Molecular Regulation of the Annual Growth Cycle in *Populus* Trees

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Abstract

Adaptation to the change of seasons is essential for tree survival. Here I show that the phenology of hybrid aspen is regulated by three *FLOWERING LOCUS T* (*FT*) genes.

FT1, *FT2a* and *FT2b* are the result of both a whole genome and a local duplication. All three *FTs* are highly similar in sequence but their expression patterns and functions have diverged over time. *FT1* expression is drastically induced by cold temperatures during winter in vegetative and reproductive buds, while *FT2a* and *FT2b* are expressed in leaves during spring and summer. I used CRISPR/Cas9 gene editing tools to generate individual and specific knockout mutants of *FT1* and *FT2*. *FT1* mutants showed no defects in vegetative growth during the first year. However, their bud flush was severely delayed, indicating a role of *FT1* in dormancy release during winter. In contrast, knock-out of both *FT2s* greatly impaired growth and lead to early growth cessation, showing their importance for vegetative growth during summer.

Additionally, I investigated the regulation of *FT* and possible mechanisms that can fine-tune the response to seasonal changes. I show that the timing of both bud set and bud flush is regulated by the photoreceptor Phytochrome B and its interacting factor *PHYTOCHROME INTERACTING FACTOR 8* trough *FT2* and probably also *FT1*.

Furthermore, I show that growth cessation is induced in response to SD by *SHORT VEGETATIVE PHASE LIKE*, which represses the expression of *FT2* and gibberellin metabolism genes in the leaves.

Keywords: Poplar, FLOWERING LOCUS T, CRISPR-Cas9, Phenology, Phytochrome B, SHORT VEGETATIVE PHASE, Flowering

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Molekylär reglering av den årliga tillväxtcykeln hos asp/poppel (*Populus*)-träd

Sammanfattning

Anpassning till de olika årstiderna är nödvändigt för ett träds överlevnad. Här visar jag att hybridaspens fenologi kontrolleras av tre *FLOWERING LOCUS T* (*FT*)-gener.
FT1, *FT2a* och *FT2b* har uppkommit efter dels en helgenomduplicering och dels efter en lokal duplikation. Alla tre *FT*-gener har mycket likartade DNA-sekvenser men deras uttrycksmönster och funktioner har med tiden förändrats åt olika håll. Uttrycket av *FT1* är kraftigt inducerat av låga vintertemperaturer i både vegetativa och reproduktiva knoppar, medans *FT2a* och *FT2b* är uttryckta i blad under vår och sommar. Jag har använt CRISPR/Cas9-medierad geneditering för att specifikt slå ut funktionen hos de olika *FT1* och *FT2*-generna. *FT1*-mutanter uppvisade en normal vegetativ tillväxt under den första tillväxtsäsongen. Deras knoppbrytning var dock extremt försenad vilket indikerar att *FT1* har en roll i att bryta trädens vintervila. I motsats till detta så ledde en förlorad *FT2*-funktion till kraftigt reducerad tillväxt och ett tidigt tillväxtavslut, vilket visade hur viktiga dessa gener är för sommarens vegetativa tillväxt.

Jag har också undersökt *FT*-genernas reglering och möjliga mekanismer som kan finjustera trädens respons till årstidsförändringar. Jag visar att tidpunkten för både knoppsättning och knoppbrytning regleras av att uttrycket av *FT2*, och förmodligen också av *FT1*, kontrolleras av fotoreceptorn Fytokrom B och dess interagerande protein PHYTOCHROME INTERACTING FACTOR 8.

Dessutom så visar jag att tillväxtavslutet, som stimuleras av en kort dagslängd, delvis kontrolleras av transkriptionsfaktorn SHORT VEGETATIVE PHASE-LIKE, som håller nere uttrycket av *FT2* och av gener som kontrollerar bildandet av tillväxthomonet gibberellin i bladen.

Nyckelord: Poppel/Asp, FLOWERING LOCUS T, CRISPR-Cas9, Fenologi, Fytokrom B, SHORT VEGETATIVE PHASE, Blomning

Contents

List of publications

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

I. Jihua Ding, Bo Zhang, Yue Li, **Domenique André**, Ove Nilsson (2021). Phytochrome B and PHYTOCHROME INTERACTING FACTOR8 modulate seasonal growth in trees. New Phytologist https://doi.org/10.1111/nph.17350

II. **Domenique André**, Keh Chien Lee, Daniela Goretti, Alice Marcon, Bo Zhang, Nicolas Delhomme, Markus Schmid and Ove Nilsson. FLOWERING LOCUS T Paralogs Control the Annual Growth Cycle in *Populus* Trees (manuscript)

III. **Domenique André**, José Alfredo Zambrano, Bo Zhang, Mark Rühl, Ove Nilsson. SHORT VEGETATIVE PHASE-LIKE Modulates Short Day-Induced Growth Cessation in *Populus* Trees (manuscript)

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The contribution of Domenique André to the papers included in this thesis was as follows:

I. DA performed experiments, read and edited the manuscript.

II. DA planned and executed experiments, analyzed the data and wrote the manuscript.

III. DA performed experiments, analyzed the data and wrote the manuscript.

List of figures

Abbreviations

ABA	Abscisic acid
AGL20	AGAMOUS-LIKE 20
AGL24	AGAMOUS-LIKE 24
AIL1	AINTEGUMENTA-LIKE 1
ANT	AINTEGUMENTA
AP	APETALA
BRC1	BRANCHED 1
CAL	CAULIFLOWER
CALS1	CALLOSE SYNTHASE 1
Cas9	CRISPR associated
CCA1	CIRCADIAN CLOCK ASSOCIATED
CDF	CYCLING DOF FACTOR
CDL	Critical day length
cDNA	Complementary DNA
CENL	CENTRORADIALIS LIKE
CO	CONSTANS
COP1	CONSTITUTIVELY PHOTOMORPHOGENIC 1
CRISPR	Clustered regularly interspaced short palindromic repeats
CRY	Cryptochrome

CT	Cold treatment
CYCD3	CYCLIN D 3
DNA	Deoxyribonucleic acid
ER	Endoplasmic reticulum
FDL	FD-LIKE
FKF1	FLAVIN BINDING, KELCH REPEAT, F-BOX PROTEIN 1
FLC	FLOWERING LOCUS C
FLM	FLOWERING LOCUS M
FRI	FRIGIDA
FT	FLOWERING LOCUS T
FTP1	FT-INTERACTING PROTEIN 1
FUL	FRUITFULL
GA	Gibberellic acid
GA20ox	GA20 oxidase
GFP	GREEN FLUORESCENT PROTEIN
GH17	glucan hydrolase family 17
GI	GIGANTEA
GID1	GIBBERELLIN INSENSITIVE DWARF1
GIL	GIGANTEA-LIKE
Gln	Glutamine
Glu	Glutamate
GM	Genetically modified
H3K27	Histone 3 lysine 27
HOS1	HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE 1
KO	Knock-out

LAP1	LIKE-AP1
LD	Long day
LFY	LEAFY
LHY	LATE ELONGATED HYPOCOTYL
MADS	MCM1, AGAMOUS, DEFICIENS, SRF
miRNA	Micro ribonucleic acid
mRNA	Messenger ribonucleic acid
MS	Murashige and Skoog
N	Nitrogen
NCED3	NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 3
NPK	Nitrogen, phosphorous, potassium
PAM	Protospacer-adjacent motif
Pc-G	Polycomb-group
PCR	Polymerase chain reaction
Pfr	Far red-absorbing phytochrome form
PFT1	PHYTOCHROME AND FLOWERING TIME 1
PHY	Phytochrome
PIF	PHYTOCHROME INTERACTING FACTOR
PKL	PICKLE
Pr	Red-absorbing phytochrome form
PRC2	Polycomb-Repressive Complex 2
R:FR	Red to far-red light ratio
RCAR/PYL	Regulatory components of ABA receptor / Pyrabactin-like
RNAi	RNA interference
qPCR	Quantitative PCR
SAM	Shoot apical meristem

SAR	Shade avoidance response
SD	Short day
sgRNA	Single guide RNA
SNP	single-nucleotide polymorphism
SOC1	SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1
SPA1	SUPPRESSOR OF PHYTOCHROME A 1
SPL	SQUAMOSA PROMOTER BINDING-LIKE
SVL	SHORT VEGETATIVE PHASE-LIKE
SVP	SHORT VEGETATIVE PHASE
SwAsp	Swedish Aspen Collection
T89	*Populus tremula x tremuloides* clone T89
TCP4	TEOSINTE BRANCHED 1/ CYCLOIDEA/ PROLIFERATING CELL NUCLEAR ANTIGEN FACTOR
TEM	TEMPRANILLO
TFL1	TERMINAL FLOWER 1
TOC1	TIMING OF CAB EXPRESSION 1
TOE1	TARGET OF EARLY ACTIVATION TAGGED (EAT) 1
TPM	Transcripts per kilobase million
TPS1	TREHALOSE-6-PHOSPHATE SYNTHASE 1
TSF	TWIN SISTER OF FT
UFO	UNUSUAL FLORAL ORGANS
UV	Ultra violet
WT	Wild type
ZTL	ZEITLUPE

1. Introduction

More than half of Sweden's land is covered by forest and forestry business is an important part of the national economy. Swedish forests provide pulp, paper and timber, as well as material for the production of biofuels[1]. Second generation biofuels are considered as major contributors to renewable energy (Ragauskas *et al.*, 2006). The demand for renewable energy and thus forest products is growing fast, but growing trees takes time. Especially in northern countries the growth of trees is slow, since the growing season is much shorter compared to the one in regions close to the equator. Trees growing in the North stop growing early in the year in order to prepare for the coming winter. Also, the continuation of growth in the next season starts late due to a long period with cold temperatures. Another problem, which is not specific to the North, is that it takes a long time to introduce new genetically improved plant material. Tree breeding is a very longsome process spanning decades. Furthermore, climate change is rapidly changing the environment and both natural populations and elite trees may not be adapted to new challenges, e.g., prolonged droughts and flooding.

In order to sustain or ideally increase the yield of plantations, several aspects of tree growth could be targeted; first, the growing season could be extended by manipulation of the timing of growth cessation and growth initiation without increasing the risk of frost damage. Secondly, acceleration

[1] https://www.sveaskog.se/en/forestry-the-swedish-way/short-facts/brief-facts-1/

of flowering in elite lines could fasten the process of breeding and thus creating genetically superior individuals quicker.

Understanding the above-mentioned processes is as an important step towards being able to manipulate them according to our wishes. My thesis focuses on major regulators of both the annual growth cycle and flowering: *FLOWERING LOCUS T* (*FT*) genes.

1.1 Poplar as a model species

Angiosperms or flowering plants presumably evolved between 140 and 190 million years ago (Bell *et al.*, 2005). Since then, they diversified tremendously and with more than ~290.000 extant species are now the most abundant plants on earth (Christenhusz & Byng, 2016). As the name suggests, they are distinguished from other groups of plants by their development of flowers; modified shoots that bear the reproductive organs. Their seeds are produced within a carpel and their ovaries later develop into fruits. The induction of flowering at the right time is not only crucial for the plant in order to secure the offspring's survival, but also for agriculture. Since we absolutely rely on plants' fruits or seeds for our food, their healthy development is of great importance. Therefore, there is enormous interest in understanding and optimizing the processes that lead to flowering and subsequent seed production. Apart from genuine curiosity and the desire to understand life, this is the reason why the study of plant biology exists in its modern form.

Because complex organisms are difficult to study, researchers use so-called model species. These species represent a wider group of related species and are usually easier to study practically. For example, medical research is mostly done on mice and yet those results can be used to derive medications and treatments for humans. For plant research, the most used model species is *Arabidopsis thaliana* (hereafter Arabidopsis), the thale cress. It is a small weed that any gardener would probably remove without batting an eye. For research purposes, however, it is absolutely invaluable. Because of its small size, it is easy to grow in large quantities. Its genome has been fully sequenced and is very dense, meaning there is not a lot of "useless" DNA around to complicate things (Kaul *et al.*, 2000). And very importantly: It is easy to transform (Zhang *et al.*, 2006). Making genetically modified (GM) plants is necessary to understand how they work. One can

remove or “knock-out” one gene and see where the plant has trouble developing normally. Or one can add a marker to a protein of interest and see where it goes in the plant or within a single cell. There are many possibilities, but easy transformation and following propagation are crucial for all of them. Furthermore, Arabidopsis itself is of no commercial interest, lowering the chance of conflicts of interests by funding bodies and sponsors (in contrast to research on tobacco for example, where the tobacco industry has great interest in getting certain results).

Research with Arabidopsis has increased our understanding of plant biology immensely, which now is being transferred and applied to other more economically important plants such as rice, wheat, barley and even trees like *Populus*.

When transferring the findings of Arabidopsis research into poplar and looking for possibly conserved mechanisms, one has to keep in mind several things: First, Arabidopsis and *Populus* are only somewhat closely related (their lineages diverged 100 to 120 million years ago (Tuskan *et al.*, 2006)) and an absolute 1:1 conversion is unlikely. Second, both species may use conserved mechanisms for different purposes and thus will have adapted them accordingly. And third, *Populus* underwent a recent whole-genome duplication (Tuskan *et al.*, 2006). This means that in many cases where there is one gene in Arabidopsis, there are two orthologs in poplar. This is true for many of my genes of interest, including but not limited to, *FT*.

Additionally, studying trees makes things more complicated and their much bigger size is only one reason. Many tools that are readily available for Arabidopsis research do not exist for poplar. There is no catalogue from where you can order mutants of your genes of interest. Making double or triple mutants is very difficult if not impossible with standard techniques because crossing two GM poplars would take several years. With new advances in biotechnology, such hurdles might be overcome (more on that later), but it is still far from being common.

The most important difference, however, lies in the plants themselves.

1.2 The life of a perennial

A major difference between a small weed like Arabidopsis and a tree like poplar is that a tree does not grow within just one season. While annual weeds complete their life cycle (from germination to senescence and death) typically within one spring or summer period, trees live much longer, some of them having the potential to live for several hundred years. This means that annual and perennial plants must have different life strategies and to have different ways to deal with their environments. While annual summer weeds mainly have to overcome overshadowing by other plants and short stretches of bad weather, trees also have to withstand the change of seasons. Reproduction is also more complicated: In contrast to Arabidopsis, poplar trees remain in a vegetative state for several years before they can flower and they are able to resume vegetative growth after sexual maturity.

The further away the plant is growing from the equator, the more extreme the difference between seasons become. In temperate climates, the biggest obstacle to overcome is wintertime, when low temperatures are suboptimal for any metabolic activities and frozen soil makes the water uptake (nearly) impossible.

Perennials, including trees, shrubs and herbaceous plants, have developed two different strategies to face these challenges. Most angiosperm perennials (including the genus *Populus*) are so called deciduous plants, meaning that they can lose their leaves. In temperate and boreal zones, leaf abscission usually coincides with the onset of winter. The loss of leaves reduces the force, with which water is "sucked" from the soil and transported through the plant body. This reduces the risk of collapsing xylem vessels, when no water is to be extracted from the ground.

While the leaves are dropped, the remaining tissues need to be protected from freezing temperatures. Sensitive tissues like meristems, which harbor stem cells, enter a state of dormancy and increase their cold hardiness. Shoot apical meristems are additionally enclosed by bud scales, "specialized" stipules, and overwinter in buds. The tree then needs to experience a prolonged time of low temperatures in order to be responsive again to favorable conditions in the next season (**Figure 1**).

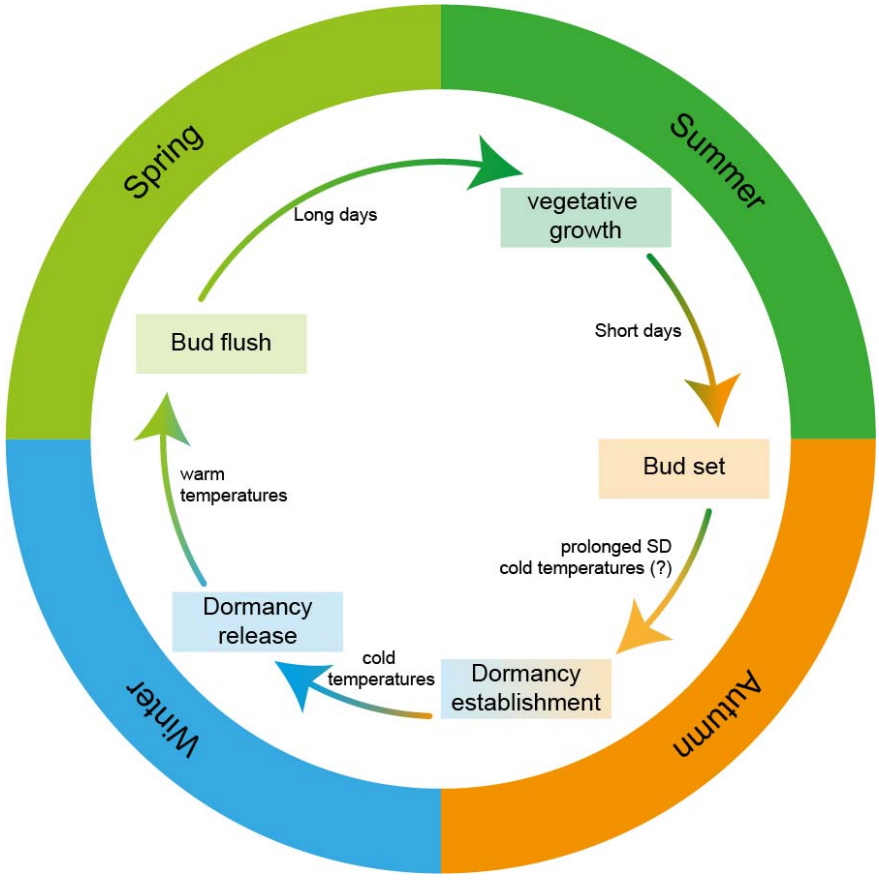

Figure 1: Annual growth cycle of *Populus* trees.
The different seasons and the respective growth stages are indicated in the same colors. Arrows indicate the environmental signals that induce physiological changes in the plant. After Singh *et al*., 2016

In poplar, the timing of growth cessation, bud set and subsequent dormancy is controlled by a trait called critical day length (CDL). The CDL marks the minimal day length that does not cause the short day-induced growth cessation. Photoperiod is an environmental cue that is stable at the same place and time over several years and the response to it is highly variable among plants from different latitudes (Böhlenius *et al.*, 2006).

1.3 Flowering in Arabidopsis

Sensing of day length and the distinction between long days (LD) and short days (SD) is critical information. While it regulates growth cessation in polar, Arabidopsis uses it for the correct timing of flowering,
But how do the plants sense light? And how can they "calculate" the length of the day? And how is this in turn transmitted into a flowering/growth-promoting signal? Interestingly, both plants utilize similar mechanisms. Below I will first summarize what is known from Arabidopsis and then compare it to our understanding of poplar.

1.3.1 Light dependent flowering of Arabidopsis

Arabidopsis is a facultative long day plant, which means that long days are strongly promoting flowering, but it can also occur under other conditions. Also, the light quality has a strong influence on flowering time. Photoperiod, the length of light and dark cycles, and light quality, the light's wavelength(s), are perceived in the leaves. Light is sensed by photoreceptors and different types sense different wavelengths: phytochromes that absorb red/far-red light and cryptochromes, which absorb blue/UV-A light (**Figure 2**; Lin, 2000). UV-B is perceived by the UVR8 protein (Rizzini *et al.*, 2011).

Light perception

Phytochromes are photochromic proteins, which exist in two photo-interconvertible isomeric forms: a red-light absorbing (Pr) form and a far-red light absorbing form (Pfr). Absorption of red light causes a conformational change in Pr and converts it into Pfr. This activates the protein and also reveals a nuclear localization sequence and the active Pfr form is transported into the nucleus, where it can trigger a change in gene expression (Lin, 2000). Two types of phytochromes exist in plants: Type I-phytochromes, which are light labile, and Type II-phytochromes, which are light stable. There are five phytochromes (*PHY*) in Arabidopsis, *PHYA* to *PHYE* (Quail *et al.*, 1995). *PHYA* and *PHYB* were found to make the biggest contributions to phytochrome signaling regarding flowering time, but despite the fact that they both can absorb the same wavelengths, they have different functions. *PHYA* mainly acts in far-red light, while *PHYB* is responsible for red light responses (Quail *et al.*, 1995). Consistent with that, the import of phyA into the nucleus is possible under far-red light, while phyB is imported only under red light (Kircher *et al.*, 1999). Import of phyA is also much faster than that

of phyB (de Lucas & Prat, 2014). *PHYA* was found to have a positive effect on flowering, as *phyA* mutants flower late in long days and *PHYA* over-expressers flower early under long and short days (Bagnall *et al.*, 1995). *PHYB* (and to a small extent *PHYD* and *PHYE*) on the other hand negatively regulate flowering, as *phyB* mutants flower early regardless of day length (Lin, 2000). PHYTOCHROME INTERACTING FACTORs (PIFs) are negative regulators of phytochrome signaling. Their physical interaction with phytochromes leads to PIF phosphorylation and subsequent degradation (Al-Sady *et al.*, 2006). Phytochromes also have a protein kinase function and can phosphorylate themselves and other proteins. It has been shown that phyA can interact with and phosphorylate one of the cryptochromes (Ahmad *et al.*, 1998). Arabidopsis has two cryptochromes (CRY), which are nuclear proteins associated with a flavin chromophore (Lin, 2000). CRY1 and CRY2 have a positive effect on flowering and their actions are partially redundant, as *cry1 cry2* double mutants flower significantly later than either single mutant (Liu *et al.*, 2008). *CRY1* is also important for the entrainment of the circadian clock (Somers *et al.*, 1998).

The circadian clock

Plants have an internal timekeeper called the circadian clock, which allows them to synchronize physiological processes with the correct time of the day, but also to anticipate the change of seasons. The circadian clock can control the expression of individual genes as well as larger processes like photosynthesis, leaf movement and stomatal opening. These outputs have a daily rhythm of roughly 24 hours and this rhythm persists even after the plants are transferred from day/night cycles to constant light or dark. Furthermore, they are temperature compensated and keep their periodicity in cold as well as hot weather. However, they can eventually be reset by certain stimuli to adapt to new conditions (Harmer, 2009). The circadian clock is not just a simple hourglass timer, but rather a complex network with interlocked feedback loops and different in-/outputs, which themselves can influence each other (Harmer, 2009). A highly simplified model is shown in **Figure 3**. The genes involved in the circadian clock are regulated on several levels, including transcriptional and post-transcriptional regulation as well as protein stability.

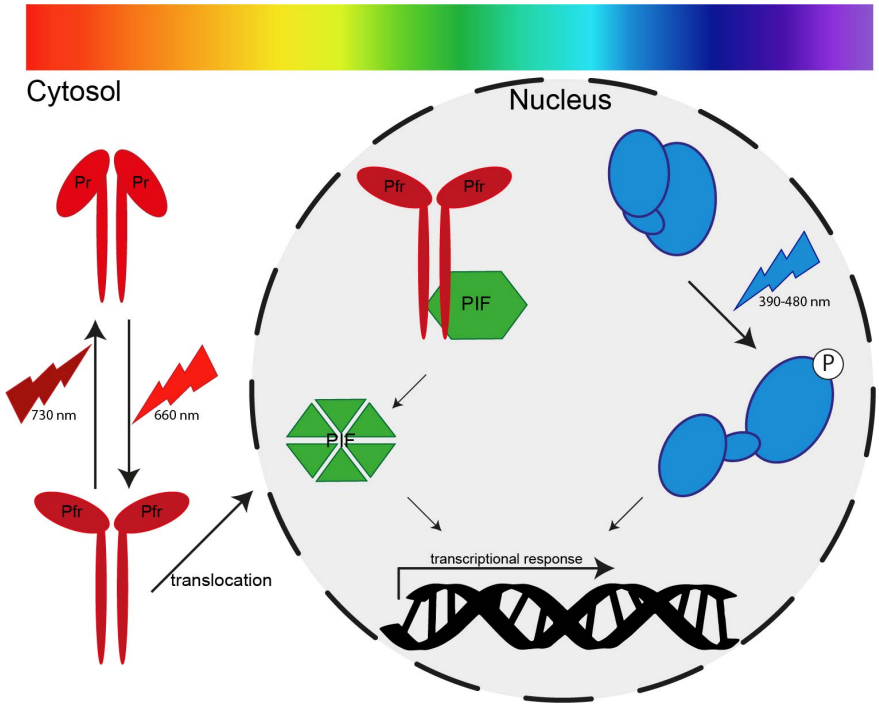

Figure 2: Light perception in Arabidopsis.
Red/Far-red receptors (colored in red) are located in the cytosol and switch between Pr and Pfr forms depending on the wavelength they absorb. The Pfr form can translocate to the nucleus, where it facilitates the degradation of PIFs. Blue light receptors (colored in blue) are located in the nucleus and are phosphorylated upon absorbing blue light. The phosphorylated form can induce transcriptional changes.

The very core of the circadian oscillator is a negative feedback loop, a balancing feedback that stabilizes the output of a system, of two MYB transcription factors, CIRCADIAN CLOCK ASSOCIATED1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), and a transcriptional repressor TIMING OF CAB EXPRESSION1 (TOC1). *CCA1* and *LHY* are expressed in the morning and inhibit the expression of *TOC1*, as well as their own. Their down-regulation leads to de-repression of evening phased genes like *TOC1* (Shim & Imaizumi, 2014). TOC1 suppresses the expression of *CCA1* and *LHY*, but gets degraded in the dark, leading to an increased *CCA1*/*LHY* expression towards the morning (Huang *et al.*, 2012). Many more factors are involved in this process, which support the timed expression of *CCA1*/*LHY* and *TOC1* (Shim & Imaizumi, 2014). One of these factors is ZEITLUPE

(ZTL), which is part of an E3 ligase complex and responsible for the degradation of TOC1. During the day, it interacts with another clock component called GIGANTEA (GI) in a blue light- dependent manner and this interaction prevents the degradation of TOC1 until nightfall. Like many other clock related genes, *GI* also has functions unrelated to the clock (Harmer, 2009).

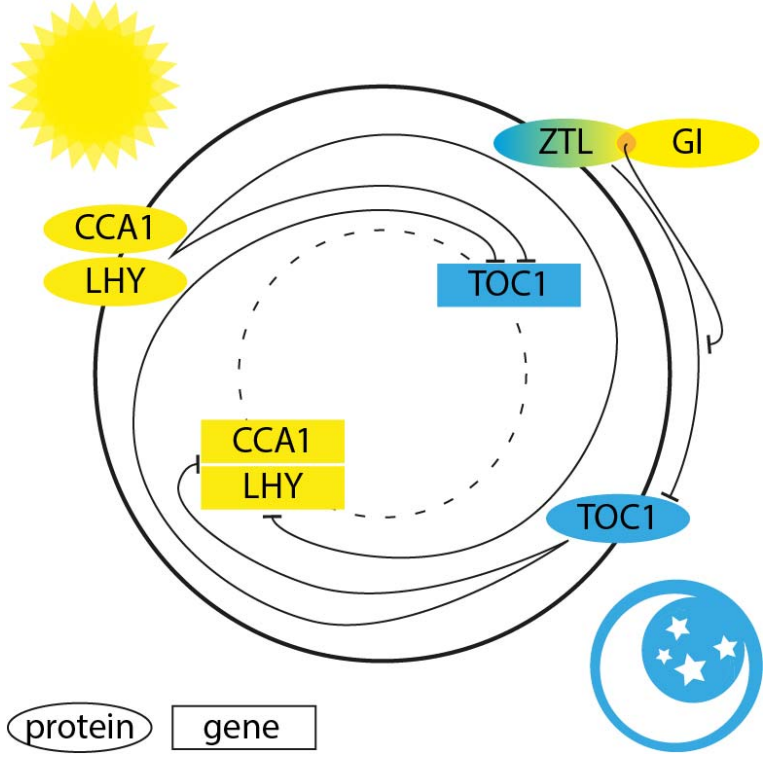

Figure 3: Simplified model of the circadian clock.
Genes that are active during the day are indicated in yellow, while genes that are active during the night are indicated in blue. ZTL is active during both the light and dark periods. Boxes indicate genes, ovals indicate proteins.

Internal and external coincidence model

Two models have been proposed to explain how measuring the daylength works: the "Internal coincidence" and the "External coincidence" model (**Figure 4**; Davis, 2002). The internal coincidence model describes two

distinct circadian oscillators being entrained by light in a manner that in long days they peak at the same time, while in short days their expression patterns are shifted and their peaks do not coincide. Only the joint action of both oscillators triggers a response. In the external coincidence model, the expression of a circadian oscillator exceeds a certain threshold at a certain time, but a response is triggered only if light is perceived simultaneously (Davis, 2002). In Arabidopsis, the molecular bases for both models have been (at least partially) described and it seems that a combination of both is responsible for long day induced flowering. A central role in both models plays *CONSTANS* (*CO*), a gene that acts between the photoperiod perception and the generation of *florigen* (Ayre & Turgeon, 2004).

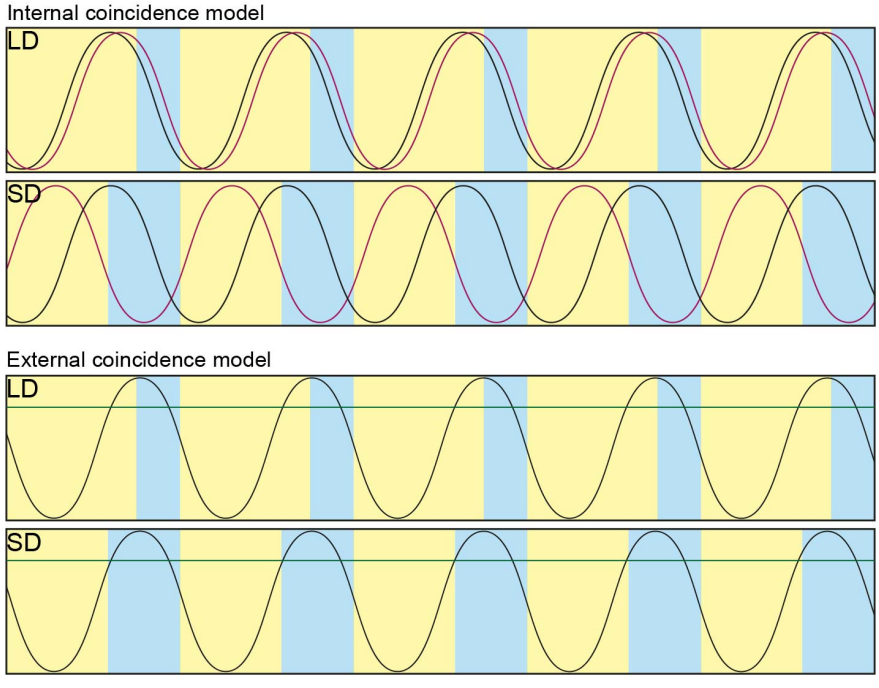

Figure 4: Schematics of the internal and external coincidence models.
The curves are representing the expression patterns of oscillators and the green line a certain threshold. Yellow boxes represent light period, while blue boxes indicate dark periods.

The photoperiodic pathway

The name CONSTANS derives from the fact that *co* mutants always take the same time to flower regardless of day length. Their flowering time is delayed

under long day conditions as if the plants had grown in SD (Rédei, 1962). CO is a nuclear zinc-finger protein (An *et al.*, 2004) and expressed at the site of light perception: in the main veins of cotyledons (Takada & Goto, 2003) and minor veins of mature leaves (Ayre & Turgeon, 2004). This expression is tightly regulated on the transcriptional, as well as on the posttranslational level (**Figure 5**). In LD, it shows a diurnal transcriptional expression pattern with a broad peak between 12 and 20 hours after dawn (Suárez-López *et al.*, 2001). This expression pattern is similar but generally at a lower level in SD (Suárez-López *et al.*, 2001). *CO* mRNA is expressed early in the morning, but the resulting proteins are inhibited in their function by TARGET OF EAT1 (TOE1) and related proteins, which bind to the transcriptional activation domain (Zhang *et al.*, 2015). In the late morning, *CO* transcription is redundantly repressed by CYCLING DOF FACTORs (CDFs) 1, 2, 3 and 5. At least for CDF1 it has been shown that it can bind to several DOF consensus sequences in the *CO* promoter (Imaizumi *et al.*, 2005). CO proteins resulting from this very low transcriptional expression are destabilized by phyB and HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE1 (HOS1; Valverde *et al.*, 2004; Lazaro *et al.*, 2012).

During the light phase, transcriptional repression is damped by the degradation of the CDFs. FLAVIN BINDING, KELCH REPEAT, F-BOX PROTEIN 1 (FKF1) can interact with CDF1 (and possibly other CDFs) in order to ubiquitinate and thus target them for degradation via the 26S proteasome (Imaizumi *et al.*, 2005). This activity depends on the interaction with GI in light (Sawa *et al.*, 2007). FKF1 and GI also interact and stabilize the CO protein. Expression of *FKF1* and *GI* is controlled by the circadian clock and peaks 12 hours after dawn in LD. However, in SD, *GI* expression is shifted towards the morning. Therefore, *GI* and *FKF1* expression peaks do not coincide in SD and their ability to form complexes is impaired (Sawa *et al.*, 2008). These features of photoperiod controlled *FKF1* and *GI* expression match very well the proposed “internal coincidence” model (Davis, 2002) and the resulting lower expression of *CO* partially explains the inhibition of flowering in SD. After release of its repression, *CO* is additionally transcriptionally activated by the TCP4 complex and GI (Kubota *et al.*, 2017), and *CO* mRNA accumulates at the end of the day.

In SD, this happens in the dark and accumulation of CO protein is prevented by their rapid degradation (Valverde *et al.*, 2004). COP1 is an E3 ubiquitin ligase and targets CO for degradation via the 26S proteasome (Liu *et al.*,

2008). SPA1, also a negative regulator of light signaling, interacts with COP1 and enhances its activity (Laubinger *et al.*, 2006). Therefore, no stable CO proteins are present to induce transcription of *FT*. In the light evening of long days, the interaction of SPA1 and COP1 is prevented by CRY1 and CRY2 (Zuo *et al.*, 2011; Lian *et al.*, 2011), which are activated by blue light. Also, phyA was shown to affect the stability of the CO protein (Valverde *et al.*, 2004). As proposed in the "external coincidence" model, the high (transcriptional) expression of *CO* can only trigger a response, when it coincides with light. Even though several early targets of CO have been identified (Samach *et al.*, 2000), *FT* is the only one that responds differentially in leaves of wild type and *co* mutants already in the first long day (Wigge *et al.*, 2005).

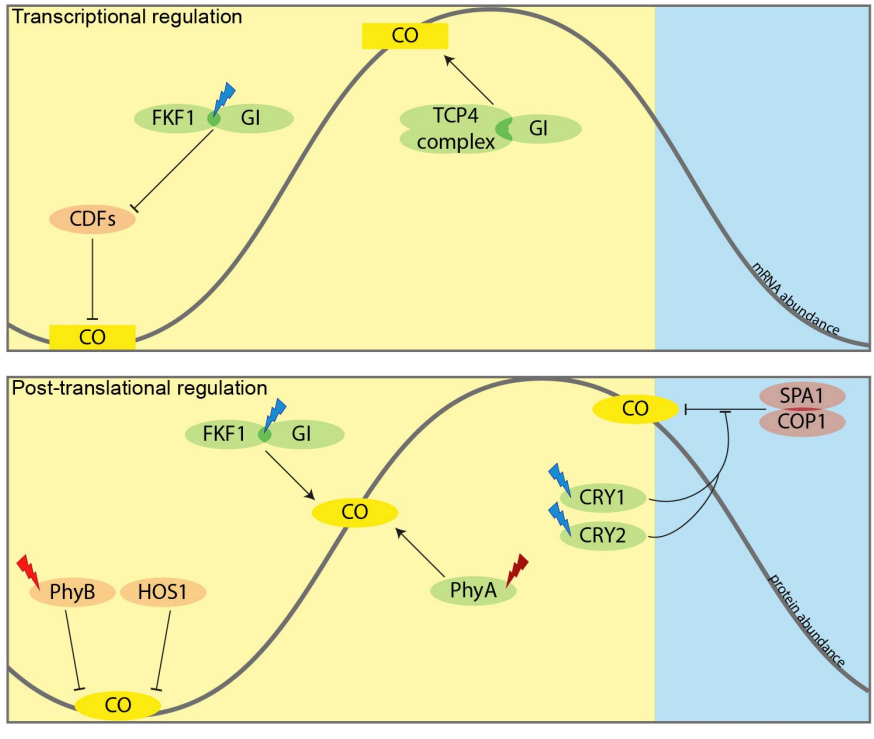

Figure 5: Regulation of *CO* in the photoperiodic pathway.
The yellow background indicates light period and blue background indicates dark period. Genes in green indicate positive effects on *CO* expression, while red genes symbolize *CO* repression. Blue and red lightning bold indicate blue and red light, respectively.

Flowering in shade avoidance response

The day length gives indication to plants about the current season. The quality of the light, however, may give indications about their situation regarding competitors. When a plant grows in the shadow of another one, the spectrum of light is different compared to an open field. The shading leaves absorb red light, but far-red light is either reflected or shines through. Thus, the shaded plant senses a drop in red to far-red light (R:FR) ratio. Subsequently the ratio between the Pfr form to total phytochromes ($Pfr:P_{total}$) decreases. This triggers changes in the plants development, which are known as the shade avoidance response (SAR). Upon sensing neighbors, the plant elongates its stem in order to outgrow the competition and get more direct sunlight. This happens on the cost of leaf expansion and branching. Furthermore, flowering is accelerated to secure reproductive success before the plant is outcompeted.

Of the five phytochromes present in Arabidopsis, *PHYB* plays the most prominent role during SAR (Cerdán & Chory, 2003), as *phyB* mutants display a constitutive SAR (Endo *et al.*, 2005). As described earlier, *PHYB* has a negative effect on flowering and its expression only in the mesophyll cells of cotyledons has been shown to be sufficient for *FT* repression (Endo *et al.*, 2005). In light with a low R:FR ratio, however, the levels of active Pfr are decreased and repression is less efficient. Also, the absence of active phyC, phyD and phyE contributes to accelerated flowering (Wollenberg *et al.*, 2008).

It has been shown that increased levels of *FT* cause the rapid flowering as part of the SAR. Two pathways downstream of *PHYB* cause this up-regulation. One of them partially relays on factors of the photoperiod pathway, such as *CO* and *GI*, as far-red enriched light accelerates flowering only in LD and not under unfavorable photoperiods (Wollenberg *et al.*, 2008). Far-red light leads to a small increase in *CO* transcript levels (Wollenberg *et al.*, 2008) and a significant increase in CO protein (Kim *et al.*, 2008). This in turn causes elevated *FT* transcript levels. Transcription of *GI* is not increased, but its expression peak is shifted in long days towards the end of the day (Wollenberg *et al.*, 2008). The other pathway is through *PHYTOCHROME AND FLOWERING TIME1* (*PFT1*), an important regulator of the light quality pathway (Cerdán & Chory, 2003). It has later been identified as the Med25 subunit of the plant mediator (Bäckström *et al.*,

2007). Consistent with its role as a transcriptional co-activator, activation of PFT1 induces the transcription of *CO* and *FT* (Iñigo *et al.*, 2012).

CO-independent pathway

Apart from its function in *CO*-transcription, GI has been shown to activate *FT* in other ways. First, its interaction with FKF1 to degrade CDFs releases some repression of *FT*, not only *CO* (Song *et al.*, 2012). Second, it can bind to *FT* promoter regions that contain binding sites for repressors like SVP (Sawa & Kay, 2011). Furthermore, GI can directly bind SVP, TEM1 and TEM2, suggesting that GI regulates *FT* transcription by blocking repressors' access to the promoter and/or affecting their stability/activity (Sawa & Kay, 2011). Third, some data indicate that *GI* regulates the abundance of microRNA 172 (miRNA172; Jung *et al.*, 2007). miRNA172 represses AP2-like genes, which repress *FT* (Jung *et al.*, 2007). Thus, expression of *GI* leads to higher levels of miRNA172 and consequently reduced repression of *FT* by AP2-like genes (Jung *et al.*, 2007).

1.3.2 Thermosensory pathway

Besides light, temperature is another obvious factor that can affect the plant. Too high temperatures stress plants due to water loss or damage to proteins. Too low temperatures on the other hand can lead to a slowed metabolism, reduced photosynthesis and more rigid membranes. Warm temperatures generally induce flowering (Balasubramanian *et al.*, 2006), while cold temperatures delay it (Posé *et al.*, 2013). SHORT VEGETATIVE PHASE (SVP) has been identified as a floral repressor (Hartmann *et al.*, 2000) in the thermosensory pathway (Lee *et al.*, 2007). *svp* mutants flower early and even more so at lower ambient temperatures, while overexpressers show a stronger late-flowering phenotype at warmer temperatures (Lee *et al.*, 2007). SVP regulates flowering both directly through the binding to *FT* and *SOC1* promoters (Lee *et al.*, 2007; Li *et al.*, 2008) and indirectly by repression of gibberellin biosynthesis (Andrés *et al.*, 2014). SVP acts together with FLM-β, a complex that is more stable at lower temperatures (Lee *et al.*, 2013). TEMPRANILLO1 (TEM1) and TEM2 were identified as direct targets of SVP (Tao *et al.*, 2012) and *tem* mutants are less temperature sensitive than wild type (Marín-González *et al.*, 2015). Like SVP, TEM can repress *FT* transcription and GA biosynthesis (Osnato *et al.*, 2012).

1.3.3 Other flowering pathways

While the above-described pathways are the most relevant ones here, they are not the only factors contributing to the regulation of flowering. Plant age, nutrient/energy status and other internal factors can contribute to the adjustment and fine-tuning of flowering time by modulating *FT* expression.

Flowering by vernalization

Arabidopsis accessions differ in their flowering behaviors. Some are rapid cycling, while winter annuals are late flowering even under favorable conditions. A process called vernalization, the exposure to cold temperature for several weeks, eliminates this late flowering phenotype (Wang, 2014). The late flowering is greatly dependent on FLOWERING LOCUS C (FLC), which suppresses the expression of *FT* in the leaf and *SOC1* in the shoot apex (Searle *et al.*, 2006). FRIGIDA (FRI) activates *FLC* (Choi *et al.*, 2011) in winter annuals, but is mutated in rapid cycling ecotypes (Johanson *et al.*, 2000). During vernalization, *FLC* is first repressed by mechanisms involving non-coding RNAs. Later, histones 3 at the *FLC* locus are modified by tri-methylation of lysine at position 27 (H3K27me3; Angel *et al.*, 2011). These modifications and higher order chromatin assembly stabilize this repression in order to fully silence the gene (Wang, 2014; Andrés & Coupland, 2012). After vernalization the plants respond with rapid flowering to inductive long days. FLC interacts with SVP and its function is greatly dependent on it (Li *et al.*, 2008). However, they are also able to function autonomously and their complex regulates a specific set of genes that are not affected by either transcription factor alone (Mateos *et al.*, 2015).

Flowering dependent on age

Another pathway controlling floral induction depends on the age of the plant. This age-pathway is mediated by another microRNA, namely miRNA156 (Wang, 2014). miRNA156 expression is temporally regulated and high in young seedlings, but decreases with age. Targets of miRNA156 are a family of 11 *SQUAMOSA PROMOTER BINDING LIKE* (*SPLs*) genes. They can be divided into two groups by size of their gene products; proteins of one group are larger than 800 residues and proteins of the other are less than half the size (Xing *et al.*, 2010). *SPL*s are floral promoters and expressed in the shoot apex as well as in the leaves. In the shoot apex they induce *SOC1*, *AP1* and *LFY*, while in the leaves they indirectly promote *FT* expression by inducing

miRNA172 (Wang *et al.*, 2009). Thus, the balance between miRNA156 and miRNA172 shift towards the latter with age (Wu *et al.*, 2009).

Flowering dependent on carbohydrate- and nutrient status

Trehalose-6-phosphate is a signaling molecule relaying information about the carbohydrate status of the plant and its amount correlates with sucrose availability (Lunn *et al.*, 2006). *FT* expression is greatly reduced in its absence and the absence of its producer TREHALOSE-6-PHOSPHATE SYNTHASE 1 (TPS1; Wahl *et al.*, 2013). However, overexpression of *TPS1* at the shoot meristem directly induces very early flowering, indicating that it can work *FT*-independently. Like the age pathway, it affects the expression of *SPL* genes and in turn meristem identity genes like *AP1* and *LFY* (Wahl *et al.*, 2013).

Besides carbohydrates, nitrogen (N) is an important macronutrient for plants and often a limiting factor for growth. Low nitrate accelerates flowering in SD, but not LD and independent of *FT* (Marín *et al.*, 2011).

Gibberellins

Gibberellic acids or gibberellins (GA) are plant hormones regulating a variety of developmental processes from seed germination to flowering (Hedden & Sponsel, 2015). There are many different GAs, which are synthesized in a series of oxidations (**Figure 6**), but only GA_1 and GA_4 are bioactive (Yamaguchi & Kamiya, 2000). Their role in LD-induced flowering is less pronounced compared to SD (Wilson *et al.*, 1992), but GAs do contribute to the regulation both in the leaf and the shoot apex (Galvão *et al.*, 2012; Porri *et al.*, 2012).

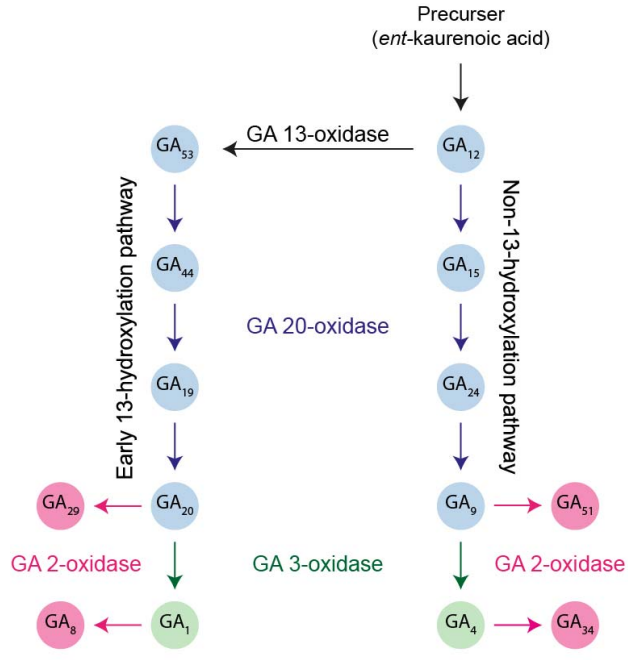

Figure 6: Biosynthesis pathway of gibberellins.
GA precursors are indicated in blue, active gibberellins in green and inactive GA forms in pink. The enzymes involved in their synthesis are indicated in the same colors.

GA signaling is relayed through DELLA[2] proteins, which regulate both gene expression and transcription factor activity (Davière *et al.*, 2008). Bioactive GAs bind to the receptor GIBBERELLIN INSENSITIVE DWARF1 (GID1) and cause conformational changes. This modification facilitates interaction with the DELLA proteins, which ultimately leads to their degradation, thus activating GA signaling (Griffiths *et al.*, 2006). Among the DELLA-regulated genes are enzymes involved in GA biosynthesis, creating feedback loops between GA synthesis and perception (Hedden & Sponsel, 2015). The GA pathway also integrates other pathways; DELLA proteins can interact with PIFs (De Lucas *et al.*, 2008; Feng *et al.*, 2008) and SVP and TEM repress the biosynthesis genes GA20-oxidase and GA3-oxidase, respectively (**Figure 7**; Osnato *et al.*, 2012; Andrés *et al.*, 2014).

[2] Named after a highly conserved amino acid sequence in the N-terminus (Peng *et al.*, 1997)

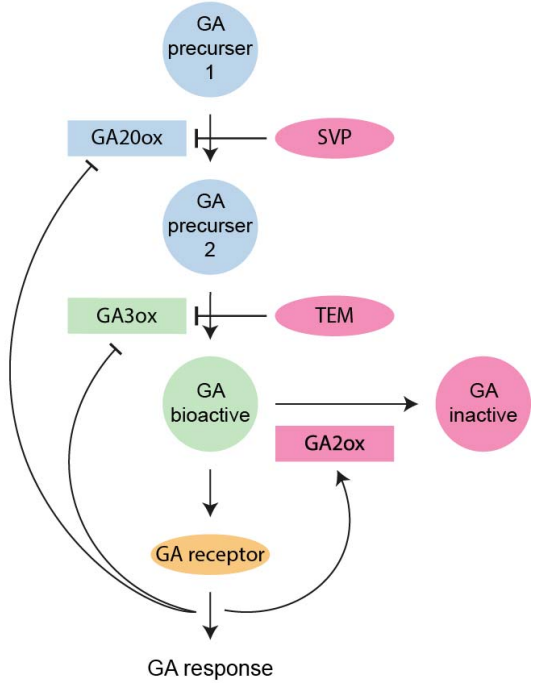

Figure 7: Feedback regulation on GA biosynthesis.
GA precursors are indicated in blue, active gibberellins in green and inactive GA form in pink. Enzymes involved in their synthesis are indicated in the same colors and genes that have a negative effect on GA biosynthesis are indicated in pink as well. Circles indicate GA forms, boxes indicate GA biosynthesis enzymes and ovals indicate other proteins.

1.3.4 *FT* as the merging point of different pathways

FLOWERING LOCUS T (*FT*) is *the* regulator of floral transition. The *ft* mutation causes late flowering in long days (Koornneef *et al.*, 1991) and overexpression of *FT* causes early flowering independent from day length (Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999). Even though *FT* was originally identified as an actor of the photoperiodic pathway, it has become clear that it integrates signals from all other pathways described above (**Figure 8**).

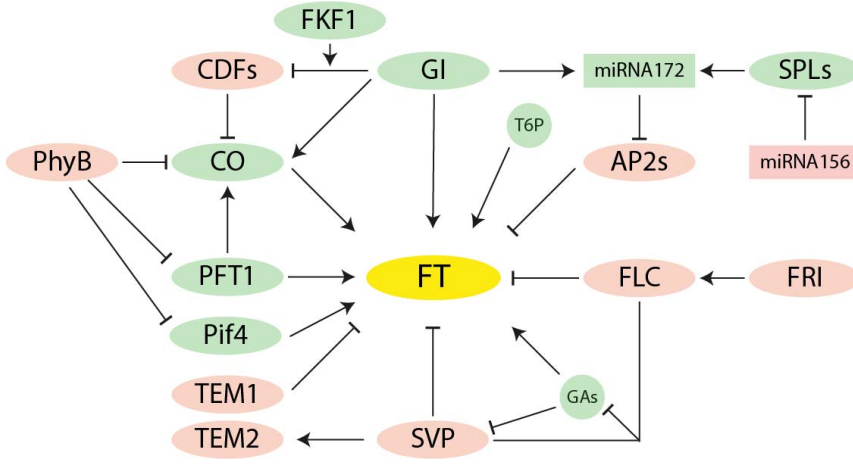

Figure 8: *FT* is the merging point of many pathways.
Factors in green indicate a positive effect on *FT* expression, while red factors are repressors of *FT*. Ovals indicate genes, boxes indicate miRNAs and circles indicate hormones/metabolites.

Interestingly, *FT* is not expressed where the floral transition takes place, i.e., the shoot apex, but in the vasculature of leaves, more specifically in the phloem companion cells (Takada & Goto, 2003; An *et al.*, 2004). This means that the FT protein has to travel through the plant in order to induce its downstream targets.

1.3.5 FT is the plant *florigen*

Already in the 1930s it has been demonstrated that exposure of leaves, but not the shoot apex, to flower-inducing photoperiods is sufficient as a trigger of flowering (Kobayashi & Weigel, 2007). This led to the hypothesis that *florigen*, a floral inducing stimulus, is produced in the leaves and then transported to the shoot apex. In 2007, large pieces of evidence were obtained that this long-range signal is indeed the FT protein. However, it is still possible that other factors contribute as well (Corbesier *et al.*, 2007; Jaeger & Wigge, 2007; Mathieu *et al.*, 2007).

Three different approaches were taken to investigate the ability of the FT protein to move. First, fusions of FT and the GREEN FLUORESCENT PROTEIN (GFP) were specifically expressed in the phloem companion cells, but green fluorescence was anyway found in the shoot apex of plants, which were just about to flower, as well as in sink tissues of receiver plants

after grafting (Corbesier *et al.*, 2007). Another approach consisted of the block of putative FT transport, which was achieved by targeting FT to the nucleus of phloem companion cells. This resulted in a late flowering phenotype despite high FT expression (Jaeger & Wigge, 2007). A similar method was used to demonstrate that the release of the transportation block was sufficient to restore the flowering time phenotype (Mathieu *et al.*, 2007). TWIN SISTER OF FT (TSF), which can act redundantly with FT (Mathieu *et al.*, 2007), but mostly in SD (Hiraoka *et al.*, 2013), can also travel through the plant. However, it seems both less mobile and less stable (Jin *et al.*, 2015).

Consistent with the hypothesis that the FT protein is transported from the leaves to the shoot apex via the phloem sap, a putative transporter has been identified. Like *FT*, *FT INTERACTING PROTEIN 1* (*FTP1*) is expressed in the phloem, but its mRNA levels are not regulated in the same way. They are unaffected by day length and do not follow a circadian rhythm. The FTP1 protein is localized in the membrane of the endoplasmic reticulum (ER), especially at plasmodesmata between phloem companion cells and sieve elements. In the *ftp1* mutant, FT:GUS protein fusions are barely detectable in the shoot apical meristem, while they are clearly visible in the wild type. Together with the localization of FTP1, this suggests that FTP1 is required for FT transport (Liu *et al.*, 2012).

1.3.6 Changes in the shoot apical meristem (SAM)

The FT protein moves to the SAM to fulfil its function. There, it greatly depends on a bZIP protein called FD, as *fd* mutants can at least partially suppress the early flowering of *35S::FT* (Abe *et al.*, 2005; Wigge *et al.*, 2005). *FD* is expressed in the shoot apex, already before floral induction (Wigge *et al.*, 2005). It does not show any distinct circadian oscillation, nor is it affected by photoperiod and CO activity (Abe *et al.*, 2005). The FD protein is constitutively located in the nucleus and also FT seems to be targeted to the nucleus in the shoot apex and interactions between both proteins have been observed (Abe *et al.*, 2005). The protein complex induces the expression of several downstream targets.

Integration of several pathways at the SAM

One of the first targets up-regulated by FT and FD is *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), also known as *AGAMOUS-LIKE 20* (AGL20; Borner *et al.*, 2000; Searle *et al.*, 2006). It is named after the ability of *soc1* to partially suppress the early flowering phenotype of CO-overexpressers (Onouchi *et al.*, 2000). Thus, *SOC1* must act downstream of *CO* and it has indeed been identified as one of COs early targets (Samach *et al.*, 2000). However, in contrast to CO, it is mainly expressed in the shoot apex and is induced by FT/FD (Yoo *et al.*, 2005), as *SOC1* expression is severely delayed in *ft fd* double mutants (Searle *et al.*, 2006). Thus, induction of *SOC1* by CO is through FT (Yoo *et al.*, 2005). However, *SOC1* expression in the meristem is not able to overcome the late flowering of *co ft* double mutants, indicating that FT must have additional targets (Searle *et al.*, 2006). *SOC1* is a MADS domain gene and its overexpression is sufficient to induce flowering (Borner *et al.*, 2000). Several flowering pathways converge at this point, as *SOC1* is induced by *FT*, but the *soc1* mutant delays flowering independently of day length (Onouchi *et al.*, 2000). It has been shown that *SOC1* is also regulated by gibberellins (Borner *et al.*, 2000) and *FLC* (Searle *et al.*, 2006). *SOC1* acts partially redundantly with *FRUITFULL* (*FUL*), which is also induced by the FT/FD complex (Wang *et al.*, 2009). They are involved in the control of flowering time, but also in the determinacy of the inflorescence meristem. In *soc1 ful* double mutants, inflorescence meristems revert into vegetative meristems, resulting in a prolonged lifespan with several waves of growth (Melzer *et al.*, 2008). *SOC1* can induce the expression of another MADS-box transcription factor called *AGAMOUS-LIKE 24* (*AGL24*), which in turn promotes the expression of *SOC1*, thus engaging them in a positive feedback loop at the floral transition stage (Liu *et al.*, 2008). SOC1 by itself is located in the cytosol, but is translocated into the nucleus by AGL24, which is constitutively located in the nucleus. SOC1 and AGL24 form a heterodimer and together induce the transcription of downstream targets (**Figure 9**; Lee *et al.*, 2008).

Meristem identity genes

A well-studied target of SOC1 and AGL24 is the transcription factor *LEAFY* (*LFY*; Lee *et al.*, 2008). *LFY* exists in most land plants only as a single-copy gene (Sayou *et al.*, 2014) and is an important switch in floral development (Weigel & Nilsson, 1995). *LFY* is a meristem identity gene and plants with

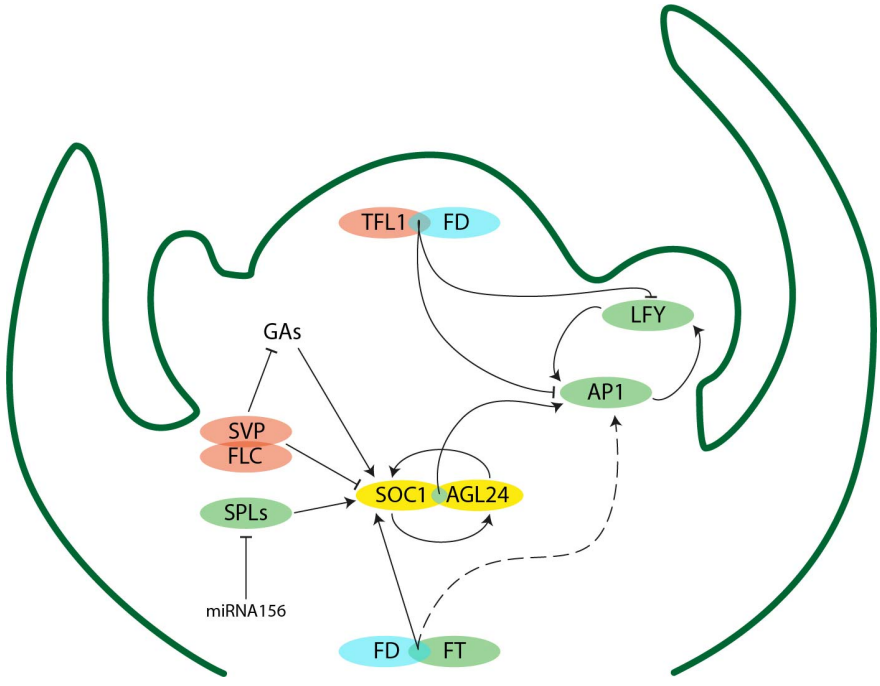

Figure 9: Floral regulation at the shoot apex.
Genes indicated in green are floral activators, while red ones are floral repressors. Activity of FD depends on its interactor. Dashed line indicates indirect activation.

lfy mutations never produce normal flowers and are typically sterile (Schultz & Haughn, 1991). The meristems that are supposed to become flowers initiate shoot development instead. Constitutive expression of *LFY* on the other hand causes all shoots to turn into flowers; the shoot has a terminal flower and solitary flowers develop in the axils of leaves (Weigel & Nilsson, 1995). Another meristem identity gene called *APETALA1* (*AP1*) has a similar function, as loss-of-function as well as gain-of-function of *AP1* causes a similar phenotypes to *lfy* and *35S::LFY*, respectively (Bowman *et al.*, 1993; Mandel & Yanofsky, 1995). *AP1* expression is delayed in *lfy* mutants and ectopic in *LFY*-overexpressers. Conversely, *LFY* is pre-maturely expressed in *AP1*-overexpressers (Liljegren *et al.*, 1999). It has been hypothesized that *LFY* and *AP1* are engaged in a positive feedback loop, which is initiated by *LFY* inducing *AP1*. Indeed, a binding site for LFY has been found in the *AP1* promoter (Parcy *et al.*, 1998). However, *AP1* is still expressed in *lfy* mutants, but on a lower level (Wagner *et al.*, 1999) and therefore must be induced by

at least one other factor. *LFY* and *AP1* are assigning the floral fate to lateral meristems together with other genes like *CAULIFLOWER* (*CAL*), *AP2* and *UNUSUAL FLORAL ORGANS* (*UFO*; Weigel & Nilsson, 1995; Liljegren *et al.*, 1999). *CAL* acts redundantly with *AP1* and its mutation enhances the phenotype of *ap1* (Bowman *et al.*, 1993; Ferrándiz *et al.*, 2000). Additionally, *LFY* and *AP1* seem to be involved in the regulation of floral organ identity; flowers of *lfy* mutants lack petals and stamens (Schultz & Haughn, 1991) and *ap1* mutants have flowers with disrupted sepal and petal development (Mandel *et al.*, 1992; Bowman *et al.*, 1993). Organ size throughout shoot development, including floral organ growth, is mediated by *AINTEGUMENTA* (*ANT*), another AP2-like transcription factor (Elliott *et al.*, 1996). *ANT* acts upstream of cell cycle genes like *CYCD3*, which themselves regulate the cell cycle and proliferation (Mizukami & Fischer, 2000; Dewitte *et al.*, 2003).

1.3.7 *TFL1* as antagonist of *FT*

The phenotypes of *35S::LFY* and *35S::AP1* somehow resemble the phenotype caused by mutations in *TERMINAL FLOWER 1* (*TFL1*), leading to early flowering and the development of a flower at the shoot apex (Mandel & Yanofsky, 1995; Liljegren *et al.*, 1999). *TFL1* belongs to the same family as and is very similar to *FT* (Kobayashi *et al.*, 1999). Despite their sequence similarity, *TFL1* and *FT* are antagonists; mutations in one enhance the effect of overexpression of the other gene. However, it seems that *FT* is more important for the timing of flowering (Kobayashi *et al.*, 1999), while *TFL1s* primary function is to maintain the inflorescence meristem identity (Bradley *et al.*, 1997). *TFL1* mRNA is expressed just below the apical dome of inflorescence and coflorescence meristems (Bradley *et al.*, 1997). The TFL1 protein on the other hand is evenly distributed within the entire meristem, but excluded from floral primordia (Conti & Bradley, 2007). *TFL1* over-expressing plants are late flowering, suggesting a role for *TFL1* as a floral repressor (Kobayashi *et al.*, 1999). This repression is on a transcriptional level, *as tfl1* mutants can only be rescued by native TFL1 or TFL1 fused to a transcriptional repressor domain. TFL1 fused to a transcriptional activator domain still results in a terminal flower (Hanano & Goto, 2011). Like the flowering promoting function of *FT*, the repressing function of *TFL1* depends on *FD* (Hanano & Goto, 2011). Given that the FT/TFL1 ratio rather than absolute amounts seems to determine the phenotype (Kobayashi *et al.*,

1999), it has been suggested that FT and TFL1 compete for the binding of FD. Depending on which protein is bound to FD, it can act either as a repressor or a promoter of flowering genes (Ahn *et al.*, 2006). Consistent with this hypothesis, flower meristem identity genes like *AP1* and *LFY* are ectopically expressed in *tfl1* mutants (Bradley *et al.*, 1997). However, *AP1* expression has a similar effect on *TFL1*; *TFL1* is ectopically expressed in *ap1* mutants (Conti & Bradley, 2007) and drastically down-regulated in *AP1* overexpressing plants (Liljegren *et al.*, 1999). Therefore, the expression of *TFL1* and *AP1*/*LFY* is mutually exclusive and defines the fate of the meristem.

1.3.8 Maintenance of flowering

Once induced, the identity of the meristem changes and in most species, including Arabidopsis, this makes the plant commit to flowering. Reversions from inflorescence meristems back to vegetative meristems are usually not possible. However, they do exist in a few species and may occur if the inductive signals are not maintained (Tooke *et al.*, 2005). Under certain circumstances, exposure of Arabidopsis plants to a single long day can be sufficient to induce flowering and makes the plants committed to it (Corbesier *et al.*, 1996). Thus, the plant must be able to "memorize" the inductive stimulus and continue the process even in unfavorable conditions. This is similar to the process of vernalization, in which the plant can "remember" that it went through a cold phase. Some mutants of Arabidopsis, however, are unable to fully commit and reversions to an earlier meristem state can occur (Melzer *et al.*, 2008; Müller-Xing *et al.*, 2014).

There are two types of mutants, in which these reversions can happen. In the first ones, reversions are due to compromised function of mutated meristem identity genes, which therefore fail to maintain the correct identity. One example is the *ap1* mutant, in which the floral meristem partially reverts into an inflorescence meristem and secondary flowers develop within a flower (Mandel *et al.*, 1992). A second example is the *soc1 ful* double mutants, whose inflorescence meristems revert into vegetative meristems and develop rosettes on lateral branches (Melzer *et al.*, 2008). In both cases the reversion is independent from day length and can occur in LD as well as SD.

The second possibility is that the plants "forget" the inductive stimulus and continue their vegetative growth when returned to non-inductive conditions (Müller-Xing *et al.*, 2014). In LD, *FT* is expressed in the leaves and triggers flowering, but within one day after the shift back to SD its expression decreases drastically (Corbesier *et al.*, 2007). However, *FT* seems to be differentially regulated in the vasculature of pedicels, where it is strongly expressed even in SD and independent from CO (Liu *et al.*, 2014). This expression seems to be necessary for the maintenance of flowering, as floral reversion has been observed in *ft* mutants (Liu *et al.*, 2014; Müller-Xing *et al.*, 2014). It was found that epigenetic repression of *FLC* is necessary to enable this *FT* expression. The epigenetic regulation of *FLC* is facilitated by Polycomb-group (Pc-G) proteins; the Polycomb Repressive Complex 2 (PRC2) facilitates H3K27me modifications at the *FLC* locus in order to block transcription (Müller-Xing *et al.*, 2014). If the epigenetic regulation is lost, also the "memory" of the flowering stimulus is lost und *FT* cannot be expressed.

1.3.9 Other functions of *FT*

Research in Arabidopsis and also other species has revealed additional functions of *FT* that are not all directly related to flowering time. For example, *FT* functions in the cell autonomous timekeeping of stomatal guard cells for the correctly timed opening and closing of the stomata (Kinoshita *et al.*, 2011). There, *FT* transcript levels were found to correlate with the activity of H+-ATPases and therefore might fulfil a broader function in growth (Kinoshita *et al.*, 2011; Pin & Nilsson, 2012). Rather closely related to flowering is the role of *FT* and its close homolog *TSF* in the branching of the Arabidopsis shoot; they have an influence on number of axillary shoots and also their elongation. Interestingly, *FT* and *TSF* function in different photoperiods with *FT* mainly acting in LD and *TSF* in SD (Hiraoka *et al.*, 2013). Both proteins are able to interact with BRANCHED 1 (BRC1), which prevents premature floral transition of axillary meristems. This secures proper elongation of lateral shoots in order to have enough space for the optimal number of flowers (Niwa *et al.*, 2013). It is also an example of the sub-functionalization of *FT*-like genes, which is common in species that have more than one *FT*.

1.4 The role of *FTs* in poplar

Research about *Populus FT* genes was first published in 2006, when two independent groups showed that both *Populus FT* paralogs (*FT1* and *FT2*) can induce early flowering when overexpressed (Böhlenius *et al.*, 2006; Hsu *et al.*, 2006). This could decrease the flowering time of poplar from several years to a few months (in extreme cases even weeks). An unexpected result at the time was that trees with a milder phenotype grew normally in LD, but were insensitive to changes in photoperiod (Böhlenius *et al.*, 2006). This established a role of *FT* in the photoperiodic pathway and regulation of SD-induced growth cessation. Both *FTs* have completely opposite expression patterns; *FT1* being expressed in buds during winter and *FT2* in leaves during the summer (Hsu *et al.*, 2011). Therefore, *FT1* is likely not involved in photoperiod control of growth and has instead been hypothesized to act in flowering and/or dormancy release (Hsu *et al.*, 2011; Rinne *et al.*, 2011). Its function will be discussed later. Recently it was also found that *FT2* had undergone another, local duplication and at least in *Populus tremula* (European aspen and parent of our model species *Populus tremula x tremuloides*) *FT2* is entirely duplicated (Wang *et al.*, 2018), resulting in three *FT* paralogs total: *FT1*, *FT2a* and *FT2b* (**Figure 10**).

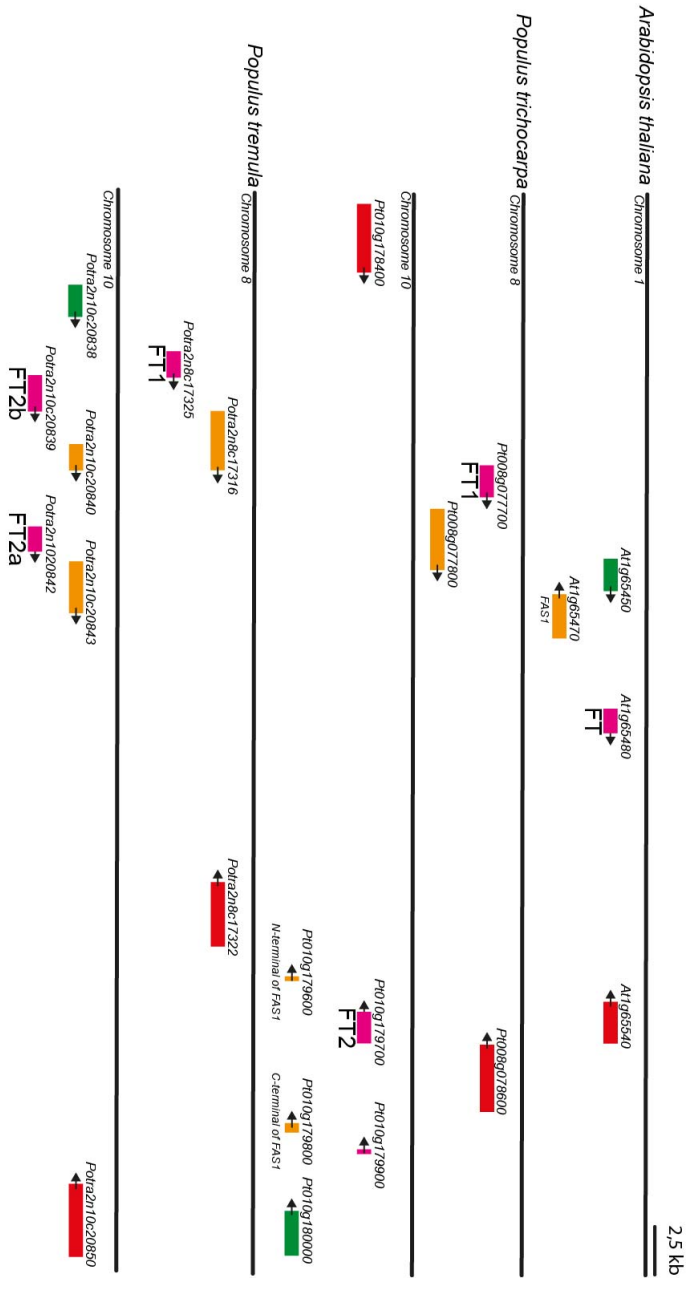

Figure 10: Synteny of the *FT* locus in *Arabidopsis thaliana*, *P. trichocarpa* and *P. tremula*.

Orthologous genes are indicated in the same colors. Arrowheads indicate the orientation of the gene.

However, all three FTs are extremely similar, differing only by a few amino acids (**Figure 11**).

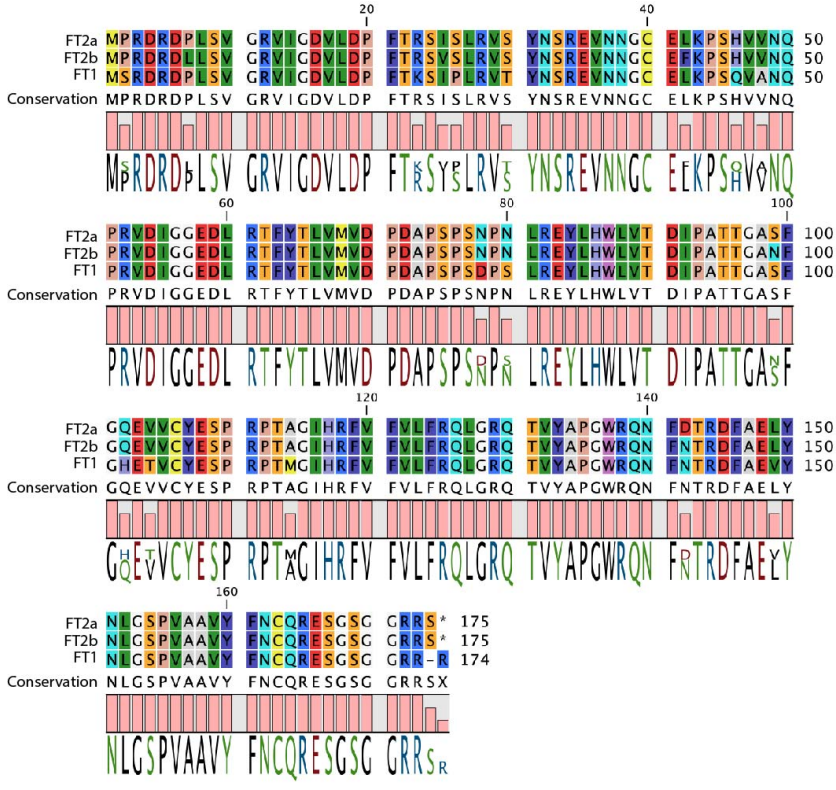

Figure 11: Protein alignment of *P. tremula* FT1, FT2a and FT2b.
Red bars indicate level of conversation between the proteins.

1.4.1 SD-induced growth cessation and bud set

What is considered a “short day” is defined by the CDL and differs in trees from different latitudes; trees in Umeå require >21h of light for growth, while trees from Germany can grow continually with only 17h (Böhlenius *et al.*, 2006). It needs to be pointed out that this is “deliberate” regulation. Trees in Umeå are not lacking the light or resources to grow at 17h light, there are no physiological constraints, only the prospect of approaching winter. This “safety mechanism” can be overridden by overexpression of *FT*; these plants are perfectly able to grow even under very short light regimes; however, they are unable to prepare for low temperatures and will suffer greater freezing

damage. Conversely, low *FT2* expression as that in *FT* RNAi trees will lead to early growth cessation even in ideal growth conditions (Böhlenius *et al.*, 2006).

The fact that trees are adapted to their "home" environment can be seen in common garden experiments. The Swedish Aspen Collection (SwAsp) is an initiative, which collected more than 100 genotypes of *Populus tremula* across Sweden and planted clones of them in two common gardens; one in the South in Ekebo and one in the North in Sävar (Luquez *et al.*, 2008). Trees originating from higher latitudes set bud earlier compared to their Southern relatives, despite being exposed to the same conditions (Luquez *et al.*, 2008).

The CONSTANS/FT regulon

Consistent with this observation, northern SwAsp clones have a much lower *FT2* expression than southern ones even in growth chamber experiments (Wang *et al.*, 2018). A possible explanation for this is the timing of *CONSTANS* expression. In accordance with the external coincidence model, *FT2* is only expressed when *CO* peaks during the light period. Indeed, the timing of the *CO* peak differs between individuals from different latitudes with it peaking later in northern than in southern populations (Böhlenius *et al.*, 2006). Therefore, sunset must be very late in the north for sunlight to coincide with *CO* expression.

The CO-independent pathway

In Arabidopsis, LD-induced *FT* expression is greatly dependent on *CO*. In poplar, however, downregulation/overexpression of *CO* orthologs has a much smaller effect than that of *FT* (Böhlenius *et al.*, 2006; Hsu *et al.*, 2012). Since *FT* is a hub in Arabidopsis, it is likely that there are other factors controlling poplar *FT2* expression. However, not much is known about upstream regulation of either *FT*. In poplar, both *GIGANTEA* orthologs (*GI* and *GIL*) have been found to have a strong effect on phenology and their downregulation leads to the complete abolishment of *FT2* expression while barely affecting *CO* expression (Ding *et al.*, 2018). Like in Arabidopsis, GIs can interact with FKF1b and CDFs and directly regulate the expression of *FT2*. However, it seems that in the case of poplar, by-passing of *CO* in this CO-independent pathway is more important for the regulation of *FT2* than in Arabidopsis and previously thought (Ding *et al.*, 2018). However, so far it is unclear whether (and if so, how) miRNA172 also contributes to *FT2*

regulation by *GI*, since poplar miRNA172 has a negative effect on *FT2* expression (Sane, 2020).

Phytochromes also play a role SD-induced growth cessation. Overexpression of *PHYA* prevents bud set through induction of *FT2* (Olsen *et al.*, 1997; Kozarewa *et al.*, 2010) and several SNPs have been identified in the *PHYB2* locus that associate with variation in bud set (Ingvarsson *et al.*, 2006; Ingvarsson *et al.*, 2008).

Furthermore, *FT2* expression ceases very quickly in SD, which cannot be fully explained by decreased induction. This suggests that *FT2* is actively down-regulated by a so far unknown repressor.

Gibberellins

Studies in other tree species have shown that exogenous GA application can prevent growth cessation in SD (Junttila & Jensen, 1988). Similarly, perturbance of GA biosynthesis affects growth cessation in poplar (Eriksson *et al.*, 2015). In hybrid aspen, steps catalyzed by the GA20-oxidase are rate limiting in the production of GA_1 and GA_4 (Israelsson *et al.*, 2004). Plants with increased GA content due to overexpression of GA20-oxidase are less sensitive to the SD signal, while the opposite is true for plants with reduced GA content. In wild type plants, the amount of bioactive GAs is also reduced in SD leaves compared to LD (Eriksson *et al.*, 2015). The effect on growth cessation can only be partially explained by GAs effect on *FT2*. While *FT2* expression is generally higher in plants with higher GA concentration, it is still strongly down-regulated when exposed to SD (Eriksson *et al.*, 2015). Therefore, GAs must affect other targets, presumably directly in the shoot apex as is the case in Arabidopsis.

FTs mode of action at the shoot apex

Arabidopsis FT is a mobile agent and the same has been shown for poplar FT1 (Miskolczi *et al.*, 2019), and is likely also the case for both FT2. FT can travel from its site of expression through the phloem to the shoot apex, where is interacts with FD-like proteins (FDL; Tylewicz *et al.*, 2015). There are two paralogs, FDL1 and FDL2, which can both interact with FT, but only the FT-FDL1 complex is able to induce the expression of downstream targets (Tylewicz *et al.*, 2015). These downstream targets are *Like-AP1* (*LAP1*) and *AINTEGUMENTA-LIKE* 1 (*AIL1*), orthologs of *AP1* and *ANT*, respectively (Karlberg *et al.*, 2011; Azeez *et al.*, 2014). *FT2* induces the expression of

LAP1 in the shoot apex, which in turn induces *AIL1* (**Figure 12**). *AIL1* can control the expression of D-type cyclins and thus the cell cycle and proliferation (Karlberg *et al.*, 2011). While *FT2* expression ceases quickly in SD, expression of *LAP1* and *AIL1* is still detectable for some time, but will eventually cease as well (Karlberg *et al.*, 2011; Azeez *et al.*, 2014). *LAP1* also represses *BRANCHED 1* (*BRC1*), which is a known repressor of bud outgrowth in Arabidopsis (Aguilar-Martínez *et al.*, 2007). BRC1 physically interacts with FT2 at the shoot apex and antagonizes its function (Maurya *et al.*, 2020), leading to lesser induction of *LAP1* and thus creating a negative feedback loop. In SD, *FT2* expression therefore not only ceases but also its protein function is inhibited, making it both less abundant and less efficient.

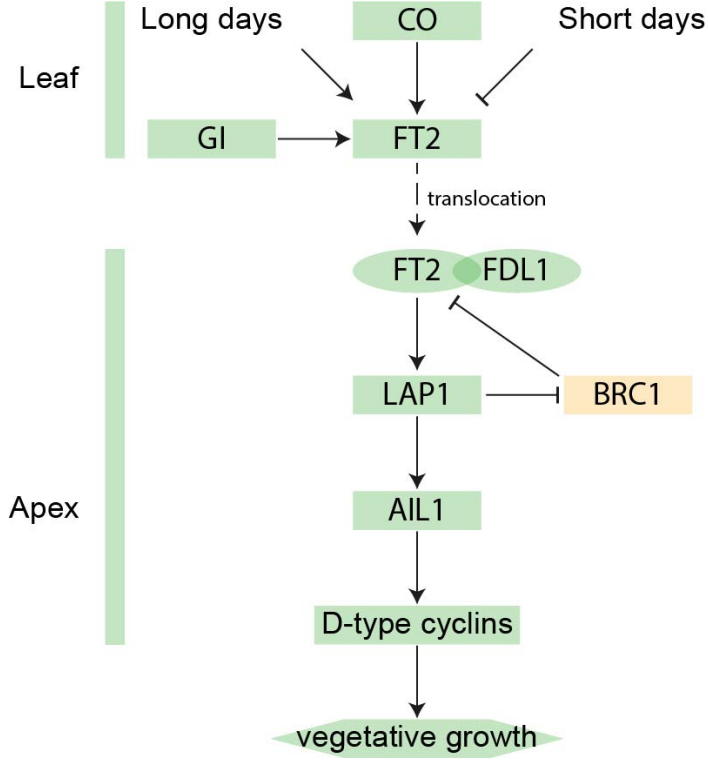

Figure 12: *FT2* regulates vegetative growth.
Genes indicated in green are promoters of vegetative growth while orange genes are repressors. Ovals indicate proteins and boxes indicate genes.

1.4.2 Bud formation and dormancy establishment

Once growth, i.e., the formation of new leaves at the shoot apex, ceases, stipules are re-purposed and form bud scales. These will form a hard bud around the meristem and protect it from harsh winter conditions. Even though the first signs of bud formation are only visible after a few weeks of SD treatment, changes in the shoot apex to develop bud scales and embryonic leaves are rapid (Ruttink *et al.*, 2007). Abscisic acid (ABA) and GAs are essential players in seed dormancy (Penfield & King, 2009) and also affect bud set and dormancy (Eriksson *et al.*, 2015; Singh *et al.*, 2019). However, decreasing GA levels seems to regulate bud set rather than dormancy *per se*, but formation of a bud is necessary for bud dormancy.

ABA on the other hand accumulates in the apex upon SD exposure and ABA-insensitive plants set bud normally, but cannot establish bud dormancy (Singh *et al.*, 2019). If these plants are exposed again to growth promoting conditions, they can flush their buds and revert to vegetative growth without the normally required cold exposure (Singh *et al.*, 2019). Dormancy is thought to be established by shutting off the shoot apex from growth promoting signals like FT and GAs. This is done by closing plasmodesmata, which connect neighboring cells, with so-called dormancy sphincters (Rinne *et al.*, 2011). These consist of callose, which is deposited by CALLOSE SYNTHASE 1 (CALS1; Singh *et al.*, 2019). This closure will impact the inter-cellular transport of nutrients, hormones and transcription factors and thus cell-to-cell signaling necessary for growth (Singh *et al.*, 2016).

ABA signaling is upstream of plasmodesmata closure and relies on both *PICKLE* (*PKL*) and *SHORT VEGETATIVE PHASE LIKE* (*SVL*; Tylewicz *et al.*, 2018; Singh *et al.*, 2019). *PKL* is an antagonist of the Polycomb group and its down-regulation is necessary for dormancy establishment (Aichinger *et al.*, 2009; Tylewicz *et al.*, 2018). *PKL* suppresses the expression of *SVL*, which similarly to its Arabidopsis ortholog represses GA biosynthesis. It also induces *CALS1*. Therefore, *SVL* expression promotes and maintains dormancy by both positive regulation of plasmodesmata closure and negative regulation of growth promoting signals (Singh *et al.*, 2019; **Figure 13**). At the same time SVL induces the expression of NCED3 and RCAR/PYL, which are ABA biosynthesis enzymes and receptors, respectively. This creates a positive feedback loop that ensures high ABA concentration and sensitivity (Singh *et al.*, 2018).

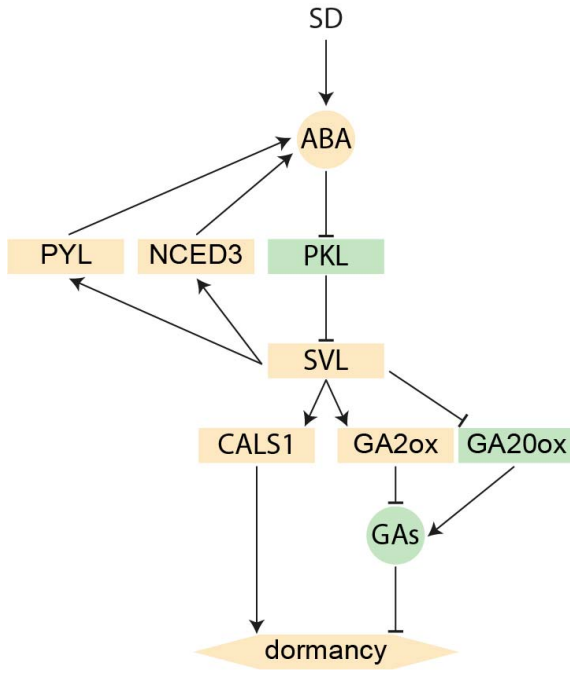

Figure 13: Regulation of dormancy establishment.
Factors indicated in green are promoters of vegetative growth and need to be downregulated for dormancy establishment. Factors indicated in orange are positive regulators of dormancy establishment. Boxes indicate genes and circles represent hormones.

Different types of dormancies

When poplars develop buds in response to SD, for a short time they are able to revert to vegetative growth if exposed to the right conditions. This state has previously been termed "ecodormancy", because the repression of growth is maintained by unfavorable environmental conditions. Establishment of true "endodormancy" follows after prolonged exposure to these unfavorable conditions. "Endodormant" buds are unable to resume growth even in the best conditions because dormancy is maintained by endogenous signals. "Endodormancy" needs to be released by prolonged exposure to cold temperatures for the buds to be responsive again. In nature, "endodormancy" is released during the winter, but growth does not continue until spring. Therefore, another "ecodormancy", maintained by low or freezing temperatures and short photoperiod, prevents bud flush. As Singh and colleagues (2016) point out, this terminology of eco- and endodormancy

can be misleading. It suggests that the states of "ecodormancy" are similar both pre- and post-endodormancy, but several studies have shown that this is not the case. Indeed, transcriptomic and metabolic profiles and even chromatin modifications differ significantly (Karlberg *et al.*, 2010; Howe *et al.*, 2015; Singh *et al.*, 2016; **Paper II**). Furthermore, seed dormancy has been defined as "the inability to germinate in growth inductive conditions" (Bewley, 1997). Similarly, only "endodormant" buds are unable to respond to growth inductive conditions. "Ecodormant" buds are kept in their state because of environmental conditions, therefore precisely not because of an inability. A third type of dormancy, namely "paradormany", describes the dormant state of lateral buds, whose flush is prevented by hormonal control and apical dominance. Even though it is controlled by endogenous signals, it is not the same as "endodormancy", since the signals come from different parts of the plant. It has been suggested to be evolutionary older and a precursor to "endodormancy" (Rohde & Bhalerao, 2007). However, any kind of dormancy is hard to assess in both seeds and buds, since the only readouts are germination/bud flush, processes which can happen much later and can be regulated independently. Here, when talking about dormancy, it refers to endodormancy.

1.4.3 Bud dormancy release and bud flush

Dormancy is mostly necessary during the autumn to prevent regrowth in case of mild weather and to make sure that cold hardiness will be established before winter. It is released by prolonged exposure to cold but non-freezing temperatures, which already prevail during autumn. Therefore, bud dormancy can already be released before winter (Rinne *et al.*, 2011). On the other hand, these temperatures promote cold hardiness and freezing tolerance (Rinne *et al.*, 2001). Cold hardiness is affected by the circadian clock, with its members *LHY* and *TOC1* having positive and negative effects, respectively (Ibáñez *et al.*, 2010).

The regulation of dormancy release involves some of the same players as its establishment, including *SVL*, ABA and gibberellins. While *SVL* expression and ABA concentrations are increased during SD, cold temperatures lead to their decrease. EARLY BUD BREAK 1 (EBB1) is induced by cold and repress *SVL*, breaking the positive feedback loop of ABA and *SVL* that maintained dormancy previously (Singh *et al.*, 2018; Azeez *et al.*, 2021; **Figure 14**). Reduction of ABA signaling then leads to an

induction of EBB3, which in turn induces cell proliferation though cyclins like CYCD3.

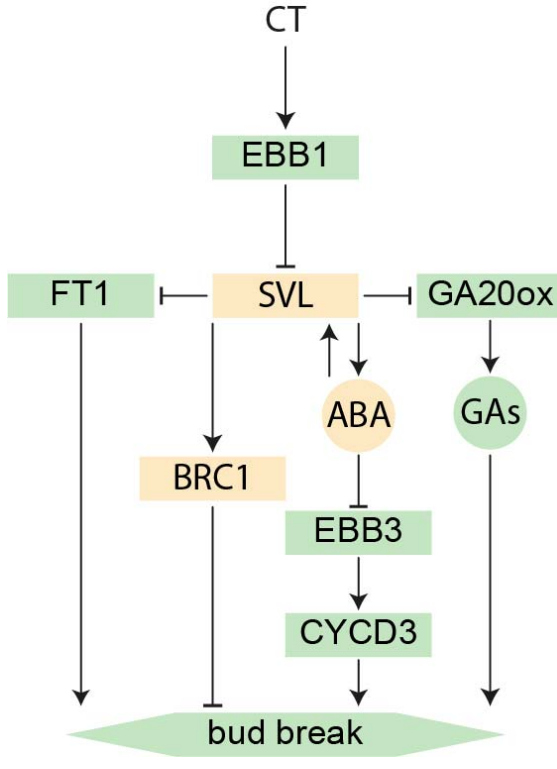

Figure 14: Regulation of bud break.
Green factors indicate a positive effect on bud break, while orange factors represent negative regulators of bud break. Boxes indicate genes and circles indicate hormones.

With lowered SVL levels, *CALS1* is less induced. This leads to an overall decrease of callose deposition and shifts the balance to callose degradation and opening of the plasmodesmata (Rinne *et al.*, 2011; Singh *et al.*, 2019). Hydrolyzation of callose is hypothesized to be done by GH17 proteins, some of which are induced by chilling, but no experimental evidence for this action *in vivo* has been found so far (Rinne *et al.*, 2011). Furthermore, GA biosynthesis is less repressed by SVL and GA levels rise during chilling (Singh *et al.*, 2019). GAs are important for dormancy release in seeds (Penfield & King, 2009) and in buds exogenous GA application is able to substitute for chilling (Rinne *et al.*, 2011). Exposure to low temperatures also causes a hyper induction of *FT1* (Rinne *et al.*, 2011). Even though its precise function is so far unknown, it seems to be a growth promoter like *FT2*. *SVL*

is a repressor of *FT1* and with its downregulation in cold, *FT1* levels can rise (Singh *et al.*, 2019). However, the absence of a repressor alone cannot explain the very high *FT1* expression and other positive regulators that are active in chilling buds are so far not known. It has been hypothesized that *FT1* is expressed in the embryonic leaves and, once the plasmodesmata are open again, can travel to the shoot apex where it drives bud flush and shoot elongation (**Figure 15**; Rinne *et al.*, 2011). Another factor controlling bud break is again *BRC1*, whose expression is positively regulated by *SVL* and ceases together with the repression of *SVL* by cold temperatures (Singh *et al.*, 2019).

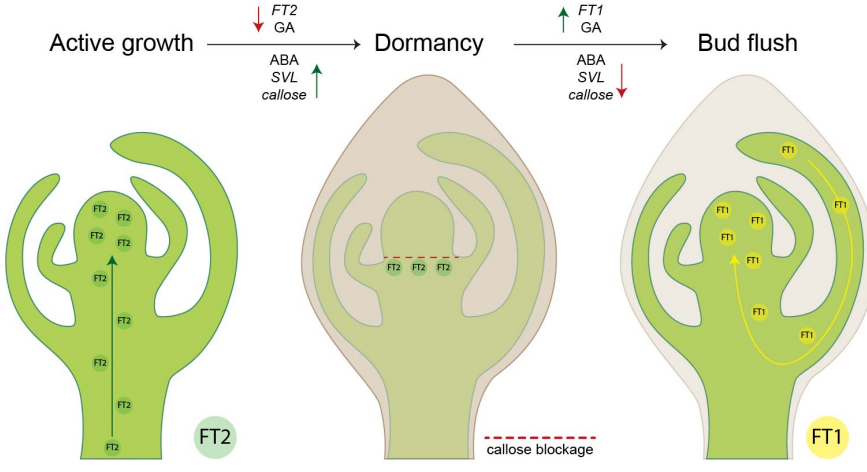

Figure 15: The SAM is isolated by callose blockage during dormancy.
Schematic representation of a poplar shoot apex during vegetative growth (left), enclosed in a bud during dormancy (middle) and during bud flush (right). FT1 and FT2 proteins are indicated by yellow and green circles, respectively, and travel to the SAM. During dormancy, access to the SAM in blocked by callose in the plasmodesmata, indicated by a dashed red line. Genes/hormones that are up/downregulated in between the stages are indicated by green and red arrows, respectively.

1.4.4 Flowering in poplar

In contrast to Arabidopsis, poplar trees remain in a vegetative state for several years before they can flower and they are able to resume vegetative growth after sexual maturity. This is possible because only a limited number of meristems transform into inflorescence meristems during one flowering event, while all apical meristems and many lateral meristems stay in the vegetative state (Albani & Coupland, 2010). So far it is still unknown which

FT is responsible for flowering in nature. *FT2* expression seems to increase with age, but *FT1* is highly expressed in reproductive buds during winter (Böhlenius *et al.*, 2006; Hsu *et al.*, 2011). Deciphering which one sends the floral signal is difficult because it takes almost one year from floral initiation to actual anthesis (**Figure 16**; after Pin & Nilsson, 2012). Experiments with weak overexpression of either *FT* showed that *FT1* has a higher potential for floral induction and that downregulation of *FT2* did not diminish this effect (Hsu *et al.*, 2011). However, in these experiments *FT1* was not expressed in its natural tissues, nor was flowering induced during the correct "season". In nature, floral buds develop during the early growing season, just when *FT2* starts being expressed. The best way to find out which one, if any, is required for flowering would be comparing the flowering time of specific knock-out mutants. However, this would take many years and can be complicated by additional effects of the mutations.

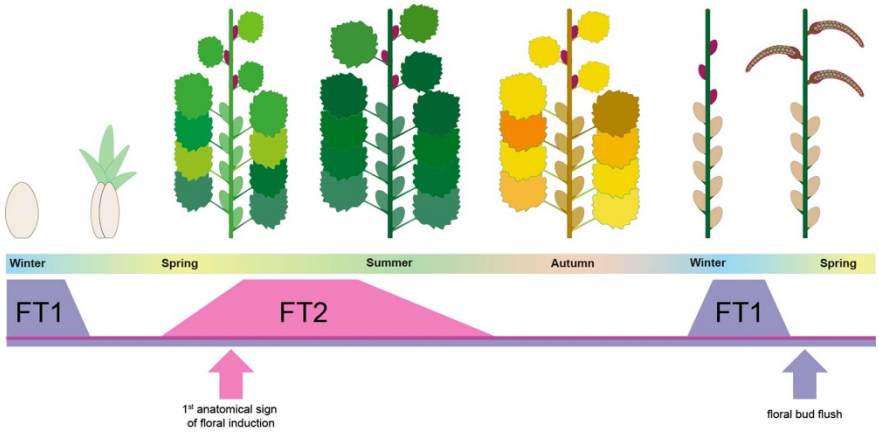

Figure 16: Expression of *FT1* and *FT2* coincides with different stages of flowering. Upper panel represents growth stages of poplar over the course of the seasons (bar below). Purple and pink lines represent the level of *FT1* and *FT2* expression, respectively. Arrows indicate important time points for flowering. (After Pin and Nilsson, 2012)

Morphogenesis of flowering shoots

For *Populus deltoides* (eastern cottonwood), a 3-year flowering cycle has been proposed. In the first year of the cycle, early preformed leaves develop within the terminal bud of a shoot, bearing axillary vegetative buds. Subsequently in the same season, late preformed leaves develop, which form reproductive buds in their axils during the growing season of the second year.

The floral bud meristems give rise to scale leaves and floral meristems in the axils of bracts, which subsequently form floral organs. They overwinter in this state and anthesis follows in spring of the third year (Yuceer *et al.*, 2003; **Figure 17**).

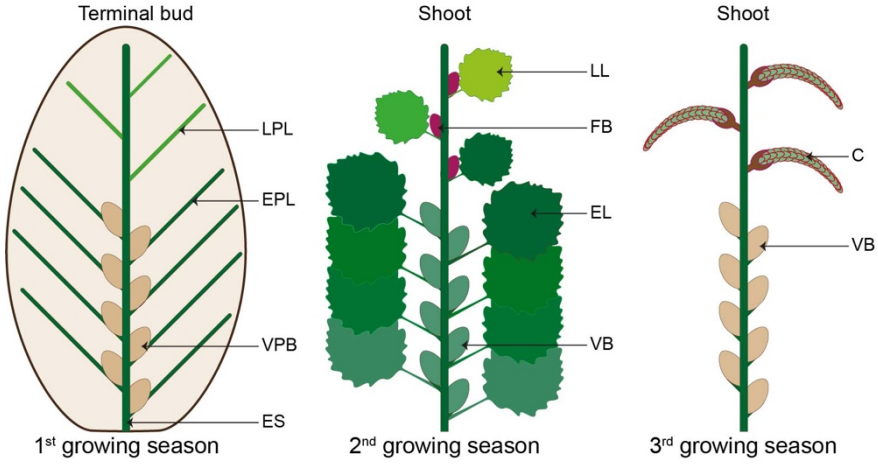

Figure 17: Flower development in *Populus deltoides*.
Schematic representation of a poplar shoot over three seasons from an embryonic shoot (left) to a flower bearing shoot (right). ES = embryonic shoot, VPB = vegetative preformed bud, EPL = early preformed leaf, LPL = late preformed leaf, VB = vegetative bud, EL = early leaf, FB = floral bud, LL = late leaf, C = catkin

CENL genes as FT antagonists

Just as Arabidopsis *FT* has its antagonist in *TFL1*, poplar *FT* have *TFL1* orthologs as their antagonists. Here they are called *CENTRORADIALIS LIKE 1* and *2* (*CENL1* and *CENL2*; Mohamed *et al.*, 2010). While *CENL2* is expressed in leaves (Mohamed *et al.*, 2010), *CENL1* is localized in the rib meristem of the shoot apex (Ruonala *et al.*, 2008). Overexpression of *CENL1* leads to a slightly advanced bud set and a more significantly delayed bud flush (Mohamed *et al.*, 2010), even though *CENL1* is naturally induced during bud flush, probably to ensure indeterminacy of the meristem (Rinne *et al.*, 2011). *CENL1* RNAi plants also have a significantly higher number of short shoots (Mohamed *et al.*, 2010). The growth of short shoots is determined, in contrast to long shoots that will grow as long as conditions are permissive (Critchfield, 1960). Short shoots are also primarily the bearers of floral organs and flowering time is negatively correlated with *CENL1* expression (Mohamed *et al.*, 2010). The function of *CENL2*, however, is so far unknown.

2. Objectives

The aim of my thesis project was to elucidate the roles of poplar *FLOWERING LOCUS T (FT)* genes in the annual growth cycle. This includes finding out which specific processes they are involved in, as well as how they are regulated.

While *FT1* has been suggested to be responsible for flowering, it is also expressed in buds of juvenile trees and likely fulfils other functions there. A role in dormancy release/bud flush has been hypothesized, but never shown. The role of *FT2* in vegetative growth and SD-induced growth cessation has been established, but much of its upstream-regulation was not well understood. Especially since it seems to deviate from the dominant regulation by *CO* as in Arabidopsis. The recently identified *FT2b* has also not received much attention and its function(ality) was unclear.

In this thesis I explore *FT* regulation and function in three different studies.

- How do light and temperature affect the regulation of the annual growth cycle and how are their signals translated through *FT* regulation?
- Which specific roles do the three *FT* paralogs play and which physiological processes are affected in their knock-out mutants?
- How is *FT2* expression fine-tuned in the leaf in response to the SD signal?

3. Material and Methods

This is a general description of our plant material, growth conditions and experimental procedures. For specifics, please refer to the individual manuscripts.

3.1 Plant material

Most experiments performed here used our model organism hybrid aspen, a cross between European aspen (*Populus tremula*) and American aspen (*Populus tremuloides*), more specifically clone T89, which originates from the Czech Republic (Nilsson *et al.*, 1992). T89 has been established because of its high transformation efficiency compared to *P. tremula*. It is easy to keep as an *in vitro* culture and can be amplified through stem cuttings.

However, the fact that T89 is a hybrid can make things more complicated. For example, looking at the different loci and alleles is already not easy, especially with highly conserved regions as in the *FT* loci. Usually, for each locus there are two alleles, a maternal and a paternal one. If they are the same, the locus is homozygous, if they are different, it is heterozygous. When looking at a data base, one would expect two sequences for each locus, but with sequencing methods that produce short reads, this distinction is not possible. Even though there is some variation between individuals, coding sequences are usually well preserved and, in most cases, it will not be a problem if both alleles get merged into one sequence. But when crossing two species, these differences might be more substantial. It might also happen that certain genes/loci do not exist in one parent, making those loci hemizygous.

In earlier versions of the T89 genome (version 1, www.popgenie.org), both *FT2a* and *FT2b* were collapsed into one sequence because of their high

similarity. Only once longer reads could be produced, *FT2b* was found upstream of *FT2a* (Wang *et al.*, 2018). This was possible because both loci were located on the very same read. This needed no assembly, which could potentially combine both loci. This was a problem because of the local duplication and not T89-specifc. The complication in T89 was the question whether *FT2b* also existed in the *P. tremuloides* part of the genome and if so, what its sequence was. New sequencing technologies eventually made it possible to distinguish both haplotypes in T89 and we have six distinct sequences for our three *FT* genes in T89 (beta.popgenie.org).

3.2 Design and application of CRISPR-Cas9

As mentioned earlier, there is no catalogue of knock-out mutants for poplar, but thanks to recent advanced in genome editing techniques, it is now possible to create knockout mutants by "cutting out" parts of the genome or creating small insertions/deletions. The development of the CRISPR-Cas9 technique has been awarded the 2020 Nobel Prize in chemistry[3].

CRISPR-Cas systems are part of prokaryotes' natural defense system that can cleave the DNA of invaders. Stretches of viral DNA, which have been integrated into the host genome during a previous infection, are flanked by clustered regularly interspaced short palindromic repeats (CRISPR). The pieces of viral DNA allow the recognition in case of a repeated infection and provide some sort of "catalogue" of known invaders. They are transcribed into long RNAs, which are bound and processed by CRISPR associated (Cas) proteins. Together they can target and cleave foreign DNA that is identical in sequence. To protect the host genome from cleavage by its own defense system, an additional PAM sequence is required for the system to work. This NGG must be located adjacent to the recognition site in the host DNA, but is absent in the host genome (Hille *et al.*, 2018).

In 2012 a technique was developed, which simplified the natural system and made it programable and versatile (Jinek *et al.*, 2012) and most importantly transferable to other organisms. This opened the door to genome editing in a range of species. Nowadays one can design a single guide RNA (sgRNA), which will lead the Cas9 protein, an endonuclease, to the desired spot in the genome and induce a double strand break. The natural DNA repair

[3] https://www.nobelprize.org/prizes/chemistry/2020/press-release/

system is error prone in these cases and will likely insert or delete a few bases at the cutting site. If this happens in an exon of a gene, a frameshift during translation is almost guaranteed and will cause a non-functional protein. The only restrictions there are now is the requirement of the PAM sequence close to the sgRNA target.

In the case of highly homologous genes like the *Populus FTs*, it can be difficult to find gene specific sgRNAs that target exons. Therefore, we decided to use two sgRNAs at the same time that cause two double strand breaks and thus a deletion of a bigger genomic fragment (**Figure 18**). In this case it is not a problem if the sgRNAs target introns or non-transcribed regions around the gene. To ensure disruption of the protein function, one can target the translational start site or the functional domain.

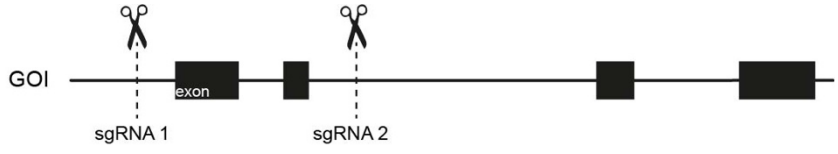

Figure 18: Graphic representation of the CRISPR design.
GOI = gene of interest; line represents UTRs and introns, boxes represent exons; dotted line marks sgRNA target and cutting site by CRISPR-Cas9 complex

3.3 Growing conditions

In vitro cultures of the trees are grown on MS medium (Murashige & Skoog, 1962) in closed jars until they are transferred to soil for the experiments. Once transferred, the pots are covered with plastic bags to slowly let the trees adapt to the much lower humidity. Corners of the bags are cut after one week and the bags are completely removed two weeks after potting. During the time, the plants hardly grow but rather establish themselves in their new environment. The day of bag removal is considered day 1 of the experiment. To simulate a change of season in our growth chambers, we use three different growing conditions; long day (LD) for spring and summer, short day (SD) for autumn and cold treatment (CT) for winter (**Figure 19**). Standard LD growing conditions in our greenhouse are 18h of light and 6h of darkness at ~20°C. Trees are also treated weekly with NPK-Rika S fertilizer. For phenotyping purposes (measuring heigh, diameter, number of leaves, etc.) trees are grown in LD until they reach ~2 meters in height. If they are to be subjected to SD treatment, this starts after 4 weeks or when the

trees are between 1 and 1.5 meters tall. The critical day length of T89 is around 15.5 hours (Olsen *et al.*, 1997). We use a cycle of 14h light/ 10h dark for our SD treatment. This mild SD treatment leads to a rather slow growth cessation and bud set, which allows the detection of even small phenotypic differences between T89 and genetically modified lines. SD treatment lasts for 10-15 weeks, depending on genotype and swiftness of the response, to ensure proper bud set and dormancy establishment. Fertilization is stopped after the shift, since it delays the SD response. To release dormancy, plants are subjected to CT; 6°C and an even shorter photoperiod of 8h light and 16h dark. Dormancy can be released after as little as 5 weeks (Singh *et al.*, 2018), but we keep them there for at least 8 weeks. To initiate growth, plants are returned to the same LD conditions as before, but fertilization starts only after buds have fully flushed.

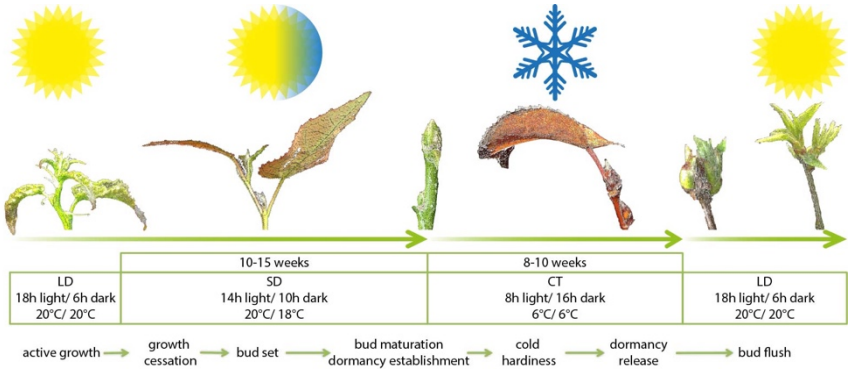

Figure 19: Illustration of the growing conditions used to simulate a change of seasons.
Boxes represent the different treatments. Photographs show representative growth stages of each treatment.

3.4 Bud set and bud flush scoring

To assess the speed of SD/LD response, we use score sheets for bud set and bud flush, respectively (Ibáñez *et al.*, 2010).

For bud set, the stages range from 3 to 0 (**Figure 20**). Stage 3 is active growth with generation of new leaves at the shoot apex. Stage 2 defines growth cessation; internode elongation has stopped and no new leaves are being produced. Stage 1 describes bud formation, where stipules start to be arranged into bud scales. Stage 0 and thus complete bud set are reached when

the bud is fully closed, has hardened and optionally is colored more reddish. Stages are scored once a week during SD treatment. Since not all buds look perfectly like the guide or perfectly alike, 0.5 increments can be scored in case of doubt. Furthermore, height can be measured to better capture the time when internode elongation stops and leaves can be counted to see when their production stops. A combination of all three will give the most accurate picture of the SD response and can allow a distinction between effect on growth cessation and effect on bud set.

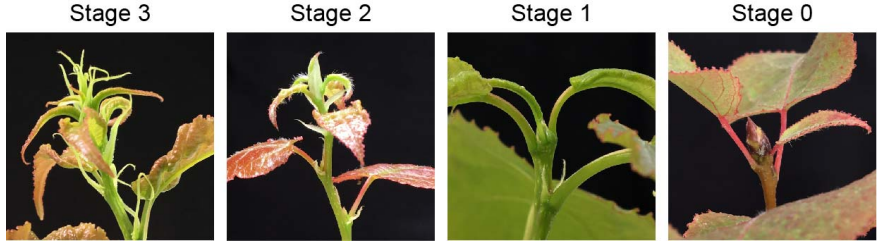

Figure 20: Bud set stages in T89.
Photographs of shoot apices over the course of growth cessation and bud set.

Bud flush can be divided into six stages, which are less broadly defined than bud set stages (**Figure 21**). The scale starts with stage 0, when buds are completely enveloped by their scales and are red-brown in color. At stage 1, buds are starting to swell and become green rather than brown. Score 2 describes the emergence of leaf tips between the scales. At stage 3, buds are opened but leaves are still clustered together. Leaves are diverging at score 4 and are completely unfolded at stage 5, when shoot elongation has visibly started. The scoring is done both for apical buds and the most advanced lateral bud. However, flush of the apical bud is considered more meaningful, since lateral buds could be influenced by a loss of apical dominance if the apical bud has died or been damaged (Singh *et al.*, 2016).

Figure 21: Bud flush stages in *Populus tremula*.
Photographs of shoot apices over the course of bud flush.

3.5 RNA sequencing and bioinformatic analyses

Usually, when checking gene expression of our genes of interest, we use quantitative real time PCR (qPCR). However, qPCR is inadequate to investigate global changes in the transcriptome. For that purpose, we use RNA sequencing instead.

Total RNA is extracted from homogenized tissues (leaf, bud or apex). Because mRNA makes up only a small fraction of the total RNA, it has to be enriched. This can be done by polyA-selection. For library preparation, the RNA is fragmented and reverse transcribed into double stranded cDNA. The resulting 300-500 bp long fragments are ligated to adapter sequences, which then bind to the flow cell.

Illumina sequencing generates two reads per fragment of around 150 bases, starting from each end. When the data is delivered by the sequencing company, it includes the sequences of the reads plus a quality score for each base, which indicates how trustworthy the result is.

During the preprocessing, several rounds of quality assessment are performed. This includes the quality check of the sequence, removal of

residual rRNA sequences and elimination of contaminations (e.g., from organelles). Adapter sequences from the library preparation are also trimmed.

The goal of the analysis is to estimate expression levels. For that, the reads are mapped to a reference transcriptome. We use Salmon (Patro *et al.*, 2017), which is fast, but disregards splice variants and no new genes/isoforms can be found. To account for biases in sequencing depth (how many reads are generated from a library) and length of a gene (more reads will map to longer genes), the counts are normalized. A metric called "Transcripts per Kilobase Million" (TPM) allows for the comparisons of different samples. For each gene, it indicates how much it contributes to the overall transcriptome. However, since this is not an absolute quantification, some assumptions need to be made. First, we assume that the number of expressed genes does not change between samples and treatment. This can be checked during the quality assessment to find the number of genes that are not expressed at all. Second, we assume a binomial distribution of read coverage and that very lowly/highly expressed genes are the exception.

Additionally, a variance stabilizing transformation corrects for the fact that there is a mean-variance relationship; genes with low expression have a high variance and genes with high expression have a low variance. The data becomes homoscedastic and usable for most parametric statistic tests. It covers the whole range of expression, where the lowest expression is defined/limited by sequencing depth.

The data can now be plotted in heat maps or simple expression over time/treatments (**Figure 22**).

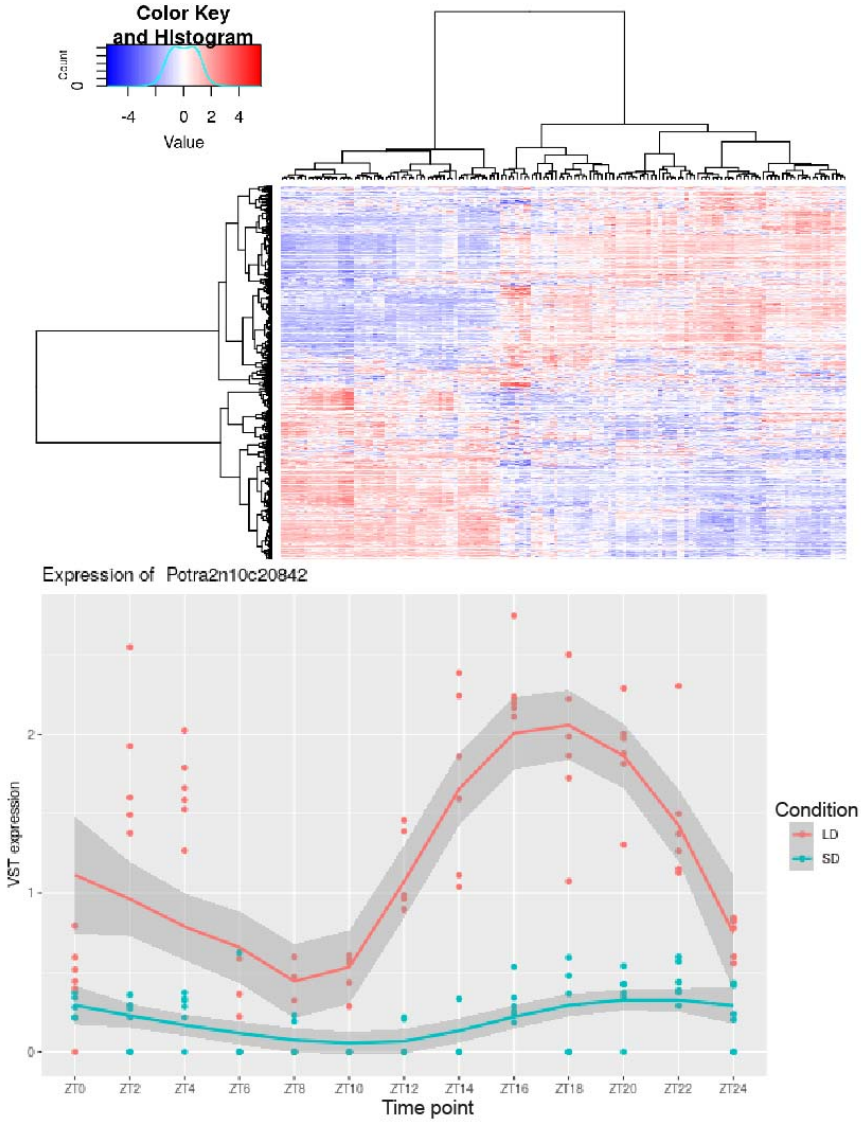

Figure 22: Typical display of RNAseq results.
Heatmap of all genes in all samples of the data set (top) and VST expression of one gene in two different growing conditions (bottom). Both plots were generated in R.

4. Results and discussion

4.1 Paper I

Phytochrome B and PHYTOCHROME INTERACTING FACTOR8 modulate seasonal growth in trees

In **Paper I** we examine the role of phytochrome B (phyB) and PHYTOCHROME INTERACTING FACTORS (PIFs); how they perceive environmental signals and how they control physiological responses through *FT*. We show that phyB controls both shade avoidance response (SAR) and vegetative growth through PIFs with PIF4 being mostly involved in SAR and PIF8 regulating both SAR and seasonal growth through FTs (Ding *et al*., 2021*)*.

Since both light and temperature change drastically over the course of one year, their perception and the subsequent signaling plays a major role in the regulation of the annual growth cycle. Night breaks with either red or far-red light can inhibit SD-induced growth cessation (Howe *et al.*, 1996), showing that phytochromes play an important role in the SD response. In Arabidopsis, phyA and phyB play opposite roles in the regulation of *FT*; phyA stabilizes CO, while phyB destabilizes it. Since *CO* appears to play only a minor role in the regulation of *Populus FT*, the photoperiodic pathways of both species seem to have diverged. It is therefore of interest to understand how phytochromes control seasonal growth in *Populus* independently of *CO*. phyB has been associated with phenology before (Frewen *et al.*, 2000), but the mechanisms by which it controls growth were poorly understood.

Phytochrome signaling goes through PIF proteins, which inhibit phytochrome-induced responses while themselves being inhibited by phytochromes (Leivar *et al.*, 2008). In Arabidopsis, PIFs are involved in SAR as well as thermo-morphogenesis. Here we characterize *PHYB* and *PIF4* and *PIF8* and investigate their roles in the regulation of the annual growth cycle.

The genome of *Populus tremula* contains three phytochrome-like genes: *PHYA*, *PHYB1* and *PHYB2* (Paper I; Figure S1). We generated transgenic lines that either downregulated both *PHYB1* and *PHYB2* expression together (*PHYB*RNAi) or overexpressed each of them individually (oePHYB1 and oePHYB2) and examined their effect on growth. High *PHYB* expression had a negative effect on shoot elongation during LD growth (Paper I, Figure S4), while absence of phyB led to elongated internodes (Paper I, Figure 1), a typical shade avoidance response known from *phyB* mutants in Arabidopsis. Consistent with their roles in other species (Franklin & Quail, 2010), these results suggest that *P. tremula* phyBs play a role in SAR and are negative regulators of shoot elongation during vegetative growth.

We next investigated the role of phyBs in SD-induced growth cessation by subjecting the transgenic lines to our standard SD treatment. *PHYB*RNAi plants were hypersensitive to the change in photoperiod and responded with growth cessation two weeks earlier than the wild type (WT; Paper I, Figure 1). Overexpression of either *PHYB* caused hyposensitivity to the SD signal and plants ceased growth later than WT (Paper I, Figure S4). Thus, both phyB1 and phyB2 can act as suppressors of the SD response.

In contrast to bud set, bud flush is triggered by warm temperatures (regardless of day length) and phyB has been shown to be a thermosensor in Arabidopsis. Therefore, we investigated whether phyB plays a role in temperature-mediated bud break. Indeed, after chilling and return to warm temperatures, *PHYB*RNAi plants flushed their buds later than WT, while oePhyB flushed earlier than WT (Paper I, Figures 1 & S4). This suggests that phyB promotes vegetative growth also during spring.

To investigate whether phyB1 and phyB2 act redundantly or have specific functions, we generated individual knock-out (KO) lines with CRISPR-Cas9. Since only *PHYB2*KO plants showed strikingly different phenotypes compared to WT in height growth, growth cessation and bud break (Paper I, Figures 1 & S5), phyB2 seems to be the dominant phyB in *Populus*.

However, double knock-out of both *PHYB1* and *PHYB2* resulted in very sick plants, most of which died shortly after transformation. The few surviving shoots terminated growth and set terminal buds already in tissue culture (Paper I, Figure S5). This suggests that phyB1 may have a smaller role but nevertheless can compensate partially for the lack of phyB2 activity.

In Arabidopsis, *PIF4* is a central hub integrating environmental cues like light and temperature downstream of the phytochromes. Therefore, we investigated its role in *Populus*. Of two *PIF4* genes, only *PIF4a* encodes a protein with an active phyB binding domain (Paper I; Figure S6). *PIF4a* overexpressing plants had poor survival on soil (Paper I; Figure S9). Downregulation of *PIF4* expression on the other hand had only a small effect on vegetative growth and no effect on SD-induced growth cessation and bud break (Paper I, Figure S9). Instead, *PIF8* expression levels greatly affected these processes. Overexpression of *PIF8* (oePIF8) showed a strong SAR, mimicking the phenotype of *PHYB*RNAi (Paper I, Figure 2, S3). In contrast to *PHYB*, downregulation of *PIF8* delayed growth cessation, but promoted bud flush (Paper I, Figure 2), suggesting that their negative relationship is conserved. Next, we wanted to investigate whether the *PHYB/PIF8* regulon acts through the regulation of *FT* and *CENL* genes. *FT2* was downregulated in *PHYB*RNAi and oePIF8 plants already in LD. The normally drastic decrease of *FT2* expression upon shift to SD was attenuated in *PIF8*RNAi lines (Paper I, Figure 3). This shows that *PHYB* promotes vegetative growth in the autumn through *FT2*. During bud break, *PHYB* expression was negatively correlated with *FT1* and *CENL1*. Both genes are induced by cold and quickly repressed in warm temperatures, but maintained higher expression in *PHYB*RNAi and oePIF8 (Paper I, Figure 3).

Since *PHYB* regulates both SAR and seasonal growth, we investigated how these different pathways are coordinated. Using wild type and *PHYB*RNAi plants, we compared leaf and shoot samples from both LD and SD. During growth in LD, *PHYB*RNAi seemed to affect the leaf transcriptome much more than the shoot transcriptome (~1000 vs ~150 differentially expressed (DE) genes; Paper I, Figure 4). Upon shift to SD, however, the number of differentially expressed genes increased in both tissues. Since many of the DE genes were tissue specific, it indicated that phyB regulates the photoperiodic response in a spatial manner. Three different groups of DE genes were identified; group A specifically differentially regulated in leaves, group B in shoots and group C that was

shared between both tissues and time points. Gene ontology analysis showed that group A genes were mainly related to response to shade, e.g., photosystem, response to light and hormone regulation. Group B genes on the other hand were involved in processes that change during growth cessation like cell cycle/division and cell wall organization. Group C genes have been associated with both SAR and growth cessation (Paper I, Figure 4). These results suggest that phyB can regulate SAR and growth cessation by both common and distinct pathways, the latter being separated by tissue and photoperiod.

Lastly, we investigated how the *PHYB/PIF8* regulon controls seasonal growth. We compared dormant buds from *PHYB*RNAi with those from oePIF8 plants and found a set of common DE genes, whose promoter regions were significantly enriched for potential PIF binding sites (Paper I, Figure 5. Table S6). Down-regulated genes were associated with growth related processes, such as cell proliferation and meristem activity. Many of these genes have opposite expression patterns during bud set and bud flush (Ruttink *et al.*, 2007), suggesting that the *PHYB/PIF8* regulon controls both processes through common genes. As an example, we confirmed the expression patterns of *BRC1* and *AIL1*. Consistent with its role as growth suppressor *BRC1* is upregulated during growth cessation and decreases during bud flush. Its expression is increased in both *PHYB*RNAi and oePIF8 lines, correlating with their early bud set/late bud flush phenotype. The opposite was the case for *AIL1*.

We propose a model for the *PHYB/PIF* regulon as depicted in **Figure 23**.

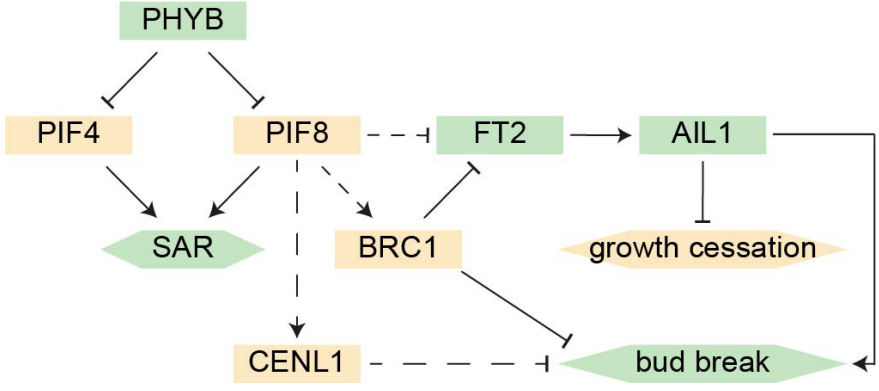

Figure 23: Model for the mode of action of the *PHYB/PIF* regulon.
Green color indicates growth promoting factors, while orange color indicates repression of vegetative growth.

4.2 Paper II

FLOWERING LOCUS T Paralogs Control the Annual Growth Cycle in *Populus* Trees

In **Paper II** we show that the three *Populus* three *FLOWERING LOCUS T* (*FT*) paralogs are important regulators of phenology and essential for vegetative growth, albeit in different tissues at different times of the year. *FT2a* has been established as an important regulator of autumn phenology previously, but not much was known about *FT2b*. It is now clear that both *FT2s* act together to regulate SD-induced growth cessation. The role of *FT1* during winter was so far unclear and we show that *FT1* is indispensable for the continuation of growth after winter, probably through the release of dormancy.

Since *FT2b* was recently identified in *Populus tremula*, we made a phylogenetic analysis of *FT* genes in other species, including *P. trichocarpa* and *P. tremuloides.* This revealed that *FT2b* was indeed present in other *Populus* species (Paper II, Figure S1A). However, comparing the genomic regions surrounding *FT* in Arabidopsis and *Populus* showed that *FT2b* is truncated in *P. trichocarpa* (Paper II, Figure 1A). This raised the question whether *FT2b* is required or even functional in *P. tremula*. When analyzing the expression of all three *FT* genes in both greenhouse-grown hybrid aspen (*Populus tremula x tremuloides*) and field-grown *Populus tremula*, we found that *FT2a* and *FT2b* have very similar expression patterns; they were expressed in leaves during LD with a peak at the end of the light period (Paper II, Figure 1B-D). *FT2b* was somewhat higher expressed than *FT2a* in growth chamber conditions. Given the close proximity of *FT2a* and *FT2b*, one could hypothesize that they share common regulatory elements that facilitate these very similar expression patterns. However, this question will be addressed in a different study (Lee *et al*., unpublished). Overexpression of *FT2b* lead to a very early flowering phenotype, like it has been reported for *FT1* and *FT2a* (Paper II, Figure S1C; Böhlenius *et al.*, 2006; Hsu *et al.*, 2006). *FT1* expression corresponded to previously published reports (Hsu *et al.*, 2011) and was limited to cold exposed buds (Paper II, Figure C, D). I*n situ* hybridization showed that expression was mainly localized to the embryonic leaves and vasculature within the buds (Paper II, Figure S1D).

To study the individual functions of the three *FT* genes, we generated specific knock-out mutants. Previous attempts to downregulate *FT1* and *FT2* individually failed due to their high sequence similarity and this became only more difficult with the discovery of *FT2b.* However, the new CRISPR-Cas9 technique made it possible to target specific genomic regions and distinguish between the paralogs. Remarkably, knock-out of *FT2a* had no effect on vegetative growth or growth cessation (Paper II, Figure S2B) despite it harboring several significant SNPs for bud set (Wang *et al.*, 2018). Knock-out of *FT2b* had a more significant effect with plants setting bud after two months in LD (Paper II, Figure S2C). Double *FT2* knock-out (hereafter FT2 CRISPR) plants were severely affected in their vegetative growth; dwarfed and setting bud shortly after potting or even in tissue culture (Paper II, Figure 2). After cold treatment, FT2 CRISPR plants flushed their buds at the same time as the WT. Nonetheless they set bud again shortly afterwards. This confirms that *FT2* indeed plays an important role in the regulation of growth cessation, but as a combination of the activities of both *FT2a* and *FT2b*.

FT1 CRISPR plants on the other hand grew normally during LD and responded to SD treatment as the WT did. However, their bud flush after cold treatment was strongly impaired and started only months after WT trees had fully flushed (Paper II, Figure 2). In contrast to WT, FT1 CRISPR plants also did not flush all of their buds, but only a few per tree. This indicated that *FT1* was necessary for the continuation of growth after winter. To exclude the possibility that FT1 CRISPR buds simply died during cold treatment, we performed a viability staining that showed that cold treated buds were still alive and metabolically active (Paper II, Figure S2D).

We also tested whether the phenotypes of either CRISPR line could be restored by grafting. FT2 CRISPR shoots could grow when supplied FT2 from WT rootstocks until the shoots grew too tall, confirming previous reports of *FT*s mobility (Paper II, Figure S3A; Miskolczi *et al.*, 2019). FT1 CRISPR buds on the other hand could not flush, regardless of their position on rootstock or scion (Paper II, Figure S3B). This suggests that FT1 function is restricted to the bud it is produced in. However, it was still unclear which process it affects; whether it was dormancy release or bud flush itself.

To address this question, we designed an RNA sequencing experiment to get an overview of the transcriptome of WT and FT1 CRISPR plants at different stages during the cold treatment. The goal was to identify the time point at which both genotypes diverge in their gene regulation. This analysis

showed that big transcriptomic changes are happening after 4 weeks in cold treatment in both genotypes. However, FT1 CRISPR trees were unable to transition to the next state and did not change their expression profile between 4 and 8 weeks of cold treatment (Paper II, Figure 3A). This is also reflected in the number of differentially expressed genes between both genotypes (Paper II, Figure 3B). Since plants typically release dormancy in our growth conditions between four and eight weeks of cold treatment, this suggests that *FT1* is required for dormancy release rather than bud flush. This conclusion is supported by the fact that FT1 CRISPR plants can flush normally when decapitated or transferred back to LD before dormancy establishment (Paper II, Figure S4). Dormancy release has been associated with the removal of callose plugs (Rinne *et al.*, 2011). We analyzed the expression patterns of *PICKLE*, a negative regulator of dormancy establishment during autumn, and several *GH17* genes, which are hypothesized to hydrolyze callose. Expression of both *PICKLE* and *GH17_101* (as an example) rose significantly in WT after four weeks of cold treatment, but not in FT1 CRISPR (Paper II, Figure 3C, D), suggesting that callose plugs might still have been in place. However, it is still unclear how lack of FT1 can affect these processes in the apex. So far, it has been believed to act downstream and to be enabled to move there from the embryonic leaves. Additionally, *FT1* expression in field-grown trees peaked in the midst of winter (Paper II, Figure 1D), long after dormancy has been released. On the other hand, *FT1* transcript was already detectable during the autumn. It might fulfil functions both in dormancy release and subsequent bud flush, but we might miss the latter in our analysis, because the FT1 CRISPR plants got 'stuck' in an earlier phase. In conclusion, *FT1* is indispensable for the continuation of growth after winter and likely affects dormancy release.

The duplication of *FT* in *Populus* has offered a convenient way to express growth promoters at different times in different tissues and regulate their function individually. Since both FT1 and FT2 can interact with FD-like proteins (Tylewicz *et al.*, 2015) to induce downstream targets like *LAP1*, one can speculate that their downstream pathways are conserved. Growth cessation and bud flush are regulated by many of the same factors, including *LAP1* and *AIL1*. Since *FT1* is still strongly expressed after dormancy release, it might still promote growth during bud flush through this same pathway through which *FT2* regulates growth during the season (**Figure 24**).

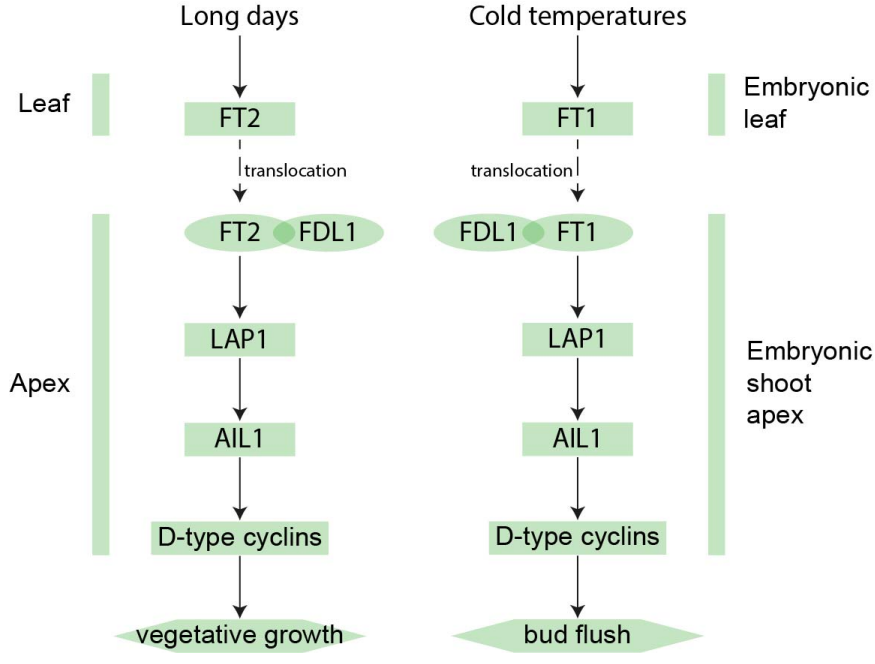

Figure 24: Potential parallels between *FT1* and *FT2* pathways.
Boxes indicate genes, ovals indicate proteins.

4.3 Paper III

SHORT VEGETATIVE PHASE LIKE Modulates Short Day-Induced Growth Cessation in *Populus* Trees

Downregulation of *FT2* in the leaf is necessary for SD-induced growth cessation. So far, *FT2* regulation is mostly understood through promotors, but the speed of *FT2* decrease in SD suggests influence of repressors in addition to reduced induction. In Paper I we have already identified *PIF8* as a negative regulator of *FT2*. In **Paper III**, we show that the previously described *SHORT VEGETATIVE LIKE* (*SVL*) is not only involved in dormancy establishment in the apex (Singh *et al.*, 2019), but also plays a role in growth cessation by repressing *FT2* and gibberellin biosynthesis.

Poplar *SVL* is related to Arabidopsis *SVP* and the *DAM* genes of peach (Paper III, Figure S1A) and is able to rescue the Arabidopsis *svp-32* mutant phenotype (Paper III, Figure S3). It has been described before that *SVL* expression increases in the apex after exposure to SD (Singh *et al.*, 2018). We show that the same is true for *SVL* expression in leaves (Paper III, Figure 1). In field grown *Populus tremula*, *SVL* expression peaks at the end of summer (Paper III, Figure 1A), after *FT2* (Paper III, Figure S2). In controlled conditions, *SVL* has a strong morning peak in SD, but not LD (Paper III, Figure 1B). Additionally, we show that more SVL protein accumulates over the course of SD treatment and at lower temperatures (Paper III, Figure 1D, E). This offers the possibility to integrate photoperiod and temperature, which both decrease during autumn. To investigate *SVL*s role in leaves further, we generated overexpression (SVLoe) and *SVL* RNAi lines (Paper III, Figure S4). In LD, no significant changes in vegetative growth could be observed and transgenic lines were indistinguishable from wild types (Paper III, Figure 2A). We then subjected them to our standard SD treatment and examined their SD response. *SVL* RNAi lines showed a subtle delay in growth cessation, while SVLoe lines ceased growth significantly earlier than WT (Paper III, Figure 2B, C). It should be noted that the *SVL* RNAi phenotype can easily be missed when more drastic shifts in photoperiod are used for SD treatment as they were by Singh and colleagues (2019). The shorter the photoperiod, the faster the response and smaller effects will be undetectable. In our conditions, we can show clearly that *SVL* affects growth cessation.

Next, we investigated through which pathway *SVL* acts. A major regulator of SD-induced growth cessation is *FT2* (Paper II) and given that Arabidopsis SVP targets *FT* transcription (Hartmann *et al.*, 2000), *FT2* was an obvious candidate to test. Indeed, after shift to SD, *FT2* expression ceases in WT and SVLoe, while it was still detectable in *SVL* RNAi lines (Paper III, Figure 3A). SVLoe lines had a lower *FT2* expression already in LD (Paper III, Figure S5A). Expression of *LAP1*, a downstream target of *FT2*, was also significantly increased in *SVL* RNAi lines (Paper III, Figure S5B). In addition to *FT*, SVP is known to affect gibberellins in both Arabidopsis and poplar (Andrés *et al.*, 2014; Singh *et al.*, 2019) and previous studies show that high GA content delays growth cessation (Eriksson *et al.*, 2000). Therefore, we examined the expression of the rate limiting GA biosynthesis enzyme *GA20 oxidase* (*GA20ox*). Its expression was similarly but less severely affected than *FT2*; *SVL* RNAi showed higher *GA20ox* expression than WT. Expression levels were also very similar over the whole day. SVLoe plants, on the other hand, had lower *GA20ox* expression and the morning peak at ZT4 was abolished completely (Paper III, Figure 3B). This indicated that GA metabolism was altered in the transgenic lines. Since GA biosynthesis is subject to several layers of feedback regulation and the amount of GAs cannot easily be inferred from expression data only, we measured and compared the contents of active GAs in leaves of *SVL* RNAi and WT. In both LD and SD, GA_1 contents were significantly elevated in *SVL* RNAi (Paper III, Figure S6). These results indicate that *SVL* regulates growth cessation through the repression of both *FT2* and GA biosynthesis.
To understand how SVL regulates the expression of target genes, we performed chromatin immunoprecipitation using myc-tagged SVL overexpressers. Analysis showed that the SVL protein associated with the promoter regions of both *FT2* and *GA20ox* (Paper III, Figure 3C, D). Previous studies did not identify *GA20ox* as a direct target of SVL (Singh *et al.*, 2018), but it should be noted that they used apices in their analysis and we used leaves. It is possible that other factors affect binding of SVL, which are present/absent in either tissue.

Last, we compared the transcriptomes of WT and *SVL* RNAi before and during SD treatment. We performed RNA sequencing on leaves during LD and after several weeks of SD (Paper III, Figure 4). The leaf transcriptome of both genotypes changed drastically upon shift to SD, but was stable afterwards (Paper III, Figure S7). It changed again after ten weeks of SD

treatment, but at that point the leaves were old and senescing. Overall, changes between WT and *SVL* RNAi were moderate with less than 100 differentially expressed genes (DEG) in LD and SDW1 (Paper III, Figure 4B). *FT2* and *GA20oxidase* expressions, however, were similar to previous qPCRs (Paper III, Figure 4C). Gene ontology enrichment did not yield any specific terms with such few DEG. This indicated that the role of *SVL* in leaves in limited to the regulation of *FT2* and gibberellin biosynthesis.

We conclude that *SVL* first plays a role in leaves during SD-induced growth cessation by repressing *FT2* and GA biosynthesis and then in the apex during dormancy establishment/release by repressing GAs and *FT1* (**Figure 25**).

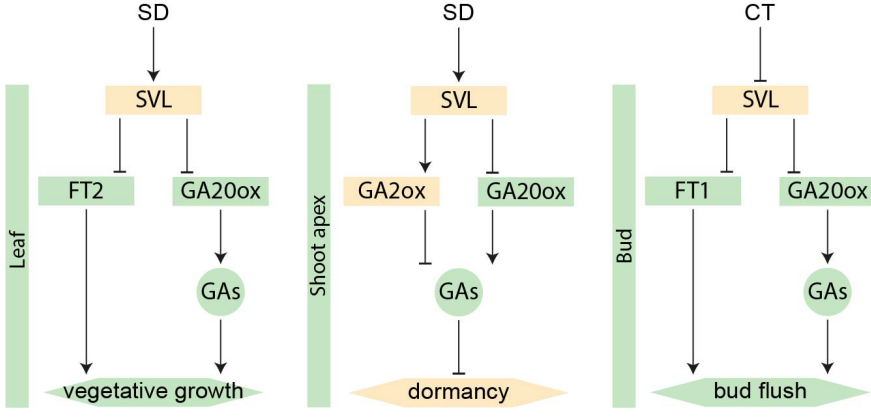

Figure 25: Different roles of *SVL* in the annual growth cycle.
Green color indicates growth promoting factors, while orange color indicates repressors. Boxes represent genes and circles represent hormones.

5. Conclusions

In this thesis I investigated how the annual growth cycle of *Populus* is regulated on a molecular level.

Vegetative growth is promoted during the spring and summer by long days and warm temperatures. I show in **Paper II** that the *FLOWERING LOCUS T2* (*FT2*) genes are indispensable for this process, since knock-out mutants are unable to grow. **Paper I** establishes the regulon of the light receptor phytochrome B (phyB) and its interacting factor PIF8 as upstream regulators of *FT2* expression. Reduction in the ability to sense light leads to reduced *FT2* expression.

Growth cessation in autumn is a response to reduced day length. Short days (SD) induce *SHORT VEGETATIVE PHASE-LIKE* (*SVL)*, a negative regulator of growth. In **Paper III** I show how *SVL* expression in the leaf represses both *FT2* and the biosynthesis of gibberellins, thereby removing two growth promoting factors. While photoperiod is the main factor controlling growth cessation, the timing can be fine-tuned. With decreasing day length temperatures drop as well. SVL seems to be more stable in cold temperatures and growth may cease early in response to a cool late summer/early autumn.

Once growth ceased and dormancy has been established it takes prolonged exposure to cold temperatures for the trees to be able to respond again to growth promoting conditions. I show in **Paper II** that *FT1* is required for dormancy release and that it acts locally in the buds where it is expressed. **Paper I** shows that *FT1* is also under control of the *PHYB*/*PIF8* regulon. But in contrast to *FT2*, *PHYB* seems to negatively regulate *FT1*.

The importance of *FT* in the regulation of the annual growth cycle is undeniable. However, there are still many aspects that are not fully understood. In juvenile trees, growth cessation is induced by short days and regulated through *FT2*. But how important is this in older trees that mainly form short shoots with predetermined growth? Does photoperiod matter for those? Short shoots are also the bearers of floral buds. How are these initiated? **Paper II** shows that *FT2b*, like *FT1* and *FT2a*, is able to induce flowering when overexpressed, but whether that is the case in nature is still unknown.

While I establish the role of *FT1* in dormancy release, its mode of action remains unclear. Does it move from the embryonic shoots to the apex as hypothesised? What are its targets? How much is actually going on in buds during winter? Are they metabolically active at -20°C and how much of transcription/translation is happening?

If the complexity of Arabidopsis *FT* regulation is of any indication, there is still a lot to be discovered and understood about *Populus FT*.

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Popular science summary

Plants need to adapt to their environment to survive. Trees in temperate and boreal regions face big challenges: the environment can change significantly depending on the season. From dry, hot summers to freezing winters, trees need to adapt. And because these adaptations take time, they need to prepare in advance.

During the summer months, growth is promoted by warm temperatures and long days. While temperatures are highly variable between years, the day lengths in the same season are stable over the life time of the tree. Once it falls under a certain threshold, summer is officially over and preparations for winter begin. This includes the cessation of growth and the formation of terminal buds to protect sensitive tissues. Afterwards dormancy and cold hardiness are established to ensure winter survival. After winter, warm temperatures promote bud flush and the continuation of growth.

In my thesis I investigated how these adaptations are regulated on a molecular level. Three closely related and very similar genes called *FLOWERING LOCUS T* are major regulators of bud set and flush. I show how these genes are controlled and what happens when they are not functional.

Populärvetenskaplig sammanfattning

Växter måste kunna anpassa sig till sin omgivning för att kunna överleva. Träd som växer i tempererade och boreala klimat måste klara stora utmaningar eftersom tillväxtmiljön ändras dramatiskt beroende på årstid. Träden måste kunna anpassa sig till både torra, heta somrar och iskalla vintrar. Och eftersom dessa anpassningar tar tid så måste de förbereda sig i tid för en ny årstid.

Under sommaren så stimuleras tillväxten av varma temperaturer och långa dagar. Medans temperaturerna kan variera kraftigt mellan olika år, så är längden på dagen alltid densamma vid samma datum varje år. När längden på dagarna blir kortare än en viss kritisk dagslängd så är det en signal som talar om att sommaren är över och att trädet måste börja förbereda sig för vintern. Detta inkluderar att trädet slutar växa och att skottspetsarna bildar knoppar för att skydda de känsliga tillväxtzonerna. Sedan utvecklar träden köldhärdighet och går in i en djup vila för att kunna överleva vintern. Efter att vinterkylan hjälpt till att bryta vilan så är det de varmare temperaturerna på våren som stimulerar knoppbrytning och ny tillväxt.

I min avhandling har jag studerat hur dessa anpassningar styrs på molekylär nivå hos aspträd. Aspen har tre stycken mycket likartade gener som kallas för *FLOWERING LOCUS T*. Det är dessa gener som huvudsakligen styr när träden sätter knopp och när knopparna brister. Jag visar hur dessa gener styrs och vad som händer när de tappar sin funktion.

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