Regulation of Phenology and the Juvenility-to-Maturity Transition in Trees

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Cover: *Populus tremula* at Flåsjön, Alanäs
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Abstract

The juvenile reproductive phase refers to the period when young plants are unable to respond to inductive environmental signals to induce flowering. The length of this phase varies considerably between different species. In annuals, e.g., Arabidopsis, the juvenile phase is typically very short, whereas in perennials, e.g., hybrid aspen, it is several years. Apart from juvenile and mature reproductive phases, the plant can also display juvenile and mature vegetative phases, distinguished by morphological changes in growth pattern, leaf shape, trichome distribution, etc.

miR156 is a primary regulator of the juvenile phase in Arabidopsis, whereas *TEMPRANILLO* (*TEM*) plays a more minor but still important role in regulating the length of the juvenile phase in Arabidopsis.

In the work described in this thesis, I investigated the function of the closest *Populus* homologs of both miR156 and *TEM* in hybrid aspen with respect to their involvement in regulating the juvenile vegetative phase in *Populus* as well as their effects on phenology.

The results showed that miR156 regulates the juvenile vegetative phase in *Populus* as hybrid aspen overexpressing *PttmiR156e* exhibits a severely prolonged juvenile phase. In addition, both PttmiR156e and the *TEM* homologs *PttRAV1* and *PttRAV2* affect sylleptic branching, possibly by changing the dormancy of the axillary bud. Interestingly, they also affect bud set. This indicates that similar genetic pathways are involved in the control of aging and phenology in *Populus*.

I also studied the biochemical evolution of the angiosperm *FT* lineage. My data show that *FT*-like genes are absent in gymnosperms and suggests that the FT-like function emerged at an early stage during the evolution of flowering plants as a means to regulate flowering time.

Keywords: maturity, juvenility, flowering, miR156, FLOWERING LOCUS T (FT), TERMINAL FLOWER 1 (TFL1), SQUAMOSA PROMOTER BINDING PROTEIN LIKE (SPL), RAV, TEMPRANILLO (TEM)

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Till mina två fantastiska familjer.

Men are not prisoners of fate, but only prisoners of their own minds.
Franklin D. Roosevelt

Contents

List of Publications

This thesis is based on work contained in the following papers, referred to by Roman numerals in the text:

I Maria Klintenäs, Peng Chen, Shashank Sane and Ove Nilsson. *miR156* overexpression in transgenic *Populus tremula x tremuloides* reveals novel aspects of miR156 regulation in trees. (Manuscript).

II Maria Klintenäs, Pierre A. Pin, Reyes Benlloch, Pär K. Ingvarsson and Ove Nilsson (2012). Analysis of conifer *FLOWERING LOCUS T/TERMINAL FLOWER1*-like genes provides evidence for dramatic biochemical evolution in the angiosperm *FT* lineage. *New Phytologist*, Vol 196, Issue 3, 1469-8137.

III Maria Klintenäs, Esther Marín-González, Soraya Pelaz and Ove Nilsson. The *Populus* homologs of the Arabidopsis *TEMPRANILLO* genes are regulators of bud set and early bud dormancy. (Manuscript).

The contribution of Maria Klintenäs (MK) to the papers included in this thesis was as follows:

I Maria Klintenäs, Peng Chen, Shashank Sane and Ove Nilsson. *miR156* overexpression in transgenic *Populus tremula x tremuloides* reveals novel aspects of miR156 regulation in trees.

-MK planned the work together with ON. MK performed the majority of the practical work and wrote the article together with ON.

II Maria Klintenäs, Pierre A. Pin, Reyes Benlloch, Pär K. Ingvarsson and Ove Nilsson (2012). Analysis of conifer *FLOWERING LOCUS T/TERMINAL FLOWER1*-like genes provides evidence for dramatic biochemical evolution in the angiosperm *FT* lineage.

-MK planned the work together with ON and PAP. MK performed the majority of the practical work and was responsible for writing the article together with PAP.

III Maria Klintenäs, Esther Marín-González, Soraya Pelaz and Ove Nilsson. The *Populus* homologs of the Arabidopsis *TEMPRANILLO* genes are regulators of bud set and early bud dormancy.

-MK planned the work together with ON. MK performed the practical work together with EM and was responsible for analyzing and writing of the article.

Abbreviations

Abbreviations are explained at their first mention in the main text or figure legends in addition to the list below.

35S	cauliflower mosaic virus 35S promoter
ABA	abscisic acid
AG	AGAMOUS
AGL24,42	AGAMOUS LIKE 24,42
AP1-3	APETALA1-3
ATC	ARABIDOPSIS THALIANA CENTRORADIALIS
BFT	BROTHER OF FT AND TFL1
CAL	CAULIFLOWER
CCA1	CIRCADIAN CLOCK ASSOCIATED 1
CDF1	CYCLING DOF FACTOR 1
CDS	coding sequence
CEN	CENTRORADIALIS (TFL1 ortholog)
CO	CONSTANS
Col	*Arabidopsis thaliana* Columbia ecotype
Col-0	*Arabidopsis thaliana* Columbia ecotype wild type
COP	CONSTITUTIVE PHOTOMORPHOGENIC
CRY1-2	CRYPTOCHROMES 1-2
DNA	deoxyribonucleic acid
EBS	EARLY BOLTING IN SHORT DAYS
ELF3,4	EARLY FLOWERING 3,4
fd, fe and *fwa*	flowering time mutants with unspecified name
Fig.	figure

FKF1	FLAVINBINDING KELCH REPEAT F BOX PROTEIN 1
FLC	FLOWERING LOCUS C
FLM	FLOWERING LOCUS M
FRI	FRIGIDA
FT	FLOWERING LOCUS T
ft10	Arabidopsis Col *FT* mutant
FUL	FRUITFULL
GA2,3,20OX	gibberellin 2,3,20-oxidase
GFP	green fluorescent protein
GI	GIGANTEA
Hd1	Heading date 1 (CO ortholog)
Hd3a	Heading date 3a (FT ortholog)
kDa	kilo Dalton
LFY	LEAFY
LHP1	LIKE HETEROCHROMATIN PROTEIN 1 (also known as TFL2)
LHY	LATE ELONGATED HYPOCOTYL
LKP2	LOV KELCH PROTEIN 2
LUX	LUX ARRHYTHMO
Ma	million years ago
MFT	MOTHER OF FT AND TFL1
miR	microRNA
miRNA	microRNA
mRNA	messenger RNA
nt	nucleotides
PCR	polymerase chain reaction
PDF1	PROTODERMAL FACTOR 1
PEBP	phosphatidylethanolamine-binding protein
PEP1	PERPETUAL FLOWERING 1 (FLC ortholog)
PHYA-E	PHYTOCHROME A-E
PI	PISTILLATA
PRR3,5,7,9	PSEUDO-RESPONSE REGULATOR 3,5,7,9
QPCR	quantitative PCR
RAV	RELATED TO ABI3/VP1
RFP	red fluorescent protein
RNA	ribonucleic acid

SBP	SQUAMOSA-PROMOTER BINDING PROTEIN
SFT	SINGLE FLOWER TRUSS (FT ortholog)
SMZ	SCHLAFMUTZE
SNZ	SCHNARCHZAPFEN
SOC1	SUPRESSOR OF OVEREXPRESSION OF CONSTANS 1
SPA	SUPPRESSOR OF PHYA
SPL	SQUAMOSA PROMOTER BINDING PROTEIN LIKE
SUC2	SUCROSE-PROTON SYMPORTER 2
SVP	SHORT VEGETATIVE PHASE
T89	*Populus tremula x tremuloides* male clone T89
TEM1,2	TEMPRANILLO 1,2 (also known as RAV2like and RAV2)
TFL1	TERMINAL FLOWER 1
TFL2	TERMINAL FLOWER 2 (also known as LHP1)
TOC1	TIMING OF CAB 1
TOE1-3	TARGET OF EAT 1-3
TSF	TWIN SISTER OF FT
UTR	untranslated region
VIN	VERNALIZATION INSENSITIVE
VRN	VERNALIZATION
WT	wild type
ZCN8	ZEA CENTRORADIALIS 8 (FT ortholog)
ZT	zeitgeber time
ZTL	ZEITLUPE

1 Introduction

During evolution, plants have become more and more advanced and diverse; from green algae to non-vascular land plants (e.g., mosses, liverworts), non-seed vascular plants (e.g., clubmosses), non-flowering (naked) seed plants/gymnosperms (e.g., conifers, cycads, ginkgo) and lastly, flowering (covered) seed plants/angiosperms, which now make up the vast majority of all living plant species on Earth. Although having many characteristics in common, angiosperms exist in a wide range of shapes colors and sizes. Some are annuals (germinate, flower and die within one year) some biennials (grows vegetatively during the first year, flower and set bud in the following year) and others are perennials (live for more than two years).

The perennials include both herbaceous plants, which usually have a short life span, and woody plants (including the gymnosperms) like shrubs and trees, which can live for hundreds or even thousands of years. Unlike annuals, most herbaceous and woody perennials don't flower in their first growing season. Instead, they have a long juvenile period during which they grow vegetatively to collect energy and develop an appropriate size prior to flowering.

The length of the juvenile period varies enormously. In fact, it even occurs in annuals, though it only lasts for weeks, while in perennials, it can last from a few weeks or months (as in lupins) to decades, as in some tree species (reviewed in (Bergonzi & Albani, 2011)). When plants reach maturity and are competent to flower, they need to match their flowering time to the most suitable season, as well as synchronize their flowering with other plants of the same species. The preferred flowering season depends on the plant's requirements for light and pollination assistance from specific animal species, as well as the time needed to produce rape seeds, fruits or nuts. This timing is crucial for successful reproduction, and thus a large number of endogenous and exogenous (environmental) cues are sensed and integrated by the plant prior to flowering (Fig. 1).

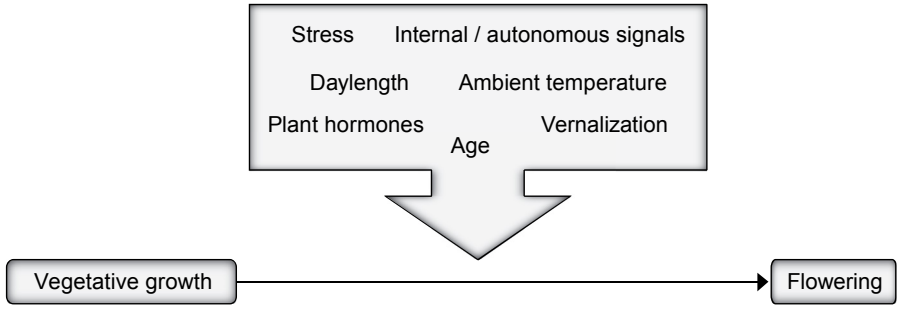

Figure 1. Information analyzed by plants prior to inducing and producing flowers. A more complete model based on the model plant *Arabidopsis thaliana* is shown in figure 4.

Perennial plants in temperate regions also need to recognize the upcoming winter in time to stop growing and acquire cold tolerance to prevent frost damage.

In the work described in this thesis, I attempted to gain a deeper understanding into the regulation of the length of the juvenile phase in woody species and the evolution of flowering with respect to the *FLOWERING LOCUS T* (*FT*) gene, which has been shown to be of general importance to flowering in Angiosperms (Paper I, II and III). In addition, Bohlenius *et al.* (2006) found that the *Populus FT* ortholog is involved in short-day-induced growth cessation and bud set in *Populus* (Section 2.11). Based on this finding, I investigated whether other genes known to be involved in flowering and maturation in Arabidopsis, could possibly have an effect on *Populus* phenology, e.g., bud set and bud burst (Paper I and III).

2 Background

Before presenting my results, I will first introduce the concepts of juvenility and maturity, bud set, bud burst and flowering, both from a general and an evolutionary perspective. Unless otherwise indicated, the previous research discussed below was performed on the herbaceous, annual, long-day plant, *Arabidopsis thaliana* (Section 2.2).

2.1 Evolution of flowering plants

About 250 million years ago, by the end of the Paleozoic era, gymnosperms had already evolved, but it took a further 120 million years before the angiosperm (flowering plants) explosion occurred (Fig. 2) (Labandeira, 2010). This explosion of species is commonly known as Darwin's abominable mystery (Friedman, 2009; Davies *et al.*, 2004; Crane *et al.*, 1995). Darwin was perplexed by the fossil records of the 1870's, showing that angiosperm diversification had been remarkably rapid; it was virtually non-existent in the early Cretaceous period, but was suddenly dominant in the late Cretaceous period (reviewed in (Friedman, 2009)). Approximately 90% or 352 000 of all living plant species on earth today are angiosperms (http://www.theplantlist.org/browse/), while there are only about 1026 living species of gymnosperms (http://www.catalogueoflife.org/). Both gymnosperms and angiosperms produce seeds, but while gymnosperms have naked seeds, angiosperms have seeds enclosed within an ovary.

Eudicots or the "true dicotyledons" make up the largest contingent of angiosperms and include the model organisms *Arabidopsis thaliana* (Section 2.2) and *Populus trichocarpa* (Section 2.11.1). The second largest group of angiosperms is the monocots, which includes many important crops like wheat, barley and rice. The remaining angiosperm lineages are fairly small in comparison (http://www.theplantlist.org/browse/).

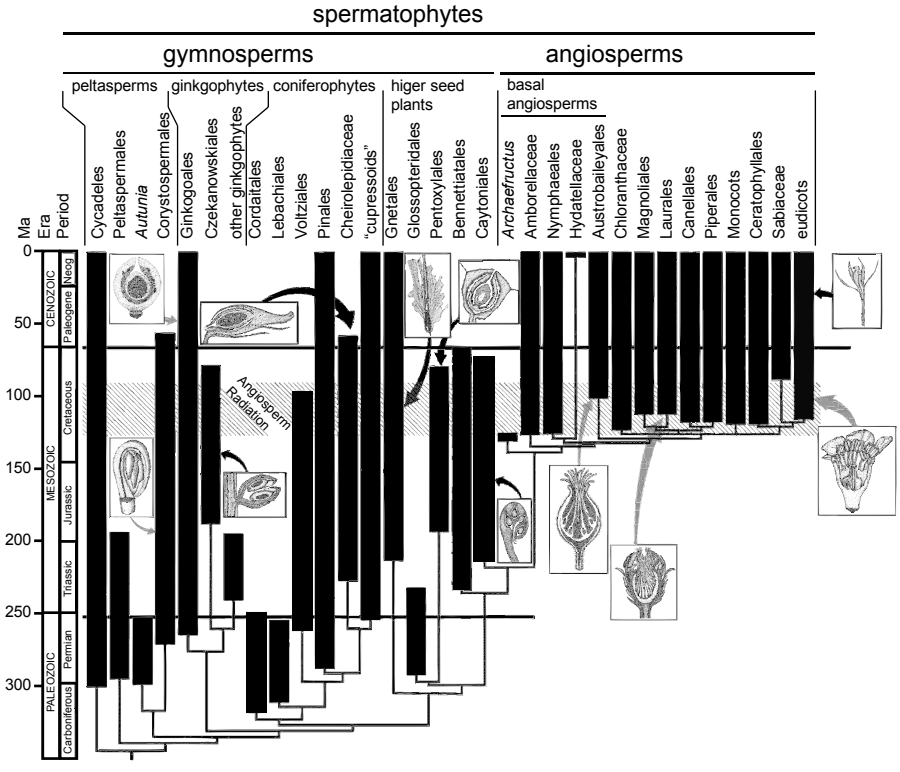

Figure 2. The evolution of seed plants (spermatophytes). Seed plants began evolving about 350 million years ago (Ma) and the gymnosperm lineages we know today, the Cycadales, Ginkgoales, Pinales (e.g. spruce and pine), cupressoids and Gnetales were already present 250 million years ago. The angiosperm lineages of today appeared much later. The huge explosion in the numbers of angiosperms occurred approximately 90-130 Ma. Examples of ovulate structures are shown next to some of the lineages of seed plants. The part of the Paleozoic Era shown in this figure ranges from about 360-250 Ma, the Mesozic Era from 250-60 Ma and the Cenozoic Era from 60 Ma until the present day. Major extinction events are marked as horizontal lines. The figure is simplified and modified from Labandeira (2010) using Gradstein *et al.* (2008) and Friedman and Floyd (2001).

The oldest known living angiosperm species is *Amborella trichopoda*, the only member of the family *Amborellaceae*. *Amborella* is a shrub or small tree that grows in the rainforest of New Caledonia located east of Australia in the Pacific Ocean. Since *Amborella* diverged near the base of the flowering plant lineage (Fig. 2) (Soltis *et al.*, 2008; Albert *et al.*, 2005), it is the closest known relative to the common gymnosperm/angiosperm ancestor, the proto-angiosperm (Chanderbali *et al.*, 2009). Another evolutionarily ancient but more well-known species is the water lily (*Nymphaeales*), which together with *Amborella*, the small aquatic family *Hydatellaceae*, and the *Austrobaileyales* (exemplified by star anise) make up the basal angiosperms (Fig. 2), a group

that is sometimes expanded to include the *Magnoliales* (Avocado, Tulip tree, etc.) as well (Chanderbali *et al.*, 2009).

2.2 *Arabidopsis thaliana*

As in many areas of plant research, flowering has been most thoroughly described in the well-established model organism *Arabidopsis thaliana* (hereafter called Arabidopsis) whose genome was completely sequenced in the year 2000 (Kaul *et al.*, 2000). Arabidopsis self-pollinate and has a very short generation time; germination to seed production only takes about 6-8 weeks. In addition, exogenous genes can be readily introduced into it using *Agrobacterium tumefaciens*.

Arabidopsis is commonly found in temperate regions all over the world. It is a small (approx. 35 cm) weed with a leaf rosette and a primary inflorescence from which develops secondary inflorescences subtended by cauline leaves. Arabidopsis exhibits indeterminate growth; its inflorescence meristem does not produce a terminal flower but generates floral meristems from its periphery (Bradley *et al.*, 1997).

Prior to flower induction, two developmental phases can be clearly distinguished from the leaf morphology: juvenile and adult. The cotyledons are initiated during the embryonic phase (reviewed in (Huijser & Schmid, 2011)). Juvenile leaves, which develop early during the juvenile vegetative phase, are small, round, have long petioles and lack trichomes on their abaxial (lower) surface, whereas adult leaves are larger, have elongated serrated blades with short petioles and trichomes on both adaxial and abaxial leaf surface (Telfer *et al.*, 1997). In addition, many other characteristics, such as leaf initiation rate (plastochron), internode length, adventitious root production and cell size, change as the plant matures (reviewed in (Huijser & Schmid, 2011)). The transition from juvenile to adult leaves is gradual.

Adult plants continue to grow vegetatively in a similar way to juvenile plants, but they have the ability to induce flowering whenever the environmental conditions become appropriate (reviewed in (Martin-Trillo & Martinez-Zapater, 2002)). Flowering time in Arabidopsis is commonly measured by counting the total number of rosette leaves as well as cauline leaves formed on the main inflorescence. Northern populations flower later than southern and some northern populations even require a vernalization period prior to flowering (Section 2.11.5). Flowering time is also affected by external factors, such as day length, water and nutrient availability (Marin *et al.*, 2011; Kolar & Senkova, 2008), temperature (Balasubramanian *et al.*, 2006; Blazquez *et al.*, 2003) and other stress factors.

2.3 Photoperiodic dependence - the circadian clock

As discussed in the introduction, it is vitally important that plants are able to recognize seasons and coordinate developmental processes with them. Temperature, water and nutrient availability vary from year to year, but day length at a given date and growth location does not. This fact is used by plants, not only to synchronize photosynthesis to the light period of the day (reviewed in McClung and Kay (1994)), but also to coordinate flowering based on the individual species' requirements, and, in the case of perennials in temperate regions, acquire cold tolerance prior to exposure to freezing conditions (Sections 2.11.3 and 2.11.4).

In plants that in contrast to Arabidopsis do not self-pollinate, the ability to synchronize flowering is essential. Plants have different preferences as to the most suitable time to flower; some need to coordinate it to the insect or bird pollinators they depend on, others prefer to flower in the spring to minimize competition for light with other plants or so that they have all summer to collect energy to invest in their fruits, nuts or berries. Still others prefer to grow vegetatively during the summer and flower in the fall when the day length starts to shorten.

Plants exhibit three major variants of day-length-dependent flowering: short- and long-day-induced flowering or day neutral, i.e., plants are insensitive to day length (reviewed in Kobayashi and Weigel (2007)). Some plants require a dual combination of day lengths, i.e., short/long day or long/short day, where the former possibly involves down-regulation of a repressor and the latter causes up-regulation of a flower promoter (Zeevaart, 2008; Heide, 2004). The terms short day and long day are relative, and thus a "short day" for one species might constitute a "long day" for another.

As mentioned above, Arabidopsis is a long-day plant, to be more specific it is a quantitative long-day plant. That means that Arabidopsis flowering does not depend on a specific critical day length, but the speed of flower induction increases with the length of day. 16 hours of light is commonly regarded as a long day for *Arabidopsis thaliana*. Flowering occurs even under continuous short-day conditions, but it takes longer and is regulated by a different mechanism than under long days (Section 2.8).

Day-length-dependent flowering can sometimes cause problems when plants are transferred to new latitudes. For example, barley, natively grown in the Middle East, was originally a long-day-induced plant. This meant its growth was very inefficient in northern Europe since it flowered too early in the season to acquire any feasible size, resulting in very low yields. Luckily, a point mutation in the gene corresponding to Arabidopsis *PSEUDO-RESPONSE REGULATOR 7* (*PRR7*) (Fig. 3) gave rise to a photoperiod-

insensitive variant that could be cultivated in northern Europe (Turner *et al.*, 2005).

In order to respond to variations in photoperiod (daily patterns of light and dark), plants have developed an internal timing mechanism, i.e., circadian clock (Fig. 3), which comprises interlocking feedback loops with a core oscillator that receives input signals and generates output responses (Harmer, 2009; Mas & Yanovsky, 2009; McClung, 2006).

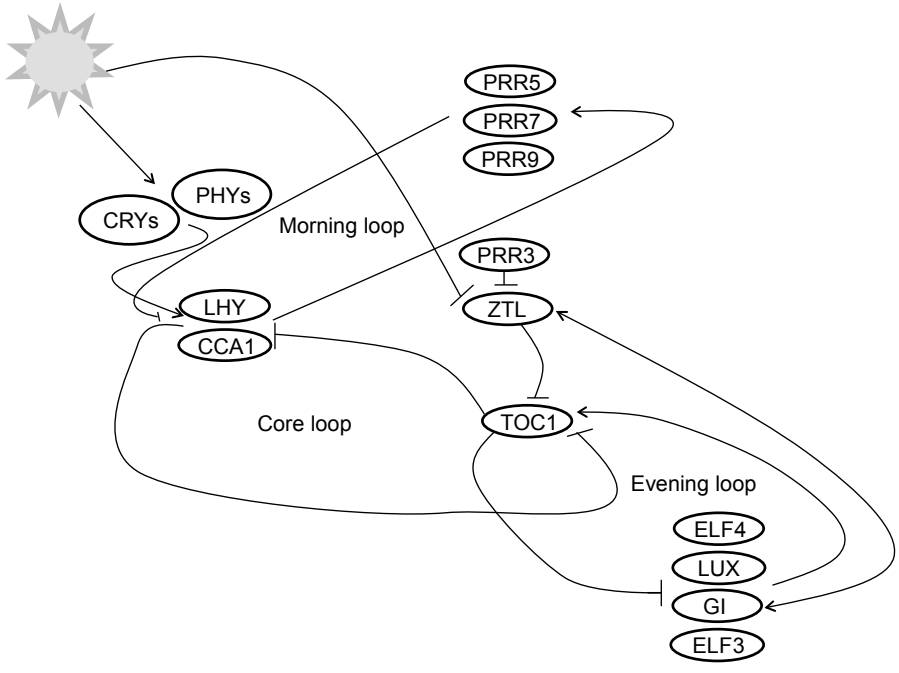

Figure 3. Simplified model of the circadian clock in Arabidopsis. The circadian clock is made up of interlocking feedback loops. Lines with arrowheads shows direct or indirect positive regulation, while lines with perpendicular lines show direct or indirect negative regulation. The core loop is made up of CIRCADIAN CLOCK ASSOCIATED 1 (CCA1), LATE ELONGATED HYPOCOTYL (LHY) and TIMING OF CAB 1 (TOC1). *CCA1* and *LHY* levels peak at dawn. CAA1 and LHY bind to the *TOC1* promoter and repress expression of *TOC1*. In the morning loop, CCA1 and LHY activate expression of the *TOC1* paralogs *PRR5*, *7* and *9*, which in turn repress *CCA1* and *LHY* expression. In the evening loop, GIGANTEA (GI), EARLY FLOWERING (ELF)3, ELF4 and LUX ARRHYTHMO (LUX) promote *TOC1* expression, while TOC1 represses *GI*, *ELF3*, *ELF4* and *LUX* expression. TOC1 acts as a general repressor of oscillator gene expression (Gendron *et al.*, 2012; Huang *et al.*, 2012). *TOC1* expression peaks in the evening/dusk and prevents activation of morning-expressed genes *CCA1* and *LHY* at night. ZEITLUPE (ZTL) inhibits TOC1 by proteosomal degradation and its activity is regulated by GI and PRR3. The photoreceptors perceive light and entrain the circadian clock. I have made no distinction between genes and proteins in this figure. (Reviewed in (Harmer, 2009; Mas & Yanovsky, 2009; McClung, 2006; Yanovsky & Kay, 2003).) For a more detailed overview of the clock see Nagel and Kay (2012).

The clock "ticks" with approximately the same period (24 h) in the absence of environmental cues. To be able to function as expected throughout the year and respond to seasonal changes, the clock needs to be daily entrained with the ambient light and temperature conditions under which the plant is growing (reviewed in (Harmer, 2009; Jiao *et al.*, 2007; Salome & McClung, 2005)). The input to the clock comes from photoreceptors, which are abundant in Arabidopsis; PHYTOCHROMES (PHYA-PHYE) that recognize red and far red light, CRYPTOCHROMES (CRY1-CRY2), PHOTOTROPINS (phot1-phot2) and the ZTL/LOV KELCH PROTEIN 2 (LKP2)/FLAVIN BINDING KELCH REPEAT F-BOX 1 (FKF1) family that recognize ultraviolet A and blue light, as well as unidentified ultraviolet B photoreceptors (reviewed in Yanovsky and Kay (2003) and Jiao *et al.* (2007)). The output is delivered in the form of oscillating gene expression with a diurnal or circadian rhythm.

Diurnal rhythms that have approximately the same period between peaks even if the plant is put under continuous light or dark conditions are classed as being circadian. The circadian clock keeps approximately the same rhythm in the absence of environmental cues; the rhythm is not created by the inputs of temperature, light or dark, but is an inherent characteristic of the negative and positive feedback loops within the clock itself (reviewed in McClung (2006)).

Oscillating expression of the gene *CONSTANS* (*CO*), which is expressed in the phloem companion cells of leaves (An *et al.*, 2004) and for which the corresponding protein is only stable in light, is regulated by the circadian clock and works as an important mediator between the clock and the flowering mechanism (Section 2.5) (Suarez-Lopez *et al.*, 2001).

There seem to be many similarities, but also some major differences in the function of the circadian clock in trees; these are discussed in Section 2.11.

2.4 Competence to flower / reaching reproductive maturity

The juvenile phase following germination, as well as the adult vegetative phase following the *vegetative phase transition* or as it is also called the juvenile-to-adult phase transition, is a time for the plant to increase its size, mass and photosynthetic capacity (reviewed in (Huijser & Schmid, 2011)). The plants are not able to respond to inductive environmental conditions and express flower meristem identity genes, e.g., *APETALA1* (*AP1*) and *CAULIFLOWER* (*CAL*) (Figs. 1 and 4), until they have passed through a second transition, namely the *reproductive phase transition* (reviewed in (Bergonzi & Albani, 2011; Huijser & Schmid, 2011; Boss *et al.*, 2004; Martin-Trillo & Martinez-Zapater, 2002). An enormous amount of information is analyzed and integrated in the decision to allow flowering. This includes internal signals concerning

age, as well as growth conditions, with temperature and day length being the most important (Figs. 1 and 4).

MicroRNAs (miRNAs) are short (20-24 nt) non-coding RNAs (Reinhart *et al.*, 2002) that regulate genes post-transcriptionally by complementary binding to their target mRNA, resulting in target degradation or gene silencing (Fig. 8) (reviewed in (Bartel, 2004)). miR156 (Section 2.10) and miR172 are two important miRNAs involved in the juvenility-to-maturity transition. miR156 is highly expressed during juvenility and its expression decreases in adult plants, whereas miR172 exhibits the opposite trend. This is the case in Arabidopsis (Wu *et al.*, 2009) as well as several tree species, such as *Acacia confusa*, *Acacia colei*, *Eucalyptus globulus*, *Hedera helix*, *Quercus acutissima* and *Populus deltoides x nigra* (Wang *et al.*, 2011a). Arabidopsis miR156 targets the majority of the *SQUAMOSA PROMOTER BINDING PROTEIN LIKE* (*SPL*) family members (Section 2.10.2) (*SPL2*, *SPL3*, *SPL4*, *SPL5*, *SPL6*, *SPL9*, *SPL10*, *SPL11*, *SPL13A*, *SPL13B* and *SPL15*) (Wu *et al.*, 2009; Schwab *et al.*, 2005; Rhoades *et al.*, 2002b). (In *Antirrhinum* the *AP1* (Fig. 4) homolog is called *SQUAMOSA*.)

Since several *SPL* genes induce expression of flowering-promoting genes, such as *LEAFY* (*LFY*), *AP1*, *CAL* and *FRUITFULL* (*FUL*) (Fig. 4), high miR156 levels retains the plant in a juvenile or vegetative state, incompetent to respond to flower induction, whereas down-regulation of miR156 with age allows maturation and subsequent flowering to occur. Thus the *SPL* genes and their post-transcriptional regulation by miR156 incorporate endogenous aging signals into the flowering system (Wang *et al.*, 2009b; Yamaguchi *et al.*, 2009).

miR172 acts downstream of and is negatively regulated by miR156 (through the action of SPL9) (Fig. 4) (Wu *et al.*, 2009). The up-regulation of miR172 with age promotes adult epidermal phenotypes in Arabidopsis by targeting six APETALA2 (AP2)-like transcription factors, (*AP2*, *TARGET OF EAT* (*TOE*)*1*, *TOE2*, *TOE3*, *SCHLAFMUTZE* (*SMZ*) and *SCHNARCHZAPFEN* (*SNZ*)), each encoding proteins with two AP2 DNA-binding domains (Wu *et al.*, 2009; Schmid *et al.*, 2003). miR172 seems to monitor the developmental age rather than the chronological age because it increases more slowly in short-day-grown plants than in long-day grown plants (Jung *et al.*, 2007). The targets of miR172 regulate both the transition to flowering and the flower development itself (Fig. 4). Both miR156 and miR172 are positively regulated by their targets, possibly contributing to the stability of the juvenile and adult phases in Arabidopsis (Wu *et al.*, 2009).

FT, which is a powerful flower inducer, has also been found to be up-regulated with age (Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999). The

chromatin status influences the accessibility of transcription factors (like CO) to the FT promoter (Adrian *et al.*, 2010). *FT* expression is transcriptionally repressed by chromatin re-modeling through TERMINAL FLOWER 2 (TFL2) (also known as LIKE HETEROCHROMATIN PROTEIN 1 (LHP1)) (Kotake *et al.*, 2003), EARLY BOLTING IN SHORT DAYS (EBS) (Pineiro *et al.*, 2003; Gomez-Mena *et al.*, 2001) and others (Adrian *et al.*, 2010; Yang *et al.*, 2010) as well as being directly repressed by TEMPRANILLO 1 (TEM1) (Castillejo & Pelaz, 2008) and at least one of the previously mentioned miR172-targeted AP2-like proteins, namely SMZ (Mathieu *et al.*, 2009). The increase in *FT* levels with age could be a result of decreasing levels of any of its above mentioned repressors, or decreasing levels of miR156 (Wu *et al.*, 2009) (Paper I) resulting in increasing levels of the SPL3 protein, which has been suggested to bind directly to the *FT* promoter, activating transcription of *FT* (Kim *et al.*, 2012), or any combination of the above-mentioned factors.

A recent study has shown that even a modest change in growth temperature affects flowering time. Arabidopsis flowers later when grown at 16 ºC than at 23 ºC, and this effect is even stronger in *miR156*-overexpressing plants (Kim *et al.*, 2012). It is not just flowering time that is affected by lower ambient temperature but also leaf phenotype (Section 2.2), indicating that lower temperatures keep the plant juvenile for an extended period of time (Kim *et al.*, 2012). *ft* mutants were also found to flower later at lower ambient temperatures (Blazquez *et al.*, 2003), and thus the temperature effect on flowering, or possibly maturation, cannot be solely due to the FT integrator (Blazquez *et al.*, 2003).

Figure 4 shows that approximately five of the six flower-inducing pathways present in Arabidopsis, i.e., the photoperiodic, the autonomous, the warm ambient temperature, the vernalization and at least parts of the age-dependent pathways, all converge on FT, being a flower integrator (Section 2.6). It is still unclear to what extent the gibberellin pathway acts through FT (Section 2.8).

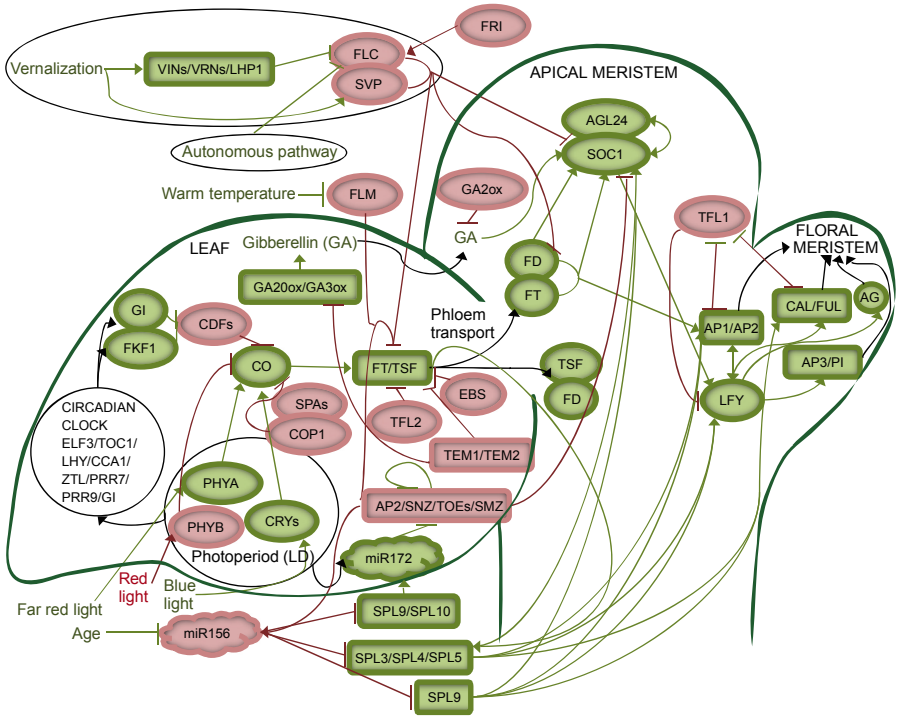

Figure 4. An overview of the gene regulatory network involved in the decision to flower consisting of at least six pathways; photoperiod, vernalization, warm ambient temperature, gibberellin, age dependent and autonomous (involving RNA modification and chromatin remodeling). Direct or indirect positive regulation is indicated by arrows and direct or indirect negative regulation by perpendicular lines. Genes, proteins or actions ultimately causing floral activation are marked green and those leading to floral repression are marked pink. Signaling starts in the leaf where the timing of flowering is decided based on external conditions, such as photoperiod, light quality, ambient temperature and vernalization, as well as internal conditions, such as age and gibberellin levels. Far red and blue light, increased age and growth temperature are programmed to promote flowering, whereas red light, low age and growth temperature prevent flowering and promote vegetative growth. All this information is registered through different pathways and integrated to give an FT signal that passes through the phloem to the meristem (described in detail in Section 2.6). Under favorable flowering conditions, the gene flow in the meristem first of all activates SUPRESSOR OF CONSTANS OVEREXPRESSION 1 (SOC1) and eventually comes to the meristem identity genes, which include LFY and the MADS-box genes AP1, CAL and FUL. SOC1 and FT can bind directly to the promoters of *SPL3*, *SPL4* and *SPL5* to induce flowering under inductive conditions and SOC1 performs the same function under non-inductive short-day conditions (Section 2.8). The meristem identity genes initiate a phase change of the meristem from vegetative to reproductive or floral. Subsequently, floral development takes place through flower organ identity genes, e.g., AP1, AP2 (dual function, in the leaf AP2 acts as a floral inhibitor, whereas in the floral primordial, it is one of the ABC genes (Section 2.7)), APETALA3 (AP3), PISTILLATA (PI), among others. Under short-day conditions, flowering is dependent on the gibberellin and possibly temperature pathways. In this figure, I have made no distinction between gene and protein function. (Jung *et al.*, 2012; Osnato *et al.*, 2012; Srikanth & Schmid, 2011; Amasino, 2010; Fornara *et al.*, 2010; Wellmer & Riechmann,

2010; Mathieu *et al.*, 2009; Wang *et al.*, 2009b; Yamaguchi *et al.*, 2009; Castillejo & Pelaz, 2008; Blazquez, 2000; Samach *et al.*, 2000; Wilson *et al.*, 1992)

Ultimately, the maturation process in Arabidopsis enables the meristem to respond to inductive growth conditions and change from a vegetative to a reproductive stage. Arabidopsis (ecotype Landsberg erecta) can respond to long days only four days after germination, but the sensitivity to induction increases with age, indicating that the process of maturation is gradual (Mozley & Thomas, 1995). As in Arabidopsis (reviewed in (Baurle & Dean, 2006)), certain environmental conditions can effectively shorten the juvenile period in some woody species like birch, camellia and azalea (reviewed in (Bergonzi & Albani, 2011)), pine, blackcurrant, willow and oak (reviewed in (Wareing, 1956)). In addition, gibberellins which actually inhibit flowering in many trees like cherry, peach, apricot, almond and lemon, is frequently used to stimulate flowering in conifers (reviewed in (Brunner & Nilsson, 2004)).

To complicate matters further, a few species, e.g., *Eucalyptus risdonii*, flowers during the juvenile vegetative phase. Thus, the plant exhibits juvenile as opposed to adult characteristics when flowering occurs (reviewed in (Poethig, 2010)). There are still quite a few loose ends to unravel on the subject of maturation in Arabidopsis, and even more so in trees, which I have tried to address in Papers I and III.

2.5 Flower induction - external coincidence model

Once the Arabidopsis plant reaches adulthood (Section 2.4) and recognizes that it grows under inductive long-day conditions (Section 2.3), it induces flowering. As discussed in Section 2.3, *CO*, which encodes a B-box-type zinc finger transcription factor (Putterill *et al.*, 1995), is an important mediator between the circadian clock and the flowering mechanism. GI, which is under control of the circadian clock, peaks in the afternoon and forms a complex together with FKF1, which peaks at the same time as *GI* (internal coincidence) under long-day conditions (Sawa *et al.*, 2007). The GI-FKF1 complex degrades an important *CO* repressor, CYCLING DOF FACTOR 1 (CDF1) (Fig. 4), and thus *CO* gene expression peaks in the late afternoon (Sawa *et al.*, 2008) as an indirect result of light through the action of the circadian clock. CO is also directly affected by light via a range of photoreceptors through post-translational modifications of the CO protein. The CO protein is unstable in dark or red light but stable during daytime or in blue light (Valverde *et al.*, 2004).

The combined effects on *CO* expression and CO protein stability ultimately leads to active CO protein being present only under long-day conditions when

the expression peak of *CO* mRNA coincides with factors that stop the protein being degraded (reviewed in Valverde (2011)). This is known as the external coincidence model (Fig. 5) and was first proposed in the 1930's by Erwin Bünning (reviewed in Saunders (2005)), although the details were reported much later (Valverde *et al.*, 2004; Yanovsky & Kay, 2002). Arabidopsis needs the CO protein to exceed a certain threshold value to induce flowering through the photoperiod pathway (Fig. 4), and this is only achieved during long days (Fig. 5) (reviewed in (Imaizumi & Kay, 2006)).

CO promotes flowering by activating expression of *FT*, a small transcriptional cofactor (reviewed in (Kobayashi & Weigel, 2007)) found in the vascular tissue of leaves (Wigge *et al.*, 2005; An *et al.*, 2004; Samach *et al.*, 2000). Both *CO* and *FT* are expressed in the phloem companion cells of leaves (Yamaguchi *et al.*, 2005; An *et al.*, 2004; Takada & Goto, 2003). FT is then transported to the apical meristem (Section 2.6) (Corbesier *et al.*, 2007; Jaeger & Wigge, 2007; Mathieu *et al.*, 2007; Tamaki *et al.*, 2007) where it interacts with the transcription factor FD (Section 2.7).

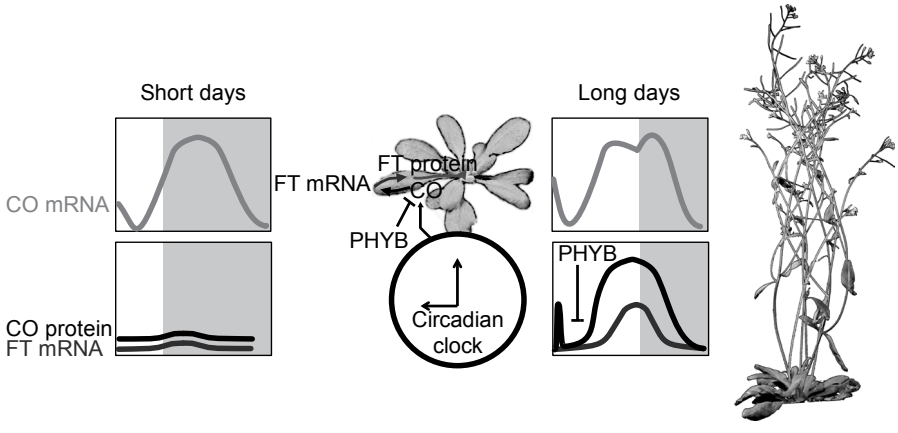

Figure 5. CO mRNA (light grey) is regulated by the circadian clock through GI and FKF1, resulting in highest expression in the late afternoon under long days. Light stabilizes CO protein (black) through the photoreceptors CRY1, CRY2, FKF and PHYA. In the dark, the CO protein is degraded, (possibly dark-dependent ubiquitin ligases target CO for degradation). In the morning, PHYB causes proteosomal degradation of the CO protein, preventing it from inducing expression of *FT* (dark grey). *FT* is only induced when the endogenously clock-controlled *CO* expression-peak coincides with the external light signal that stabilizes the CO protein, hence the name external coincidence model. The FT protein and the *FT* mRNA can then move through the phloem to the apex to induce flowering (Fig. 4, Section 2.6) The figure is modified from Imaizumi and Kay (2006) incorporating additional information from (Valverde, 2011; Jarillo *et al.*, 2008; Corbesier *et al.*, 2007; Jaeger & Wigge, 2007; Valverde *et al.*, 2004; Yanovsky & Kay, 2002)).

Once flowering has been induced under long-day conditions, this process is irreversible. Even if the plant is moved back to non-inductive short-day conditions and *FT* expression in the leaves ceases, the plant will still continue to flower (Corbesier *et al.*, 2007).

In plants that flower during short days, e.g., rice, the mechanism is similar to that in Arabidopsis, but instead of flowering as a response to high CO protein levels, the rice CO homolog Heading-date1 (Hd1) inhibits flowering under long days and promotes flowering under short days when Hd1 peaks in the absence of PHYs (reviewed in Hayama and Coupland (2004) and Jarillo *et al.* (2008)).

Zea CENTRORADIALIS 8 (ZCN8) has been suggested to be the most likely FT ortholog in the day-neutral plant maize (Danilevskaya *et al.*, 2008). ZNC8 expressed under a phloem companion-specific promoter could completely rescue the *ft* mutant in Arabidopsis, indicating that ZNC8 could function as florigen in maize (Lazakis *et al.*, 2011). Exactly how flowering is regulated in day neutral plants is not well characterized, but it is plausible that FT has an important function just like in Arabidopsis.

2.6 The flower former - florigen

Since the mid-1930's, it has been known that the flower induction signal is produced in the leaves and transported to the apex. Knott (1934) showed that the long-day plant spinach flowered if the leaves were placed under inductive long-day conditions and the shoot tips under non-inductive short-day conditions, but not the other way around, suggesting that this was caused by a mobile substance produced in the leaves.

Chailakhyan (1937) showed that in several plant species, a graft-transmittable signal he called florigen (and believed to be a hormone) was produced in the leaves under inductive conditions and transported to induce flowering in both long- and short-day-induced plants. Florigen, which means flower former, was even found to be graft transmittable between different species, indicating that the substance must be very evolutionarily conserved (Chailakhyan, 1937).

During the following decades, a great deal of effort was devoted to finding the identity of the florigen but without success (reviewed in (Zeevaart, 1976)). The florigen was thought to be transported in vascular tissue (King & Zeevaart, 1973), but since it was able to induce flowering in graft combinations between different families with no apparent functional vascular connections, (reviewed in (Zeevaart, 1976)), albeit taking a longer time, it was also suggested to be transferable from cell to cell in the absence of phloem tissue (Zeevaart, 1976).

In 2005, it became clear that the FT protein had a very important function in the shoot apex to induce flowering (Abe *et al.*, 2005; Wigge *et al.*, 2005), far from the leaf where it was expressed (Yamaguchi *et al.*, 2005; An *et al.*, 2004; Takada & Goto, 2003). It wasn't until 2007, exactly 70 years after the florigen term was coined (Chailakhyan, 1937) that the FT protein, in both Arabidopsis and rice, was shown to function as a mobile flowering signal (Corbesier *et al.*, 2007; Tamaki *et al.*, 2007).

Pinpointing FT as being the long sought florigen naturally put a lot of focus on the gene. Recently, it has been shown that FT from a wide range of species, including apple, maize, gentian, grape vine, lettuce, barrel clover, orchid, Japanese morning glory, poplar, potato, rice, sugar beet, sunflower and squash causes early flowering if overexpressed in Arabidopsis (Danilevskaya *et al.*, 2011; Fukuda *et al.*, 2011; Hsu *et al.*, 2011; Imamura *et al.*, 2011; Laurie *et al.*, 2011; Lazakis *et al.*, 2011; Meng *et al.*, 2011; Navarro *et al.*, 2011; Blackman *et al.*, 2010; Kotoda *et al.*, 2010; Pin *et al.*, 2010; Traenkner *et al.*, 2010; Hou & Yang, 2009; Jang *et al.*, 2009; Komiya *et al.*, 2009; Carmona *et al.*, 2007; Hayama *et al.*, 2007; Lin *et al.*, 2007; Bohlenius *et al.*, 2006; Hsu *et al.*, 2006; Sreekantan & Thomas, 2006; Izawa *et al.*, 2002; Kojima *et al.*, 2002). This proves that the FT function is extremely conserved between species ((Pin & Nilsson, 2012), Paper II), which is another important criteria of the florigen. In addition, data indicating that the *FT* mRNA might also have a function in the florigen signal have been reported (Lu *et al.*, 2012; Li *et al.*, 2011; Li *et al.*, 2009).

Thus, the present situation is that whereas there is no doubt that FT works as a florigen (reviewed in (Pin & Nilsson, 2012)), it is less clear whether this function is only performed by the protein or if the *FT* mRNA, and possibly some transporter proteins, are involved as well. Since FT has a prominent and central role in this thesis, I will provide a thorough review of this subject (Sections 2.6.1-2.6.4).

2.6.1 Florigen movement in the vascular system

The xylem, phloem, their meristem tissues (the vascular cambium) and some supporting cells make up the vascular bundles or vascular tissue in Arabidopsis and other vascular plants. The xylem transports water and minerals, whereas the phloem transports sugars and other organic nutrients dissolved in water, to different parts of the plant.

The sieve elements of the phloem are responsible for the conduction of the florigen; they are connected end-to-end, making up a system of tubes throughout the plant. Each sieve element is connected to a companion cell, which takes care of its cellular functions. The companion cells are connected to

the sieve tubes by plasmodesmata (vanBel, 1996). The vascular tissue does not extend all the way to the shoot apical meristem in Arabidopsis, but there are several cell layers separating the two. Closest to the apex is a region of rapid growth known as the primary phloem or protophloem, which is made up of thin-walled sieve cells that often lack companion cells and usually only function for short periods before being eradicated.

Since the florigen, assuming it is FT, is foremost expressed in the companion cells of phloem (Yamaguchi *et al.*, 2005; An *et al.*, 2004; Takada & Goto, 2003) but the active protein is found in the shoot apical meristem (Section 2.7) (Abe *et al.*, 2005; Wigge *et al.*, 2005), it must be transported through the plasmodesmata of the companion cells into the sieve elements, further transported in the sieve tubes through the phloem and protophloem, out of the protophloem and into the apical meristem, and lastly, through the lower cell layers of the apical meristem, until it reaches the upper part where FD is expressed (Section 2.7) (Abe *et al.*, 2005; Wigge *et al.*, 2005), possibly by cell-to-cell movement. Exactly how far from the protophloem into the apex the florigen needs to reach to be able to interact with FD is unclear. Wigge *et al.* (2005) localized FD expression to the very top of the shoot apex, especially in the leaf and floral anlagen, by means of *in situ* hybridization. The same analysis performed by Abe *et al.* (2005) showed FD expression in pretty much the whole apex.

2.6.2 FT protein movement

FT is a small globular protein of 175 amino acids or 20 kDa consisting almost entirely of a phosphatidylethanolamine-binding protein (PEBP) domain (Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999) belonging to a family of closely related genes that will be thoroughly described in Section 2.9. *FT* is primarily expressed in the phloem companion cells of the distal parts of source leaves (Yamaguchi *et al.*, 2005; An *et al.*, 2004; Takada & Goto, 2003) of plants grown under long-day conditions.

Stadler *et al.* (2005) have shown that proteins smaller than 67 kDa are non-specifically loaded through the plasmodesmata of the companion cells to the sieve elements of the phloem and then transported through the phloem by diffusion in Arabidopsis. However, they did not observe the same non-specific unloading out of the phloem. Free green florescent protein (GFP) (27 kDa) and small GFP-fusion proteins expressed in the companion cells were found to be transported through the phloem and were also detected in the adjoining cell layer, but not beyond, suggesting that the latter cells control which macromolecules are unloaded from the phloem (Stadler *et al.*, 2005). Thus, it is

likely that the FT protein, which is a very small soluble cytoplasmic protein, can enter the sieve elements and move through the phloem by diffusion.

When the phloem companion cell specific SUCROSE TRANSPORTER 2 (SUC2) (Stadler & Sauer, 1996; Truernit & Sauer, 1995) as well as the endogenous FT promoters were used to express *FT-GFP* in an *ft* mutant background, both caused early flowering in Arabidopsis (Corbesier *et al.*, 2007). *In situ* hybridization and confocal microscopy, (Corbesier *et al.*, 2007) showed that *SUC2::FT-GFP* mRNA was only detectable in mature phloem where the SUC2 promoter is active, whereas FT-GFP protein was detected in the protophloem and at the base of the shoot apex. They also performed grafting experiments, which revealed that the FT-GFP protein, but not *FT-GFP* mRNA, crossed the graft union. Despite the fact that neither *FT-GFP* mRNA nor protein was found in the upper part of the shoot apex in either experiment (graft or SUC2), flowering was induced in both, implying that FT in some form must have been transported there. Corbesier *et al.* (2007) concluded that the FT protein is the florigen signal since it was the detectable FT signal closest to the area of *FD*-expression.

Hd3a is the FT ortholog in rice (Kojima *et al.*, 2002). It causes early flowering if overexpressed and exhibits phloem- and xylem-specific expression (Tamaki *et al.*, 2007). Using confocal laser scanning microscopy, the Hd3a-GFP fusion protein was found to move from the leaf phloem and xylem parenchymal cells through the vascular bundles in the stem, ending up just below the meristem inside the shoot apical meristem (Tamaki *et al.*, 2007). Tamaki *et al.* (2007) suggested that the Hd3a protein is transported from the vascular bundles, through the basal cells and into the shoot apical meristem aided by intercellular transport proteins, and that the Hd3a protein could act as the rice florigen.

The conclusions reported in Tamaki *et al.* (2007) and Corbesier *et al.* (2007) are based on small FT/Hd3a-GFP fusion proteins. Considering that Stadler *et al.* (2005) have shown that free GFP as well as small GFP-fusion proteins can move from the companion cells into the sieve elements and through the phloem in Arabidopsis and that Tamaki *et al.* (2007) have pointed out that free GFP protein diffuses in many tissues in rice, it seems difficult to distinguish whether the FT/Hd3a-GFP fusion proteins move as a consequence of being fused to the GFP protein or not.

Therefore, it was originally not clear whether the movement of FT/Hd3a-GFP fusion protein observed by Tamaki *et al.* (2007) and Corbesier *et al.* (2007) would occur with the native FT protein. However, native FT protein has since been detected by mass spectrometry in the phloem of a non-induced scion after grafting it onto an induced stock, and the grafting resulted in

flowering in *Curcurbita* (Lin *et al.*, 2007). Likewise, Giavalisco *et al.* (2006) detected native FT-like proteins in the phloem of *Brassica napus* inflorescence stems, and members of the FT-, TFL1- and MFT-subfamilies have been detected in the phloem sap of rice (Aki *et al.*, 2008). Thus, these studies suggest that native FT proteins are able to travel through the phloem, as implied by Tamaki *et al.* (2007) and Corbesier *et al.* (2007). In agreement with this, Western blotting has revealed that phloem-specific expression of *Myc-FT* gives rise to Myc-FT protein beyond the phloem into areas involved in flower initiation (Jaeger & Wigge, 2007). Finally, flower induction was found to be severely delayed if movement of the FT protein was inhibited by introducing large C-terminal fusions and/or nuclear localization, further indicating that the FT protein is the florigen (Jaeger & Wigge, 2007; Mathieu *et al.*, 2007). Neither Jaeger and Wigge (2007) or Mathieu *et al.* (2007) examined whether the engineered *FT* mRNA movability was affected in addition to the protein movability.

2.6.3 *FT* mRNA movement

In wild type Arabidopsis, *FT* transcripts as well as FT protein are in low abundance (Giakountis & Coupland, 2008; Corbesier *et al.*, 2007; Wigge *et al.*, 2005; An *et al.*, 2004; Kotake *et al.*, 2003), suggesting that just a small amount of expression is sufficient to induce flowering (Kotake *et al.*, 2003). The failure to demonstrate the presence of either *FT* mRNA or FT protein might thus not necessarily be taken as proof of its absence, nor of its inability to induce flowering. This can be exemplified by the inability of *in situ* hybridization studies (An *et al.*, 2004) to visualize *FT* mRNA in wild-type leaf vasculature from long-day-grown Arabidopsis, where it most certainly is expressed (Yamaguchi *et al.*, 2005; Takada & Goto, 2003).

Neither Tamaki *et al.* (2007) nor Corbesier *et al.* (2007) ruled out the possibility that *FT* mRNA could function as a florigen signal (Li *et al.*, 2009). In fact, the Arabidopsis *FT* mRNA coding sequence, nucleotides 1-102, forms a domain shown to be responsible for RNA mobility (Li *et al.*, 2009). In addition, hundreds of RNA transcripts (Deeken *et al.*, 2008; Kehr & Buhtz, 2008; Lough & Lucas, 2006; Haywood *et al.*, 2005; Kim *et al.*, 2001) have been found to travel through the phloem in a wide range of plants, acting as long distance signaling molecules. It was recently discovered that the transcription factor WEREWOLF (WER) could indirectly stabilize *FT* mRNA, and thus extend the life span of the *FT* transcript (Seo *et al.*, 2011).

FT mRNA has been detected in many organs where, according to studies of its promoter, it is not expressed. By analyzing GUS expression under the *FT* promoter, it was demonstrated that *FT* is expressed in the vascular tissues of

cotyledons and the vascular tissues of the apical, but not basal or main veins of leaves from mature seedlings, as well as in the vascular tissues of inflorescence stems, pedicels and floral organs (Yamaguchi *et al.*, 2005; Takada & Goto, 2003). Furthermore, the *FT* promoter was found to be inactive in the leaf primordia, shoot apical meristems, inflorescence meristem, hypocotyls and roots (Takada & Goto, 2003).

Although the highest levels of *FT* mRNA have been found in the phloem companion cells in the distal parts of the oldest leaves in plants grown under long-day conditions (Wigge *et al.*, 2005; Yamaguchi *et al.*, 2005; Takada & Goto, 2003), *FT* has in fact been found in a variety of tissues in Arabidopsis, e.g., shoot apex, hypocotyls, cotyledons, roots, floral buds, flowers, immature siliques, mature siliques, stems, rosette leaves and bracts (Kobayashi *et al.*, 1999). *FT* mRNA has been shown to be present in proximal and distal (but not petiole) parts of leaves of different ages by microarray studies, as well as in the apex and leaves of Arabidopsis grown under both long- and short-day conditions using quantitative PCR (QPCR) (Wigge *et al.*, 2005). Whereas the microarray analysis was too insensitive to find *FT* mRNA in the apex, the QPCR results showed a threefold increase of *FT* mRNA in the apex and an approximately 70-fold increase in leaves of plants grown under inductive long-day conditions compared to plants grown under short-day conditions (Wigge *et al.*, 2005). In agreement with this, low levels of *Hd3a* mRNA were detected in the shoot apical meristem of rice by QPCR, in spite of the fact that the Hd3a promoter is not active in rice shoot apical meristems (Tamaki *et al.*, 2007).

The finding that *FT* mRNA is present in the apex (Wigge *et al.*, 2005; Kobayashi *et al.*, 1999) clash with results showing that the *FT* promoter is inactive in the shoot apical meristem (Yamaguchi *et al.*, 2005; Takada & Goto, 2003). If *FT* mRNA is not expressed in the apex, but still found there, it must almost certainly be transported there. Recently, several studies have shown that *FT* mRNA is able to move inside plants of different species. Jaeger and Wigge (2007) used *in situ* hybridization to show that Myc-tagged *FT* mRNA expressed in the phloem companion cells can be transported through the plasmodesmata, but it was not found in the shoot apex. Li *et al.* (2009) reported that the Arabidopsis *FT* mRNA can move over long distances inside both Arabidopsis and *Nicotiana benthamiana* plants and later demonstrated that it can move into the shoot apical meristem of *Nicotiana* plants (Li *et al.*, 2011). They showed that Arabidopsis *FT-GUS* mRNA (but not *GUS* mRNA) allowed the PVX virus to overcome the viral meristem exclusion mechanism and move into the shoot apical meristem of *Nicotiana benthamiana* independently of the presence of the FT protein.

The fact that the FT protein clearly interacts with FD in the apex (Abe *et al.*, 2005; Wigge *et al.*, 2005) suggests that *FT* mRNA would need to be translated in the shoot apical meristem to affect flowering. Translation of *FT* mRNA in the shoot apical meristem has been demonstrated by Wigge *et al.* (2005); they expressed *FT* under the shoot apical meristem specific FD promoter, causing early flowering. In addition Abe *et al.* (2005) showed that *FD::FT* as well as PROTODERMAL FACTOR 1 *(PDF1)::FT*, which only expresses *FT* in the outermost cell layer of the shoot apical meristem, could complement the *ft* mutant phenotype.

However, even non-translated *FT* mRNA seems to have a function in flowering as non-translatable *FT-GUS* mRNA has been shown to slightly promote flowering in *Nicotiana tabacum*, under inductive short-day conditions, although the plants did not flower as early as when they were provided with translatable *FT-GUS* mRNA (Li *et al.*, 2011). This suggests that *FT* mRNA may play a role in flowering, even if it is not translated into protein. In contrast to translatable *FT* mRNA, non-translatable *FT* mRNA does <u>not</u> induce flowering under non-inductive conditions, but only speeds up the flowering process under inductive conditions (Li *et al.*, 2011). Somewhat in agreement with the slight flowering promotion function of *FT* mRNA suggested by Li *et al.* (2011) are the results of Jaeger and Wigge (2007), who showed that expression of nuclear-tagged *FT* in an *ft* mutant background grown under inductive conditions flowers slightly earlier than *ft* even though the protein is presumably unable to move to the site of floral induction. Li *et al.* (2011) speculated that *FT* mRNA might function as a protein transporter to facilitate efficient transport of the FT protein to the shoot apical meristem.

Very recently, advanced grafting methods and RED FLORESCENCE PROTEIN (RFP) fusions have revealed that *SUC2::RFP-FT* can induce early flowering even though the RFP-FT protein is unable to move from the phloem cells (Lu *et al.*, 2012). *ft10* scions grafted onto *SUC2::RFP-FT* stocks flowered considerably earlier than *ft10*, proving that *FT* mRNA is sufficient to promote flowering (Lu *et al.*, 2012). In addition, Lu *et al.* (2012) have shown that endogenous *FT* mRNA can travel through the plant.

2.6.4 Summary concerning FT as a florigen signal

Assuming that both the FT protein and *FT* mRNA can move from the site of expression in the leaves to the site of *FD* expression in the apex and that *FT* mRNA is translated into FT protein and interacts with FD, as the above findings suggest, raises the question, which would be the most important florigen - FT protein or *FT* mRNA?

Two articles have shown that apex-expressed artificial miRNA against *FT* mRNA did not affect flowering time in wild-type, suggesting that *FT* mRNA in the apex is *not* necessary for flowering (Lu *et al.*, 2012; Mathieu *et al.*, 2007). However, artificial miRNA delayed flowering in *FT* mRNA overexpressing (*SUC2::RFP-FT*) plants by several days (Lu *et al.*, 2012).

In contrast, an artificial miRNA targeting *FT* mRNA and expressed under a global (*35S*) or phloem companion cell specific (*SUC2*) promoter, which theoretically exclude both FT protein and *FT* mRNA in the shoot apical meristem, severely delayed flowering (Mathieu *et al.*, 2007).

Thus, the available data suggests that even if *FT* mRNA functions as a florigen redundantly with the FT protein, its effect is not as strong as the FT protein.

Further, the fact that *FT* mRNA does not seem to be required in the shoot apical meristem (Lu *et al.*, 2012; Mathieu *et al.*, 2007) does not in any way contradict the hypothesis that *FT* mRNA could have a function in facilitating effective export of the FT protein to the shoot apical meristem (Li *et al.*, 2011).

2.7 FT-FD interactions and the ABC model

The shoot apical meristem contains stem cells that divide to form leaf primordia, stem tissue and new meristematic cells. Once the FT protein is present in the apex, it interacts with the bZIP transcription factor FD (Abe *et al.*, 2005; Wigge *et al.*, 2005). FD is only expressed in the shoot apical meristem. Its expression is induced under long-day conditions (Wigge *et al.*, 2005), but it is not affected by CO activity, nor is it clock regulated. However, it does increase with time after germination (Abe *et al.*, 2005). Together, the FT-FD complex/heterodimer, is recruited into the nucleus (Abe *et al.*, 2005) where it coordinates the transition to flowering by binding to the promoters of the MADS-box genes *SOC1* (reviewed in Valverde (2011)) and *AP1* (Abe *et al.*, 2005; Wigge *et al.*, 2005). (MADS-box genes encode a large family of transcription factors that contain the highly conserved DNA-binding MADS domain.) These changes induce the reproductive phase, flower initiation and subsequent flower development (Fig. 6) (reviewed in Valverde (2011)).

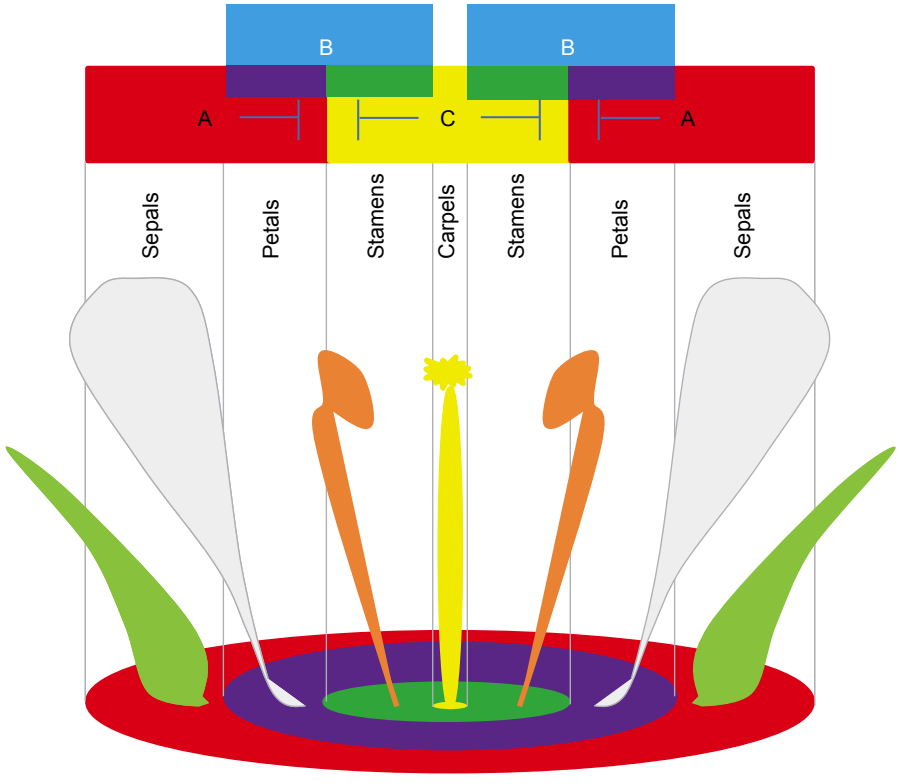

Figure 6. The classical ABC model showing how the "A"- "B"- and "C" genes specify floral organ identity. The organization of the sepals, petals, stamens and carpels are shown as distinct color-coded whorls; red, purple, green and yellow respectively. The "A" function is probably specific to Arabidopsis, whereas the "B"- and "C" gene functions are supposedly conserved in most eudicot species (Causier *et al.*, 2010; Rijpkema *et al.*, 2010) but need a bit of adjustment (not explained here) to apply to monocots (Johansen *et al.*, 2006) and the basal angiosperms (Soltis *et al.*, 2007).

When flowering is induced, the apical meristem stops producing leaf primordia and instead produces an inflorescence meristem producing flower primordia. The flowers consist of sepals, petals, stamens and carpels. The genes involved in producing the different parts of the flower (floral organ identity genes) have since the 1990's been referred to as the ABC genes (Fig. 6) (Weigel & Meyerowitz, 1994; Coen & Meyerowitz, 1991). Classically, the "A" function is performed by AP1 and AP2, the "B" function by AP3 and PI and the "C" function by AGAMOUS (AG) (reviewed in (Causier *et al.*, 2010; Rijpkema *et al.*, 2010)).

The "B-" and "C-" genes are exclusively MADS-box transcription factors that have both individual and combined roles in floral organ identity establishment (Causier *et al.*, 2010). It has been suggested that the "A"

function in the ABC model should be redefined from an organ identity function to a role in establishing floral meristem identity as well as enabling and restricting the functions of "B" and "C" (reviewed in (Causier *et al.*, 2010; Rijpkema *et al.*, 2010)). It is thought that most of the ABC genes exist in gymnosperms as well, and that reprogramming of these in an ancestral gymnosperm species could have produced the first perfect flower (Melzer *et al.*, 2010; Theissen & Melzer, 2007). More recently, a fourth class of genes involved in all four whorls has been added to the ABC model. These are known as the E-class function genes and consist of *SEPALLATA* (*SEP*)*1*, *SEP2*, *SEP3* and *SEP4* ((Ditta *et al.*, 2004) as reviewed in (Huijser & Schmid, 2011; Tan & Swain, 2006).

2.8 Flowering under short days

As previously mentioned, flowering in Arabidopsis occurs even in the absence of inductive long days, but it takes a considerably longer time. In addition, short-day-grown plants produce leaves with abaxial trichomes at later internodes (Telfer *et al.*, 1997), indicating that short days keep Arabidopsis plants juvenile for a longer period of time *and* size. Exactly how short-day-induced flowering is regulated in Arabidopsis is not well understood, but gibberellins play a very important role (Wilson *et al.*, 1992).

The pathways involved in flowering under inductive long days and non-inductive short days operate separately in Arabidopsis; some mutations strongly affect flowering under short days but only have a slight effect on flowering time under long days *ga* (Wilson *et al.*, 1992) and vice versa *co*, *gi, cry2, fd, ft, fe and fwa* ((Koornneef *et al.*, 1991; Redei, 1962), as reviewed in (Turck *et al.*, 2008))). Flowering in Arabidopsis, ecotype Columbia, occurs approximately 45 leaves later if the plants are grown under non-inductive short-day conditions in comparison to inductive long-day conditions (Jang *et al.*, 2009). This is considerably later than the difference in flowering time of Columbia (Col) wild type and *ft10* grown under long-day conditions, which only differ by about 25 leaves (Yoo *et al.*, 2005). Thus, even though the *ft10* mutant does not flower significanlty later than the wild type under short days (Jang *et al.*, 2009), it is clear that it is not just *FT* expression that differs between long and short-day-induced flowering.

GA promotes *FT* expression under long-day conditions (Hisamatsu & King, 2008) and *ga* mutants flower slightly later than wild type under inductive conditions (Wilson *et al.*, 1992). Thus gibberellins have a clear effect but are not essential for flowering in plants growing under long days (Hisamatsu & King, 2008; Wilson *et al.*, 1992). However, the fact that strong gibberellic acid

deficient mutants are unable to flower under short-day conditions, indicates that the gibberellin pathway (Fig. 4) is required for flowering under non-inductive conditions (Wilson *et al.*, 1992). In addition gibberellins appear to have an age-related function in Arabidopsis as *ga* mutants form abaxial trichomes later than wild type, whereas applied GA speeds up the formation of abaxial trichomes (reviewed in (Bergonzi & Albani, 2011; Huijser & Schmid, 2011)).

The *LFY* promoter is regulated by GA (Blazquez & Weigel, 2000) and GA has been shown to induce flowering through the induction of *SOC1* and *LFY*, but not *FT*, if applied to Arabidopsis plants grown under short-day conditions (Hisamatsu & King, 2008; Moon *et al.*, 2003; Blazquez & Weigel, 2000). GAs induction of *SOC1* precedes SOC1s direct binding to and subsequent induction of *SPL3*, *SPL4* and *SPL5* (Jung *et al.*, 2012). Increasing SPL activity eventually causes the plant to flower without photoperiod-dependent FT activity (reviewed in (Huijser & Schmid, 2011)). SOC1 in association with the transcription factor AGAMOUS LIKE 24 (AGL24) can also bind to and induce expression of *LFY* under both long- and short-day conditions (Lee *et al.*, 2008; Liu *et al.*, 2008).

GI overexpression (35S) or expression specifically in mesophyll or vascular tissue under short-day conditions has recently been found to increase *FT* but not *CO* levels, thus allowing photoperiodic and CO independent transcriptional activation of *FT* (Sawa & Kay, 2011). However, since *gi* and *ft* mutants only have a slight effect on flowering under short days (Jang *et al.*, 2009; Koornneef *et al.*, 1991), *GI* is unlikely to play a major role in short-day-induced flowering. The *FT* repressor *TEM1* has been found to directly repress *GA3OX1* and *GA3OX2,* thus decreasing the levels of bioactive GA (Osnato *et al.*, 2012). *TEM1* peaks in the early night under both long- and short-day conditions, but the highest expression levels occur under short days and declines gradually with age (Osnato *et al.*, 2012; Castillejo & Pelaz, 2008). This could lead to higher levels of GA with increased age, eventually inducing flowering under short days, but this hypothesis has not been confirmed. The effect of the *tem1tem2* mutant has not yet been examined in the *ft* mutant background under short days, and thus it is not possible to uncouple the effect on GA from the possibility that the absence of TEM in the *tem1tem2* mutant could cause *FT* up-regulation under short-day conditions.

There is still a lot to work out on the subject of short-day-induced flowering. Possibly there is no need for an FT-like function under short-day conditions but the direct effect of GA on *SOC1* and *LFY* is sufficient to induce flowering.

2.9 The PEBP family in Arabidopsis

As mentioned in Section 2.6.2, FT belongs to a family of closely related proteins, the PEBPs. In Arabidopsis, this family comprises six members (Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999), namely; FT, TERMINAL FLOWER 1 (TFL1), ARABIDOPSIS THALIANA CENTRORADIALIS (ATC), BROTHER OF FT AND TFL1 (BFT), TWIN SISTER OF FT (TSF) and MOTHER OF FT AND TFL1 (MFT). The FT family in angiosperms is grouped into three distinct sub families, FT-like (FT and TSF), TFL1-like (TFL1, ATC and BFT) and MFT-like (MFT) proteins.

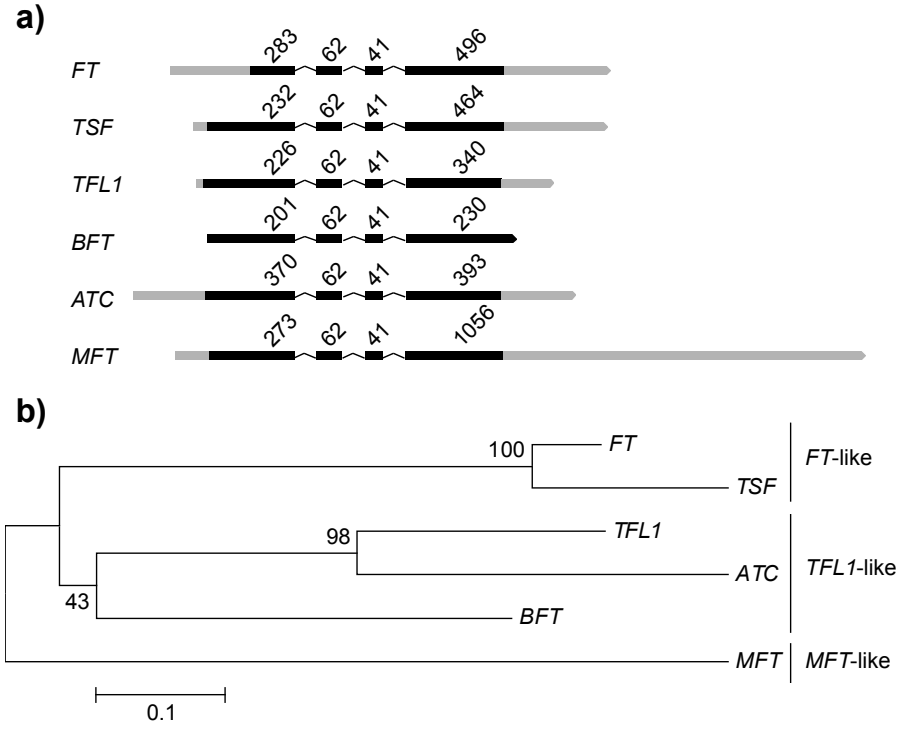

Figure 7. The PEBP gene family in Arabidopsis. a) Intron exon structure of the Arabidopsis PEBP genes, each consisting of four exons, of which exons two and three are identical in length in all cases. Squares mark exons and lines introns (of 87-852 nucleotides), black represents translatable CDS and grey 5' and 3' UTR. Exon length is indicated above. b) Phylogenetic tree showing the three subfamilies that the angiosperm PEBP genes can be divided into. The maximum likelihood tree was constructed using a manually adjusted codon alignment, (CLUSTAL W (Thompson *et al.*, 1994)) Kimura 2-parameter model, with invariant sites and tested using 1,000 bootstrap replications, (MEGA5 (Tamura *et al.*, 2011)). TAIR gene model names: *FT* (AT1G65480.1), *TFL1* (AT5G03840.1), *ATC* (AT2G27550.1), *BFT* (AT5G62040.1), *TSF* (AT4G20370.1) and *MFT* (AT1G18100.1).

The members of the FT family are not transcription factors but protein binding transcriptional cofactors (reviewed in (Kobayashi & Weigel, 2007)). They contain a eukaryote PEBP domain which takes up about 75-80% of the central region of the proteins, contributing to their high sequence homology. In line with this, all members of the PEBP-like family show extensive fold conservation and two conserved central regions, CR1 and CR2, that form part of the ligand-binding site. The N- and C-terminal parts are less conserved. The PEBP proteins are present in eukaryotes, bacteria and archaea and are represented in both animals and plants (in Paper II, I only considered proteins with a eukaryotic PEBP domain).

Despite the family's conserved structure and high sequence homology (Fig. 7), the members have quite different expression patterns and functions. Table 1 summarizes their known main expression, natural function and function with regard to flowering.

2.9.1 FT-like sub-group

An interesting curiosity about FLOWERING LOCUS T is that it was originally given the name FT (Koornneef *et al.*, 1991). The name "FLOWERING LOCUS T" was assigned years later by the group of Detlef Weigel (Kardailsky *et al.*, 1999) because the journal they wished to publish in insisted on a spelled out name (reviewed in (Kobayashi & Weigel, 2007)). In addition to its important role in flowering, (thoroughly described in Sections 2.4-2.7) Arabidopsis FT has also been implicated in the timing of stomata opening and closing (Kinoshita *et al.*, 2011).

TSF is the closest sequence homolog to FT in Arabidopsis (Mimida *et al.*, 2001; Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999). *TSF* has a similar expression pattern to *FT,* with diurnal oscillation peaking at dusk during long days (Yamaguchi *et al.*, 2005). *TSF* expression is higher than *FT* immediately after germination, but rapidly becomes much lower than *FT* (Yamaguchi *et al.*, 2005; Kobayashi *et al.*, 1999). Although both are expressed in cotyledons of seedlings, *FT* is mostly expressed in the distal parts of the leaf and *TSF* in the proximal parts of the leaf and hypocotyl (Yamaguchi *et al.*, 2005). Using reverse transcription PCR, low levels of *TSF* have been detected in shoot apex and rosette leaves of young long-day-grown seedlings (Yamaguchi *et al.*, 2005).

Like *FT*, *TSF* is suggested to be negatively regulated by FLOWERING LOCUS C (FLC) (Fig. 4 and Section 2.11.5), and in plants defective in the autonomous pathway, both *TSF* and *FT* mRNA levels are reduced (Yamaguchi *et al.*, 2005). Under short-day conditions, *FT* is repressed by EBS (Pineiro *et al.*, 2003), and *ebs* mutants show increased levels of both *TSF* and *FT*

(Yamaguchi *et al.*, 2005). By contrast, *FT* and *TSF* do not show the same response to the TFL2 repressor. Only *FT* levels were increased in the *tfl2* mutant under short-day conditions, whereas *TSF* expression was not affected (Yamaguchi *et al.*, 2005), indicating that TFL2 does not operate as a repressor of *TSF* under short days. TEM1 is another repressor of *FT* (Castillejo & Pelaz, 2008), but it is not known if TEM1 represses *TSF*.

Another difference between *FT* and *TSF* expression is that whereas *TSF* mRNA levels increase at low ambient temperatures, *FT* levels exhibit the opposite trend and decrease if the plant is moved from 23 °C to 16 °C (Blazquez *et al.*, 2003). *TSF* but not *FT* levels were also found to be markedly induced during drought stress conditions (Chung *et al.*, 2010).

The *ft tsf* double mutant is quite insensitive to long days. Whereas it is clear that *FT* and *TSF* work redundantly in the photoperiodic pathway because the *ft tsf* double mutant flowers later than both *ft* and *tsf* single mutants under long-day conditions, there is only a small difference in flowering time compared to Col-0 of both the single *ft* or *tsf* mutants and double *ft tsf* mutant under short days (the double mutant is in fact significantly, but only very marginally, later than Col-0) (Jang *et al.*, 2009). *35S::TSF* has elevated expression of *SOC1* and *LFY* as do *35S::FT* plants (Yamaguchi *et al.*, 2005) and plants to which GA has been applied (Moon *et al.*, 2003; Blazquez & Weigel, 2000). This indicates that TSF can work redundantly with FT in the shoot apical meristem to induce flowering through SOC1.

TSF mRNA has been found in the apex of Arabidopsis (Yamaguchi *et al.*, 2005), but in contrast to the *FT* promoter, the *TSF* promoter is active in a number of cells between the shoot apical meristem and leaf primordial (Yamaguchi *et al.*, 2005). Despite this, Giavalisco *et al.* (2006) detected native TSF (as well as FT) proteins in the phloem of *Brassica napus* inflorescence stems, and it has been suggested that TSF protein and/or mRNA is able to move through the phloem just like FT (Fornara *et al.*, 2010) (Section 2.6). TSF can bind to FD (Jang *et al.*, 2009) and it is conceivable that the pathways thereon after are very similar to those of the FT-FD complex under long-day-induced flowering (Fig. 4). At least the SOC1 activation of *SPL3*, *SPL4* and *SPL5* seem to be in common to the downstream pathways of the two genes (Jung *et al.*, 2012).

2.9.2 MFT-like sub-group

MFT-like proteins have been found in green algae, nonvascular land plants, as well as all gymnosperm and angiosperm species with available sequence data and is for sure the most ancient of the eukaryotic PEBPs ((Hedman *et al.*, 2009), Paper II). MFT has been shown to be expressed in gametophytes and

developing seeds (Xi & Yu, 2010), as well as being highly expressed in siliques, more moderately expressed in roots and rosette leaves and weakly expressed in cauline leaves, young inflorescences and open flowers (Xi *et al.*, 2010). Constitutive overexpression of *MFT* under long-day conditions result in a weak early flowering phenotype (Yoo *et al.*, 2004). The *mft* loss-of-function mutant has no obvious phenotypes in flowering or meristem development (Xi *et al.*, 2010; Yoo *et al.*, 2004), but is hypersensitive to abscisic acid (ABA), a phytohormone that regulates seed germination in response to stress (Xi *et al.*, 2010). The natural function of MFT is not well known, but it is possible that MFT promotes seed germination and fertility under environmental stresses (Xi *et al.*, 2010; Xi & Yu, 2010).

2.9.3 TFL1-like sub-group

TFL1, BFT and ATC belong to a clade that has a repressing function with respect to flowering despite their close sequence homology to FT and TSF. Most likely, *BFT* originates from an early gene duplication of the *TFL1* ancestor, whereas *ATC* originates from a much more recent gene duplication of *TFL1*.

TFL1 is without a doubt the most well studied of these repressing members of the FT family in Arabidopsis. FT and TFL1 have remarkably contrasting functions; overexpressing one or down-regulating the other produce very similar phenotypes with respect to flowering (although they are not exact mirror images of each other (Ahn *et al.*, 2006)). Nevertheless, their amino acid sequences are extremely alike, (60 % identical (Paper II)). Like *FT* (Section 2.5), *TFL1* expression is activated by CO (Simon *et al.*, 1996). In addition, it has been shown that TFL1 binds to FD in yeast cells but with a much weaker interaction than FT-FD (Abe *et al.*, 2005), thus supposedly competing with FT for FD interaction. It has been suggested that *FT* and *TFL1* can act antagonistically to fine-tune the response to flower inductive signals (Kardailsky *et al.*, 1999).

The *tfl1* mutant bolts early compared to wild type and its inflorescence only produces a few flowers before a terminal flower is formed (Bradley *et al.*, 1997). *35S::TFL1* plants bolt later than the wild type and produce a large number of side shoots with initially leaf-like and eventually normal flowers, giving the plant a highly branched architecture (Ratcliffe *et al.*, 1998). Whereas the *tfl1* mutant has a very short life span (~30 days) and produces few seeds compare to wild type (~45 days), *35S::TFL1* plants have a long life span (~80-100 days) and produce more seeds than wild type (Ratcliffe *et al.*, 1998).

TFL1 is expressed in shoot apical as well as axillary meristems under both long- and short-day conditions (Conti & Bradley, 2007; Bradley *et al.*, 1997).

Prior to flowering, during vegetative growth, *TFL1* is weakly expressed in all meristem cells, probably to delay commitment to flowering (Bradley *et al.*, 1997). However, after the transition to flowering, the expression increases substantially and becomes restricted to the lower regions of the shoot apical meristem (Conti & Bradley, 2007; Bradley *et al.*, 1997), probably to maintain inflorescence meristem identity (Bradley *et al.*, 1997). Whereas *TFL1* expression is restricted to the center of the mature shoot meristem, below the apical dome (Bradley *et al.*, 1997), the TFL1 protein has a more broad distribution pattern and is present throughout the meristem but not in the lateral or flower meristem primordia (Conti & Bradley, 2007).

TFL1 has an important function in controlling plant architecture through spatial regulation of the meristem identity genes *AP1* and *LFY* (Liljegren *et al.*, 1999). Whereas FT promotes *LFY* and *AP1* expression in the floral anlagen or primordia (Fig. 4), LFY and AP1 repress *TFL1* in the emerging floral primordia. The TFL1 protein represses *LFY* and *AP1* in the center of the inflorescence meristem, resulting in high levels of *LFY* and *AP1* expression on the sides of the meristem that develop into floral meristems or floral primordia, allowing the center of the shoot meristem to continue its indeterminate growth (Conti & Bradley, 2007; Bradley *et al.*, 1997).

In addition, it has been proposed that TFL1 is involved in trafficking to the protein storage vacuole (Sohn *et al.*, 2007) (not discussed in this thesis).

As previously stated, *BFT* probably originates from an early gene duplication of *TFL1*. It is present in many species and is quite commonly put in a separate, smaller clade, adjacent to the TFL1-likes (Fig.1 and Fig. S1 in Paper II).

BFT is the member of the TFL1-likes that has strongest sequence homology to the FT-like proteins (Fig. 7). QPCR, GUS staining and *in situ* hybridization have revealed *BFT* expression in all examined tissues, but higher levels were found in the aboveground tissues, specifically leaf and mature flowers, although it was also detected in the shoot apical meristem using all three methods (Yoo *et al.*, 2010).

BFT exhibits a diurnal expression pattern similar to that observed for *FT*, with a peak 12 hours after lights on (ZT12) (Yoo *et al.*, 2010) or ZT16 (Ryu *et al.*, 2011). In addition, *BFT* expression is higher in long than in short days for plants of the same age, as is the case for *FT*, *TFL1*, *TSF* and *MFT*, but in contrast to *ATC* (Yoo *et al.*, 2010).

35S::BFT plants are late flowering and look very similar to *35S::TFL1* plants (Yoo *et al.*, 2010), exhibiting leaf-like flowers (Chung *et al.*, 2010; Yoo *et al.*, 2010). However, overexpression of *BFT* only partly rescues the *tfl1* mutant phenotype as *35S::BFT* in a *tfl1* background flower later and have a

longer inflorescence than *tfl1* but still produce a terminal flower (Yoo *et al.*, 2010). The *bft* mutant does not exhibit an altered phenotype compared to wild type under normal long-day conditions (Ryu *et al.*, 2011; Yoo *et al.*, 2010).

BFT expression has been shown to be induced under drought stress (Chung *et al.*, 2010) and high salinity (Ryu *et al.*, 2011) conditions, but unlike wild-type Col, the flowering time of the *bft* mutant was not affected by high salinity (Ryu *et al.*, 2011). The function of BFT is not well known, but it has been proposed to delay flowering under stress conditions (Ryu *et al.*, 2011; Chung *et al.*, 2010). It has also been suggested that BFT could have a redundant role with TFL1 in the development of axillary meristem and in the control of plant architecture (Yoo *et al.*, 2010).

Table 1. *Expression pattern, natural function and flowering phenotype of the Arabidopsis PEBP family members.*

Gene	Mainly expressed in	Natural function	Knockout effect on flowering time	Overexpression phenotype regarding flowering time
FT	Phloem tissue of cotyledons, leaves and inflorescence stems [1, 2]	Induce flowering under long-day conditions [3, 4]	Late flowering under long days [5]	Early flowering [3, 4]
TSF	Phloem tissue of hypocotyl, flowers and developing siliques [1]	Induce flowering redundantly with FT under long-day conditions [6]	Weakly late flowering under long days [6]	Early flowering [4]
TFL1	Shoot apical and axillary meristems, below the apical dome [7]	Repress flowering and controls plant architecture [8]	Early flowering under both long and short days [9, 10]	Late flowering [8]
ATC	Hypocotyl of young plants [11]	Unknown [11, 12]	None [11]	Late flowering [11]
BFT	All examined tissue [13]	Possibly inhibits flowering under drought and salt stress conditions [14, 15]. Might be involved in plant architecture [13]	None [13, 14] unless at high salinity when it is earlier than wild type [15]	Late flowering [13-15]
MFT	Gametophytes and developing seeds [16] siliques, roots and rosette leaves etc. [17]	Possibly regulates seed germination and fertility under stress conditions [16, 17]	None [17, 18]	Weakly early flowering [18]

1 Yamaguchi *et al.* (2005), 2 Takada and Goto (2003), 3 Kardailsky *et al.* (1999), 4 Kobayashi *et al.* (1999), 5 Koornneef *et al.* (1991), 6 Jang *et al.* (2009), 7 Bradley *et al.* (1997), 8 Ratcliffe *et al.* (1998), 9 Liljegren *et al.* (1999), 10 Shannon and Meekswagner (1991), 11 Mimida *et al.* (2001), 12 Benlloch *et al.* (2007), 13 Yoo *et al.* (2010), 14 Ryu *et al.* (2011), 15 Chung *et al.* (2010), 16 Xi and Yu (2010), 17 Xi *et al.* (2010), 18 Yoo *et al.* (2004)

ATC is the closest homolog to the *CENTRORADIALIS* (*CEN*) gene from *Antirrhinum* and thereof given its name (Mimida *et al.*, 2001). Expression of *ATC* is very low and it has not been detected (using *in situ* hybridization) in the inflorescence meristem, only in the hypocotyl of young plants, suggesting it exhibits a different expression pattern than *TFL1* (Mimida *et al.*, 2001). Arabidopsis plants constitutively overexpressing *ATC* (*35S::ATC*) look very similar to *35S::TFL1* plants, displaying late flowering and formation of terminal flowers, but the loss-of-function *atc* mutants are not early flowering (Mimida *et al.*, 2001). This difference in expression pattern and mutant phenotype suggests that ATC and TFL1 have different functions despite the fact that *ATC* can rescue the *tfl1* phenotype if constitutively overexpressed (*35S::ATC*) (Mimida *et al.*, 2001). However, the exact function of ATC is not yet known (reviewed in (Benlloch *et al.*, 2007))

2.10 miRNA156 and its targets in Arabidopsis

2.10.1 miR156

miR156 is one of the most abundant miRNAs in Arabidopsis and has been detected in a large number of evolutionarily old species, such as moss, ferns and gymnosperms, indicating that it has an important and relatively basal function (reviewed in (Huijser & Schmid, 2011)). As previously mentioned, (Section 2.4), miR156, which post-transcriptionally regulates the majority of the *SPL* gene family members, is an important regulator of the juvenility-to-maturity switch. miR156 expression is high during juvenility and gradually decreases during maturation in several species, e.g., Arabidopsis (Wu *et al.*, 2009; Wu & Poethig, 2006), eucalyptus, oak, acacia, ivy (Wang *et al.*, 2011a), poplar ((Wang *et al.*, 2011a) and Paper I) and maize (Chuck *et al.*, 2007), integrating aging as a component in the flowering pathway (Fig. 4) (Wang *et al.*, 2009b; Yamaguchi *et al.*, 2009). Neither photoperiod, vernalization nor gibberellins have any obvious effect on miR156 levels in Arabidopsis seedlings (reviewed in (Huijser & Schmid, 2011)). Wu *et al.* (2009) go as far as to suggesting that; “miR156 is both necessary and sufficient for the expression of the juvenile phase, and that it functions as a master regulator of this phase”.

A pri-miRNA is generally thought to be a long sequence that is cleaved into a precursor or pre-miRNA (Fig. 8), consisting of a stem-loop or hairpin structure (Fig. 12d). The pre-miRNA is cleaved into the mature miRNA of 20-24 nt (Fig. 11) (Bartel, 2004; Reinhart *et al.*, 2002). There are ten miR156 in Arabidopsis (AtmiR156a-j) (http://www.mirbase.org) (Kozomara & Griffiths-Jones, 2011; Griffiths-Jones *et al.*, 2008; Griffiths-Jones *et al.*, 2006; Griffiths-

Jones, 2004). Six of them, AtmiR156a-f, have the same mature, target recognition sequence of 20 nucleotides (UGACAGAAGAGAGUGAGCAC) (Fig. 11). miRNAs in general, as well as miR156, recognize their targets through imperfect base pairing. In the case of AtmiR156a-f, it seems that only certain bases can vary, whereas others must be perfect matches (Paper I).

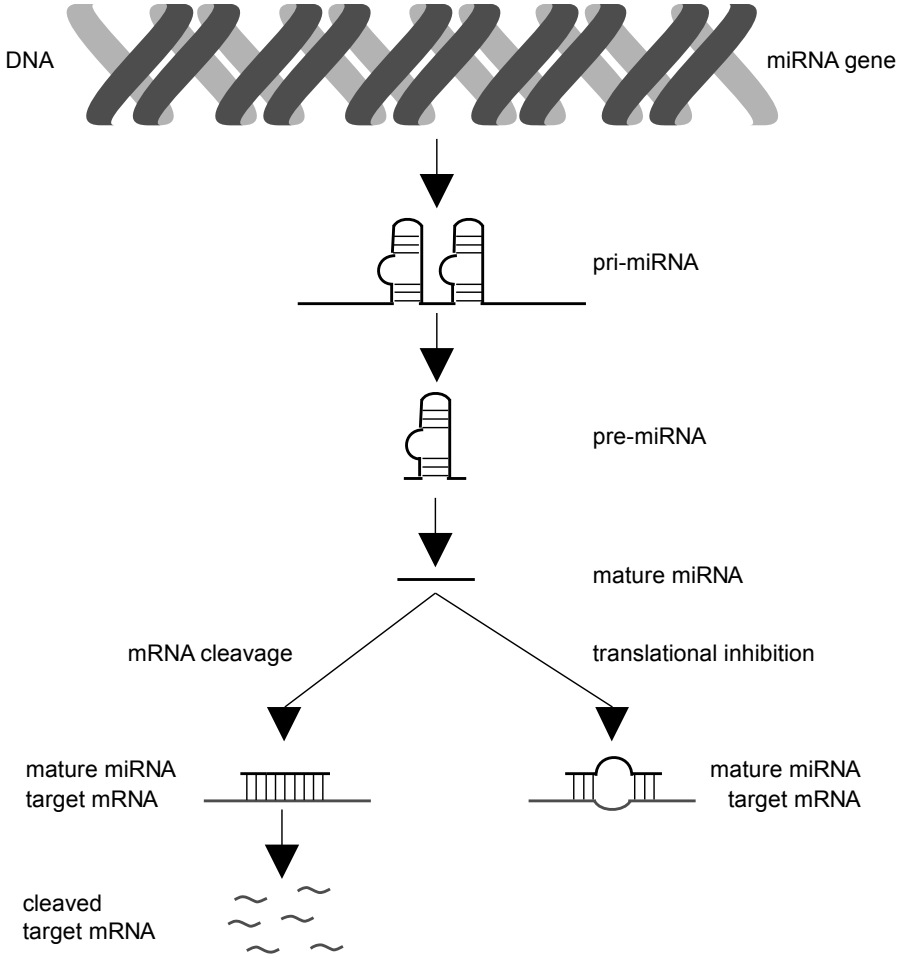

Figure 8. Mechanisms by which miRNAs inhibit target gene expression.

miR156a- and *miR156b*-overexpressing plants exhibit prolonged expression of juvenile vegetative traits and flower late (Schwarz *et al.*, 2008; Wu & Poethig, 2006). In one study under short-day conditions, *miR156a*-overexpressing plants produced approximately 90 leaves with juvenile

characteristics, compared to ~7.5 for wild type (Wu *et al.*, 2009). *35S::miR156* have also been reported to exhibit reduced apical dominance (Huijser & Schmid, 2011) and *35S::miR156f* plants have a faster leaf initiation rate than wild type (Wang *et al.*, 2008).

As might be expected, almost the opposite phenotypes are exhibited by plants with reduced levels of miR156. Plants reduced in miR156b, generated by mimicry constructs, flowered at the same time as wild type, but with considerably fewer leaves due to them having very long plastochrons (Franco-Zorrilla *et al.*, 2007). Huijser and Schmid (2011) generated a mimicry *miR156*-overexpressing Arabidopsis strain that only produced a few adult leaves prior to flowering under long-day conditions. Similarly, plants with a non-cleavable miR156 target site, only produce adult leaves if grown under short days (Wu *et al.*, 2009).

The factor(s) responsible for the down-regulation of miR156 with age under natural conditions are not yet known. A study by Yang *et al.* (2011) excluded the root system as a possible source of a juvenilizing factor as their results showed that mutant Arabidopsis without roots mature and flower. Instead, they suggested that the leaf primordia produce some kind of aging factor that inhibit *miR156* expression, but the identity of this factor remains unknown (Yang *et al.*, 2011).

2.10.2 The SPL family in Arabidopsis

SPL transcription factors were originally isolated from *Antirrhinum majus* and bind to the *AP1* ortholog *SQUAMOSA* (*SQUA*), inducing its transcription (Klein *et al.*, 1996). There are 17 *SPL* genes in Arabidopsis; (*SPL1-12*, *13A*, *13B*, *14-16*). *SPL13A* and *SPL13B* are identical but encoded twice in the genome. Of the 17 *SPL* genes, 11 have been predicted to be targeted by miR156 (Gandikota *et al.*, 2007; Rhoades *et al.*, 2002b), and have also been experimentally verified as targets (Schwab *et al.*, 2005). Hence, miR156 targets the majority of the *SPL* genes in Arabidopsis (Wu *et al.*, 2009).

The *SPL* family in Arabidopsis (Fig. 9) can be divided into two major groups based on protein size and structural organization (Xing *et al.*, 2010). The first group of genes (Group A: *SPL1*, *-7*, *-12*, *-14*, and *-16*) consists of ten or more exons and proteins of more than 800 amino acids (Xing *et al.*, 2010). The second group (Group B) encodes for proteins of less than half the size and contains only two to four exons (Xing *et al.*, 2010). The two main groups can be sub-divided into seven smaller groups (I-VII) based on phylogenetic reconstruction of the *SPL* gene family of Arabidopsis together with moss and rice *SPL* genes (Riese *et al.*, 2007). Group A is divided into I (*SPL7*) and II (*SPL1*, *-12*, *-14*, *-16*), while Group B is divided into III (*SPL8*), IV (no *SPLs*

from Arabidopsis or poplar), V (*SPL2*, *-10*, *-11* and *SPL9*, *-15*), VI (*SPL3*, *-4*, *-5*) and VII (*SPL6*, *-13A*, *-13B*).

Sub-groups V, VI and VII are targeted by miR156, but while sub-group V and VII have their target sites in the coding region, sub-group VI has their target sites in the 3'UTR ((Wu & Poethig, 2006) and Paper I). The remaining sub-groups are not targets of miR156. *SPL8* is the only member of Group B that does not carry a miR156/miR157 response element ((Xing *et al.*, 2010) and Paper I). No further details about the non-targeted SPL transcription factors are provided in this thesis.

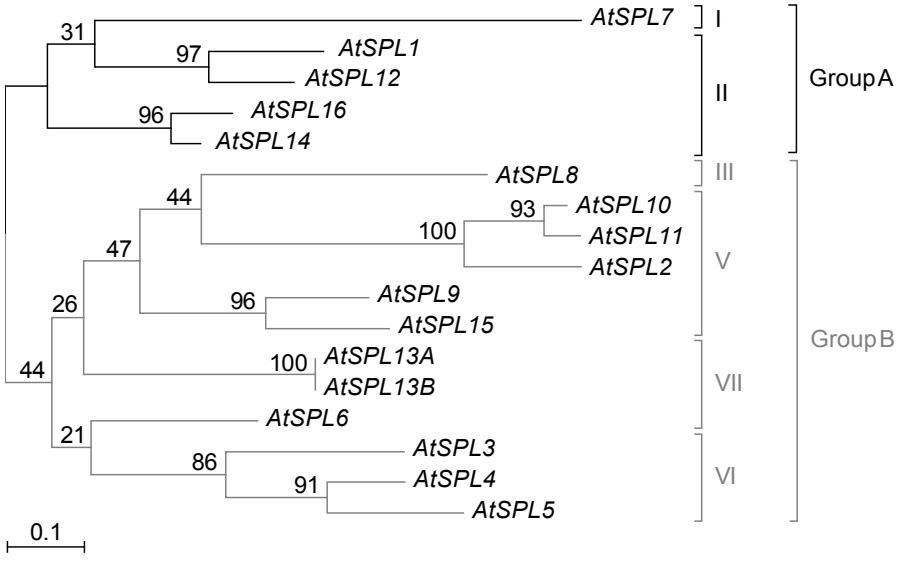

Figure 9. Phylogenetic reconstruction of the *SPL* gene family in Arabidopsis, which can be divided into two main groups (A and B) based on protein size and structural organization (Xing *et al.*, 2010) and subdivided into smaller groups (roman letters I-VII) based on their amino-acid sequences and phylogenetic reconstruction together with moss and rice *SPL* genes (Riese *et al.*, 2007). The codon alignment was made using CLUSTAL W (Thompson *et al.*, 1994), the phylogenetic reconstruction using complete deletion, maximum likelihood method based on the best-fit substitution model (Kimura 2-parameter with invariant sites), nodal support was estimated using 1,000 bootstrap re-samplings (MEGA5 (Tamura *et al.*, 2011)).

In contrast to miR156, expression of the target *SPL* genes increases with age (at least *SPL3*, *SPL4*, *SPL5* and *SPL9*) and/or flower induction *SPL2*, *SPL3*, *SPL4*, *SPL5*, *SPL6*, *SPL9*, *SPL10*, *SPL11*, *SPL13* and *SPL15* (Jung *et al.*, 2012; Wang *et al.*, 2009b; Wu & Poethig, 2006; Schmid *et al.*, 2003; Cardon *et al.*, 1999; Cardon *et al.*, 1997). Expression of the *SPL3* and *SPL9*

genes has also been found to increase with age in other species, e.g., *Populus deltoides x nigra* and *Eucalyptus globulus* (Wang *et al.*, 2011a).

The miRNA-targeted *SPLs* are involved in both flowering and other developmental processes, such as the juvenility-to-maturity and vegetative-to-reproductive phase transitions (Wang *et al.*, 2009b; Yamaguchi *et al.*, 2009; Schwarz *et al.*, 2008; Gandikota *et al.*, 2007; Wu & Poethig, 2006; Cardon *et al.*, 1997). High miR156 levels retain the plant in a juvenile or vegetative state, whereas down-regulation of miR156 with age allows maturation and subsequent flowering (Fig. 4).

Arabidopsis *SPL* genes have been shown to promote flowering under both inductive long-day and non-inductive short-day conditions (Jung *et al.*, 2012; Wang *et al.*, 2009b). This occurs through (i) SPL inducing flowering-promoting genes like *LFY*, *AP1*, *CAL* and *FUL* (Fig. 4) (Wang *et al.*, 2009b; Yamaguchi *et al.*, 2009), (ii) SOC1-SPL-GA (Jung *et al.*, 2012) and (iii) SPL promoting miR172 expression (Wu *et al.*, 2009).

Plants with loss-of-function mutations in single *SPL* genes, i.e., *spl2*, *spl3*, *spl9*, *spl10*, *spl11*, *spl13*, and *spl15,* are phenotypically normal with respect to plastochron and flowering time, indicating that several of the SPLs have redundant functions (Wang *et al.*, 2008).

Group V (SPL2, -10 and -11)

Using semi-quantitative reverse transcription, *SPL2*, *SPL10* and *SPL11* have been detected in a large range of tissues, including roots, juvenile, adult and cauline leaves, inflorescence stems, flowers and fruits, with the highest expression being found in stem and flowers (Shikata *et al.*, 2009). However, *SPL10* could not be detected in shoot apices using RNA *in situ* hybridization (Wu *et al.*, 2009). Further, it has been shown that *SPL10* expression is not affected in *35S::FT* plants or *ft10* loss-of-function mutants (Jung *et al.*, 2012).

As mentioned above, single *spl2*, *spl10*, *spl11* loss-of-function mutants have no obvious phenotypes (Wu *et al.*, 2009; Wang *et al.*, 2008). In addition, the double loss-of-function mutants *spl2 spl10* and *spl2 spl11* exhibit a wild-type phenotype with no effect on flowering, but they produce more trichomes than wild type on both cauline leaves and flowers (Shikata *et al.*, 2009). The *spl2* mutant weakly enhances the double *spl9 spl15* mutant (Schwarz *et al.*, 2008).

Plants overexpressing miRNA-insensitive variants *SPL2*, *SPL10* and *SPL11* all produced adult rosette leaves at an earlier stage than wild type (Shikata *et al.*, 2009). Plants overexpressing miRNA-insensitive variants of *SPL10* displayed long plastochron (Shikata *et al.*, 2009; Wang *et al.*, 2008), but there were no apparent effects on flowering time (Shikata *et al.*, 2009). To summarize, *SPL2*, *SPL10* and *SPL11* only seem to have a minor role in the

vegetative phase change (reviewed in (Huijser & Schmid, 2011)), both with respect to flowering and leaf phenotypes.

Group V (SPL9 and SPL15)

The sequences of *SPL9* and *SPL15* are very similar (Schwarz *et al.*, 2008). *SPL9* is expressed in pre-emergent and expanding leaf primordia but not in the central part of the meristem (Wu *et al.*, 2009; Wang *et al.*, 2008). SPL9 homologs have been identified in *Populus deltoides x nigra*, rice and maize (reviewed in (Huijser & Schmid, 2011)). As previously stated, both *spl9* and *spl15* single mutants are phenotypically normal considering plastochron and flowering time (Wang *et al.*, 2008). However, whereas the *spl15* loss-of-function mutation has no obvious effects on other characteristics, the *spl9* loss-of-function mutant exhibits delayed abaxial trichome production (Wu *et al.*, 2009). The double *spl9 spl15* mutants produce abaxial trichomes later than the single *spl9* mutant (Wu *et al.*, 2009) and exhibit short plastochrons (Schwarz *et al.*, 2008; Wang *et al.*, 2008), i.e., it produces more leaves during a certain period of time than does wild type Arabidopsis.

SPL9 overexpression increases leaf size and reduces plastochron (leaf initiation rate) (reviewed in (Bergonzi & Albani, 2011)). Overexpression of miRNA-insensitive *SPL9* results in plants that almost completely skip the juvenile phase (reviewed in (Huijser & Schmid, 2011)).

In terms of flowering, SPL9 has a double role. It binds to the promoters of *FUL*, *AP1* and possibly also *SOC1* and *AGL42,* inducing their expression (Wang *et al.*, 2009b). In addition, it regulates flowering by promoting miR172 expression (Wu *et al.*, 2009), which leads to down-regulation of several *FT* repressors (Fig. 4). *SPL9* and *SPL15* do not seem to be targets of the photoperiod or GA pathways in the same way as *SPL3*, *SPL4* and *SPL5* are (discussed in the following section) since neither *SPL9* nor *SPL15* levels are affected in activation-tagging or loss-of-function *soc1* mutants, or in loss-of-function *ft10* mutants or *35S::FT* plants (Jung *et al.*, 2012). Further, it has been shown that *SPL9* levels are not affected by GA treatments (Wang *et al.*, 2009b).

To summarize, *SPL9* and *SPL15* probably have redundant functions on abaxial trichome formation (Wu *et al.*, 2009), leaf initiation rate (Schwarz *et al.*, 2008; Wang *et al.*, 2008) and flowering promotion (Schwarz *et al.*, 2008).

Group VI (SPL3,-4 and -5)

The Arabidopsis members of sub-group VI, i.e., *SPL3*, *-4* and *-5,* are small proteins (Cardon *et al.*, 1999), which are only about twice the size of the SBP DNA binding domain. *SPL3* is expressed in the apical vegetative and

inflorescence meristems, leaf- and floral organ primordia (Cardon *et al.*, 1997), expanding leaf primordia as well as throughout the shoot apex (Wu *et al.*, 2009). *SPL3* sequence homologs have been identified in *Populus deltoides x nigra*, tomato, maize and *Antirrhinum* (reviewed in (Huijser & Schmid, 2011)).

As previously mentioned, *SPL3*, *SPL4* and *SPL5* are all up-regulated with age and such up-regulation correlates with the juvenility-to-adult transition (Jung *et al.*, 2012; Wu *et al.*, 2009; Wu & Poethig, 2006; Schmid *et al.*, 2003; Cardon *et al.*, 1999). Overexpression of *SPL3* was first reported to cause dramatically early flowering (Cardon *et al.*, 1997), although the strong phenotype observed was probably due to a truncated miR156 target site in the 3' UTR (Wu & Poethig, 2006). Huijser and Schmid (2011) even renamed the construct as a miR156-resistant *SPL3*. Since then, overexpression of *SPL3, SPL4* or *SPL5* has been shown to cause only slight early flowering in Arabidopsis, whereas overexpression of miRNA156-insensitive variants of *SPL3*, *SPL4* or *SPL5* cause significant early flowering compared to wild type in addition to accelerating the production of abaxial trichomes and short petioles (Wu & Poethig, 2006). Loss-of-function *spl3* mutants have no obvious phenotype (Wu *et al.*, 2009), but adult leaves appear significantly earlier in miRNA156-insensitive variants of *SPL3*, *SPL4* or *SPL5* than in wild type (Gandikota *et al.*, 2007; Wu & Poethig, 2006). Thus, *SPL3*, *SPL4* and *SPL5* seem to have redundant functions with respect to flowering and maturation (Wu & Poethig, 2006).

SPL3, *SPL4* and *SPL5* promote flowering under both long- and short-day conditions (Jung *et al.*, 2012; Wang *et al.*, 2009b). *SPL3*, *SPL4* and *SPL5* have been found to be activated by FT-mediated photoperiod signals under long-day conditions, possibly via SOC1 (Jung *et al.*, 2012; Schmid *et al.*, 2003). *SPL3* expression even peaks in the dark (Jung *et al.*, 2012) under long-day conditions, probably as an effect of FT activation since miR156 expression does not oscillate (Jung *et al.*, 2012). *SPL3*, *SPL4* and *SPL5* expression are up-regulated in *35S::FT*, *35S::FD*, *35S::CO* and *35S::GI* and down-regulated in *ft*, *fd*, *co* and *gi* (Jung *et al.*, 2012).

Arabidopsis plants expressing *35S::miR156* or *35S::SPL3* exhibit reduced or increased levels of *AP1*, *CAL* and *FUL,* respectively, but in both transgenic plants, neither *SOC1* nor *FT* expression were affected (Jung *et al.*, 2012). *SPL3* thus seems to activate *FUL*, *LFY* and *AP1* transcription (Jung *et al.*, 2012; Yamaguchi *et al.*, 2009) (Fig. 4), but work downstream of FT and SOC1.

Short-day-induced flowering has been found to occur through the GA pathway (Fig. 4 and Section 2.8), involving direct binding and activation of *SPL3*, *SPL4* and *SPL5* by SOC1 (Jung *et al.*, 2012).

In summary, these data suggest that *SPL3*, *SPL4* and *SPL5* function in both the long- and short-day flowering pathways (Jung *et al.*, 2012).

Group VII (SPL6, -13A and -13B)

No loss- or gain-of-function for *SPL6* have been reported, and the *spl13* single mutant is phenotypically normal with regard to plastochron and flowering time (Wang *et al.*, 2008). However, miR156-resistant *SPL13* has been found to exhibit a slight delay in the emergence of the first true leaves (reviewed in (Huijser & Schmid, 2011)).

In one of its most recent articles on the subject, the group of Scott Poethig concluded that *SPL3*, *SPL4* and *SPL5* probably have a more limited role in adult leaf development than previously thought, whereas *SPL9* possibly promotes most, if not all, phenotypic traits associated with an adult leaf phenotype (Wu *et al.*, 2009), (Section 2.2). In addition, they conclude that *SPL3*, *SPL4, SPL5, SPL9* as well as *SPL10* all promote flowering under long-day conditions. Since floral induction has a major effect on leaf development, this may have resulted in an over-estimation of adult leaf phenotypes being attributed to the *SPL3*, *SPL4* and *SPL5* genes in previous studies (Wu *et al.*, 2009).

2.11 Maturation, flowering and growth cessation in *Populus*

In this section, I will explain some of the major differences and possibly unexpected similarities between herbaceous annual plants like Arabidopsis and woody perennials, with special emphasis on *Populus* as this is the main species considered in this thesis besides Arabidopsis. The focus will be on phenology, i.e., seasonal changes in plant growth, e.g., flower initiation, short-day-induced bud set, temperature-induced bud burst as well as heteroblasty, i.e., the ability to grow vegetative and reproductive shoots on the same plant.

2.11.1 *Populus trichocarpa*

Arabidopsis and *Populus* are both rosids, and thus closely related from an evolutionary perspective, (as is most lineages of angiosperm forest trees, since the rosids include more than one quarter of all angiosperm species) (Wang *et al.*, 2009a). *Populus trichocarpa* (black cottonwood or western balsam poplar) was the first tree to have its genome completely sequenced. The species is native to western North America, where it is grown for timber. *P. trichocarpa* grows to about 30 m height and < two meters in diameter. It reaches maturity about 10-15 years after germination in natural populations, and within 4-8

years in intensively managed plantations (reviewed in (Slavov & Zhelev, 2010)). Once mature, *P. trichocarpa* annually forms unisexual catkins, prior to the leaves appearing in early March to mid-June depending on growth conditions.

P. trichocarpa has a genome of over 500 million base pairs divided into 19 chromosomes, making it four times the size of the Arabidopsis genome but still quite small compared to the gymnosperms; the pine genome is, for example, 50 times larger (Tuskan *et al.*, 2006). The *P. trichocarpa* genome has been subjected to two whole-genome duplications, resulting in 8000 pairs of duplicated genes from the most recent one (Tuskan *et al.*, 2006). The oldest gene duplication coincided with the divergences of the *Populus* and Arabidopsis lineages (Tuskan *et al.*, 2006).

2.11.2 Flowering in *Populus*

Flower initiation or development of reproductive meristems in *P. trichocarpa* is thought to occur in the early spring (Boes & Strauss, 1994). When the flower buds containing next year's flowers appear in the axils of leaves on extending shoots, the buds already show morphological changes, indicating that flower initiation occurs earlier (Boes & Strauss, 1994). The flower buds develop during spring, then enlarge during the summer and enter dormancy in the fall, bursting finally before the vegetative buds early the following spring (Boes & Strauss, 1994). *Populus* are normally dioeceous, meaning trees are either male or female (reviewed in (Jansson & Douglas, 2007)).

As in Arabidopsis, FT plays an important role in flowering in *Populus* (Bohlenius *et al.*, 2006; Hsu *et al.*, 2006), there are two *FT* genes in *Populus*, *FT1* and *FT2*. *PtFT1* is only weakly expressed under vegetative growth during the summer but is predominantly expressed in late winter, in leaves, shoots, shoot apices, reproductive and vegetative buds (Hsu *et al.*, 2011). *PtFT2*, on the other hand, is predominantly expressed during the spring and summer with abundant expression only in leaves and reproductive buds (Hsu *et al.*, 2011). The expression of both *PtFT1* and *PtFT2* is up-regulated with age under natural conditions and overexpression of either *PtFT1* or *PtFT2* causes early flowering (Bohlenius *et al.*, 2006; Hsu *et al.*, 2006).

PtFT2 has been proposed to be involved in active growth, whereas PtFT1 has been suggested to be responsible for flower induction (Hsu *et al.*, 2011). Hsu *et al.* (2011) have suggested that as high *PtFT1* expression is induced by cold temperatures, it may trigger flower initiation during the winter in some of the axillary buds next to the pre-formed leaves inside the dormant bud. Thus, flower initiation may not occur in early spring as postulated by (Boes & Strauss, 1994), but in late winter. This would mean it could not be caused by a

photoperiodically induced FT signal from a source leaf as in Arabidopsis since in the winter there are no leaves present and the circadian clock does not operate to detect day length (see Section 2.11.3). However, it is of course conceivable that a photoperiodic-insensitive PtFT1 signal is sent during the winter from a pre-formed leaf inside the dormant bud or other tissue (*PtFT1* is expressed in many tissue types in *Populus* during winter (Hsu *et al.*, 2011)), more than a year before the catkin is visible.

Considering that *FT* expression is up-regulated as trees age and that overexpression of either *PtFT1* or *PtFT2* causes early flowering, it has been hypothesized that *Populus* must reach a threshold level of *FT* to induce flowering (Bohlenius *et al.*, 2006; Hsu *et al.*, 2006).

As in Arabidopsis (Section 2.9.3), TFL1 seems to have a repressing function on flowering in *Populus*. *Populus tremula x alba* with reduced expression of the *Populus TFL1* homologs *PopCEN1* and *PopCEN2*, flowers early and to a higher degree than wild type (Mohamed *et al.*, 2010). In addition, it commits more shoots to reproductive growth when grown outside under natural conditions (Mohamed *et al.*, 2010). In line with this, plants overexpressing *PopCEN1* flowered late under the same conditions (Mohamed *et al.*, 2010).

Flower induction in *Populus* may or may not be day length induced, but the fact that some trees, e.g., the Swedish aspen (*Populus tremula*), do not flower every year once mature despite the day length remaining the same each year, is a very interesting matter that has not been tackled in this thesis.

In contrast to flower initiation, the actual flowering process (flower emergence) has been well defined. With the exception of some subtropical species, flowering in *Populus* is regulated by temperature-mediated bud flush prior to the tree producing leaves in the early spring (reviewed in (Slavov & Zhelev, 2010; Jansson & Douglas, 2007)). The trees flower for one to two weeks, and both pollen and seed are wind-dispersed (Slavov & Zhelev, 2010).

2.11.3 Bud set

To avoid frost damage during the winter period, it is essential for the meristems of woody perennial plants in temperate regions to cease growth (Fig. 10) and acquire cold hardiness in the form of bud scales, dehydration and accumulation of freeze-tolerable proteins and sugars prior to the first frost (reviewed in (Eriksson & Webb, 2011; Kozlowski & Pallardy, 2002)).

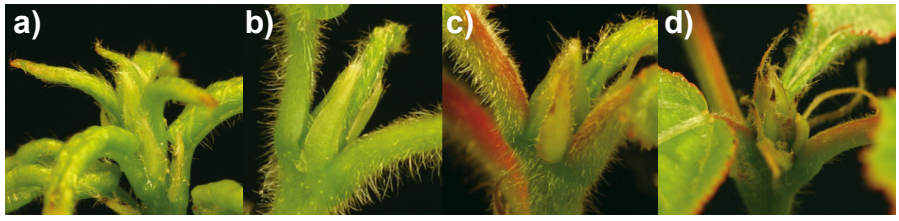

Figure 10. Short-day-induced bud set in *PttmiR156e*-overexpressing trees: (a) active growth, (b) growth cessation (internode elongation has ceased but there is no visible bud yet), (c) visible open/soft bud, (d) green closed/hard bud.

Angiosperm trees in temperate regions, like *Populus*, set buds when the day length falls below a critical value (defined as the longest photoperiod that induces growth cessation) (Howe *et al.*, 1995; Nitsch, 1957; Wareing, 1956), which varies depending on the latitude from which the tree originates (Howe *et al.*, 1995). The critical day length is under genetic control and inherited as a quantitative trait (reviewed in (Rohde *et al.*, 2000)). For example, in Northern Sweden, the trees stop growing when the days are still quite long, whereas trees in Germany do not cease growth until the days become quite short (Bohlenius *et al.*, 2006). Temperature has also been found to play a minor role in bud set in *Populus* (Rohde *et al.*, 2011), and trees that have not set bud by the time the temperature falls below zero, will quickly do so (reviewed in (Jansson & Douglas, 2007)). In contrast, bud set in apple and pear is only induced by low temperatures (< 12ºC), while photoperiod has no effect at all (Heide & Prestrud, 2005).

Photoreception in *Populus* seems to work in a similar manner to that in Arabidopsis. Both PHYA (Kozarewa *et al.*, 2010) and PHYB (Ingvarsson *et al.*, 2006) have been found to be involved in sensing the day length and timing of bud set in *Populus*. In addition, the circadian clock in chestnut (*Castanea sativa*) seems to have a similar construction and function as in Arabidopsis, with cyclical expression of *CsTOC1* and *CsLHY* in both leaf and stem (Ramos *et al.*, 2005). Remarkably, this cycling pattern disappears under cold temperatures (+4ºC), for both chestnut and *Populus* (Ibanez *et al.*, 2010; Ramos *et al.*, 2005). Thus, the circadian clock ceases to function during the winter season in at least two angiosperm tree species. The clock components *LHY* and *TOC1* (Fig. 3) have been shown to stabilize at high levels during dormancy, (Ibanez *et al.*, 2010; Ramos *et al.*, 2005), and the high *LHY* levels are reportedly needed to obtain full freezing tolerance (Ibanez *et al.*, 2010).

The florigen FT, which is regulated by the circadian clock though CO, has been shown to have an additional function in bud set in *Populus* (Bohlenius *et al.*, 2006). Trees overexpressing *PtFT1* are incapable of setting bud under inductive short-day conditions, whereas trees with reduced *PtFT* and *PtCO*

expression set buds faster than wild type (Bohlenius *et al.*, 2006). In other words, *FT* must be down-regulated for short-day-induced growth cessation and bud set to occur in *Populus*. The other FT paralog, *PtFT2*, was later shown to be down-regulated during growth cessation and bud set under natural conditions (Hsu *et al.*, 2011). The difference in critical day length for trees originating from different latitudes can be explained by variations in the phase of expression of *PtCO*, i.e., northern trees exhibit a later peak than southern trees, and thus set bud at a longer day length (see Section 2.5 for a discussion on the link between CO and FT) (Bohlenius *et al.*, 2006).

Hypothetically, photoreceptors in the leaf could induce the production of *PtFT* through PtCO, which in turn could move to the apex. As long as the apex is supplied with PtFT, it continues growing, but when the PtFT supply diminishes, it stops growing and eventually sets bud, explaining why PtFT may be both necessary and sufficient to promote active growth.

To sum up, FT has at least two important functions in *Populus* - to induce flower initiation and prevent growth cessation.

2.11.4 Dormancy

At the start of dormancy, it is possible for the bud to revert to active growth under favorable conditions without a period of cold. This state is called ecodormancy. Later in the process, the bud needs a period of prolonged chilling to be able to flush, which is known as endodormancy or deep dormancy (reviewed in (Rohde *et al.*, 2000)). Chilling usually refers to temperatures below 10 ºC, and the optimum chilling temperature is usually 2-4 ºC (reviewed in (Battey, 2000)). Once the required amount of chilling (a cumulative sum) is reached, the trees once again revert to an ecodormant state and stay as such until a feasible temperature or temperature sum, is reached for bud flush (reviewed in (Rohde & Bhalerao, 2007; Rohde *et al.*, 2000)) regardless of the day length (reviewed in (Wareing, 1956)).

The fact that bud burst is not day-length but temperature dependent in *Populus* suggests that temperature has two opposing roles in dormancy release and budburst (Heide, 1993); dormancy release requires low temperatures, whereas increasing temperatures promote growth when the chilling requirements are fulfilled (Heide, 1993).

As previously mentioned the circadian clock, which is active in both stem and leaf, was found to be out of order at +4 ºC in chestnut and *Populus* (Ibanez *et al.*, 2010; Ramos *et al.*, 2005). The buds of *P. tremula* burst in the first week of May in southern Norway (Ås) (Heide, 1993), where the average temperature during that period is quite close to +4 ºC. In Ås, the average monthly temperature for April and May (1961-1990) was +4.1 ºC and +10.3 ºC,

respectively (http://www.yr.no/place/Norway/Akershus/%C3%85s/%C3%85s~60637/statistics.html). Thus, depending on the exact temperature when the circadian clock starts cycling again, it is possible that day-length sensing is not even an option for the timing of bud burst. However, long days significantly increase bud flush at any given flushing temperature (9 ºC, 15 ºC, 21 ºC) for *Betula*, *Populus* and *Alnus* (Heide, 1993).

Optimizing bud set and bud burst to the environmental conditions under which the tree grows, gives it the longest possible growth period and a competitive advantage (Wareing, 1956). Although temperature may seem a risky method to base such an important decision as budburst on, it has the advantage that the tree is ready to start accumulating the sun's energy as soon as the weather starts to improve in the spring, even if it's critical day length is not reached.

The *Populus TFL1* homolog *PopCEN1* has been shown to control the amount of chilling needed before bud flush. Trees overexpressing *PopCEN1* need a lot longer period of chilling than wild type trees, and trees with reduced levels of *PopCEN1* and *PopCEN2* need a much shorter period of chilling than wild type. Thus, TFL1 seems to have an important function in *Populus* dormancy release, as well as in inhibiting reproductive or promoting vegetative growth (Section 2.11.2) (Mohamed *et al.*, 2010).

In this context, it is important to consider that *Populus* produce two types of shoots. The short shoot, has a pre-determined size (it is pre-formed inside the bud) and grows independently of photoperiod; buds will burst even in continuous darkness (reviewed in (Wareing, 1956)), but if days are extremely short it will not elongate at all, else it will. Note: trees overexpressing the *Populus TFL1* homolog *PopCEN1* don't extend shoots after flushing (Mohamed *et al.*, 2010). Gene expression studies of leaves during bud burst have indicated that the trees seem to be mainly under a developmental program until one month after bud burst, at which time environmental input becomes more important again (reviewed in (Jansson & Douglas, 2007)).

Some of the shoots, especially in the top of the crown and on young trees, will not terminate growth but continue to elongate into long shoots. However, for that to happen, long days seem to be required else growth cessation is induced. Thus, *Populus* can break its buds on short days as soon as suitable temperature conditions are reached, but it will stop growth and set bud again, entering ecodormancy, if long days do not occur during the time the short shoots expands and possibly elongates (Klintenäs and Böhlenius, unpublished results). Long shoots will continue to grow in long days.

2.11.5 Polycarpic growth causes heteroblasty in *Populus*

When Arabidopsis starts to flower, its shoot apical meristem converts to an inflorescence where the lateral primordia develop into flowers. Since wild-type Arabidopsis is incapable of reverting to vegetative growth, it continues flowering until senescence. The majority of perennials, including all trees, only commit some meristems to reproductive development, whereas other meristems maintain vegetative growth during and after flowering. Thus, trees can flower and set seeds repeatedly during their lifetime. This is called polycarpic growth (reviewed in (Bergonzi & Albani, 2011)).

Studies on different annual and perennial herbs have provided some insights into the effect of vernalization and the prevention of all mature meristems flowering once inductive environmental signals are perceived, something that would efficiently obstruct perennial growth.

Arabidopsis FLC is a MADS-box transcription factor that has been found to activate the same genes that are targeted by miR172; the AP2-like flower repressors SMZ and TOE3 (Fig. 4) (Deng *et al.*, 2011). High *FLC* expression occurs naturally in many winter annual Arabidopsis accessions. These elevated *FLC* levels render the plant unable to respond to environmental flower induction (reviewed in (Baurle & Dean, 2006)). Thus, it behaves as a juvenile plant prior to vernalization. *FLC* reduction and flower induction through vernalization have been thoroughly studied in *Arabis alpina*, ecotype Pajares. In *A. alpina*, the *FLC* ortholog *AaPEP1* is reduced during vernalization in both young and old plants (Wang *et al.*, 2011b). Despite this, the young plants do not flower (in contrast to young Arabidopsis plants with reduced *FLC* levels) due to high *AaTFL1* expression which prevents *AaLFY* expression (Wang *et al.*, 2011b). AaTFL1 is also involved in extending the duration of vernalization needed for flower induction to occur (Wang *et al.*, 2011b). After vernalization, *AaPEP1* expression increases again, blocking flowering in meristems where it is not already initiated, thus contributing to its perennial life cycle (Wang *et al.*, 2011b). This relationship is not conserved in Arabidopsis, where *FLC* expression levels stay low after vernalization (reviewed in (Wang *et al.*, 2011b)).

No *FLC* ortholog has been identified in *Populus trichocarpa* (Brunner & Nilsson, 2004), but it is likely that regulation of meristem commitment to flowering occurs in a similar way and it might involve TFL1. Decreasing levels of *TFL1* in both *Populus* and apple have been found to induce early flowering and are suggested to have a role in the juvenility-to-maturity phase shift in these woody perennial species (Mohamed *et al.*, 2010; Kotoda *et al.*, 2006).

Many plants e.g. Arabidopsis exhibit heteroblasty, a condition where juvenile and mature traits appear simultaneously on the same plant because different parts of the plant exist in different developmental phases (reviewed in (Huijser & Schmid, 2011)). This is usually obvious in trees. Shoots initiated when the plant was juvenile often keep producing leaves with juvenile traits, whereas shoots created after the plant becomes adult exhibit adult traits. Thus, shoots close to the base of the tree behave and look juvenile (for example, they don't flower and it is possible to take cuttings from them, which is normally not possible from the branches at the top of the tree), whereas shoots at the top of the tree behave and look adult (reviewed in (Poethig, 2010; Brunner & Nilsson, 2004)). Grafting top and bottom shoots from a mature apple tree on the same stock will result in flowering on the graft from the top, but no flowering on the graft from the bottom (juvenile part) of the tree. Thus, the shoots express a stable developmental state even when removed from their original position (reviewed in (Poethig, 2010)).

The same phenotypes of heteroblasty are also exhibited due to phenotypic plasticity in response to growth conditions (reviewed in (Huijser & Schmid, 2011)), which sometimes makes it hard to distinguish juvenile/mature phenotypes from growth-/stress-induced phenotypes. Possibly this could explain why the small round dark green leaf phenotype naturally found in *Populus tremula x tremuloides* male clone T89 (hereafter referred to as T89) in the second growth season (Fig. 2a in Paper I) and onwards frequently occur in the first growth season if the T89 plants are cut and kept in a greenhouse for too long (unnaturally extended growth season) (Fig. 14b).

3 Objectives

This thesis contributes to the understanding of genetic regulation of the length of the juvenile period in trees, the regulation of phenology in *Populus* as well as the evolutionary aspect of the florigen gene *FT*.

Several questions were addressed:

Paper I: Does native *Populus* miR156 have a function in the juvenility-to-maturity switch or vegetative phase change in *Populus*? Does *Populus* miR156 have an effect on phenology or other novel functions in trees not yet detected?

Paper II: Are there *FT*-like genes in gymnosperms? Ectopic or conditional *FT* expression has already been suggested as a tool to shorten the generation time in many species, e.g., *Populus*, soybean, tobacco and Chinese bitter orange breeding (Yamagishi & Yoshikawa, 2011; Zhang *et al.*, 2010; Lewis & Kernodle, 2009; Endo *et al.*, 2005). Could ectopic or conditional *FT* expression be an option for gymnosperm breeding?

Paper III: What is the function of the *Populus* homolog of the FT inhibitor TEMPRANILLO? Does the *Populus* homolog of TEM have an effect on phenology in *Populus*?

4 Results and discussion

To avoid confusion in this chapter Arabidopsis, *Populus trichocarpa* and *Populus tremula x tremuloides* genes and proteins will be identified by an At, Pt and Ptt prefix, respectively.

4.1 Paper I (*miR156* overexpression in transgenic *Populus tremula x tremuloides* reveals novel aspects of miR156 regulation in trees.)

Paper I addresses the juvenility-to-maturity switch in *Populus*.

4.1.1 Construction of *PttmiR156e* overexpressing *Populus tremula x tremuloides*

As previously mentioned (Section 2.10), there are ten miR156 in Arabidopsis (AtmiR156a-j). Schwab *et al.* (2005) described how overexpression of several of the family members causes similar phenotypes to overexpression of *AtmiR156b* but with differences in severity of the phenotype. Overexpressing *AtmiR156* in Arabidopsis results in prolonged expression of juvenile vegetative traits and late flowering as discussed above (Section 2.10). Similar phenotypes have been described for *Populus* overexpressing *AtmiR156b* (Wang *et al.*, 2011a). The most obvious phenotypes described for *Populus deltoides x nigra* trees overexpressing *AtmiR156b* is that they are shorter and have smaller pale-green leaves, but they have also been reported to have a faster leaf initiation rate than wild type (Wang *et al.*, 2011a). At an age of six months, *35S::AtmiR156b*-overexpressing trees resembled one-month-old wild-type *Populus deltoides x nigra* (Wang *et al.*, 2011a). Overexpression of *miR156* in rice (OsmiR156) has been shown to cause severe dwarfism, increased number of tillers and delayed flowering (Xie *et al.*, 2006).

At an early stage of our investigations, prior to the publication of Schwab *et al.* (2005), we were informed that over-expressing a short fragment of *AtmiR156c* (132 nucleotides), basically consisting only of the miRNA stem-loop, was sufficient to cause over-expression phenotypes e.g. late flowering in Arabidopsis (personal communication Ove Nilsson - Rebecca Schwab). As my objective was to investigate the regulation of juvenility in *Populus* and the role of miR156 in this process, I generated trees that overexpressed the stem-loop sequence of the *Populus* homolog of AtmiR156c in *Populus tremula x tremuloides* (T89).

There are eleven miR156 in *Populus trichocarpa* (PtmiR156a-k), of which six (PtmiR156a-f) have the same targeting site (UGACAGAAGAGAGUGAGCAC) as AtmiR156a-f (www.mirbase.org) (Fig. 11).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
AtmiR156a-f	U	G	A	C	A	G	A	A	G	A	G	A	G	U	G	A	G	C	A	C
PtmiR156a-f	U	G	A	C	A	G	A	A	G	A	G	A	G	U	G	A	G	C	A	C
PtmiR156g-j	U	G	A	C	A	G	A	A	G	A	U	A	G	A	G	A	G	C	A	C
PtmiR156k	U	G	A	C	A	G	A	A	G	A	G	A	G	G	G	A	G	C	A	C
AtmiR156g	C	G	A	C	A	G	A	A	G	A	G	A	G	U	G	A	G	C	A	C
AtmiR156h	U	G	A	C	A	G	A	A	G	A	A	A	G	A	G	A	G	C	A	C
AtmiR156i	U	G	A	C	A	G	A	A	G	A	G	A	G	A	G	A	G	C	A	G
AtmiR156j	U	G	A	C	A	G	A	A	G	A	G	A	G	A	G	A	G	C	A	C
		*	*	*	*	*	*	*	*	*		*	*		*	*	*	*	*	

Figure 11. Mature sequence of the miR156 families in Arabidopsis and *Populus*.

Phylogenetic studies and sequence alignment analysis (Fig. 12a-c) indicated that *PtmiR156c* (located on chromosome six) and *PtmiR156e* (located on chromosome 18) were the closest homologs of *AtmiR156c*. Thus, I constructed overexpressing lines of both PttmiR156c and PttmiR156e in T89, consisting almost entirely of the stem-loop sequence (Fig. 12d) and using T89 as a template for amplification. Initially, I phenotyped trees expressing both constructs, but since I found a stronger phenotype in trees expressing *35S::PttmiR156e*, we decided to focus on this aspect in Paper I.

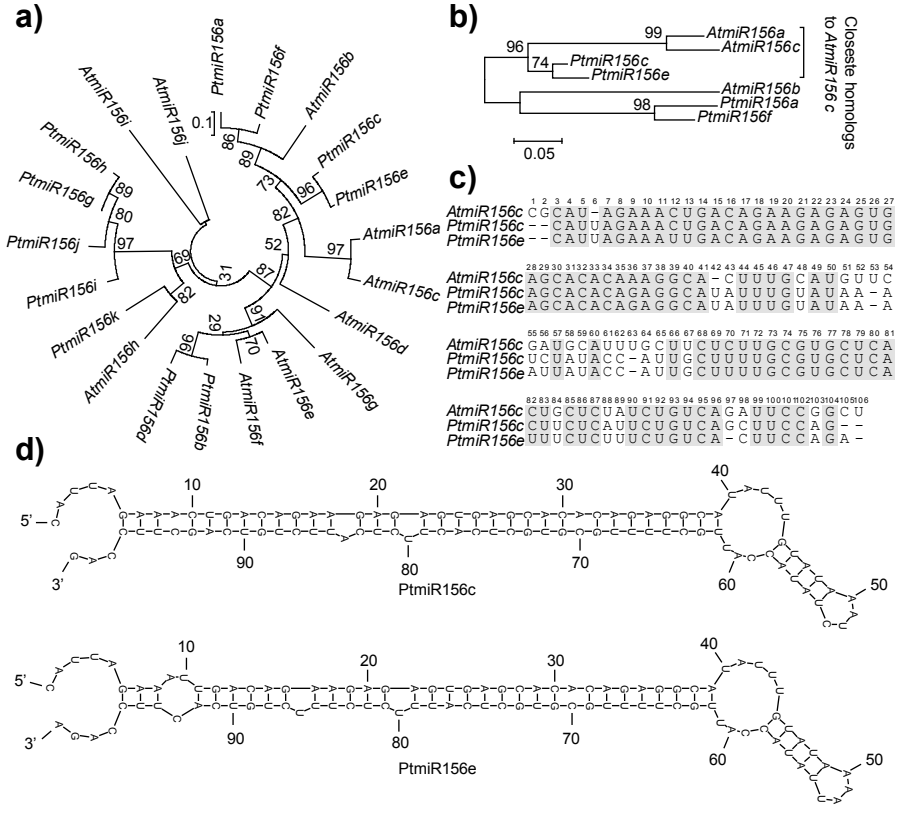

Figure 12. Determination of the *Populus* sequence homologs of *AtmiR156c*. (a, b) Phylogenetic reconstruction of the miR156 family in Arabidopsis and *Populus trichocarpa.* Stem-loop nucleotide sequences were aligned with CLUSTAL W (Thompson *et al.*, 1994), and phylogeny was inferred using the MEGA5 (Tamura *et al.*, 2011) neighbor-joining method (K2), employing pairwise deletion with 1,000 bootstrap replications. (a) *AtmiR156a-j* and *PtmiR156a-k*, (b) *AtmiR156a-c*, *PtmiR156a, c, e* and *f*, (c) nucleotide alignment (CLUSTAL W (Thompson *et al.*, 1994)) of *AtmiR156c*, *PtmiR156c* and *e* (nucleotides identical for all three genes are shaded), (d) folding of PtmiR156c and e stem-loops using mfold (http://mfold.rna.albany.edu) (Zuker, 2003).

4.1.2 Quantifying mature PttmiR156

To examine the levels of mature PttmiR156 in wild type and transgenic T89 using QPCR, we employed a specific protocol for RNA extraction and cDNA synthesis. Details on how to run QPCR on mature PttmiR156 of only 20 nucleotides are provided in figure 13.

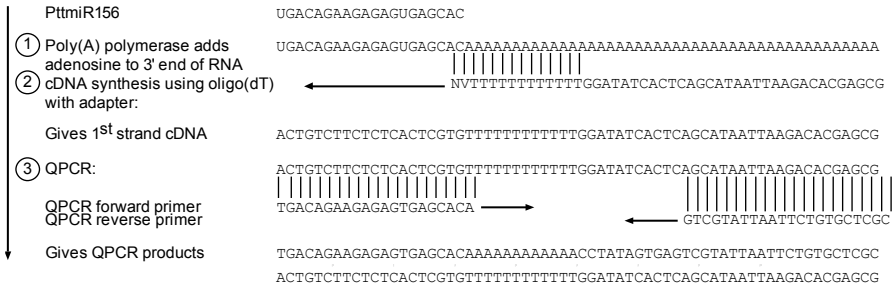

Figure 13. Polyadenylation (1), cDNA synthesis (2), and QPCR amplification (3) of mature miR156. Between step 1 and 2, there is a second RNA purification step involving phenol:chloroform extraction and ethanol precipitation to remove remnants from the DNase and polyadenylation treatments. Using a mixture of random hexamers and oligo(dT) with adapter primers, cDNA with acceptable lengths can be synthesized from both small RNAs and mRNA. The forward QPCR primer is identical to miR156, while the reverse primer is complementary to the adapter. Thus, cDNA from all small RNAs will match the reverse primer and only the forward primer is specific for miR156.

Using QPCR, I observed a trend towards lower expression of mature PttmiR156e in T89 during its second compared to its first growth season (Fig. 1 in Paper I), as seen previously when comparing juvenile and adult trees of ivy, eucalyptus or oak (Wang *et al.*, 2011a). In addition, I saw a significant increase in mature PttmiR156 in the transgenic trees expressing the *35S::PttmiR156e* construct, indicating that the construct functions as expected (Fig. 1 in Paper I). I selected three lines with increased PttmiR156e levels for our study. Although, the three chosen lines had different levels of miR156, they had indistinguishable phenotypes, indicating that the level of miR156 was sufficient in all three constructs to saturate the response.

I also tried to examine the levels of miR172 in both wild-type and *35S::PttmiR156e* trees to determine whether they increased between the first and second growth season, or were affected by *PttmiR156e* overexpression. However, the levels were below the detection limit for the method used in both cases.

4.1.3 Genotype of *35S::PttmiR156e* trees

By blasting the SBP-box domain against the *Populus trichocarpa* genome (http://www.phytozome.net/) as well as nr/nt database on NCBI (http://blast.ncbi.nlm.nih.gov), I identified 32 *SPL* genes in poplar, of which 18 were shown to have a target site for PtmiR156 using psRNATarget (http://plantgrn.noble.org/psRNATarget/) (Figs. S2 and S3 in Paper I). The putative targets were found to have recognition/target sites that varied at a maximum of two positions (Fig. S3 in Paper I), while one of the Arabidopsis

targets, *AtSPL5*, had three mismatches. In addition, mismatches were only found in the same positions as commonly observed in Arabidopsis (Fig. S3 in Paper I).

In Arabidopsis, all predicted AtmiR156 targets (*AtSPL2*, *AtSPL3*, *AtSPL4*, *AtSPL5*, *AtSPL6*, *AtSPL9*, *AtSPL10*, *AtSPL11*, two *AtSPL13* and *AtSPL15*) (Gandikota *et al.*, 2007; Rhoades *et al.*, 2002a) have been experimentally verified, as being down-regulated in response to *AtmiR156* overexpression (Schwab *et al.*, 2005).

I chose to examine the expression of seven of the predicted targets in *Populus*, representing four of the six targets sites present in *Populus SPL* genes (Fig. S3 in Paper I). Only four (*PttSPL2c*, *PttSPL3*, *PttSPL4a* and *PttSPL9*) were found to be down-regulated in *miR156*-overexpressing trees (Fig. 6 in Paper I). Similar results have been described by Xie *et al.* (2006), who found that six of the predicted target *SPL* genes were down-regulated in rice overexpressing *OsmiR156*, whereas three other of the predicted targets did not show a change in transcription level. Xie *et al.* (2006) also found that different members of the OsmiR156 family interacted with different targets in different tissue. They suggested that the targets not apparently down-regulated in the tested tissues by the two rice miR156 versions they investigated, could be targeted in other tissues not examined or by other members of the OsmiR156 family (Xie *et al.*, 2006). The same might be true for the predicted, but not yet experimentally verified, *SPL* targets in *Populus* presented in Paper I.

SPL3 and *SPL9* expression in Eucalyptus and *P. trichocarpa* has been shown to be higher in adult than in juvenile trees (Wang *et al.*, 2011a). In addition, *AtSPL3*, *AtSPL4*, *AtSPL5* and *AtSPL9* increase with age in Arabidopsis (Wang *et al.*, 2009b; Wu & Poethig, 2006; Cardon *et al.*, 1999; Cardon *et al.*, 1997). All miR156-targeted *SPL* genes in Arabidopsis are induced during flower induction (0, 3, 5, 7 days after a move to long days), the induction of *AtSPL3*, *AtSPL4* and *AtSPL5* being considerably stronger than the rest (Schmid *et al.*, 2003). In contrast, we found no up-regulation of the *PttSPL* genes during the first and second growth seasons in T89 (Fig. 6 in Paper I). This is not unexpected considering the slight difference in mature miR156 levels that we observed between the first and second growth seasons (Fig. 1 in Paper I) and is most likely due to the small age difference in our samples.

There was not a full year between the first- and second growth season samples. The samples from the first growth season were picked in the "autumn" during long days, before bud set and the second set of samples were picked in the "spring" during bud burst under long day conditions. Although fresh uncut plants were used for both, we did not use the same set of trees for "autumn" and "spring" samples. The trees sampled in their first growth season

were sampled about 2.5 months after potting at a height of ~120 cm. The trees sampled in their second growth season were sampled about 5.5 months after potting at a height of ~90-100 cm, as they were moved to 8 h short days already about one month after potting. Thus, the difference in time was only about three months and the older trees in their second growth season were in fact shorter than the younger trees. Another explanation is provided by Yang *et al.* (2011), who proposed that there is a leaf-primordia-produced signal that reduces the miR156 level in Arabidopsis. If a leaf produced signal in *Populus* cause down-regulation of miR156, it is not surprising that we saw such a small decrease in miR156 levels early in the second growth season since the trees sampled during bud burst had hardly more leaves than before bud set.

In addition to examining the expression levels of *SPL* genes, we also investigated the downstream target *SOC1*. We showed that the *P. tremula x tremuloides SOC1* homolog *PttMADS5* (XM_002302516.1) is down-regulated in *35S::PttmiR156e* trees. This confirms the data presented by Wang *et al.* (2011a), who examined the *SOC1* homolog *PcMADS4* (XM_002320711.1) levels, although they erroneously classified it as *PcFUL* (personal communication Ove Nilsson - Jia-Wei Wang). These findings also suggest a conserved function for *Populus* miR156 and Arabidopsis miR156 since SPL9 has been proposed to bind to the promoter of *SOC1* to induce expression in Arabidopsis (Wang *et al.*, 2009b).

PttFT2 expression

Tomato plants overexpressing the tomato miR156a partly phenocopy the (tomato *FT* ortholog, *SINGLE FLOWER TRUSS* (*SFT*)) *sft* mutant (Zhang *et al.*, 2011). This is expected considering Arabidopsis FT is a target of miR156 through the action of miR156-repressing *SPL9*. If *SPL9* is not repressed, it inhibits the AP2-like *FT* repressors through its activation of miR172 (Fig. 4).

Surprisingly, our results clearly indicated that *PttFT2* is up-regulated in *35S::PttmiR156e* trees (Fig. 5 in Paper I). To confirm that this was not an artifact, we investigated the relationship for a second short age series by measuring expression levels in both young (as in figure 5 in Paper I) and older (source) leaves, where *FT* is more highly expressed. Although the absolute values varied in the different experiments, the relationship was clear; *PttFT2* levels were strongly elevated in the *35S::PttmiR156e* trees. The mechanisms underlying this regulation have not yet been determined, but our results suggest an interesting difference in the regulatory networks between Arabidopsis and *Populus*.

4.1.4 Phenotypes of *35S::PttmiR156e* trees

Despite the fact that only four of the examined *PttSPL* genes could be verified as being targets of PttmiR156e, we found a wide range of deviating phenotypes in *PttmiR156e*-overexpressing trees. Many of them correlated very well with phenotypes previously described in Arabidopsis or trees overexpressing *AtmiR156b*, as well as exhibiting delayed aging in *PttmiR156e*-overexpressing trees.

The most striking phenotype observed for the *35S::PttmiR156e* trees was that they were completely covered in trichomes (Figs. 10, 14e and Figs. 3b, e and f in Paper I) on the stem, petioles and both sides of the leaves. Trichomes are epidermal cells produced to protect plants from small herbivores, excessive transpiration and UV light among others (reviewed in (Wagner *et al.*, 2004)).

Trichome production has been studied in *AtmiR156*-overexpressing Arabidopsis (but not previously reported for *Populus*-overexpressing *AtmiR156b*). In juvenile Arabidopsis, trichomes are evenly distributed on the adaxial (upper) side of the leaf, and the transition from juvenile-to-adult coincides with the initiation of trichome production on the abaxial side of the leaves (Telfer *et al.*, 1997). Once the plant enters the reproductive stage, the number of trichomes produced on the main inflorescence stem gradually decreases and almost glabrous floral organs develop (Yu *et al.*, 2010). Yu *et al.* (2010) have shown that AtmiR156 and *AtSPL9* establish a direct link between developmental programming and trichome distribution.

Wild-type tissue-cultured-amplified T89 trees are also covered in trichomes on stem, leaf and petiole when potted, but stop producing new trichomes by about four-six weeks after potting or at a height of 60-80 cm. Thus, there is a clear difference in the trichome-juvenility correlation between Arabidopsis and *Populus*, as adult Arabidopsis leaves have abaxial trichomes and juvenile do not, whereas the opposite occurs in T89. However, even though the *Populus* and Arabidopsis trichomes distribution patterns are not equivalent, there still seems to be a strong correlation between miR156 expression and juvenile phenotype with respect to trichomes in *Populus tremula x tremuloides*. Contrary to wild-type T89 trees, *35S::PttmiR156e* trees produce trichomes on stem and leaves at least up to heights of about 160 cm.

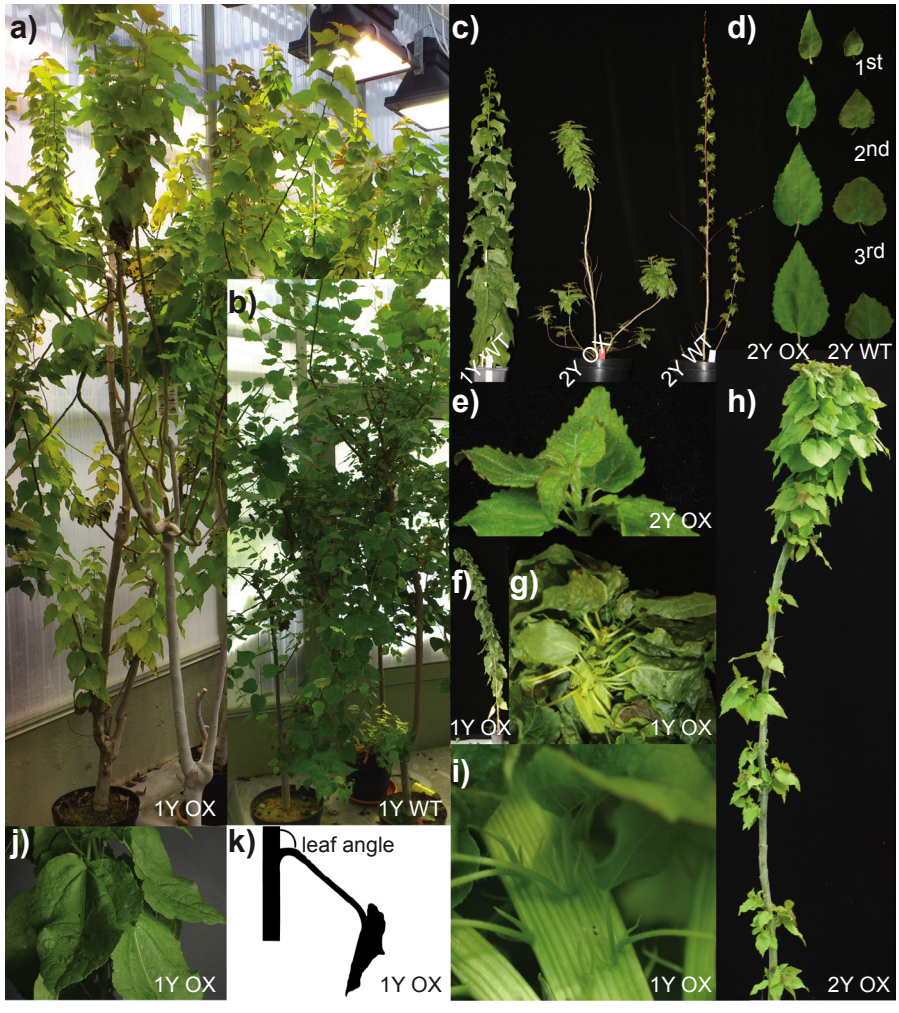

Figure 14. Phenotypes of *35S::PttmiR156e* trees. (a, b) Trees grown in a greenhouse for almost four years, but never entering dormancy, are still technically in their first growth season (1Y), (a) *35S::PttmiR156e* (OX) and (b) wild-type T89 (WT). (c) Full-length photograph of a wild-type tree grown under long-day conditions during its first season (1Y) alongside OX and wild-type trees during bud burst (second season, 2Y) after a period of dormancy. (d) Close-up of leaves from the short shoots of OX and wild type during bud burst showing the 1st, 2nd, 3rd and 4th completely unfolded leaf on the shoot. (e) Close-up of a short shoot of *35S::PttmiR156e* during the second growth season showing how it is still covered with trichomes. (f-i) Fasciation in *35S::PttmiR156e* trees (f, g) during bud set under 14 h short-day conditions. (h) Bud burst of the fascinated shoots giving the top of the plant a folding fan like appearance. (i) Fasciation initiated under long-day conditions. (j, k) Leaf shape and leaf angle of young *35S::PttmiR156e* trees growing in its first season.

The idea that down-regulation of miR156 is regulated by a signal from the leaf primordia (Yang *et al.*, 2011) or leaf, agrees reasonably well with the

phenotypes we observed in older and taller *35S::PttmiR156e* trees. Repeatedly cut *35S::PttmiR156e* trees grown over an unnatural almost four-year-long first growth season, in which they reached heights of 220-260 cm (Fig. 14a), still produced stem trichomes and exhibited leaves with abaxial trichomes on the mid nerve sporadically at high heights, but fairly evenly at lower heights and on short branches. Trees grown for this long need to be cut regularly to fit into the greenhouse. Thus it is possible that the leaves in the top of the crown with abaxial trichomes on the mid nerve were initiated by a cutting event of the tree, which then might have resulted in higher miR156 levels. Wild-type trees grown under the same conditions are completely hairless on stem, leaf and petioles (Fig. 14b and Figs. 3b, e and f in Paper I).

35S::miR156 trees growing in their second season (approximate height > 100 cm) initially only produced stem and leaves covered with trichomes (wild-type T89 did not produce trichomes during the second season), (Fig. 14c), but as they were allowed to grow, they produced some leaves without abaxial trichomes on the mid nerve.

In conclusion, there was no apparent correlation between the presence of leaves without abaxial trichomes on the mid nerve formed by *35S::PttmiR156e* trees with age (either counted as days/years since potting or the number of winters passed). However, there does seem to be a correlation with height of the plant and to some extent also length of the branches. It is quite possible that the number of leaves present on the tree at the time of leaf initiation would correlate better with the amount of abaxial trichomes on the mid nerve.

Although trichomes are supposedly a protective mechanism, I noticed that the *35S::PttmiR156e* trees were often severely infected with powdery mildew, in stark contrast to wild-type T89, which normally coped with greenhouse conditions quite well. Probably, the hairy surface makes it easy for the mildew to stick to the leaves.

It should be mentioned that formation of the stem trichomes of wild-type T89 plants ceases a couple of weeks (~four weeks after potting) before the abaxial trichomes (~six weeks after potting). Thus, the fact that tall *35S::PttmiR156e* trees (Fig. 14a) stop producing abaxial trichomes on the mid nerve of leaves but still produce large amounts of trichomes on the stem indicates that they are regulated in different ways.

Increased leaf initiation rates, as seen in our *35S::PttmiR156e* trees (Fig. 14c and Fig. 2b in Paper I), have previously been reported for *35S::AtmiR156f*-expressing Arabidopsis (Wang *et al.*, 2008) and *35S::AtmiR156b*-expressing *Populus* (Wang *et al.*, 2011a). Plastochron is thought to be affected by *SPL9* and *SPL15* since *spl9 spl15* double mutants also exhibit a fast leaf initiation rate, although not as fast as *35S::AtmiR156f* plants (Wang *et al.*, 2008). Both we and Wang *et al.* (2011a) observed down-regulation of *SPL9*, in addition to an increased leaf initiation rate. Therefore, this may indicate a conserved function for SPL9 in Arabidopsis and *Populus*.

Reduced height, as seen in our *35S::PttmiR156e* trees (Fig. 2a and Table 1 in Paper I), and even dwarf phenotypes have been observed previously in rice and maize overexpressing rice and maize miR156 as well as *Populus deltoides x nigra* expressing *35S::AtmiR156b* (Wang *et al.*, 2011a; Chuck *et al.*, 2007; Xie *et al.*, 2006).

Thus, both a positive effect on leaf initiation rate and a negative effect on height seem to be highly conserved features of miR156.

My results on leaf size, shape and color undoubtedly show that *35S::PttmiR156e* trees are more juvenile than wild-type trees at the same time after potting. The leaves of *35S::PttmiR156e* trees were lighter in color (Fig. 14a and Fig. 3a in Paper I) and clearly had a more juvenile phenotype during the second growth season than the wild type (Figs. 14d and e and Fig. 3a in Paper I); the former were larger, lighter and more oblong and were thus more juvenile in size, shape and color. As the wild-type leaves from short shoots on plants grown in their second season mature, they become a lot darker in color and retain an almost round shape (Fig. 4d and Fig. 3a in Paper I).

Apart from the extensive coverage of trichomes I interpret the severe leaf phenotypes of the second growth season as proof of the *35S::PttmiR156e* trees extended juvenile period.

Sylleptic branching or axillary shoots were a common feature of all *35S::PttmiR156e* trees (Table 1 in Paper I). As can be seen in figure 14c, there were almost no lateral buds left to break after winter dormancy in the *35S::PttmiR156e* trees since most had already broken in the first growth season. This agrees with the reduced apical dominance that has previously been observed in *AtmiR156*-overexpressing Arabidopsis (Shikata *et al.*, 2009; Schwarz *et al.*, 2008; Schwab *et al.*, 2005) and *AtmiR156b*-overexpressing *Populus deltoides x nigra* (Wang *et al.*, 2011a) as well as rice overexpressing *OsmiR156* (Xie *et al.*, 2006) and maize overexpressing *Zma-miR156b/c* (Chuck *et al.*, 2007).

Most sylleptic branches died, and many fell off after extending to about ten cm in length (Fig. 14c), possibly due to lack of light. The *35S::PttmiR156e* trees displayed extremely wide leaf angles (Fig. 14k), large, slightly miss-formed, "cupped" leaves (Fig. 14j) in addition to short internodes (Fig. 2b and Table 1 in Paper I), a combination which effectively covered the stem and young axillary shoots.

35S::AtmiR156 and *spl9 spl15* double mutants in Arabidopsis both exhibit a reduced response to photoperiodic shifts, requiring a longer period of inductive long days than wild-type Arabidopsis to induce flowering (Section 2.10) (Schwarz *et al.*, 2008). I found that hybrid aspen overexpressing *PttmiR156e* also had a reduced response to photoperiodic shifts.

When moved from long days to short days, growth cessation and bud set was delayed compared to wild type (Fig. 4a in Paper I). This agrees well with my result that the *35S::PttmiR156e* trees exhibited highly elevated *PttFT2* levels (Section 4.1.3), and the fact that *PttFT* is known to inhibit bud set in *Populus* (Hsu *et al.*, 2011; Bohlenius *et al.*, 2006) (Section 2.11.3).

When moved back to long days after a dormant period, bud burst occurred earlier than for wild type (Fig. 14c and Fig. 4b in Paper I).

Another deviating phenotype seen quite regularly in *35S::PttmiR156e* trees but not observed in wild-type hybrid aspen was fasciation of the top shoot. Fasciation was most common under 14 h short days (Figs. 14f and g), when the majority (but not all) of the transgenic trees fasciated. For some reason, it did not occur under 8 h short days, but did occur in a few long-day grown plants (Fig. 14i).

When the short-day-induced fasciated plants were put back under long-day conditions after a period of dormancy, all top shoots burst and eventually the wide fasciated stem divided into a very large number of small stems (Fig. 14h).

4.1.5 Conclusion

Taken together, my results confirm that PttmiR156e in *Populus*, just like miR156 found in other species (Arabidopsis, rice and maize), affect height, plastochron, leaf phenotype, apical dominance, trichome formation, *SPL* and *SOC1* expression and the juvenility-to-maturity switch.

As previously discussed, a *Populus FT* ortholog is involved in short-day-induced growth cessation and bud set in *Populus* (Section 2.11) (Hsu *et al.*, 2011; Bohlenius *et al.*, 2006). Because of this, we were interested in investigating if other genes involved in maturation and flowering in Arabidopsis had an effect on phenology in *Populus*. In Paper I, I show that

both bud set and budburst are severely affected in *35S::PttmiR156e* trees, indicating that in *Populus*, similar genetic pathways are involved in the control of aging, flowering and phenology. I also propose that the *Populus miR156e* may have a role in maintaining the dormancy of the axillary buds as the *35S::PttmiR156e* trees exhibited extensive sylleptic branching.

4.2 Paper II (Analysis of conifer *FLOWERING LOCUS T/TERMINAL FLOWER1*-like genes provides evidence for dramatic biochemical evolution in the angiosperm *FT* lineage.)

Paper II addresses the evolution of the PEBP gene family in gymnosperms and angiosperms. *FT*- and *TFL1*-like genes have been thoroughly studied in a wide range of angiosperms, but knowledge about this PEBP gene family in gymnosperms is extremely limited.

In the work described in Paper II, I used sequence alignments and phylogenetic reconstructions to analyze the PEBP family from gymnosperms as well as ancient angiosperm lineages (Section 2.1). I showed that *FT*-like genes are extremely sequence conserved and present in all completely sequenced angiosperms, as well as the majority of partially sequenced angiosperms, including ancestral species like amborella and avocado (Fig. 1 in Paper II). Despite this, I found no evidence of *FT*-like genes in the available sequence material of gymnosperms; instead they contain a group not present in the angiosperm lineage, i.e., the *FT/TFL1*-like group (Fig. 1 in Paper II). It is not possible to conclude whether the *FT/TFL1*-likes are a sister group of the *FT*-like or the *TFL1*-like groups, or an ancestor to the two (Paper II). However, regardless of its evolutionary origin, its biochemical function is clearly not the same as FT (Paper II). Thus, our data presented in Paper II do not support the idea of the presence of *FT*-like genes in gymnosperms.

I identified a group of *MFT*-likes in moss and gymnosperms, named "ancestral *MFT*-likes" (Paper II), which, as their name suggests seem to be an evolutionarily older version of the MFT-likes found in angiosperms, although both moss and gymnosperms also possess the more modern version of *MFT*.

To further examine the *FT/TFL1*-like, *MFT*-like and ancestral *MFT*-like genes from pine (*Pinus*) and spruce (*Picea*), I expressed them heterologously in Arabidopsis.

We also analyzed the specific amino acids necessary for FT vs. TFL1 function and suggested two evolutionary models (Fig. 7 in Paper II) that explain the formation of true FT-like function in the angiosperm lineages.

4.2.1 The *FT* gene from an evolutionary perspective

Genes change by large-scale genomic/chromosomal/gene duplications or more frequently, by small-scale alterations, i.e., spontaneous mutations, causing insertions, deletions or point mutations that either result in a change in amino acid composition (non-synonymous (dN)) or not (synonymous (dS)).

Mutations can be harmful or deleterious, in which case, they are usually selected against (purifying or negative selection). However mutations can also be beneficial, which are usually positively selected for and may in the long term become established in the population, or neutral/near neutral, which do not give the individual either a benefit or disadvantage and thus are not much affected by selection (Kimura, 1968). The fact that *FT* genes are so extremely conserved in the angiosperm lineage (Figs. 1, 6 and 7 in Paper II) implies that there are very few positions, especially in exons two and four, where non-synonymous mutations are neutral to natural selection. A ratio between non-synonymous and synonymous substitutions close to one would indicate that there is no selective pressure acting on that DNA region (reviewed in (Yang & Bielawski, 2000)), but this was not found to be the case for the *FT* gene. Instead, the measured ratio for the *FT* gene indicates that it is under strong negative or purifying selection, removing deleterious alleles (Fig. 7 in Paper II).

4.2.2 *FT*-gene duplication

The *FT* gene has been subjected to several intraspecific gene duplications. In Arabidopsis, there are two *FT*-like genes (*FT* and *TSF*), rice has at least three *FT* paralogs (*Hd3a*, *RFT1*, *FTL*) that are involved in flowering promotion (Izawa *et al.*, 2002; Kojima *et al.*, 2002) and the legumes pea and burclover (*medicago*) each have five *FT* homologs (Hecht *et al.*, 2011; Laurie *et al.*, 2011).

Gene duplication initially generates two identical copies that either both preserve the original function or one may evolve freely without much pressure from natural selection until it becomes a non-functional pseudogene or acquires a different function. This has occurred frequently in *FT*-likes in different species. Degenerative mutations in duplicated genes can lead to diverging expression domains, where a function previously performed by only one gene is divided into two separate functions (sub-functionalization) regulated by the two new genes. This could be the case for the *Populus FT1* and *FT2* genes; it has been proposed that *PtFT2* is responsible for active growth whereas *PtFT1* is responsible for flower induction (Hsu *et al.*, 2011), although the mechanism behind this is not yet clear (Section 2.11.2). Neofunctionalization, in which one copy gains a new function, has been demonstrated in sugar beet where FT1 has acquired a flower-repressing function (Pin *et al.*, 2010), whereas loss-of-function in one copy has been found in sunflower (*Helianthus annuus*), where HaFT3 seems to have lost its function (Blackman *et al.*, 2010).

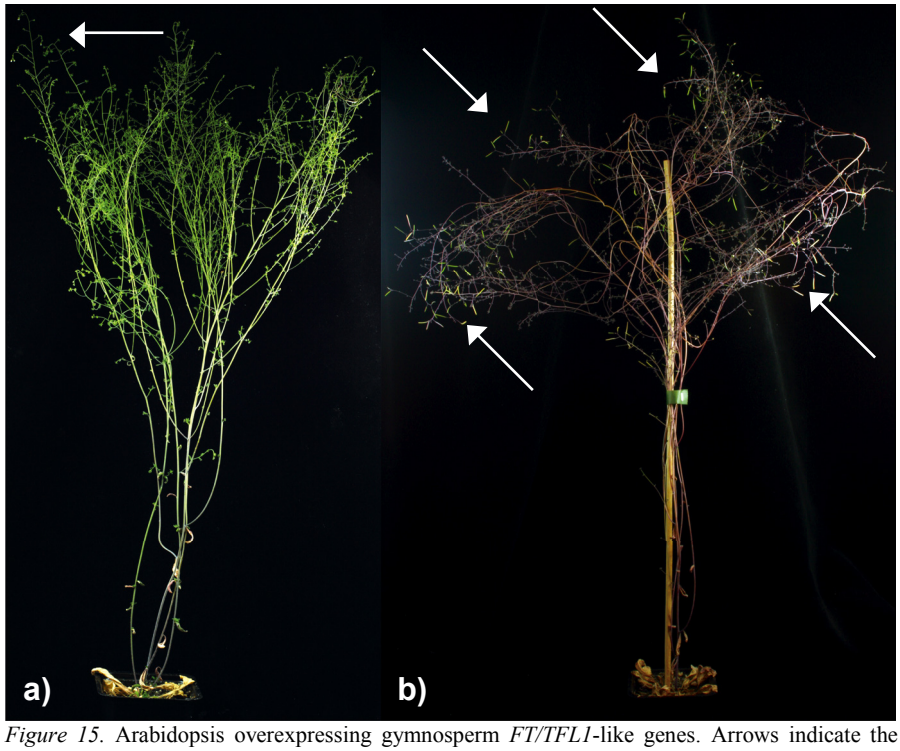

Figure 15. Arabidopsis overexpressing gymnosperm *FT/TFL1*-like genes. Arrows indicate the presence of flowers or siliques. Plants with severe overexpressing phenotypes like these grow very large before flowering, usually producing quite a lot of seeds in the end. However, some never flowered or took more than 7 months, and it was difficult to keep them in a healthy state for such a long time.

4.2.3 Are the *FT/TFL1*-like genes repressors of the vegetative-to-reproductive switch in gymnosperms?

Bradley *et al.* (1997) suggested that the mechanism for preventing terminal flowers arose very early during evolution. He based this on the fact that the sequence homologs *AtTFL1* and *Antirrhinum CENTRORADIALIS* (*CEN*) have the same function in preventing terminal flower formation, despite the fact that they belong to different groups of Eudicots, i.e., the rosids and asterids, respectively. On a somewhat similar subject, but concerning vegetative and perennial growth, it has been discussed that adulthood and flowering is the default developmental stage, and the aim of early plants was to gain the ability to postpone the vegetative-to-reproductive transition to gain more time for vegetative growth and possibly increase the chance of reproductive success (Hecht *et al.*, 2011; Baurle & Dean, 2006; Stebbins, 1974).

Gymnosperms apparently lack the FT function (Paper II) that is of such great importance to flowering in angiosperms, but they do seem to have TFL1 function in the form of *FT/TFL1*-like genes that inhibit the vegetative-to-

reproductive switch, at least when expressed in an angiosperm system (Figs. 15a and b and Fig. 5 in Paper II). This TFL1 function is however missing in moss and algae, which have neither *TFL1*-like nor *FT/TFL1*-like genes (Paper II). Is it possible that the native function of *FT/TFL1*-like genes is to inhibit the vegetative-to-reproductive switch in the gymnosperm system as well?

Naturally, since we still have neither mature *FT/TFL1*-like overexpressing nor *ft/tfl1*-like loss-of-function gymnosperms, we can't conclusively answer this question. It is possible that at the time of gymnosperm speciation more than 250 million years ago (Fig. 2) (Labandeira, 2010), the focus was still on repressing the vegetative-to-reproductive switch to allow for vegetative as well as perennial growth, and thus the FT/TFL1-like function, as we have seen it in Arabidopsis (Figs. 15a and b), would come in useful. About 120 million years later, after vegetative and perennial growth had become a factor of success, the FT-function as we know it would have become important in optimizing flowering, dividing the summer season between angiosperm species and timing flowering to specific pollinators. The TFL1 function would have still been useful in preventing premature flowering and terminal flower formation.

4.2.4 Could ectopic or conditional *FT* expression be an option for gymnosperm breeding?

We attempted to induce flowering in spruce by introducing Arabidopsis *FT* mRNA in shoot apices of spruce using a gene gun (http://skogstradsforadling.se/index.php/sidor/sektion/forskningsbidrag/projekt-176-200), and although there are several reasons for such an experiment being unsuccessful, we saw no increase in flowering following the treatment. There does not seem to be a native *FT* gene in gymnosperms (Paper II). Thus it wouldn't be surprising if the machinery used to propagate the FT signal is not present in gymnosperms. However, many of the MADS-box transcription factors involved in angiosperm flowering, e.g., the ABC genes, seem to be present in gymnosperms (Section 2.7). It is still not clear whether there is a *FD* gene present in gymnosperms or not.

It seems that an FD-TFL1 interaction is possible in angiosperms (see Section 2.9.3), but TFL1 probably enhances translational repression rather than activation (Ahn *et al.*, 2006; Abe *et al.*, 2005; Wigge *et al.*, 2005). FT and TFL1 have been suggested to act antagonistically to fine-tune the response to flower induction (Kardailsky *et al.*, 1999). Gymnosperms possess, if not *TFL1* itself, at least *TFL1* function seen from an angiosperm perspective in the *FT/TFL1*-likes. Speculating that *FT/TFL1*-likes have a somewhat conserved native biochemical function in gymnosperm and that there is a FD homolog in

gymnosperms, there is a small chance that it is just FT itself that is missing, but the machinery behind FT might be conserved after all.

However, FT may not be the best choice for increasing the rate of breeding in gymnosperms. Reducing the levels of *FT/TFL1*-like or overexpressing one of the homologs of the flower meristem identity genes *LFY*, *AP1*, *CAL* or *FUL* would be other options to induce "flowering" and speed up gymnosperm breeding. Recently overexpression of the birch gene *BpMADS4* in combination with marker-assisted selection has been used to increase the rate of breeding in apple (Flachowsky *et al.*, 2011).

4.2.5 Future perspectives

To follow up the results of Paper II, it would be interesting to examine the endogenous function of *FT/TFL1*-like genes by further evaluation of the *FT/TFL1*-like overexpressing plants (started in Paper II) as well as knocking out or reducing the expression of the *FT/TFL1*-likes in spruce.

4.3 Paper III (The *Populus* homologs of the Arabidopsis *TEMPRANILLO* genes are regulators of bud set and early bud dormancy.)

Paper III addresses the function of the *Populus* homologs of the *FT* repressor TEMPRANILLO.

The Arabidopsis *TEMPRANILLO* genes encode for transcription factors that belong to a family called RAV (related to ABI3/VP1) (Kagaya *et al.*, 1999). The *AtTEM* genes have been found to regulate the length of the juvenile phase, as well as directly repress *AtFT* (Section 2.4) (Castillejo & Pelaz, 2008). Loss-of-function *tem* mutants flower early under both long- and short-day conditions (Osnato *et al.*, 2012; Castillejo & Pelaz, 2008). Like *AtFT*, *AtTEM* has a diurnal pattern with peak expression in the early night (Castillejo & Pelaz, 2008), but in contrast to *AtFT*, *AtTEM* expression decreases with age in Arabidopsis (Castillejo & Pelaz, 2008). AtTEM1 has also been found to directly repress *AtGA3OX1* and *AtGA3OX2,* thus decreasing the levels of bioactive GA (Osnato *et al.*, 2012) (Fig. 4 and Section 2.8). *AtTEM1* is expressed under both long and short days, with higher expression levels being observed under short days (Osnato *et al.*, 2012).

Both its effect on *FT* and its ability to regulate the juvenile phase in Arabidopsis makes the *TEM* genes interesting candidates to investigate in *Populus*.

The phylogenetic reconstruction reveal that there is no clear *TEM* ortholog in *Populus* but instead two paralogous *RAV* genes (*RAV1* and *RAV2*), which share sequence characteristics with both the Arabidopsis *TEM1*/*TEM2* and the *RAV1*/*RAV1like* genes (Fig. 1 in Paper III). To characterize these genes, we generated trees with down-regulated levels of both *Populus TEM* homologs in *Populus tremula x tremuloides* (T89), called *PttRAV1&2* RNAi (Fig. 6 in Paper III).

The resulting phenotypes indicated that the *PttRAV1* and *PttRAV2* genes are primarily involved in regulating sylleptic branching and bud set (Table 1 and Fig. 9 in Paper III). Compared to the effect on *35S::PttmiR156e* trees, the effect on sylleptic branching in *PttRAV1&2* RNAi plants was less severe and significantly different from that of the wild type first around ten weeks after potting. Nevertheless, this is still an interesting result since it shows that *PttRAV1&2* inhibit sylleptic branching, whereas it was previously believed that *RAV1* would induce sylleptic branching, based on the results of Moreno-Cortes *et al.* (2012).

The *PttRAV1&2* RNAi plants did not set bud later than wild type, but I saw an extended bud set period and several cases of reverted growth, where a formed bud broke and started growing again; producing more leaves (Fig. 9 in

Paper III). In *Populus*, it is possible for the bud to revert into active growth without a period of cold at the start of dormancy (during ecodormancy), but in wild type, this only happens if the trees are moved back to favorable long-day conditions (Section 2.11.4).

For both sylleptic branching and bud set, we observed a correlation between severity of phenotype and efficiency of down-regulation, which supports the hypothesis that *PttRAV1* and *PttRAV2* have a function in bud set, preventing breaking of ecodormant buds and inhibiting sylleptic branching.

In addition to the above-mentioned phenotypes we saw a trend towards increased stem diameter and height in the *PttRAV1&2* RNAi plants (Table 1 in Paper III), indicating that *PttRAV1* and *PttRAV2* might have a role in inhibiting both primary and secondary growth in *Populus*. In Arabidopsis, *TEM* has been found to inhibit height growth as reduced levels result in plants with elongated hypocotyls, whereas increased levels cause dwarfism (Osnato *et al.*, 2012). Similarly, overexpression of soy bean *RAV* in tobacco and overexpression of chestnut *RAV1* in hybrid poplar result in reduced growth (Moreno-Cortes *et al.*, 2012; Zhao *et al.*, 2008).

Obtaining a clear result concerning the possible effects of *PttRAV1* and *PttRAV2* on *FT* proved harder than expected, mainly due to the high biological variation in expression of both *FT* and *RAV*. Throughout the experiments leading to Paper III, we aimed to examine *PttFT1*, *PttFT2*, *PttRAV1* and *PttRAV2* levels. *PttFT1* has very low expression levels, and there are contradictory data on the circadian expression peaks of *PttFT2*, *PttRAV1* and *PttRAV2*. In addition, the expression levels of the *AtTEM* genes have been found to be affected by touch, wounding, pathogen attack, cold temperature, darkness and drought in Arabidopsis (personal communication with Esther Marín-González and Soraya Pelaz). These factors would almost certainly contribute to the difficulties in obtaining stable biological replicates. As discussed in Paper III, it is possible, but remains to be proven, that PttRAV1 and PttRAV2 have a role in regulating the length of the juvenile phase and repressing *FT* in *Populus*.

In addition to examining the *PttRAV1&2* RNAi trees we studied the endogenous expression of *PttRAV1* and *PttRAV2* in wild type T89. Both genes are expressed in a variety of tissue (Fig. 2 in Paper III) and in agreement to *AtTEM* expression (Castillejo & Pelaz, 2008), both decrease with age in *Populus* (Fig. 5 in Paper III). In addition we found that *PtRAV1*, but not *PtRAV2* have a diurnal expression pattern (Fig. 3 in Paper III) and that this pattern closely resemblance that seen for chestnut *RAV1* (Moreno-Cortes *et al.*, 2012).

4.3.1 Conclusion

I have, for the first time, shown that *PttRAV1* and *PttRAV2* are inhibitors of sylleptic branching, possibly through an effect on the dormancy of the axillary bud. Interestingly, I also found that *PttRAV1* and *PttRAV2* affect the duration of the bud set period and inhibit bud-set reversion.

4.3.2 Future perspectives

To follow up the results from Paper III, it would be very interesting to examine what happens to height, stem diameter and amount of axillary shoots in older *PttRAV1&2* RNAi trees, to determine whether a reduction of *PttRAV1* and *PttRAV2* leads to significantly increased biomass production in mature trees. It would also be interesting to further investigate the effect of PttRAV1 and PttRAV2 on the expression of *FT*.

5 Conclusions

5.1 Most important findings of Paper I

Paper I reveals that native *Populus* and Arabidopsis miR156 have a conserved function and that *Populus* miR156 has a clear and strong function in retaining the tree in a juvenile state. *Populus* miR156 also seems to have a large effect on the phenology of the tree, strongly affecting the timing of both bud set and bud burst.

I can also conclude that *Populus tremula x tremuloides* is a very good species to study early juvenile characteristics in since the wild-type trees pass through several clear vegetative phase changes within the first two growth seasons.

5.2 Most important findings of Paper II

In Paper II, I provide two models for how the diversification between the *FT*-like gene function (activators of flowering) and *TFL1*-like gene function (repressors of flowering) most likely occurred at an extremely early stage in angiosperm evolution. The evolution of the flower-promoting function of the *FT*-like genes seems to coincide with the split between non-flowering seed and flowering seed plants, and hence might have contributed to the rapid evolution of flowering plants.

I also confirm that, when expressed in Arabidopsis, the gymnosperm *FT/TFL1*-like genes repress flowering, indicating that the proteins are biochemically more similar to the angiosperm TFL1-likes than the FT-likes.

5.3 Most important findings of Paper III

In Paper III, I characterize the *Populus TEM* homologs through *in silico*- as well as functional analysis in wild type and transgenic trees during various environmental conditions. I show that there is no clear *TEM* ortholog in *Populus* but instead two paralogous *RAV* genes (*RAV1* and *RAV2*), which share sequence characteristics with both the *TEM1*/*TEM2* and the *RAV1*/*RAV1like* genes. *Populus* RAV1 and RAV2 have a role in inhibiting outgrowth of sylleptic branching, possibly through an effect on the dormancy of the axillary bud.

I also show that the *Populus TEM* homologs have a role in bud set as well as in inhibiting early bud outgrowth during bud set.

6 Populärvetenskaplig sammanfattning

I den här avhandlingen har jag studerat åldrande, blomning, knoppsättning och förgrening hos träd.

Hur växter växer och när de blommar varierar enormt. En del växter blommar tidigt på säsongen, andra sent och vissa verkar kunna blomma lite när som helst. Annueller, eller ettåriga växter, växer vegetativt, blommar och dör samma säsong. Fleråriga växter, perenner, lever mellan tre och några tusen år. Att klara vintern i till exempel Sverige, utan att få allvarliga köldskador, ställer höga krav på perenna växter, som träd, att sätta knopp i god tid under hösten och förbereda sig väl för den kommande köldperioden. Det problemet är mindre för annueller, eftersom de ofta övervintrar som köldtåliga frön eller vegetativt under ett skyddande snölager.

En annan skillnad mellan annueller och perenner är tiden till blomning/könsmognad. De flesta växter kan fås att blomma tidigare än normalt under väldigt gynnsamma förhållanden, men det finns nästan alltid en gräns för *hur* tidigt efter att fröet grott som växten kan blomma. Hos annueller kan ungdomstiden eller den juvenila perioden vara så kort som några dagar. Hos träd är den normalt flera, ibland tiotals år. Hur och vad som styr trädens övergång från juvenil till vuxen, reproduktiv ålder har länge varit en gåta för forskarna.

Den långa ungdomstiden hos träd gör att arbetet med att förädla träd tar betydligt längre tid än till exempel spannmålsförädling. Gran har dessutom en mycket oregelbunden blomning även efter att den blivit vuxen, vilket ytterligare försvårar förädlingsarbetet. Med den här avhandlingen har jag bidragit till kunskapsområdet gällande längden på ungdomsperioden och blomning hos träd.

Arabidopsis, eller backtrav som den heter på svenska, är en ettårig ört, som det bedrivits mycket växtforsking på. En annan modellorganism, för trädforskning, är hybridasp. Eftersom Arabidopsis är betydligt lättare att arbeta

med än hybridasp, mest beroende på storlek och dess korta generationstid, görs vanligen de grundläggande undersökningarna av en ny gens funktion i Arabidopsis. Om genen därefter verkar intressant, letar man reda på motsvarande gen hos hybridasp och undersöker om den har samma funktion i en annuell ört och i en förvedad perenn växt. Att studera geners funktion i barrträd är ännu ett nästan helt outforskat område, som jag bara snuddat vid i den här avhandlingen.

För några år sedan upptäcktes att ett så kallat miRNA (miR156) fungerar som en övergripande regulator av längden på den juvenila perioden i Arabidopsis. I min första artikel (I) har jag undersökt om hybridasp-varianten av miR156 har samma funktion i hybridasp. Jag kan verifiera att miR156 i stora delar fungerar på samma sätt i den fleråriga hybridaspen, som i den ettåriga örten Arabidopsis, och påverkar längden på ungdomstiden även hos trädet. Jag har också visat att miR156 påverkar en mängd andra egenskaper hos hybridasp som förgrening, den dagslängdsstyrda knoppsättningen och den temperaturreglerade knoppsprickningen.

I både hybridasp, Arabidopsis och en mängd andra blommande växter finns en gen kallad *FT*, som startar blomningen hos vuxna växter då förhållandena är de rätta. *FT*-genen har ändrats extremt lite under evolutionens gång. Skälet är att plantor vars *FT*-gen muterat löper risk att inte blomma och därmed inte kunna föröka sig. Att *FT* är extremt konserverad och har bevarat sin ursprungliga funktion, även då nya arter bildats, kan visas genom att det går utmärkt att starta blomning hos Arabidopsis med hjälp av FT från helt andra arter, t.ex. ris, äpple eller potatis. I min andra artikel (II) har jag undersökt exakt hur konserverad *FT* är. Jag kan konstatera att *FT*, med dess funktion att starta blomning, till och med finns hos de evolutionärt sett allra äldsta blommande växterna vi känner till, såsom näckrosor, vars släkte delades av från övriga vanliga blommande växter för cirka 120 miljoner år sedan. Däremot verkar *FT* saknas i våra barrträd, som särskiljdes från de blommande växterna för ca 250 miljoner år sedan.

I den tredje artikeln (III) har jag studerat funktionen av en gen (*TEMPRANILLO*), som påverkar blomning och längden på ungdomsperioden i Arabidopsis. Jag kunde inte säkerställa att motsvarande hybridaspgen reglerar längden på ungdomsperioden i hybridasp. Däremot kunde jag visa att den påverkar både förgrening och knoppsättning hos hybridasp.

Även blomningsgenen *FT* har tidigare visat sig påverka knoppsättning hos hybridasp. Det verkar därmed finnas ett relativt starkt samband mellan regleringen av åldrande, blomning och knoppsättning i träd, som skulle kunna ligga till grund för framtida forskning angående utvecklingen av förvedade växters perenna livsstil.

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