowering won't take place without long days, below 10 °C the flowering is delayed regardless of the photoperiod and at intermediate temperatures the flower bud initiation is advanced by long days.

The induction has a similar effect on the differentiation of the axillary meristems between both of the flowering types. Hence when the induction of the everbearing strawberry plant happens during the long days of summer, the plant starts to produce branch crowns (Figure 2). Since this is earlier during the growing season than in the

short day accessions, everbearing strawberries produce usually more branch crowns than the seasonally flowering strawberries. And since axillary meristem can differentiate only once, the runnering is compromised by the phenomenon. Hence the main difference between these flowering types is the flower induction from which the phenotypic differences arise from.

3.3. Genetics of flowering

Flowering responses have been reported to be similar between *F. vesca* and *F x ananassa* (Sønsteby & Heide 2008). The flowering is regulated by the photoperiodic pathway that controls the switch between the vegetetative and generative development of these species although the temperature has a strong effect on flowering as well. The research of the photoperiodic pathway has been mainly done on *F. vesca* since it is a diploid species with a relatively small genome. However the pathway has been found to be functional also on *F x ananassa* cultivars although differences have also been established.

The everbearing trait has been studied comprehensively in both *F. vesca* and *F x ananassa* and the photoperiodic pathway has been found to coincide only partly between the two species. Whereas the trait in *F. vesca* has been located to a mutation in *FvTFL1* (Koskela et al. 2012), the genes behind the trait in octoploid *F x ananassa* still remain an open question. Silencing of *FaTFL1* in *F x ananassa* leads to everbearing phenotype although the regulation of *FaTFL1* has been found to vary among cultivars (Koskela et al. 2016). In addition the expression differs from other known upstream working genes such as *FaFT1* and *FaSOC1* indicating that there are other factors affecting the expression of *FaTFL1* (Koskela et al. 2016). Cultivar dependent differences in *F x ananassa* have also been found by Nakano et al. (2015).

3.3.1 The everbearing genotype

Everbearing trait has also been mapped to different locations and the nature of the trait has also been debated, however recently a common ground has been found on

chromosome IV. Gaston et al. (2013) mapped the QTL behind the everbearing trait to one dominant locus locating to the female linkage group LGIVb-f and named it *FaPFRU*. In the same study the *FaPFRU* locus was not found to be orthologous to the loci affecting flowering and runnering in *F. vesca*. Unlike the *FaPFRU* locus, the locus behind *TFL1* in *F. vesca* locates to LGVI (Koskela et al. 2016). The same major *PFRU* locus has been reported by other groups as well (Castro et al. 2015, Perrotte et al. 2016a&b, Verma et al. 2017). Moreover, Perrotte et al. (2016a) studied the several phases occurring during the flowering of everbearing strawberries and in addition to the *FaPFRU* locus they identified a locus in LG3c controlling the phases.

Perrotte et al. (2016b) managed to narrow down the *FaPFRU* region to an area which includes 15 flowering associated genes such as *FaFT2* which is the only one of the known genes on the photoperiodic pathway of flowering located in this area. They proposed that an allelic variant of *FaFT2* would act as a positive regulator of flowering and overcome the repressive effect of *FaTFL1*. Nakano et al. (2015) instead found out that *FaFT2* seems to work downstream of *FaAP1* which is a meristem identity gene expressed already after floral induction has occurred. Indeed Koskela et al. (2012) provided evidence that the expression is highest in the flower buds of *F. vesca*. Further support for the model proposed by Perrotte et al. (2016b) has not been found.

Gaston et al. (2013) found out that the dominant *FaPFRU* locus had an opposite effect on flowering and runnering which led them to hypothesize that there is probably a common physiological control behind the vegetative and generative phases. In the same study they concluded that perpetual flowering habit could be dominant in *F x ananassa* but the trait is controlled by a recessive mutation which means that either the other homeoalleles could be similarly mutated or that one of them is mutated and the rest inactivated. Instead Castro et al. (2015) came to a conclusion that the everbearing trait could be either dominant or recessive and the simplest explanation to this could be that the trait is controlled by one locus that is strongly affected by the environment. Environment indeed has a strong effect on flowering of everbearing strawberries as has been discussed in chapter 3.2.3.

3.3.2 The photoperiodic pathway

The photoperiodic pathway has a major role in regulating vegetative and generative development (Kurokura et al. 2017) hence the information of the expression of the photoperiodic pathway genes can be used as a source of information regarding the flower induction as well. Photoperiodic pathway in *F. vesca* consists of genes such as *CONSTANS (CO), FLOWERING LOCUS T1 (FT1), SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1), TERMINAL FLOWER1 (TFL1)* and inflorescence meristem identity genes such as *APETALA1 (AP1)* and *FRUITFULL (FUL)* which are activated respectively.

F. vesca CONSTANS (FvCO) is regulated by photoperiodic signals and is an important upstream-working factor in the *F. vesca* photoperiodic pathway that is expressed in leaf tissues (Kurokura et al. 2017). It resembles a zinc finger-like transcription factor and is regulated by light in leaf tissues (Putterill et al. 1995). One of its roles is to activate the bimodal expression of *F. vesca FLOWERING LOCUS T1 (FvFT1)* but since the expression peaks between the two do not entirely match, there might be other activators of *FvFT1* as well (Kurokura et al. 2017).

FT–like proteins are floral inducing proteins which are widely conserved among plants although the mechanisms controlling them vary greatly so that the response to *FT* activation is opposite between the short and long day plants (Andrés & Coupland 2012). However, there has not been found to be a difference in the expression of *FvCO* and *FvFT1* in seasonally flowering or everbearing cultivars of *F. vesca* (Kurokura et al. 2017). *FvFT1* is expressed in the leaf tissue although the protein product is subsequently moved to apical meristem (Koskela et al. 2012).

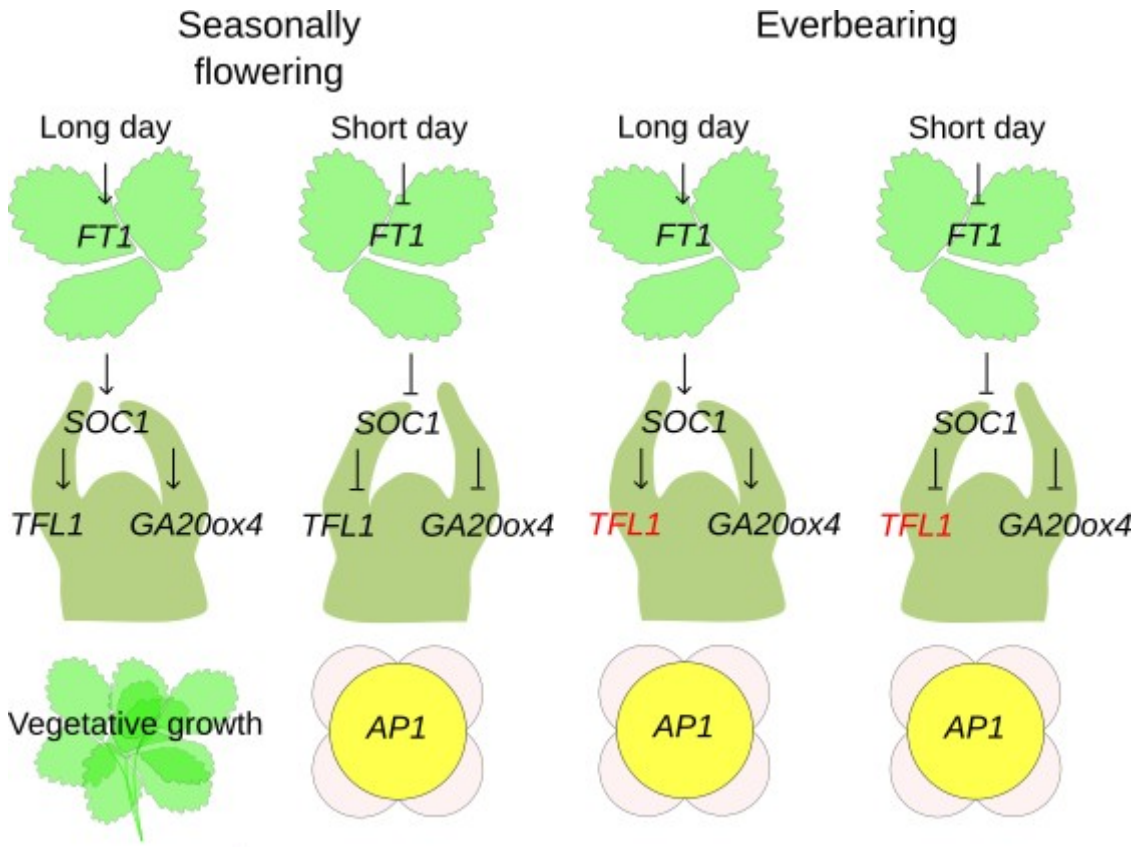

Figure 3. Overview of the photoperiodic pathway in everbearing and seasonally flowering *F. vesca* accessions. When the seasonally flowering accessions are grown under long day conditions, *FvFT1* upregulates *FvSOC1* which in turn activates the expression of floral repressor *FvTFL1* leading to inhibition of flowering. Under short day conditions the absence of FvFT1 in the apical meristem leads to repression of *FvSOC1* which then downregulates *FvTFL1*. Downregulation of *FvTFL1* subsequently leads to upregulation of *FvAP1* as the meristem commits to flowering. In everbearing accessions a frameshift mutation (in red) in *FvTFL1* leads to a non-functional protein which is incapable of inhibiting flowering and therefore floral induction can occur also in long day conditions.

Furthermore the FvFT1 mediates the photoperiodic regulation of *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (FvSOC1)* in apical meristem (Figure 3). *FvSOC1* has been found to have two roles, first of which is to activate the expression of a floral repressor *TERMINAL FLOWER 1 (TFL1)* and second of which is to mediate the differentiation of axillary buds to runners via a gene called *Gibberellin 20-oxidase4* (*FvGA20ox4)* (Figure 3) (Mouhu et al. 2013). The role of GA20-ox4 enzyme is to catalyze the synthesis of bioactive gibberellic acids (GA) which then causes the axillary

meristem differentiation to runners (Tenreira et al. 2017). *FvSOC1* and *FvTFL1* are both expressed in the shoot apical meristem when the growing conditions are not inductive for flowering (Mouhu et al. 2013).

A major gene behind the floral repression is the previously mentioned *FvTFL1* (Koskela et al. 2012). A frameshift mutation at *FvTFL1* has been shown to be behind the everbearing trait in *F. vesca* (Koskela et al. 2012). The non-functional protein caused by the mutation changes the outcome of the photoperiodic pathway which then leads to flowering in long day conditions (Figure 3) (Koskela et al. 2012). The function of *TFL1* is conserved between *F. vesca* and *F x ananassa,* hence silencing of *FvTFL1* leads to everbearing phenotype also in *F x ananassa* (Koskela et al. 2016). However the genetic control behind the perpetual flowering habit has been found to be different in *F x ananassa* as has been discussed earlier. Moreover *FaTFL1* responds also to the temperature although differences between cultivars may appear (Koskela et al. 2016).

In a nutshell the photoperiodic pathway functions as follows. *FvFT1* works by activating the *FvSOC1* under long days (Rantanen et al. 2015), whereas under short day conditions the absence of FvFT1 in the apical meristem leads to repression of *FvSOC1* which then causes the floral repressor *FvTFL1* to be downregulated. Downregulation of *FvTFL1* subsequently leads to upregulation of *FvAP1* (Mouhu et al. 2013). Upregulation of the meristem identity gene *FvAP1* causes FvAP1 to accumulate in floral meristem which makes it a good marker gene (Mouhu et al. 2013). In addition to this, downregulation of the *FvTFL1* has also been found to be linked to the upregulation of another meristem identity gene, *FvFUL1* (Koskela et al. 2012).

4. Research objectives

The aim of the project was to compare flowering and vegetative responses of in vitro propagated everbearing *F x ananassa* cultivar ‘Calypso’ and seasonally flowering

‘Hapil’ in long and short day photoperiodic conditions in order to find out differences between everbearing and seasonally flowering cultivars.

This was done by collecting data from phenotype observations linked to the vegetative and generative stages of the development of strawberries. The phenotype data was then combined with gene expression data of some of the known genes working on the photoperiodic pathway regulating the switch between the vegetative and generative development of both *F x ananassa* and *F. vesca*.

5. Materials and methods

5.1. In vitro cultivation

The plantlets of both cultivars were grown and propagated in vitro before the transfer to the greenhouses. The plantlets were grown on MS medium (Murashige and Skoog 1962) optimized for strawberry plantlets. The plantlets were transferred between shooting and rooting mediums to increase the quantity to the required 72 plantlets. The rooting medium consisted of 0.5x MS medium with 1% of sucrose and 0.7 % of agar whereas the shooting medium consisted of 1 x MS medium with 2 % of sucrose, 0.7 % of agar, 1 mg/l of BAP (6-benzylaminopurine benzyl adenine) and 0.2 mg/l of IBA (Indole-3-Butyric Acid). The plantlets were grown at room temperature with a 24-hour photoperiod. pH of the mediums was kept between 5.5–5.8.

5.2. Cultivation in the greenhouse

In vitro cultivated ‘Hapil’ and ‘Calypso’ plantlets were first taken into a greenhouse (University of Helsinki, Viikki campus) for acclimatization and pre-growing. The 72 plantlets were planted to 6 x 6 cm pots and to soil that consists of peat and sphagnum moss (Kekkilä AirBoost). The plantlets were kept under 18-hour long day conditions for five weeks. In addition to the natural irradiation, the plants were kept under 400W high pressure sodium lamps (Airam, Kerava). The temperature was kept around 20 °C. For

the first two weeks the plants were kept under additional transparent cover to keep the humidity high enough for the plantlets to acclimatize. After five weeks the plants were potted to 10 x 10 cm pots with the AirBoost soil. After the potting there were 66 plants of the cultivar ‘Hapil’and 67 of ‘Calypso’ left. Phenotypic observations were done subsequently to see if the cultivars differed in terms of phenotype. This was considered the week -1.

Half of the plants from each cultivar were moved to short day conditions on the next week (Week 0) with 12-hour photoperiod whereas the remaining plants were left to long day conditions. The temperature was kept around 20 °C. However after five weeks of the photoperiodic treatments the temperature was changed to 17°C for both of the photoperiodic treatments since the short day plants had to be moved to a different room due to practicalities. The photoperiod remained unchanged. The photoperiodic treatments lasted until week 7. Fertilization was applied to keep the electrical conductivity at around 1.5 (mS/cm x 10). The fertilizer used was Ferticare (NPK 7-4-27)(Yara, Espoo, Finland) and YaraTera Calcinit (Yara) with a ratio of 2:1. To keep the appearance of powdery mildew minimum there was occasional addition of silicon (Si 7.7%)(Kekkilä, Vantaa, Finland), given to the plants with the previously mentioned fertilizers with a ratio of 2:1:1.

5.3. Phenotypic observations

The observations started one week before the treatments began. The first observations were considered week -1 and the observations done on the day the treatments began was considered week 0. The observations were conducted every week until the flowering occurred. However the long day grown ‘Hapils’ stayed vegetative throughout the entire experiment in which case the final observations were done in week 16 (7.5.2020). The observed traits were the number of leaves, the number of branch crowns and the number of runners. The flowering dates and weeks were also recorded.

5.4. Laboratory methods

One apical meristem sample was collected from each genotype and photoperiod with three biological replicates at weeks 0, 2, 4 and 6. The samples were put to liquid nitrogen immediately after collecting and stored at -80 °C until the RNA was extracted. The total RNA was extracted using the Pine Tree method (Monte & Somerville 2002) and treated with rDNAse (Macherey-Nagel, Germany) according to manufacturer's instructions. RNA was repurified by adding 300 µl of Milli-Q water and extracted with an equal amount of chloroform:IAA (24:1). 250 µl of the aqueous solution was recovered and precipitated over night at -20 °C with 1/10 volume of 3 M NaCl, and 1000 µl of Aa EtOH. The RNA was then collected by centrifugation at +4 °C for 45 minutes at 13 000 g and then by washing the pellet with 500 µl of 70% etOH. The pellet was then dried with SpeedVac for 15 minutes (ThermoFisher Scientific, USA) and redissolved to 15 µl of Milli-Q water.

RNA concentrations were measured with Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) and diluted to 100 ng/µl with Milli-Q water. cDNA synthesis was performed using 500 ng of RNA, 2 µl of Oligoanchor (50 µM) and 1 µl of dNTP (10 mM). Milli-Q water was added to the volume of 10 µl. The mixture was heated to 65 °C for 5 minutes and incubated on ice for 1 minute. Collection of the contents was done by centrifugation. cDNA was synthetized subsequently using 1 µl of ProtoScript (II) reverse transcriptase (New England Biolabs Inc. Ipswich, MA), 4 µl of 5X Proto script buffer, 2 µl of DTT (0,1 M), 0.2 µl of RNAse inhibitor and 2.8 µl of Milli-Q water. The mix was incubated at 42 °C for 1 hour after which the reaction was terminated at 65 °C for 20 minutes. Finally the mixture was diluted to 130 µl of Milli-Q water.

The gene expression was analyzed from the samples using real time qPCR LightCycler 480 (Roche, Basel, Switzerland). The final volume of the reaction was 10 µl which included 3.5 µl of the cDNA, 5 µl of SYBR Green I Mastermix (Roche) and 1.5 µl of 3 mM primer mix (F + R). Three technical replicates were used from each sample.

FvMSI1 was used as a reference gene (Mouhu et al. 2009) against which all the expressions were calculated using relative expression equation corrected with primer efficiency as described in Pfaffl (2004). Primer efficiencies were estimated to be 2. The gene expression was analysed for *MSI1*, *FT2*, *SOC1*, *TFL1*, *GA20ox4* and *AP1*. Used primers are shown in Appendix 1.

5.5. Analysis

The phenotype data was analyzed with analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) was used as a post hoc test. The observations from week -1 were analyzed with Wilcoxon signed-rank test. Axil differentiation on weeks three and nine was analysed using logistic regression with binomial distribution. All analyses were conducted using R software (version 3.6.3, The R Foundation for Statistical Computing). The graphs were drawn using R package ggplot2 (Wickham 2016). The number of produced leaves was calculated by subtracting the number of leaves produced from the main crown on the week 0 from the number of leaves produced in total before flowering. The relative expression was calculated using LibreOffice Calc software (version 6.0.7.3, The Document Foundation, Debian and Ubuntu). Missing values were generated with Random forest method using R package missForest (Stekhoven & Buehlmann 2012).

6. Results

6.1. Phenotypic observations

6.1.1 Flowering

Each of the groups flowered at a significantly different time. On average, long day grown 'Calypsos' took only 33 days to flower whereas the short day grown 'Calypsos' flowered after 53 days (Figure 4 A). All of the 'Calypsos' in both photoperiods flowered as did all of the short day grown 'Hapils' (Table 1). Flowering of the short day grown 'Hapils' took on average 88 days (Figure 4 A). 'Hapils' did not flower during the course of the experiment (111 days) which was clearly the longest time period of the groups

(Figure 4 A). The differences between the groups and their interaction were highly significant ($p<0.001$). No flowering was observed in either of the cultivars during the in vitro cultivation.

Each of the groups produced a significantly different number of leaves before flowering which is also a sign of differential flowering times. The fastest flowerers were ‘Calypsos’ grown under long days with only four leaves produced on average before flowering whereas the short day grown ‘Calypsos’ produced on average seven leaves before flowering (Figure 4 B). ‘Hapils’ grown under short day conditions flowered after the induction had taken place in short days which was on average after producing nine leaves (Figure 4 B). ‘Hapils’ grown under long days did not flower hence the final number of produced leaves shown on the figure is the number of leaves the group produced during the experiment (Figure 4 B). The absence of flowering was also expected since the short day cultivars are obligatory regarding the photoperiod needed for flowering in intermediate temperatures. The effects of photoperiod, genotype and interaction between the cultivar and the genotype on the number of leaves were all highly significant ($p<0.001$). Strong interaction between the photoperiod and the cultivar was also expected since the plants came from short and long day cultivars.

Table 1. Means and standard deviations of the number of leaves produced from the main crown before flowering and number of days from the beginning of the treatments to flowering. The percentage of plants flowering during the experiment is also shown. Letters denote difference at $p<0.05$ level. The results were analyzed using ANOVA.

Cultivar	Photoperiod	n	Leaf number	Days to flower	Plants flowering (%)
Calypso	LD	13	4.4 (d) ± 1.3	33 (d) ± 6.1	100
	SD	13	6.6 (c) ± 1.8	53 (c) ± 13.4	100
Hapil	LD	13	> 14 (a) ± 1.3	>111 (a) ± 0.0	0
	SD	13	9.0 (b) ± 1.2	88 (b) ± 3.0	100

6.1.2 Vegetative growth characteristics

Both of the ‘Calypso’ groups produced runners regardless of the photoperiod (Figure 4 C & D). The long day grown ‘Calypsos’ produced runners steadily until the flowering, which is interesting since the differentiation of the axillary meristems into runners

usually happens before the flower induction takes place. However, after week two, the axillary meristems differentiated more into branch crowns than into runners on both of the ‘Calypso’ groups (Figure 4 C & D).

The vigorous runner production of the ‘Calypsos’ was also evident when the axillary meristem differentiation was compared on week three. ‘Hapils’s produced significantly fewer runners than ‘Calypsos’, the probability of an axil to turn into a runner in ‘Hapil’ was 48 % smaller than in ‘Calypsos’ (Figure 5 A). The difference between the cultivars regarding the differentiation into runners was highly significant ($p<0.001$) although the photoperiod did not have a significant effect on the production of runners at this stage of the growth.

When considering the relative numbers of axillary meristem differentiation of the short day grown ‘Hapils’, it can be seen that most of the axils (>75 %) remained dormant until the flower induction (Figure 4 F). There was only a minor increase in the number of runners before the flowering but basically the production of runners by short day grown ‘Hapils’ ceased in week three. Consequently it seems like ‘Calypsos’ used their axils more efficiently than the induction treated ‘Hapils’. Thus the result is a combination of dormant axils of short day grown ‘Hapil’ and a relatively larger occupancy of axils of the ‘Calypsos’.

The large fraction of dormant axils in the short day grown ‘Hapils’s was also visible on the comparison of the week three, the amount of dormant axils in ‘Hapils’ was significantly larger than in ‘Calypsos’ ($p<0.001$). (Figure 5 C). The probability of an axil to remain dormant was two times higher in ‘Hapil’ than in ‘Calypso’.

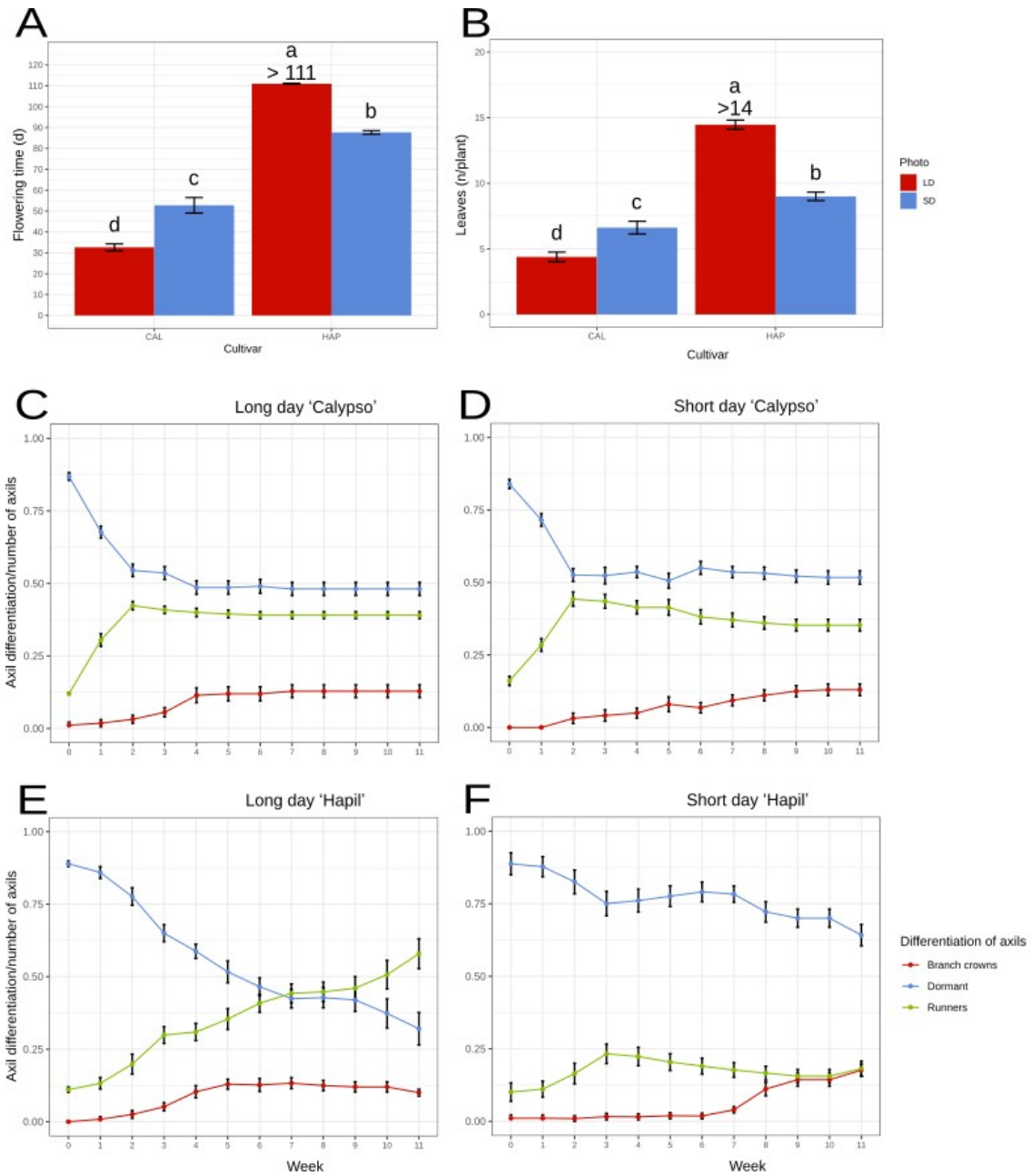

Figure 4. Days to flower (A) and the number of leaves (B) produced from the main crown before flowering. Axil differentiation of long day grown 'Calypsos' (C), short day grown 'Calypsos' (D), long day grown 'Hapils' (E) and short day grown 'Hapils' (F). Different letters in A and B denote a significant difference at $p<0.05$ level. Error bars indicate ± the standard error of mean (n=13). The results were analyzed using ANOVA.

The differentiation of the axillary buds of 'Hapils' were analysed also on week nine. Calypsos were left out of this comparison because the growth of the main crown is

terminated by the flower induction, thus the plants are not comparable after the flower induction. Interestingly the probability of an axil to remain dormant in week nine was three times higher in short day grown ‘Hapils’s than it was in long day grown ‘Hapils’ (Figure 5 F). This result confirms that most of the axils of the short day grown ‘Hapils’ remained dormant throughout the experiment.

The long day grown ‘Hapils’ remained vegetative which is why the plants produced runners steadily throughout the experiment. The steady production of runners can be seen from the relative numbers as well (Figure 4 E). Most of the axillary meristems differentiated into runners since the fraction of the dormants decreases as the number of runners increases and the fraction of the branch crowns remains stable after week five (Figure 4 E). The steady differentiation into runners was evident in week nine as well, the axils of long day grown ‘Hapils’ differentiated 22 % more likely into runners than the axils of the short day grown ‘Hapils’ (Figure 5 D).

Regarding the runner production, there was a vast difference between the cultivars already at the last week of the acclimatization period. ‘Calypsos’ had produced on average 2.5 runners before the treatments began whereas ‘Hapils’ had produced only 0.7 ($p<0.001$). Taken together, 36 % of the axillary meristems produced by ‘Calypsos’ differentiated into runners during the acclimatization period whereas the corresponding number for ‘Hapils’ was 11 %. Hence it seems like the ‘Calypsos’ were capable of producing more runners already when the growing conditions were uniform.

On the comparison of the week three axil differentiation there were no significant differences between any of the groups regarding the branch crowns (Figure 5 B & E). However, the ‘Calypso’ groups produced branch crowns from the beginning of the treatments until flowering although the curve of the branch crown differentiation on short day grown ‘Calypsos’ is less steep than on the long day grown ‘Calypsos’. Thus it can be concluded that the short day grown ‘Calypsos’ flowered later and produced branch crowns slower (Figure 4 A–D).

Axillary meristem differentiation into branch crowns on the long day grown ‘Calypsos’ was fastest between the weeks two and four which was also when the group flowered (Figure 4 A). This coincides with the runner formation of the group which slowed down at week two (Figure 4 A). Taken together the curve of the runner production and these results, it seems like the the runner production of ‘Calypso’ could have been regulated by factors outside of the photoperiodic pathway which is also why based solely on the phenotype data, it is not possible to say when were the ‘Calypso’ groups induced to flower. However, when considering the expression of *FaAP1* it seems like part of the ‘Calypsos’ were likely induced during the acclimatization period and the rest at the beginning of the treatments which will be discussed more in detail in chapter 6.3.

Branch crown production of the short day grown ‘Hapils’ started to rise at week six (Figure 4 F) which can also be seen as a sign of a prior flower induction. When considering the cessation of the runner production in week three, the likely conclusion is that the flower induction of short day grown ‘Hapils’ happened between these weeks. This observation is also backed up by the increased expression of *FaAP1*, more of which in chapter 6.3. Even though the fraction of the branch crowns started to rise after the flower induction, the vast majority of the axils still remained dormant as has been discussed earlier.

There were no significant differences in the fraction of branch crowns on the comparison of week nine (Figure 5 E). Interestingly the long day grown ‘Hapils’ produced branch crowns during the treatment period but the production ceased afterwards and subsequently there were no more observed branch crowns in this group (Figure 4 D). Considering the other indicators such as the gene expression data which did not indicate that the group would have been induced to flower and the fact that these plants did not flower, it seems odd that this flush of branch crowns happened during the treatments. Especially since there were no major fluctuations in the realized temperatures (Appendix 2).

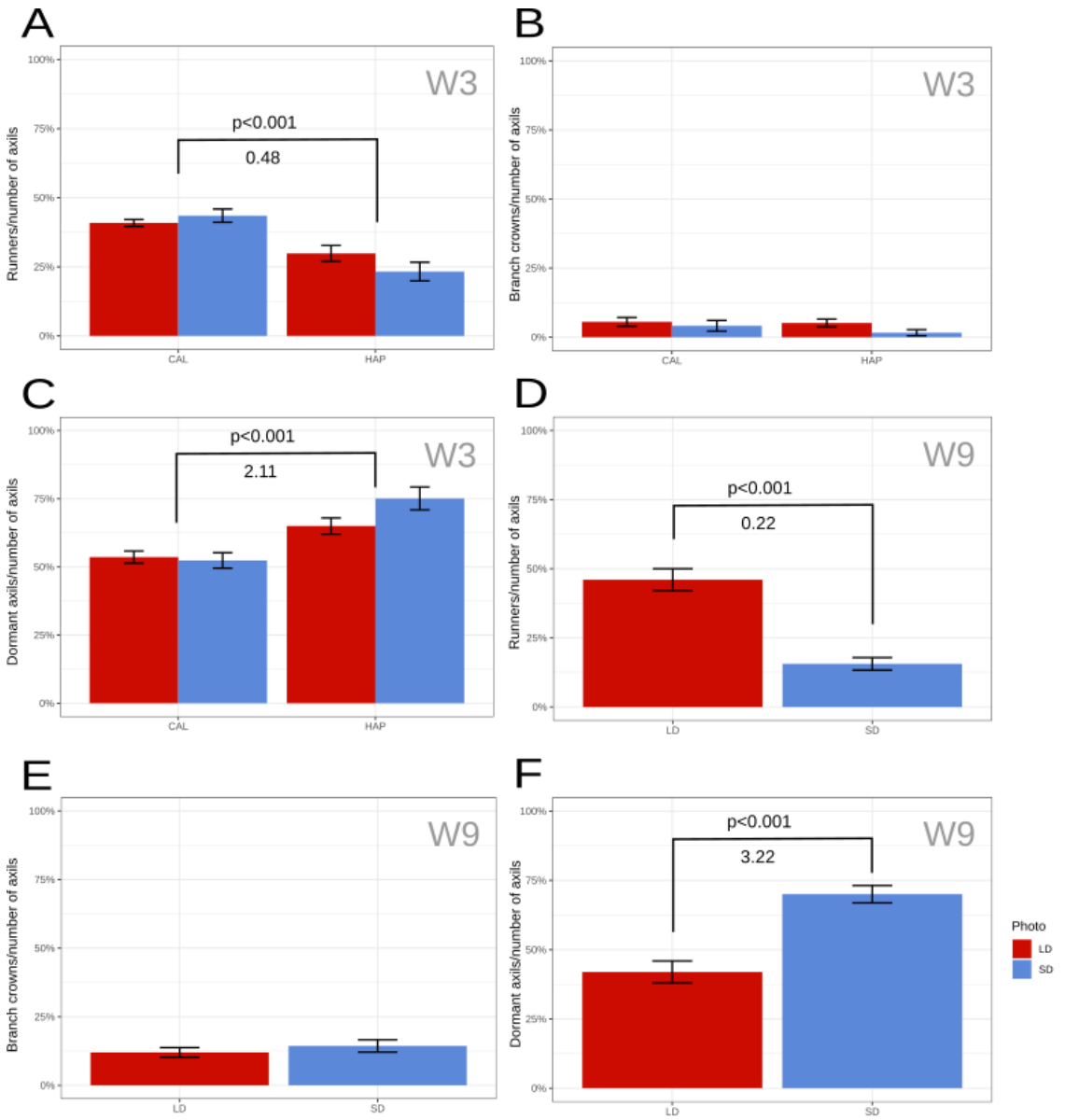

Figure 5. Axil differentiation into runners (A), branch crowns (B) and the fraction of dormant axils (C) in week three. Differentiation of the axils of Hapils into runners (D), branch crowns (E) and the fraction of dormant axils (F) in week nine. The data was analyzed using logistic regression with binomial distribution. Error bars indicate ± the standard error of mean (n=13). Numbers below the p-values indicate the odds ratios for the significant differences.

The difference between the cultivars regarding the number of produced branch crowns during the acclimatization period was not significant, however there were practically no branch crowns at this point of the experiment.

6.3. Gene expression

Since the expression of *FaAP1* is upregulated when the apical meristem commits to flowering, it can be used as a floral marker gene. Regarding the ‘Calypsos’ there was a difference in the expression pattern between the treatments (Figure 6 A). The expression was first deactivated in short day conditions after which the expression started to increase whereas the expression in the long day grown ‘Calypsos’ remained high throughout the observed period. However the deviation in the long day grown ‘Calypsos’ is high which could indicate that among the sampled plants there were induced and non-induced plants. When considering the phenotype data in the light of the expression of the *FaAP1* it seems like part of the ‘Calypso’ plants could have been induced to flower already during the acclimatization period and the rest at the very beginning of the treatment period which would have then caused the differences in the results.

The gene expression of *FaAP1* in the long day grown ‘Hapils’ remained stable throughout the experiment (Figure 6 A) which further supports the phenotypic observations regarding which the plants remained vegetative. Especially when compared to the short day grown ‘Hapils’, there was a prominent difference in the expression pattern (Figure 6 A). The expression in the short day grown ‘Hapils’ starts to increase after week two, which is only a week before the production of the runners ceased. Hence the first flower inductions probably happened already in week two and when coming to week six almost all of the plants were likely induced to flower. Week six was also the week during which the production of the branch crowns started to increase.

Expression in long day ‘Calypsos’ is much higher than in long day ‘Hapils’s which also tells about the induced nature of the former. Expression in short day ‘Calypso’ is also on a higher level already in the beginning of the experiment than in the short day ‘Hapil’ (Figure 6 A). The differences between the cultivars support the idea that the induction

could have happened for a part of the ‘Calypsos’ already during the acclimatization period.

Already in the beginning of the treatments, the *FaTFL1* expression was lower in ‘Calypso’ than in ‘Hapil’ (Figure 6 B). This also indicates that the flower induction likely happened for a part of the ‘Calypso’ plants before the treatments began since *FaTFL1* is downregulated in flower inducing conditions. In addition there was a minor difference in the expression of *FaTFL1* between the short and long day treated ‘Calypso’ groups (Figure 6 B). The expression in short days is more clearly downregulated during the treatment period whereas there is much more deviation in the long day treated ‘Calypsos’. This could indicate that the short day conditions delayed the flower inductions of the remaining non-induced plants and thus created a difference in the expression of *FaTFL1*. On the other hand, since most of the long day grown ‘Calypsos’ selected for the phenotypic observations had flowered already in week six, these expression results can deviate towards non-induced plants at the final weeks of the treatment period.

The difference in the expression of *FaTFL1* between the ‘Hapil’ groups is evident. The expression of long day ‘Hapil’ decreased only slightly during the treatment period (Figure 6 B) although the expression remained clearly higher than the expression of the other three groups which can also be considered as a sign of a vegetative state of this group. The expression of *FaTFL1* in short day grown ‘Hapils’ decreased steeply from week two on. This also coincides with the increased expression of *FaAP1* which was upregulated from week two on.

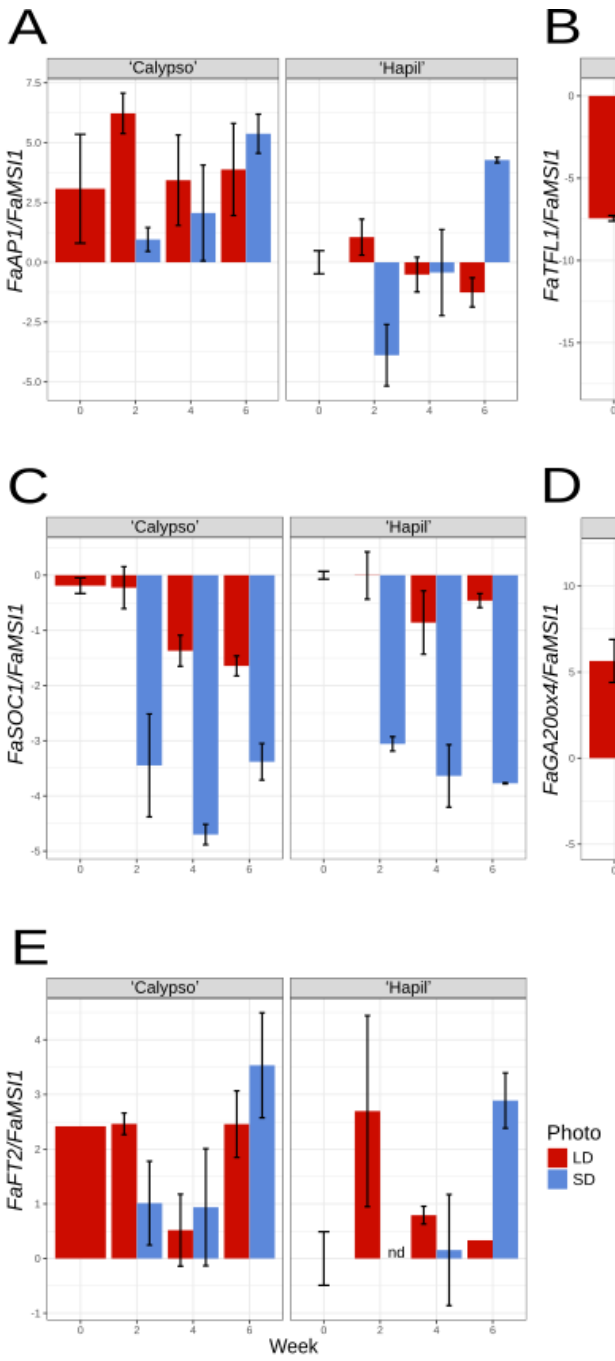

Figure 6. Expression of *FaAP1* (A), *FaTFL1* (B), *FaSOC1* (C), *FaGA20ox4* (D) and *FaFT2* (E) in relation to *FaMSI1*. Error bars indicate ± the standard error of mean (n=3).

The difference in *FaSOC1* expression was mostly between the photoperiods (Figure 6 C). *FaSOC1* was downregulated in short day conditions in both cultivars

whereas in long day conditions the expression in ‘Hapil’ remained stable throughout the experiment and in ‘Calypso’ it was only slightly downregulated after week two.

The gene expression pattern of *FaSOC1* does not entirely match with the flowering pattern (Figure 6 C & 4 A). The expression in the long day grown ‘Calypsos’ remains at a higher level than in the short day grown ‘Calypsos’ although the flowering was fastest in the long day grown ‘Calypsos’. Consequently it seems like the short days have repressed *FaSOC1* more than the long days regardless of the cultivar type.

FaSOC1 mediates the expression of *FaGA20ox4* which then causes the axillary meristem differentiation into runners although there are other factors regulating the differentiation. The expression of *FaGA20ox4* correlates with the formation of runners in both of the ‘Calypso’ groups, when the gene expression was highest the runner production was fastest (Figure 4 C–D & 6 D). *GA20ox4* is strongly deactivated on both of the photoperiods of ‘Calypso’ as was the case with *FaSOC1* as well. However as a general trend it seems like the expression of *FaGA20ox4* was higher in long days than in short days. Furthermore in week six there was no expression detected on short day ‘Calypsos’ which could indicate that by this time the expression was silenced entirely.

‘Hapil’ data however shows a lot of variation. No clear trend can be detected from the long day grown ‘Hapils’. This is possibly because the long day ‘Hapils’ remained vegetative and the expression was not clearly downregulated at any point. The trendlessness of the long day grown ‘Hapils’ is especially visible when comparing to both of the ‘Calypso’ groups (Figure 6 D). Instead the trend in short day grown ‘Hapil’ is clearer. There are missing data points already from week two on which could indicate that the expression was so low that it could not be detected. Hence this could mean that the expression in short day ‘Hapil’ was downregulated from week two on which also coincides with the cessation of the runner formation in week three. On the other hand *FaGA20ox4* is mostly expressed in axillary meristems which can cause the observed variation in the results since for this experiment, the aim was to collect apical meristem

samples. Thus, our sampling strategy was not optimal for detecting the *FaGA20ox4* transcript, causing variation among the biological replicates (Appendix 3).

The deviation in all of the groups regarding *FaFT2* was fairly large (Figure 6 E) and the expression low (Appendix 3). On short day grown ‘Calypsos’ there seems to have been activation in the expression from week four on whereas on long day grown ‘Calypsos’ and ‘Hapils’ no clear trend could be detected. However there seems to be an increasing trend on short day grown ‘Hapils’, the expression is nonexistent in week two whereas it starts to increase in upcoming weeks. However the expression levels are low all around hence it seems like the expression in the apical meristem samples was either nonexistent or then the primers did not detect the transcripts.

7. Discussion

Taken together, the data suggests that part of the plants of the cultivar ‘Calypso’ were induced already before the treatments began, after which the long day conditions promoted the flower inductions of the remaining non-induced plants. Long day conditions have also been found to promote flower bud initiation and flowering of everbearing cultivars (Sønsteby & Heide 2007a) which may have also increased the difference between the treatments when considering the time plants took to open the first flower. Sønsteby & Heide (2007b) compared the flowering responses of everbearing cultivars with similar results, long days advanced flowering at 21 °C whereas the short day grown plants flowered later.

The juvenile period of the in vitro grown strawberries was expected to be longer than that of the seed propagated (Huxley & Cartwright 1994) which is why the plants were first acclimatized in long day conditions for five weeks. However, since part of the ‘Calypsos’ were already induced at the end of the treatment period it seems like the juvenile period of ‘Calypso’ was shorter than five weeks. Having plants that are already induced has been an issue in runner propagated plants when studying the flowering of

everbearing cultivars (Sønsteby & Heide 2007b) and seems like this has been the case with these vitro propagated plants as well. Instead the ‘Hapil’ plants grown under short day conditions seem to have been induced around week six which would support the idea of a longer juvenile period.

Huxley and Cartwright (1994) observed that inductive conditions during acclimatization and in vitro cultivation periods applied for ‘Hapil’ and other short day cultivars did not induce flowering. This seems to not to have been the case with ‘Calypsos’ since part of the plants were already induced in the long days of the acclimatization period. The early flower induction could not have been prevented by growing the plants in short day conditions during the acclimatization stage since it has been shown to decrease the size and vigour of the in vitro plantlets but not to affect the number of leaves produced before flowering (Huxley & Cartwright 1994). Hence if the flower induction of ‘Calypso’ was to be caught on expression analyses and to the treatment period, lengthening of the treatment period from the beginning for one week would be beneficial. On the other hand, the differences between cultivars were nicely visible with these settings as well.

All of the ‘Calypsos’ flowered in short and long day conditions. Similar results have been obtained by Sønsteby & Heide (2007b) as well when they raised everbearing hybrid cultivar ‘Elan’ in long day conditions at 20 °C for seven weeks and then treated the plants with different temperatures in short or long day conditions. All of the plants flowered in short and long day conditions. Similar results were also obtained from this study in a sense that all of the ‘Calypso’ plants flowered in both daylengths.

For the short day cultivars such as ‘Hapil’, the critical photoperiod for floral induction is shorter when the temperature is higher but cultivar dependent differences do appear (Heide 1977, Heide et al. 2013). In this case the 20 °C and 12-hour photoperiod was enough to cause the floral induction for the short day grown ‘Hapils’. The short day grown ‘Hapils’ were induced in short day conditions between weeks three and six after

which they flowered after the treatments had ended and the plants were moved back to long day conditions.

‘Hapils’ grown under long day conditions remained vegetative throughout the experiment, which was expected since at no point it was subjected to short day nor cool conditions which is required for the flower induction to happen for a short day cultivar such as ‘Hapil’. However, the expression of *FaTFL1* was slightly downregulated on this group although no flowering was observed. Such results have been obtained before, Koskela et al. (2016) observed age-dependent reduction in the transcript level of *FaTLF1* in seasonally flowering cultivars ‘Alaska pioneer’ and ‘Honeoye’.

Strong interaction between photoperiod and temperature has been reported before regarding the flowering of both long and short day cultivars (Sønsteby & Heide 2007a, Heide & Stavang 2013) which means that both have an effect on the control of flowering. In this case the temperature was kept around 20 °C the first five weeks after which it was changed to 17 °C. At lower temperatures the flowering of ‘Calypso’ could have been delayed or even inhibited if low enough and ‘Hapils’ could have been induced regardless of the photoperiod. On the contrary, if the temperature would have been much higher, ‘Hapils’ would not have been induced at all whereas ‘Calypsos’ could have flowered only at long day conditions.

Photoperiod did not affect the runner formation of the everbearing ‘Calypso’. The runner production also continued until flowering hence it was not affected by the flower induction either. Results regarding the effect of photoperiod on runner formation have been variable between different everbearing *F x ananassa* cultivars (Bradford et al. 2010, Sønsteby & Heide 2007b). However, high temperatures have been found to increase runner development on everbearing *F x ananassa* (Bradford et al. 2010, Sønsteby & Heide 2007a). Recently it has also been found out that in *F. vesca*, temperature of 22 °C increases runner development that is likely regulated by factors independently of the photoperiodic pathway (Andrés et al. 2021). Since there are differences between different everbearing cultivars regarding the runner formation and

the high temperatures increase runner development on these cultivars, it seems possible that for Calypso, the 20 °C temperature could have been enough to cause runnering independent of the photoperiod. However more research regarding the regulation of the runner formation in different *F x ananassa* cultivars would be needed to confirm this. The need for further research around the subject has been also noted in a study by Sønsteby & Heide (2007b).

Albeit the runner production in ‘Calypso’ seemed to be independent of photoperiod, the runner production in ‘Calypso’ did correlate with the expression of *FaGA20ox4*. However, our sampling strategy was not optimal for detecting the *FaGA20ox4* transcript and therefore the utility of *FaGA20ox4* expression results are limited.

Runner production in everbearing strawberries is hindering their use in cultivation (Sønsteby & Heide 2007b) which is because the conditions that are optimal for flower induction promote the formation of branch crowns whereas the conditions that promote vegetative growth increase runnering (Hytönen et al. 2004, Kurokura et al. 2017). Having an everbearing cultivar that is capable of producing runners outside of the photoperiodic pathway in 20 °C temperature is thus a great advantage for the cultivar especially since climate change is increasing the temperatures of the growing season. For example the average temperature of Helsinki in July 2021 was 21 °C which could have then caused runner production outside of the photoperiodic pathway without compromising the flowering in long day conditions.

Most of the axillary buds in short day grown ‘Hapils’ remained dormant. Even though the runner formation is restricted in dormant plants (Sønsteby & Heide 2011) there were no other signs of dormancy in ‘Hapils’. Short day cultivars do enter dormancy at lower temperature or photoperiod than is needed for flower induction, although the dormancy requirement has been found to differ between cultivars (Heide 1977, Konsin et al. 2001). Regarding the flowering response and the growing temperature it seems unlikely that the 12-hour short day and 20 °C was enough to cause dormancy on ‘Hapil’.

Especially since the rate of produced leaves was almost identical between the ‘Hapil’ groups (data not shown) which would not have been the case with dormant plants since petiole elongation and leaf production is restricted during dormancy (Sønsteby & Heide 2011). The temperature was changed to 17 °C at week five which could have promoted the formation of branch crowns and dormant axillary buds. However, the fraction of dormant axillary buds of short day grown ‘Hapils’ was high throughout the experiment.

Andrés et al. (2021) showed recently that the conditions promoting branch crown development also promoted dormant axils in both seasonally flowering and everbearing *F. vesca* accessions. This could provide a partial explanation on why such a large fraction of the axils of short day grown ‘Hapil’ remained dormant while the axillary buds of long day grown ‘Hapils’ differentiated into runners. The large fraction of dormant axils on short day grown Hapils can not be explained by the in vitro cultivation either, since in vitro propagation has been shown to increase the branching and vegetative growth of different *F x ananassa* cultivars (Debnath et al. 2007). Auxin level has also been shown to restrict the outgrowth of axillary buds (Qiu et al. 2019) so probably the auxin level has been high in ‘Hapil’ but the reason for this is so far unknown. Other studies focusing on the dormant buds on genus *Fragaria* are scarce, hence finding out the reason behind the large fraction of dormant buds would require additional research.

In this study it was found out that the short day conditions repressed *FaSOC1* more than the long days regardless of the cultivar type and that the expression of *FaSOC1* did not correlate with the flowering pattern which could indicate that other factors contribute to the expression of *FaTFL1*. Similar results were obtained by Koskela et al. (2016) when they compared seasonally flowering ‘Elsanta’ with everbearing ‘Glima’ in short and long day photoperiodic conditions which led them to conclude that there are likely other factors than just photoperiod affecting the expression of *FaTFL1*.

Nakano et al. (2015) have suggested that *FaFT2* could work downstream of *FaAP1*. However, based on these results it is not possible to say whether *FaAP1* or *FaFT2* is first expressed in the studied cultivars. Based on these results it is neither possible to say whether *FaFT2* acts as a positive regulator overcoming the repression of *FaTFL1* as has been suggested by Perrotte et al. (2015b). The expression of *FvFT2* has been found to be highest in the flower buds of *F. vesca* (Koskela et al. 2012) and this could be the case in *F x ananassa* as well.

8. Conclusions

This project was aimed to compare the seasonally flowering *F x ananassa* cultivar ‘Hapil’ with the everbearing ‘Calypso’ to find differences in the vegetative and generative responses of everbearing and seasonally flowering cultivars. These results show that everbearing cultivar ‘Calypso’ is capable of inducing flowering at a very early age. In this case the cultivar was induced already before the photoperiodic treatments began. Since runnering and flowering are antagonistic with each other, the everbearing cultivars don’t usually produce enough runners for effective propagation. However, ‘Calypso’ was able to produce runners regardless of the photoperiod or flower induction at 20 °C. Everbearing cultivar that can produce runner regardless of the photoperiod already at intermediate temperatures and is induced at a very early age is advantageous in the rising temperatures of the future growing conditions. Flower induction-wise ‘Hapil’ behaved as would be expected from a seasonally flowering cultivar, however most of the axillary buds remained dormant to which a definite reason remains unclear. More research around the subject of everbearing strawberries will help us to find out the genes behind the everbearing trait and deepen our understanding of the vegetative and generative growth characteristics of everbearing strawberries. Improved knowledge will make it possible for us to enjoy strawberries in the future as well.

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Appendix 1. Primer sequences

Gene	Sequence from 5' to 3'
FaMSI1	(F) TCCCCACACCTTTGATTGCCA
	(R) ACACCATCAGTCTCCTGCCAAG
FaAP1	(F) AGCTCAGGAGGTTCATGACTG
	(R) TAAGGTCGAGCTGGTTCC
FaTFL1	(F) CTGGCACCACAGATGCTACA
	(R) AACGGCAGCAACAGGAAC
FaSOC1	(F) CAGGTGAGGCGGATAGAGAA
	(R) AGAGCTTTCCTCTGGGAGAGA
FaGA20ox4	(F) AGGGTGACGATGTAGCAACC
	(R) CCAGGGAAGTTTTGTGGAGA
FaFT2	(F) ACTCGGTGGCTTGTGTTTTC
	(R) ATCACTCTCCCGACGACAAG

Appendix 2. The realized temperatures of the short day rooms

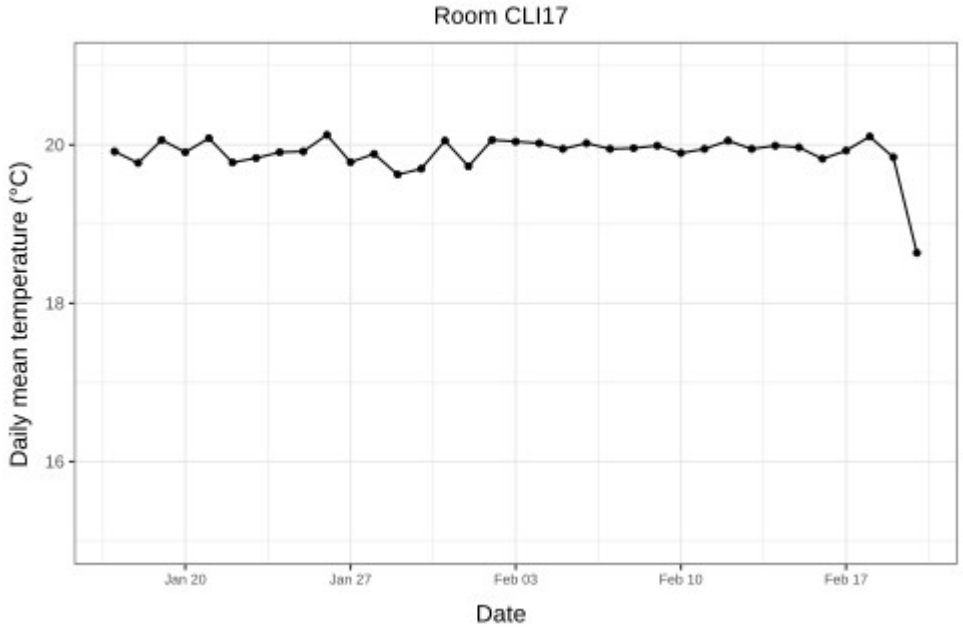

Figure 1. Daily mean temperatures (°C) of room CLI17 in which the short day grown cultivars were between 17.1.–20.2.2020.

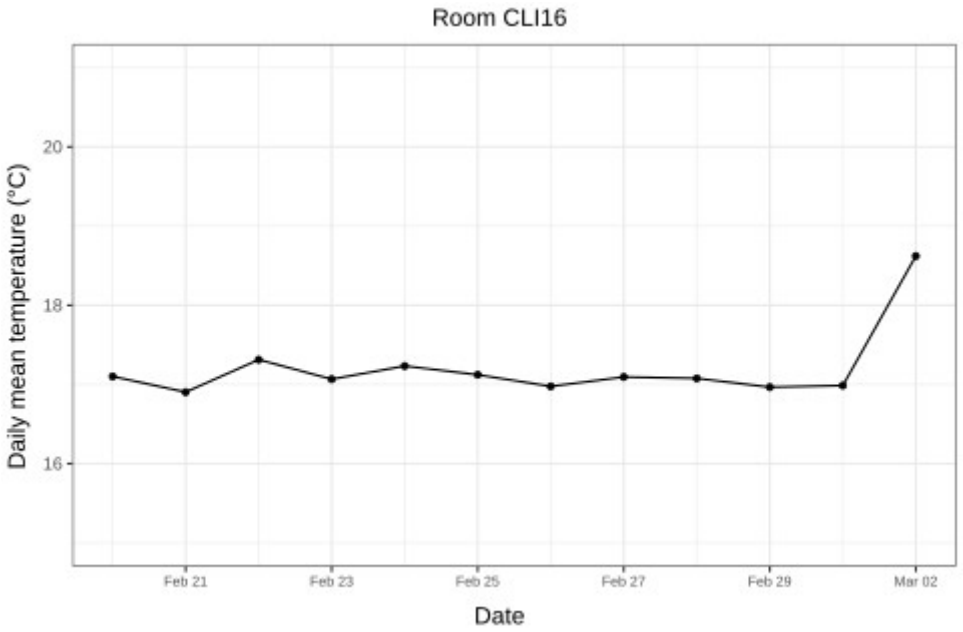

Figure 2. Daily mean temperatures (°C) of room CLI16 in which the short day grown cultivars were between 20.2.–2.3.2020.

Appendix 3. Ct values of *FaGA20ox4* and *FaFT2*

Cultivar	Photoperiod	Week	*FaGA20ox4*	*FaFT2*
Calypso	LD	0	32.82	36.27
			28.58	nd
			28.67	nd
Hapil			36.07	38.73
			36.07	38.36
			35.38	nd
Calypso	LD	2	31.63	nd
			36.94	37.55
			nd	36.15
Hapil			37.69	37.86
			nd	38.85
			25.17	nd
Calypso	SD		37.28	36.18
			38.12	38.34
			37.97	37.17
Hapil			29.48	nd
			nd	nd
			38.12	nd
Calypso	LD	4	29.89	37.20
			36.78	nd
			38.41	37.31
Hapil			nd	37.15
			29.69	37.63
			35.19	nd
Calypso	SD		37.47	34.54
			36.66	36.21
			38.20	37.88
Hapil			39.23	36.97
			nd	36.79
			39.18	38.56
Calypso	LD	6	38.08	nd
			33.61	35.06
			37.68	34.19
Hapil			38.70	nd
			36.55	nd
			30.63	37.18
Calypso	SD		nd	35.47
			nd	34.35
			nd	33.03
Hapil			nd	35.62
			38.12	34.05
			nd	34.41