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Regulation of flowering time and phenology in *Populus* trees

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Abstract

As sessile organisms, plants must adapt to a variety of environmental conditions. Perennials in particular, face prolonged exposure to both abiotic and biotic stresses. The growth of perennial plants such as poplar trees (*Populus spp.*) is dictated by the change of seasons. At northern latitudes, the growth cycle of trees alternates between a period of active growth and dormancy, during which the trees are unreceptive to any growth promoting signals to ensure that buds do not flush before winter has passed.

Previous studies of the phenology regulation in *Populus* have revealed interesting parallels to the photoperiodic flowering pathway in *Arabidopsis thaliana*.

In my work, I have investigated the role of *FLOWERING LOCUS T* (*FT*) and *TERMINAL FLOWER 1* (*TFL1*)-like genes, which in arabidopsis induce and repress flowering respectively, in the control of flowering time and phenology in the annual growth cycle of *Populus* trees. Three *FT*-like genes have been identified and have evolved to take different functions. The data show that *FT1* is involved in dormancy release while both *FT2a* and *FT2b* are crucial for vegetative growth during summer. Acting antagonistically to the *FT* genes, in a conserved mechanism to arabidopsis, two *TFL1*-like paralogues have been described. *CENL1* and *CENL2* in *Populus* are repressors of growth, although *CENL2* seems to play only a minor role. *CENL1* is involved in the maintenance of meristem indeterminacy and represses flowering, while *FT1* can induce the transition to flowering after a period of dormancy.

Furthermore, I have provided a complete transcriptional atlas of the annual growth cycle of aspen, based on RNA sequencing of samples collected from outdoor and indoor grown trees mimicking seasonal changes. This allowed me to investigate the molecular responses which regulate essential developmental processes during the growth cycle in trees.

Keywords: *Populus*, flowering, growth cycle, transcriptome, *FLOWERING LOCUS T*, *TERMINAL FLOWER 1*

Reglering av blomningstid och fenologi i (*Populus*)- träd

Sammanfattning

Eftersom de är fast rotade i marken, och därför orörliga, så måste växter anpassa sig till en mängd olika miljöförhållanden. Särskilt perenner utsätts för en långvarig exponering av både abiotiska och biotiska påfrestningar. Tillväxten av fleråriga växter som popplar och aspar (*Populus spp.*) dikteras av årstidernas växling. På nordliga breddgrader växlar trädens tillväxtcykel mellan en period av aktiv tillväxt och vila. Under vintervilan är träden inte mottagliga för några tillväxtfrämjande signaler, detta för att säkerställa att knopparna inte bryter innan vintern har passerat. Tidigare studier av fenologiregleringen i *Populus* har avslöjat intressanta paralleller till den fotoperiodiska blomningsregleringen i backtrav (*Arabidopsis thaliana*).
I mitt arbete har jag undersökt rollen av *FLOWERING LOCUS T* (*FT*) och *TERMINAL FLOWER 1* (*TFL1*)-liknande gener, vilka i arabidopsis inducerar respektive undertrycker blomning, i kontrollen av blomningstid och fenologi under *Populus*-trädens årliga tillväxtcykeln. Tre *FT*-liknande gener har identifierats och har utvecklat olika funktioner. Mina data visar att *FT1* är inblandad i regleringen av vintervilan, medan både *FT2a* och *FT2b* är nödvändiga för den vegetativa tillväxten under sommaren. Jag har också studerat funktionen och regleringen av två *TFL1*-liknande paraloger, *CENL1* och *CENL2*, som verkar antagonistiskt med *FT*-generna. *CENL1* och *CENL2* i *Populus* är undertryckare av tillväxt, även om *CENL2* bara verkar spela en mindre roll. *CENL1* är involverad i upprätthållandet av skottmeristemens vegetativa tillväxtförmåga och undertrycker blomning, medan *FT1* kan inducera övergången till blomning efter en period av vila.
Dessutom har jag utvecklat en komplett gentranskriptionsatlas under aspens årliga tillväxtcykel, baserat på RNA-sekvensering av prover som samlats in från utomhus- och inomhusodlade träd som odlats under förhållanden som efterliknar säsongsmässiga förändringar. Detta gjorde det möjligt för mig att undersöka den genreglering som kontrollerar viktiga utvecklingsprocesser under trädens tillväxtcykel.

Nyckelord: *Populus*, blomning, tillväxtcykel, transkriptom, *FLOWERING LOCUS T*, *TERMINAL FLOWER 1*

As a wise man once told me, “Mettici amore, fava!”

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I. Domenique André, **Alice Marcon**, Keh Chien Lee, Daniela Goretti, Bo Zhang, Nicolas Delhomme, Markus Schmid, Ove Nilsson (2022). *FLOWERING LOCUS T* paralogs control the annual growth cycle in *Populus* trees. Current Biology, https://doi.org/10.1016/j.cub.2022.05.023

II. **Alice Marcon**, Domenique André, Daniela Goretti, Laura García Romañach, Silvio Collani, Markus Schmid, Ove Nilsson. The interplay between *FT* and *TFL1*-like genes in the control of phenology and flowering time in *Populus* trees (manuscript)

III. Domenique André, José Alfredo Zambrano, Bo Zhang, Keh Chien Lee, Mark Rühl, **Alice Marcon**, Ove Nilsson (2022). *Populus SVL* Acts in Leaves to Modulate the Timing of Growth Cessation and Bud Set. Frontiers in Plant Science, 17;13:823019, doi: 10.3389/fpls.2022.823019. PMID: 35251092; PMCID: PMC8891642.

IV. **Alice Marcon**, Laura García Romañach, Domenique André, Jihua Ding, Bo Zhang, Torgeir R. Hvidsten, Ove Nilsson (2025). A transcriptional roadmap of the yearly growth cycle in *Populus* trees (submitted)

All published papers are published open access.

The contribution of Alice Marcon to the papers included in this thesis was as follows:

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

II. AM planned and performed experiments, analysed the data and wrote the manuscript.

III. AM performed experiments and phenotyping.

IV. AM designed experiments, analysed the data and wrote the manuscript.

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Abbreviations

ABA	Abscisic Acid
AGL24	AGAMOUS-LIKE 24
AIL1	AINTEGUMENTA LIKE 1
AP1	APETALA 1
APB	Phytochrome B-binding
APL	ALTERED PHLOEM DEVELOPMENT
ATC	*A. thaliana* CENTRORADIALIS
BFT	BROTHER OF FT AND TFL1
BiFC	Bimolecular Fluorescence Complementation
BRC1	BRANCHED 1
bZIP	Basic leucine zipper
CALS1	CALLOSE SYNTHASE 1
CaMV	Cauliflower Mosaic Virus
Cas	CRISPR associated
CBF	C-repeat/DREB binding factor
CCA1	CIRCADIAN CLOCK ASSOCIATED 1
CDF	CYCLING DOF FACTOR
CDL	Critical day length
cDNA	Complementary DNA
CENL1-2	CENTRORADIALIS LIKE 1-2
ChIP	Chromatin Immunoprecipitation
ChIP-Seq	Chromatin Immunoprecipitation Sequencing
CLF	CURLY LEAF
CO	CONSTANS
COP1	CONSTITUTIVE PHOTOMORPHOGENIC 1

COR	Cold related
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CRY	Cryptochrome
CT	Cold treatment
CYCD3	CYCLIN D 3
DAM	DORMANCY ASSOCIATED MADS-BOX
DEGs	Differentially expressed genes
DNA	Deoxyribonucleic acid
EBB1-3	EARLY BUD BREAK1-3
ELF	EARLY FLOWERING
EPS	Epoxy succinimide
ER	Endoplasmic reticulum
FD	FLOWERING LOCUS D
FDL	FLOWERING LOCUS D LIKE
FDP	FD PARALOG
FKF1	FLAVIN-BINDING KELCH REPEAT F BOX 1
FLC	FLOWERING LOCUS C
FLM	FLOWERING LOCUS M
FM	Floral meristem
FT	FLOWERING LOCUS T
FT1-2	FLOWERING LOCUS T 1-2
FTIP1	FT-INTERACTING PROTEIN 1
FUL	FRUITFULL
GA	Gibberellic acid
GA2ox	GIBBERELLIN 2-OXIDASE
GFP	Green Fluorescent Protein

GI	GIGANTEA
GID	GIBBERELLIN-INSENSITIVE DWARF
GIL	GIGANTEA-LIKE
GO	Gene Ontology
HOS1	HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES 1
IM	Inflorescence meristem
ISH	*In Situ* Hybridisation
LAP1	Like-APETALA 1
LD	Long day
LFY	LEAFY
LHY	LATE ELONGATED HYPOCOTYL
LOF1-2	LATERAL ORGAN FUSION1-2
LUX	LUX ARRHYTHMO
MADS	MCM1, AGAMOUS, DEFICIENS, and SRF
MAF	MADS AFFECTING FLOWERING
MFT	MOTHER OF FT AND TFL1
mRNA	Messenger RNA
MS	Murashige and Skoog
NCED3	9-CIS-EPOXYCAROTENOID DIOXYGENASE 3
NF-Y	NUCLEAR FACTOR Y
NGS	Next generation sequencing
PAM	Protospacer Adjacent Motif
PCA	Principal Component Analysis
PCCs	Phloem companion cells
PCD	Programmed Cell Death
PCR	Polymerase Chain Reaction

PEBP	Phosphatidylethanolamine-binding protein
PHY	Phytochrome
PIF	Phytochrome-interacting factor
PKL	PICKLE
PPIs	Protein-protein interactions
PRR	Pseudo-Response Regulator
PYL/RCAR	PYRABACTIN RESISTANCE1-LIKE/ REGULATORY COMPONENTS OF ABA RECEPTORS
QKY	QUIRKY
qPCR	Quantitative PCR
RM	Rib meristem
RNA	Ribonucleic acid
RNAi	RNA interference
RNA-seq	RNA sequencing
SAM	Shoot apical meristem
SD	Short day
sgRNA	Single guide RNA
siRNA	Small interfering RNA
SNP	Single Nucleotide Polymorphism
SOC1	SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1
SPA1	SUPPRESSOR OF PHYA 1
SPL	SQUAMOSA PROMOTER BINDING PROTEIN -LIKE
SVL	SHORT VEGETATIVE PHASE LIKE
SVP	SHORT VEGETATIVE PHASE
SYP121	SYNTAXIN OF PLANTS 121
TEM	TEMPRANILLO
TFL1	TERMINAL FLOWER 1

TOC1	TIMING OF CAB EXPRESSION 1
TSF	TWIN SISTER OF FT
WOX4	WUSCHEL RELATED HOMEOBOX 4
WT	Wild type
Y2H	Yeast 2 Hybrid

1. Introduction

Plants are sessile organisms. Plants inability to move has driven the evolution of diverse adaptation strategies enabling them to adapt to different ecosystems and environments.

In 2016, it was reported that approximately 374,000 plant species had been described (Christenhusz & Byng, 2016), with a distribution covering all types of ecosystems, from the most arid regions to wetlands, from the equator to the Artic. Adaptation to extreme environmental conditions has been particularly important for perennial plants. Unlike annual plants, which complete their life cycle in a single growing season, perennials often face prolonged exposure to both abiotic and biotic stresses. Perennial plants such as trees, which have a lifespan of many years, have evolved specific morphological and physiological traits to support growth and development and ensure reproduction. The formation of wood, which characterise trees, is a crucial adaptation which allows them to grow vertically to significant heights, accessing sunlight and establishing their place in the tree canopy. Moreover, lignified tissues serve as a protective barrier against herbivory and pathogens attacks. Another specific adaptation of trees growing in temperate and boreal regions, where environmental conditions fluctuate considerably throughout the seasons, is winter dormancy. When trees enter this physiologically dormant state, growth and metabolic activity are reduced to conserve energy to survive harsh winter conditions, such as freezing temperatures and limited resources.

In the northern hemisphere, forests are a dominant ecosystem, and in Sweden, they cover nearly 70% of the country's territory [1]. Sweden is one of the most extended countries in Europe and is situated at high latitudes, ranging from 55°N to 69°N. Because of its geographic location, daylength and temperature vary greatly during the year. The growing season of trees in Sweden, particularly in the north, is quite short since the trees must enter growth cessation earlier than trees located at southern latitudes. Also bud flush is later in the north. Despite the slow growth conditions, forestry business is of national importance. Tree breeding provides the forestry economy with improved trees through standard selection and crossing

[1] https://www.scb.se/hitta-statistik/sverige-i-siffror/miljo/marken-i-sverige/

programs which take many years. Understanding the molecular and genetic mechanisms that control trees' adaptation to seasonal environmental conditions is extremely important for enhancing forestry practices and tree breeding in the face of climate change. Optimization of flowering time for instance, could fasten the process of breeding, improving productivity. My thesis work aims at elucidating the role of some major players in the regulation of the annual growth cycle and flowering time in *Populus* trees such as *FLOWERING LOCUS T* (*FT*) and *TERMINAL FLOWER1* (*TFL1*)-like genes.

1.1 The life cycles of annual and perennial plants

Flowering plants (angiosperms) appeared approximately between 140 and 190 million years ago (Bell et al., 2005). They have evolved since into more than 290,000 known species, making angiosperms the clade with the most abundant plants species on Earth (Christenhusz & Byng, 2016).
Flowering plants have evolved three main life cycle strategies which lead to the classification of annual, biennial and perennial plants. Annual plants complete their life cycle in one year or one growing season, whereas biennials germinate and grow during the first year and reproduce in the following. Perennial plants instead, live for multiple years (Figure 1).
After germination, a young vegetative plant is referred to as juvenile; this phase is characterized by specific leaf traits and the inability to transition to reproduction even if exposed to reproduction-promoting environmental cues (Hyun et al., 2017). The duration of the juvenile phase is typically very short in annuals, facilitating a rapid transition from vegetative to reproductive phase. Once that the plant transitioned, flowering is followed by senescence and death. Perennials instead, cycle repeatedly in the vegetative phase, delaying competence to flower from months to years. The longer juvenile stage of perennials, allows plants to accumulate more biomass and more axillary meristems through branching prior to reproduction (Bergonzi & Albani, 2011). Upon maturation, adult trees initiate flowering, with vegetative and reproductive phases coexisting (Brunner & Nilsson, 2004).
In plants, the developmental switch from vegetative growth to flowering, which occurs once in annuals and repeatedly in perennials, is precisely controlled by environmental cues such as photoperiod and vernalization to generate a robust seasonal response (Andrés & Coupland, 2012).

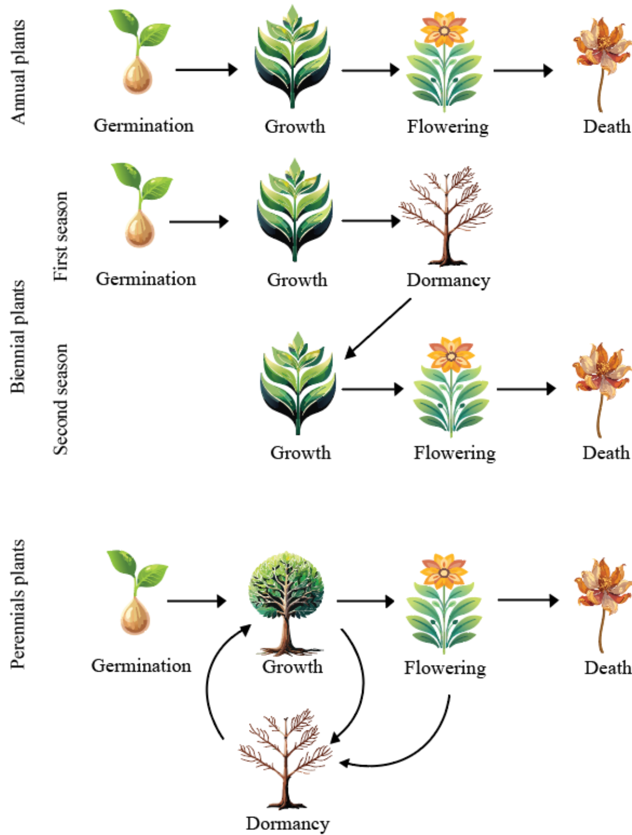

Figure 1. Life cycle strategies of annual, biennial and perennial plants.

1.2 *Arabidopsis thaliana*, a standard reference in plant biology

Decades ago, research shifted towards more interdisciplinary and multi-investigator studies, requiring extensive community resources. Plant biologists realised the need to establish a single organism as a standard reference. The choice fell on *Arabidopsis thaliana* (hereafter called arabidopsis). Arabidopsis is a member of the *Brassicaceae* family. It is a small annual plant native to Eurasia and Africa (Hoffmann, 2002). It is generally considered as a weed and does not hold any economic or agronomic value. However, arabidopsis was adopted as model species because of specific features which make it valuable for plant research (Figure 2). For instance, arabidopsis has a short regeneration time. Its life cycle, from

germination to maturation of the first seeds, takes about 6 to 8 weeks. After the leaf rosette formation, elongation of the main stem (bolting) starts after 3 weeks from planting. During the floral transition the shoot apical meristem (SAM) switches to an inflorescence meristem (IM) that forms floral meristems (FMs) on its flanks, which produces the floral primordia (Kwiatkowska, 2008). After flowering, arabidopsis self-pollinates and produces several hundred siliques with a prolific seed production.
When "floral dip", a convenient *Agrobacterium tumefaciens*-mediated transformation method was discovered, various tools for genetic analysis were developed (Clough & Bent, 1998). With a small genome of approximately 125 Mb, arabidopsis was chosen for the first plant genome sequencing project, completed in 2000 (The Arabidopsis Genome). The genome sequence, characterised by a low percentage of repetitive DNA, facilitated gene mapping and cloning efforts (The Arabidopsis Genome, 2000). Furthermore, several libraries of mutants have been generated, providing valuable resources for reverse genetic studies. For instance, mutants screening has been essential in identifying core genes of the flowering pathway, providing insights into the genetic regulation of the floral transition (Chen et al., 2018).
The release of the arabidopsis genomic sequence accelerated research in functional genomics, consolidating it as the most extensively studied species in plant biology. Comparing the total number of journal publications in arabidopsis with other plants, it is clear that wheat, maize, tomato and rice also largely investigated, all lag far behind (Marks et al., 2023).

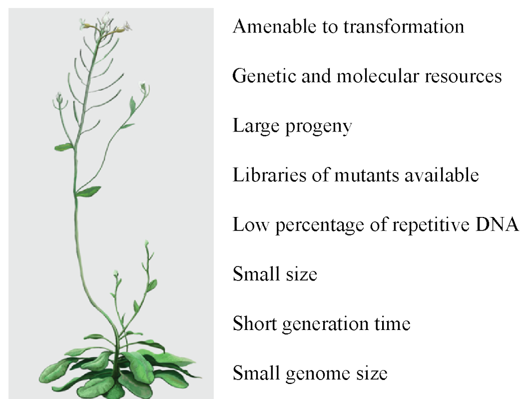

Figure 2. Important features of *Arabidopsis thaliana* as a model species.
Image of *A. thaliana* from the DataBase Center for Life Science (DBCLS).

1.3 *Arabidopsis thaliana* flowering pathways

The reproductive success of plants depends largely on their ability to synchronise developmental mechanisms precisely in response to environmental stimuli in an ecological setting.
In arabidopsis, the transition to flowering is regulated by intricate genetic networks. Screening of mutants, which display early or late flowering under various light and temperature conditions, has been crucial in shedding light on the interdependent genetic pathways regulating this process (Srikanth & Schmid, 2011).
Six primary genetic pathways have been identified: the vernalization and photoperiod pathways, which regulate flowering in response to seasonal cues; the ambient temperature pathway, which depends on temperature variation to control flowering time; the age, autonomous, and gibberellin pathways, which instead act more independently of environmental factors (Fornara et al., 2010) (Figure 3).
The integrated response of each of these pathways, to coordinate the transition to flowering, converges on a small number of developmental-transition genes, called floral-pathway integrators, which ultimately activate floral-meristem identity genes, which in turn trigger the transition from the vegetative to the reproductive phase.
In the next sections, I will focus specifically on the effect of light and temperature on the arabidopsis flower transition, to later compare it to our understanding in *Populus* species.

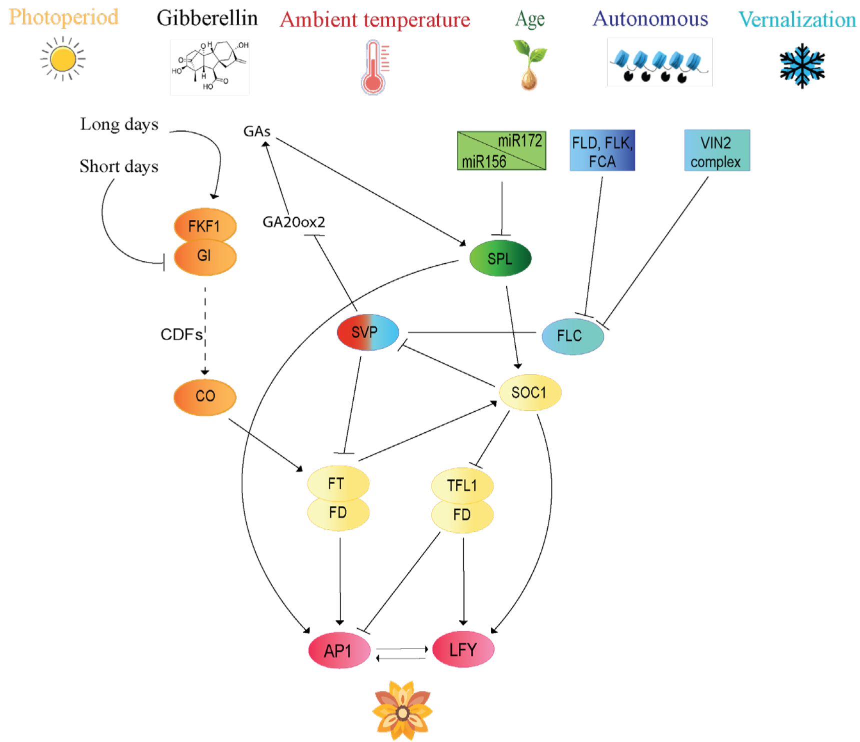

Figure 3. Flowering pathways in arabidopsis.
Main regulatory genes involved in the different flowering pathways. Solid and dotted lines indicate either direct or indirect regulation, arrows and T-ends indicate positive or negative regulation, respectively. The figure is modified from Leijten *et al.* (2018).

1.3.1 The photoperiodic pathway

In 1920, the terms ‘photoperiod’ was introduced by Wightman Garner and Henry Allard, because of the discovery that daylength is a crucial determinant of flowering response in tobacco and other plants species.
Based on photoperiodic responses, they classified plants into long day (LD), short day (SD), and day-neutral species (Garner & Allard, 1922).
Plants that flower when exposed to light periods longer than a certain critical daylength are LD plants; if flowering occurs when the light period is below that threshold, they are classified as SD plants. Flowering in day-neutral plants instead, is not regulated by photoperiodism (Garner & Allard, 1922).
A. thaliana is a facultative LD plant, which means that long days (16 h light)

promote floral transition, but eventually, it will also flower under SD conditions (8 h light).
The mechanism behind light perception and its integration into the photoperiodic pathway has been widely investigated in *A. thaliana* over the past 15 years (Hernando et al., 2017).

Light perception

Light is perceived in the leaves by photoreceptors, such as phytochromes (PHY), that absorb red/far-red light and cryptochromes (CRY), which absorb blue/UV-A light (Lin, 2000).
Five phytochromes have been identified in arabidopsis, and named PHYA to PHYE (Quail et al., 1995). Both, PHYA and PHYB have been shown to give a major contribution to light signalling in relation to flowering time. But, despite absorbing at the same wavelengths, they display different biological functions. PHYA has its main activity in far-red light and has a positive effect on flowering, which was demonstrated by the late flowering phenotype of *phyA* mutants. (Bagnall et al., 1995) PHYB instead is mainly active in red light; *phyB* mutants flower earlier regardless of the daylength, suggesting a negative effect of PHYB on flowering time (Lin, 2000).
Phytochromes are mainly acting through PHYTOCHROME INTERACTING FACTORs (PIFs). Upon the interaction with PHY proteins, PIFs are phosphorylated and then degraded (Al-Sady et al., 2006).
Cryptochromes are nuclear proteins associated with a flavin chromophore, and in arabidopsis there are two: CRY1 and CRY2 (Lin, 2000). They function redundantly, as positive regulators of flowering since the double mutants have a more accentuated late flowering phenotype than either single mutant (Liu et al., 2008).
In arabidopsis, both phytochromes and cryptochromes are responsible for resetting the circadian clock (Somers et al., 1998).

Circadian clock

The circadian clock is a time-regulating mechanism which operates with a periodicity of 24 h. It is such a pivotal system, that in arabidopsis, around a third of the genes display a diurnal expression pattern (Nohales & Kay, 2016).
It comprises interlocked feed-back loops with morning factors, CIRCADIAN CLOCK ASSOCIATED 1 (CCA1), LATE ELONGATED HYPOCOTYL (LHY) and pseudo response regulator (PRR) proteins, and

evening factors, TIMING OF CAB EXPRESSION 1 (TOC1), EARLY FLOWERING 3 and EARLY FLOWERING 4 (ELF3, ELF4) and LUX ARRHYTHMO (LUX) proteins (Alabadí et al., 2001) (Figure 4). If just one component of the evening complex is lacking, plants are photoperiod insensitive leading to longer hypocotyls and early flowering (Hazen et al., 2005; McWatters et al., 2007; Nusinow et al., 2011).
During the day, CCA1 and LHY repress *PRR7* and *PRR9* (Adams et al., 2015), as well as *TOC1*, *GIGANTEA* (*GI*), and the evening complex genes (*LUX*, *ELF3*, and *ELF4*) (Kamioka et al., 2016). *GI* is expressed at the end of the day and forms an additional negative feedback-loop with *TOC1* (Locke et al., 2006). In return, the evening complex directly inhibits the expression of *PRR9*, *PRR7* and *GI* (Mizuno et al., 2014; Nusinow et al., 2011).

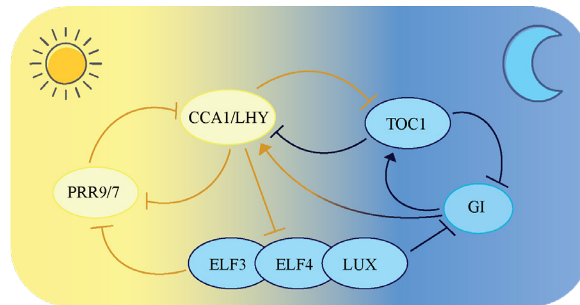

Figure 4. The circadian clock in arabidopsis.

Schematic representation of the circadian clock in arabidopsis. Main regulatory genes involved in the morning (in yellow) and evening (in blue) complexes. Arrows and T-ends indicate positive or negative regulation, respectively.

CONSTANS stability

CONSTANS encodes a B box-type zinc-finger transcription factor which induces *FLOWERING LOCUS T* (*FT*) expression in the vascular bundles to promote flowering in arabidopsis (Tiwari et al., 2010).
CO is one of the main components of the photoperiodic pathway and its expression and protein activity are greatly affected by daylength and the circadian clock entrainment.
In the morning, *CO* mRNA levels are kept low by the repressive action of CYCLING DOF FACTOR (CDF) 1, 2, 3 and 5 (Valverde, 2011). However, another level of repression is implemented post translationally by PHYB and

HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE1 (HOS1) (Lazaro et al., 2012) (Figure 5).

At the end of the light period in LD, CDF proteins are degraded through a ubiquitin-dependent mechanism, mediated by GI and FLAVIN BINDING, KELCH REPEAT, F-BOX PROTEIN 1 (FKF1), which form a complex in a blue light-dependent manner (Song et al., 2014). The same complex also stabilises CO (Hwang et al., 2019), in addition to the action of PHYA (Valverde et al., 2004). Indirectly, CRY1 and CRY2 also stabilise the CO protein by preventing its degradation from CONSTITUTIVELY PHOTOMORPHOGENIC1 (COP1) activity, enhanced by SPA1 SUPPRESSOR OF PHYA 1 (SPA1) interaction (Liu et al., 2011). In absence of light instead, without the cryptochromes presence, COP1, and SPA1 mediate CO degradation via the 26S proteasome (Zhu et al., 2008). In short days, when *CO* expression peaks in the dark phase, the protein is degraded by the COP1/SPA1 complex (Zhu et al., 2008). Thus, only when *CO* expression peaks in the light at the end of a long day, due to the degradation of the CDFs, the protein is accumulated.

The specific daytime expression of *CO* and its protein stability in LD, are the first essential steps to convey the photoperiodic response to the successive components of the flowering pathway.

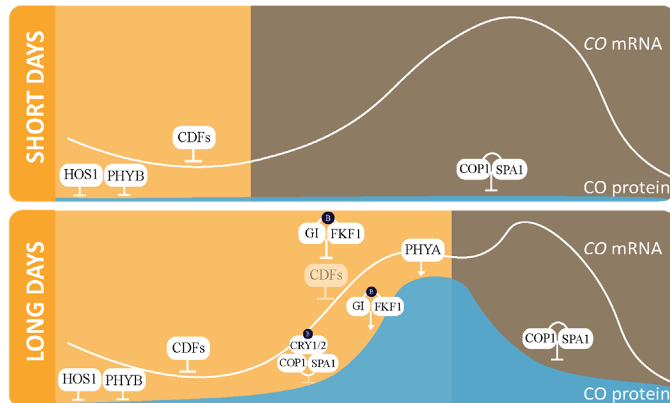

Figure 5. *CONSTANS* transcriptional and translational regulation.

Schematic representation of *CO* expression and protein regulation in long and short day. In orange are represented the hours of light, in brown the hours of dark. Arrows and T-ends indicate positive or negative regulation, respectively. The figure is modified from Bouché, Lobet *et al.* (2016).

1.3.2 Ambient temperature pathway

As for the photoperiodic signal, temperature is another environmental cue which affects flowering time. In arabidopsis, a shift to warmer temperature (23 °C to 27 °C) induces early flowering (Balasubramanian & Weigel, 2006) while reducing the temperature to 16 °C delays it (Posé et al., 2013).
A key gene involved in this pathway is the MADS box transcription factor *SHORT VEGETATIVE PHASE* (*SVP*). *SVP* has been identified as a floral repressor since *svp* mutants show an early flowering phenotype, even more accentuated at lower temperature, while overexpressing lines flower later at warmer temperature (Lee et al., 2007). Oher genes belonging to the *FLOWERING LOCUS C* (*FLC*) clade, such as *FLOWERING LOCUS M* (*FLM*/*MAF1*) and *MADS AFFECTING FLOWERING-2-4* (*MAF2–MAF4*), have crucial roles in the thermosensory pathway (Gu et al., 2013). As for *SVP,* loss-of-function of *FLM* results in early flowering even tough the plants retain temperature sensitivity below 10 °C in contrast to *svp* mutants (Lee et al., 2013). *FLM* is present in two splicing variants at different temperatures. Expression of *FLM-β* increases at low temperature (16 °C) while *FLM-δ* increases at high temperature (Posé et al., 2013). A model was proposed in which when the FLM-β protein forms a complex with SVP flowering is actively repressed, but if the splicing variant *FLM-δ* is incorporated, the complex is inactive (Posé et al., 2013). SVP controls flowering by direct binding to the promoters of *FT* and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) (Li et al., 2008) and as a repressor of the gibberellin biosynthetic pathway (Andrés et al., 2014). SVP also targets *TEMPRANILLO1* (*TEM1*) and *TEM2* (Tao et al., 2012), which can both repress *FT* transcription and gibberellin biosynthesis (Osnato et al., 2012).
Several years ago, it was observed that PIF4 can mediate flowering in response to temperature (Kumar et al., 2012) bridging the photoperiod and the ambient temperature pathway. *pif4* mutants do not show any change in flowering time in inductive LD conditions, but in SD flowering is delayed (Thines et al., 2014).

1.4 The PEBP family includes several floral integrators

The phosphatidyl ethanolamine-binding proteins (PEBPs) are a conserved family which has evolved in all taxa, from prokaryotes to eukaryotes (Karlgren et al., 2011). In plants, the proteins belonging to this

family, contain a highly conserved PEBP/RKIP domain, which covers up to 80% of the coding sequence (Chardon & Damerval, 2005). They have been connected to the determination of the plant morphological structure and the regulation of the vegetive to reproductive phase transition (Karlgren et al., 2011). The PEBPs, in plants, have been classified into three subfamilies: FLOWERING LOCUS T (FT)-like proteins, MOTHER OF FT AND TFL1 (MFT)-like proteins, and TERMINAL FLOWER 1 (TFL1)-like proteins (Chardon & Damerval, 2005) (Figure 6).

In non-seed plants, the MFT-like cluster is the only present, which suggests that the evolution of the FT-like and TFL1-like clusters coincided with the evolution of seed plants (Hedman et al., 2009).

In arabidopsis, six genes belong to this family: *MFT*, the only gene of the *MFT*-like subfamily; *FT* and *TWIN SISTER FT* (*TSF*), members of the *FT*-like cluster; *TFL1, BROTHER OF FT* and *TFL1* (*BFT*), and *A. thaliana CENTRORADIALIS* (*ATC*) belong to the *TFL1*-like subfamily (Karlgren et al., 2011).

Contrary to other genes in the family, the *MFT* effect on flowering time is quite limited, with overexpressing lines displaying slightly earlier transition (Yoo et al., 2004). It was observed that *MFT* expression is tissue specific to the seeds, and involved in seed germination in response to the abscisic acid (ABA) and the gibberellic acid (GA) signalling pathways (Xi et al., 2010).

FT and *TSF*, members of the *FT*-like cluster, are instead floral integrator genes, which regulate flowering time controlling the induction of floral meristem identity genes (Srikanth & Schmid, 2011).

FT is regulated through the photoperiodic pathway and promotes flowering in long day conditions; while TSF, whose amino acid sequence is highly similar to FT, also induce flowering but under short-day conditions activating the cytokinin metabolism (Yamaguchi et al., 2005).

The genes belonging to the *TFL1*-like subfamily display an opposite function compared to *FT*-like genes, resulting in delayed flowering time.

Therefore, despite the amino acid sequences of FT and TFL1 being highly similar (over 98% identity), the two proteins have antagonistic functions. Specific amino acid positions and segments in the protein sequences have been identified essential for the divergent function of these two genes, such as: Tyr-85 and His-99 in FT and TFL1, respectively; and segments B and C in FT (Ahn et al., 2006; Hanzawa et al., 2005).

The other two genes in the cluster with *TFL1*, *ATC* and *BFT* show a similar late flowering phenotype when overexpressed (Yoo et al., 2010).
ATC is specifically expressed in vascular tissues, but the protein is then transported to the shoot apex where it binds FLOWERING LOCUS D (FD) to inhibit floral meristem identity genes (Mimida et al., 2001).
FT and *TFL1* are the two most investigated genes belonging to the PEBP family in arabidopsis because of their key roles in regulating the transition from vegetative to reproductive phase and maintaining meristem identity.
Given the focus of my thesis on comparing arabidopsis *FT*- and *TFL1*-like genes in *Populus* trees and their regulation of the annual growth cycle a more detailed description of these genes is necessary.

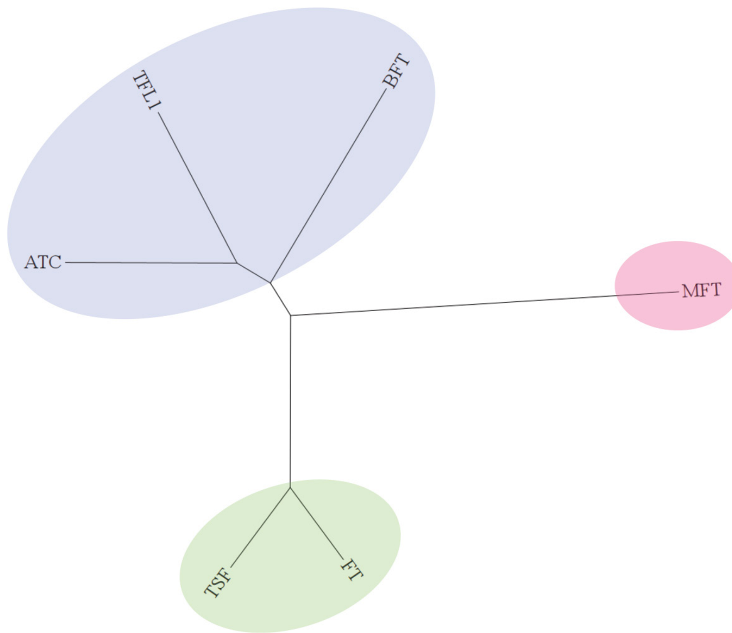

Figure 6. Phylogenetic tree of the PEBP family in arabidopsis.
Phylogenetic tree of the PEBP family in arabidopsis. The MFT-like subfamily (pink), the FT-like cluster (green) and TFL1-like subfamily (blue). The tree was generated with the neighbour joining method and a bootstrap value of 10 000.

1.4.1 *FT*, promoter of flowering

FT is a floral integrator gene, which regulates floral transition by incorporating signals from several pathways such as the photoperiodic and the ambient temperature pathway. *FT* function was investigated through the screen of flowering mutants in arabidopsis, which demonstrated that when *FT* is knocked out, plants flower late in long day, while overexpressing it causes early flowering in a photoperiodic independent manner (Kobayashi et al., 1999; Koornneef et al., 1991).

Already in the 1930s, Chailakhyan proposed the role of a hormonal florigen signal which after being synthesised in the leaves travels to the shoot apical meristem (SAM) to induce flowering under inductive day length. The theory was based on a series of grafting experiments which proved that the exposure of leaves to flower-inducing photoperiod signals is sufficient to trigger flowering (Kobayashi & Weigel, 2007). In 2007, the demonstration of FT mobility from the phloem companion cells (PCCs) in the leaf vasculature where it is expressed, to the shoot apex, confirmed that the florigen signal postulated decades before is indeed FT (Corbesier et al., 2007; Jaeger & Wigge, 2007; Mathieu et al., 2007).

Even though *FT* integrates the signal from different pathways, photoperiod has a pivotal role in *FT* activation in leaves through CONSTANS (Figure 7). In *co* mutants, *FT* is the only gene which does not respond to long day inducing flowering conditions, which makes *FT* the major target of CO (Wigge et al., 2005). Although CO is extremely important for *FT* induction it is not the only factor involved. NUCLEAR FACTOR (NF)-Y transcription factors also bind to the distal region of the *FT* promoter, while CO binds to a *cis*-element in the proximal region (Kumimoto et al., 2010). Moreover, NF-Y forms a complex with CO interacting via its conserved CCT domain (Kumimoto et al., 2010).

After *FT* is transcribed and translated the protein transport is regulated by the FLOWERING LOCUS T INTERACTING PROTEIN 1 (FTIP1) whose expression, in turn, is under control of ALTERED PHLOEM DEVELOPMENT (APL) (Abe et al., 2015). FT trafficking to the phloem sap occurs through the endoplasmic reticulum (ER) network protruding across plasmodesmata (Liu et al., 2012). More recently, a second mechanism was proposed in which FT is exported to the plasma membrane through the endosomal trafficking pathway, by the complex of the transmembrane region

protein QUIRKY (QKY), and Q-SNARE protein SYNTAXIN OF PLANTS 121 (SYP121) (Liu et al., 2020).

After reaching its destination in the SAM, FT is dependent on another protein to fulfil its function: the transcription factor FD, a basic Leucine Zipper protein (bZIP) (Wigge et al., 2005). FD is expressed in the nucleus of shoot apical cells, independently from the circadian rhythm and photoperiodic signal. *fd* mutants supress in part the early flowering phenotype of *FT* overexpressing plants (Wigge et al., 2005).

When FD and FT form a complex, which also includes 14-3-3 proteins, it generates a cascade signal inducing the expression of many downstream targets (Abe et al., 2005; Taoka et al., 2011). One of the main FT-FD complex's targets in the shoot apex, is *SOC1*. In fact, *SOC1* expression is highly downregulated in *ft fd* mutants (Searle et al., 2006). Another target of FT is *FRUITFULL* (*FUL*), which acts redundantly with *SOC1* in the control of flowering time (Wang et al., 2009). SOC1 and FUL have also a role in inflorescence meristem identity, double mutants of these genes result in a prolonged vegetative phase (Melzer et al., 2008). *SOC1* expression is also regulated in a positive feedback loop through another MADS-box transcription factor *AGAMOUS LIKE 24* (*AGL24*) (Liu et al., 2008). When co-located in the nucleus, SOC1 and AGL24 form a complex which induces expression of meristem identity genes, such as *LEAFY* (*LFY*) (Lee et al., 2008). When *LFY* is mutated, the meristems which would normally produce flowers, develop shoots. On the other hand, overexpression of the gene causes all vegetative meristems to turn into inflorescences (Weigel & Nilsson, 1995). Similar phenotypes are displayed by mutations in, or overexpression of, another meristem identity gene, *APETALA1* (*AP1*) (Mandel & Yanofsky, 1995). The expression levels of both *AP1* and *LFY* are influenced by changes in the induction or repression of either gene, suggesting that LFY and AP1 are interconnected through a positive feedback loop initiated by LFY inducing *AP1* (Liljegren et al., 1999). LFY and AP1 are involved in determining the floral identity of lateral meristems and also have a role in the regulation of floral organ identity. *lfy* mutants grow flowers which lack petals and stamens (Schultz & Haughn, 1991) while *ap1* flowers present abnormal sepal and petal development (Bowman et al., 1993).

1.4.2 *TFL1*, flowering repressor

TFL1 acts antagonistically to *FT* repressing flowering in arabidopsis. While *TFL1*-overexpressing plants flower later, *tfl1* mutants flower earlier and the inflorescence meristem is turned into a terminal flower (Shannon & Meeks-Wagner, 1991). A similar phenotype to *tfl1* mutants is displayed by overexpressing lines of *LFY* and *AP1* which are in fact downstream target genes of TFL1 (Liljegren et al., 1999).

Despite the effect on flowering time, the main function of *TFL1* is to maintain the inflorescence meristem identity (Bradley et al., 1997). In arabidopsis, *TFL1* is expressed specifically when the plant transitions to the reproductive phase and the SAM is converted into an inflorescence meristem. To fulfil its repressive function, TFL1 depends on FD and interaction with 14-3-3 proteins (Hanano & Goto, 2011). It has been proposed that both FT and TFL1 compete for the binding to FD, and the interplay between FT and TFL1 determines the promotion or repression of flowering genes (Ahn et al., 2006) (Figure 7). In the shoot apex, *TFL1* and meristem identity genes such as *LFY* and *AP1* are expressed in different domains. *TFL1* is detected just below the apical dome, in the inner part of the central zone of the SAM (Bradley et al., 1997), while *LFY* and *AP1* are located in the lateral part of the apex where the floral meristem will emerge (Baumann et al., 2015). Accordingly, in *tfl1* mutants *AP1* and *LFY* are ectopically expressed with the formation of a terminal floral meristem (Baumann et al., 2015). On the contrary, in *lfy* and *ap1* mutants the floral meristem are turned into inflorescence meristems due to expression of *TFL1* in the lateral organs (Conti & Bradley, 2007).

The spatial separation between TFL1 and the floral meristem identity genes is crucial for facilitating flower development while simultaneously ensuring the plant's indeterminate growth during the reproductive phase.

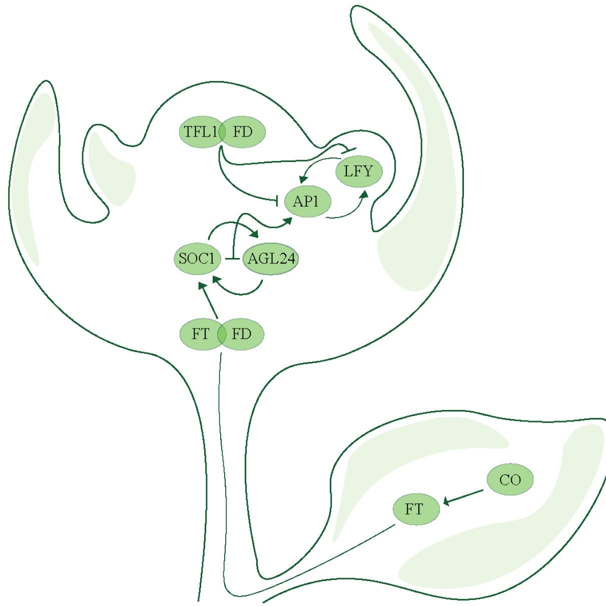

Figure 7. Floral induction in the shoot apical meristem in arabidopsis. Schematic representation of the main regulatory genes in floral induction. Arrows and T-ends indicate positive or negative regulation, respectively.

1.5 *Populus*, a model system for woody perennials

The genus *Populus* (*Salicaceae* family) comprehends six sections and twenty-nine species (Eckenwalder, 1996) (Table 1). Its members are commonly known as cottonwoods, aspens, and poplars.

Populus tree are widely spread throughout the northern hemisphere and are native to cool temperate and boreal regions of Europe, Asia and North America (MacKenzie, 2010). However some species also grow in hot and arid, desert-like regions in central Asia and Africa (Slavov & Zhelev, 2010). Because of their extensive distribution, *Populus* species have adapted to a broad range of environmental conditions and soil compositions. In the ecosystems where they live, they also fulfil a fundamental ecological role by acting as a keystone species for various microorganisms, herbivores, and insects (Kivinen et al., 2020). The European aspen (*Populus tremula*) for instance, has more host-specific species than any other boreal tree and is one of the most significant contributors to total epiphyte diversity in the boreal forest (MacKenzie, 2010).

In addition to their ecological importance, *Populus* are economically important forest trees. They fulfil the demands of veneer and pulp for paper, high-quality charcoal and chip-wood (Savill, 2013). Additionally, their rapid growth rate makes them an ideal biomass crop for energy production and carbon sequestration (Lemus & Lal, 2005). For instance, *Populus tremula* x *tremuloides*, the hybrid between the European aspen and the North American quaking aspen, is extensively used for wide-scale plantations due to its stronger vigour and higher growth rate (Wühlisch , 2009).
The interest in poplars' ecological and biological traits, such as their fast growth rate and ecological diversity, have made poplars the subjects of molecular, genetic and physiological studies (Brunner et al., 2004). In addition, *Populus* species are relatively easy to transform, regenerate and vegetatively propagate, enabling their use in species hybridization and functional genomic studies. The phenotypic diversity found in the genus lead to the mapping of economically important traits in interspecific hybrids (Tuskan et al., 2006). In 2006, *P. trichocarpa* was the first tree to have its genome sequenced (Tuskan et al. 2006), not only for its above mentioned physiological traits but also because of its modest genome size.
Due to these characteristics, genomic and molecular biology resources for this genus have rapidly increased. Thanks to the implementation of Next Generation Sequencing (NGS) techniques, extensive genetic and genomic data have accumulated, to provide an excellent model for studying how evolutionary processes affect patterns of genetic variation across genomes. Nowadays, *Populus* is a well-established model system for woody perennial plant biology.

Table 1. List of *Populus* species, their common names and distribution

Populus species	Common name	Distribution
P. adenopoda	Chinese aspen	China
P. alba	White poplar	Europe, North Africa, Central Asia
P. gamblei	Himalayan aspen	East Eurasia, India
P. grandidentata	Bigtooth aspen	North America
P. guzmanantlensis	Manantlan white poplar	Mexico
P. monticola	Baja white poplar	Mexico
P. tremula var. *sieboldii*	Japanese aspen	Japan
P.simaroa	Balsas white poplar	Mexico
P.tremula	Eurasian aspen	Europe, North Africa, Northeast Asia
P.tremuloides	Quaking aspen	North America
P.angustifolia	Narrow leaf cottonwood aspen	North America
P.balsamifera	Balsam poplar	North America
P.ciliata	Himalayan poplar	India, Pakistan, Bhutan, Nepal, Myanmar
P.laurifolia	Laurel polar	Eurasia
P.simonii	Simon poplar	Eastern Asia
P.suaveolens	Asian poplar	Northeast China, Japan
P.szechuanica	Szechuan poplar	East Eurasia
P.trichocarpa	Black cottonwood poplar	North America
P.yunnanensis	Yunnan poplar	Eurasia
P.deltoides	Eastern cottonwood	North America
P.fremontii	Fremont's cottonwood	USA
P.nigra	Black poplar	Europe, Central Asia
P.jacquemontiana	Sichuan poplar	China, USA, India
P.heterophylla	Swamp cottonwood poplar	China
P.lasiocarpa	Chinese necklace poplar	China
P.euphratica	Euphrates poplar	Northeast Africa, Asia
P.ilicifolia	Tana river poplar	East Africa
P.pruinosa	Desert poplar	Asia
P.mexicana	Mexico poplar	Mexico

1.6 The annual growth cycle of *Populus* trees

In the northern hemisphere, *Populus* trees have adapted to the boreal regions across all continents (MacKenzie, 2010). Due to the extreme seasonal fluctuations throughout the year, trees undergo remarkable phenological and developmental changes which define their annual growth cycle. Temperature and daylength are the primary environmental cues regulating these processes.

In summer, poplars experience a period of vegetative growth with the formation of new shoots and leaves (Figure 8). With the shortening of daylength and the lowering of temperature trees enter growth cessation and lose their leaves. Leaf senescence is a strategy adopted by deciduous plants, with the purpose of reducing water and nutrient uptake over winter when the ground is frozen. Moreover, trees form buds, “specialized” stipules to protect their shoot meristems. By the end of summer trees enter a dormant state which makes them unresponsive to any growth-promoting environmental signals.

In order to resume growth in the next season, dormancy is released after the trees experience a prolonged period of cold temperatures. When the conditions are favourable again, buds flush and new tissues are developed: the cycle repeats itself.

Poplars complete their annual growth cycle by producing only vegetative meristems for several years, delaying flowering until they reach maturity. Once mature, adult poplar trees initiate flowering, with vegetative and reproductive phases coexisting (Brunner & Nilsson, 2004). In early spring, axillary inflorescence buds form on individual branches. Over the following year, floral buds enlarge to protect the developing catkins within. Catkins emerge in the following spring, preceding vegetative bud burst (Yuceer et al., 2003).

Investigating the pathways regulating developmental transitional changes in the trees’ growth cycle, such as growth cessation and flowering induction, provides valuable insights into forest management and productivity in economically relevant species.

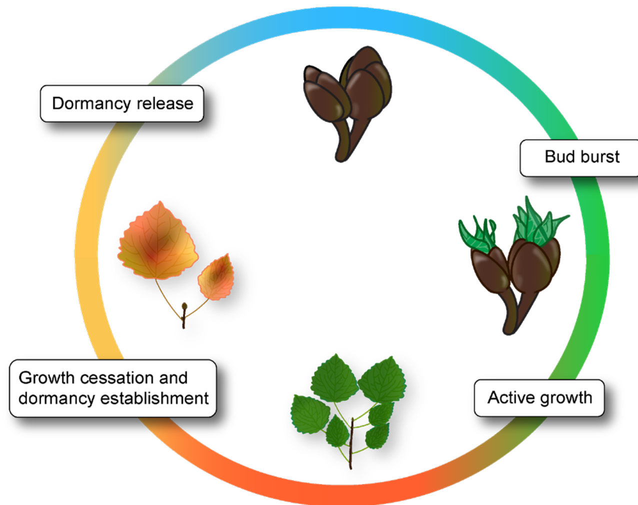

Figure 8. The annual growth cycle of *Populus* trees.

Schematic representation of the annual growth cycle of trees. During spring (green) buds flush; in summer (red) the leaves expand, and the shoots elongate; In autumn (yellow) leaves go through senescence and buds are formed; in winter (blue) unopened buds isolate the meristems from the cold temperatures.

1.6.1 Growth cessation and bud set

In boreal and temperate regions, trees' growth cessation is regulated primarily by photoperiod. Trees perceive the shortening of daylength occurring towards the end of summer and beginning of autumn, and below a certain critical day length (CDL) vegetative growth is halted (Cooke et al., 2012). The number of hours required to induce growth cessation in trees, varies depending on the latitude; trees growing at northern latitudes, normally display a longer CDL compared to southern populations (Böhlenius et al., 2006). This strategy leads the northern trees to set bud early in the season to protect their meristems from the harsh winter conditions.

Trees sense daylength through photoreceptors, such as the phytochromes (Olsen et al., 2004). Three genes have been identified in the poplar genome: *PHYA*, *PHYB1* and *PHYB2* (Howe et al., 1998). Alterations in the expression of both *PHYA* and *PHYB* affect SD-induced growth cessation and time of bud set. When downregulated, *PHYA* and *PHYB* transgenic lines display early growth cessation and bud set compared to wild type (WT) plants in SD

(Ding et al., 2021; Kozarewa et al., 2010), suggesting the role of phytochromes as promoters of growth. When phytochromes expression is altered, circadian clock genes are also affected (Figure 9). When *LHY1* and *TOC1* are downregulated the critical daylength is reduced (Ibáñez et al., 2010). The components of the circadian clock control the expression of other downstream genes such as *CONSTANS* (*CO*) and *GIGANTEA* (*GI*), which integrate the photoperiodic signal allowing trees to measure the daylength (Ding et al., 2018).

In arabidopsis, CO is at the centre of the photoperiodic response and LD induction of *FT*. However, in poplar, alterations in the expression of *CO* orthologues have minor effects on *FT* expression (Hsu et al., 2012). This suggests that there might be more important *CO*-independent pathways regulating *FT*. Two *FT* orthologues have been identified in poplar, *FT1* and *FT2*; *FT1* is induced by cold temperatures in buds during winter, while *FT2* is expressed in leaves during the growing season in summer (Pin & Nilsson, 2012). A candidate gene in controlling *FT* expression is *GIGANTEA* (*GI).*

GI and its paralogue *GIL* in poplar, have a strong effect on growth cessation and bud set when downregulated (Ding et al., 2018). Additionally, the action mechanism for GIs seems to be conserved in *Populus* species compared to arabidopsis. GIs form a complex with FKF1s and CDFs to control expression of *FT2* specifically in a CO-independent pathway (Ding et al., 2018). *FT2*, whose expression pattern resembles its homologue in arabidopsis, is a a key factor in the regulation of short-day-induced growth cessation, since its downregulation results in a faster SD response relative to the WT (Böhlenius et al., 2006). In poplar, like in arabidopsis, FT2 interacts with an FD-like protein, FDL1 (Tylewicz et al., 2015). Three *FDL* genes have been identified in *Populus* (Sheng et al., 2022), but only the interaction with FDL1 triggers the induction of downstream genes such as *Like-AP1* (*LAP1*) (Azeez et al., 2014). In fact, when *FDL1* is overexpressed, plants display an attenuated downregulation of *LAP1* in response to SD conditions (Tylewicz et al., 2015). LAP1 binds to the promoter of *AINTEGUMENTA-LIKE 1* (*AIL1*) (Azeez et al., 2014), a transcription factor whose function is to regulate proliferation through the control of D-type cyclins and thus the cell cycle (Karlberg et al., 2011). *FT2* activity is regulated not only at a transcriptional level, but also at a post-transcriptional level from other factors such as, *BRANCHED 1* (*BRC1*), which in arabidopsis represses bud outgrowth (Aguilar-Martínez et al., 2007). In the shoot apex, BRC1 physically interacts

with FT2 to repress it, and consequentially downregulating *LAP1* which in turns can repress *BRC1* generating a negative feedback loop (Maurya et al., 2020).

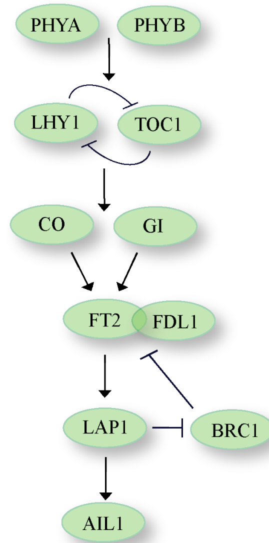

Figure 9. *FT2* promotes vegetative growth.
Schematic representation of the main regulatory genes in vegetative growth. Arrows and T-ends indicate positive or negative regulation, respectively.

1.6.2 Dormancy establishment

The term "dormancy" indicates a period in an organism's life when growth has stopped, and metabolic activities slow down. In plants, and in particular in woody perennial, three types of dormancies have been suggested: ecodormancy, endodormancy and paradormancy (Lang, 1987). Ecodormancy refers to a stage where buds have formed, but can revert to vegetative growth under favourable conditions, and only the environment conditions determine quiescence. Endodormant buds instead, are unable to resume growth even if the conditions would allow it. Endodormancy is established after a period of unfavourable conditions and maintained by endogenous signals. Finally, paradormancy refers to the negative control of hormones during apical dominance which prevent flush in lateral buds. In this thesis, the term dormancy specifically refers to endodormancy.

Dormancy is known to be established after prolonged exposure to SD conditions (Singh et al., 2017); however, the molecular mechanisms underlying this process in *Populus* trees remain poorly understood.
Studies focusing on seed dormancy have revealed the role of abscisic acid (ABA) as a key regulator of this process (Penfield & King, 2009). In trees, ABA accumulates after exposure to SDs, and ABA-insensitive trees fail to establish dormancy, which means that if the plants are exposed to growth promoting conditions buds can flush and revert to vegetative growth (Singh et al., 2019). These findings suggest a parallelism between seed and bud dormancy. Contributing to the inability of ABA-insensitive trees to establish dormancy is the absence of dormancy sphincters (Tylewicz et al., 2018).
Dormancy sphincters are depositions of callose in the plasmodesmata to impede the flow of growth promoting signals, such as hormones and transcription factors, between neighbouring cells below the shoot apex (Rinne et al., 2011). The symplastic isolation of the SAM through callose deposition is regulated upstream by ABA signalling following SD treatment (Tylewicz et al., 2018). ABA in fact, induces the expression of *CALLOSE SYNTHASE 1*(*CALS1*) and supresses glucanases, enzymes which degrade callose, to promote the production of the dormancy sphincters (Singh et al., 2019). Through the study of ABA-insensitive lines it has been possible to identify downstream components of the abscisic acid (ABA) pathway. The promotion of dormancy by ABA occurs through the downregulation of *PICKLE* (*PKL*), a chromodomain protein (Tylewicz et al., 2018) (Figure 10). In ABA-insensitive trees where *PKL* has been downregulated, the defects in dormancy are suppressed due to the induction of *SHORT VEGETATIVE PHASE LIKE* (*SVL*) in SDs (Singh et al., 2019). *SVL* is the aspen orthologue of *SVP* in arabidopsis, and it has been related to the *DORMANCY ASSOCIATED MADS* (*DAM*) genes identified for the first time in *Prunus* (Bielenberg et al., 2008). Therefore, *SVL* is induced in SD conditions by repression of *PKL* in an ABA-dependent mechanism. To promote dormancy, *SVL* positively regulates the plasmodesmata closure inducing *CALS1*, and control the gibberellin pathway repressing its biosynthesis (Singh et al., 2019). In addition, *SVL* generates a positive feedback loop inducing ABA biosynthesis enzymes and receptors, such as *9-CIS-EPOXYCAROTENOID DIOXYGENASE 3* (*NCED3*) and *PYRABACTIN RESISTANCE1 (PYR1)/PYR1-LIKE (PYL)/REGULATORY COMPONENTS OF ABA*

RECEPTORS (RCAR) (PYR/PYL/RCAR), ensuring a constant concentration of ABA in response to SDs (Singh et al., 2018).

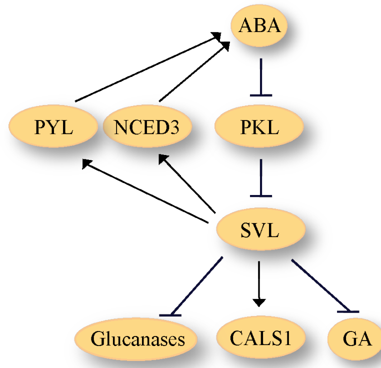

Figure 10. Regulation of dormancy establishment.
Schematic representation of the main regulatory genes in dormancy establishment. Arrows and T-ends indicate positive or negative regulation, respectively.

1.6.3 Dormancy release and bud break

Dormancy is released after trees are exposed to prolonged cold conditions. This requirement can already be fulfilled during low autumnal temperatures so that trees exit endormancy and remain in an ecodormant state until the conditions are favourable again in spring, when buds flush (Rinne et al., 2011). Despite dormancy release and bud break being two clearly separate phases in the resumption of growth, it is difficult to establish from a molecular perspective which genes are involved in either mechanism or if they have a role in both. No specific markers are associated to dormancy release; thus, trees are defined as dormant or not if under inductive conditions buds are able to flush.

If dormancy is established with deposition of dormancy sphincters, dormancy release is associated with their removal from the plasmodesmata allowing growth-promotive signals to travel back to the meristem and restore growth (Rinne et al., 2011). Some of the genes which are involved in dormancy establishment, play also a role in its release. Cold temperature represses *SVL* through the negative regulation of *EARLY BUD BREAK 1* (*EBB1*). *EBB1* encodes an APETALA2/Ethylene-responsive transcription factor (AP2/ERF), and when downregulated, aspen trees show delayed bud

break (Yordanov et al., 2014). *EBB1* is induced by cold and represses *SVL*, disrupting the positive feedback loop, which leads to ABA induction to maintain dormancy (Singh et al., 2018) (Figure 11). The decreased levels of ABA, promote the induction of another AP2/ERF transcription factor *EBB3*, which in turn acts on the cell cycle proliferation activating cyclin CYCD3 in order to restore growth (Singh et al., 2018). Simultaneously, as a consequence of the downregulation of *SVL*, *CALS1* expression drops as well and glucanases are produced so that the callose plugs are degraded (Rinne et al., 2011). *BRC1* is also downregulated in response to *SVL* repression by cold temperature leading to bud break (Singh et al., 2019). With the plasmodesmata freed by the callose plugs, the flux of growth promoting signals to the shoot apex is restored. *SVL* repression also causes an upregulation of GA biosynthetic genes and a downregulation of the catabolic ones (Karlberg et al., 2010). Gibberellins have been proven to be sufficient to release dormancy when applied exogenously even without the trees being exposed to cold treatment (Rinne et al., 2011). Moreover, overexpression of *Gibberellin 2 Oxidases* (*GA2OX*s), which are part of the catabolic pathway of gibberellin, delayed bud break, underlying the crucial role of these compounds in bud flush and growth promotion (Singh et al., 2018).

Another target of *SVL* repression is *FT1*, which is strongly induced by cold temperatures in winter; So, *FT1* is a good candidate for the regulation of dormancy release, while its paralogue *FT2* is involved in vegetative growth (Singh et al., 2019). It has been proposed that *FT1* induction occurs in embryonic leaves in the buds and and that it travels to the shoot apex upon the opening of the plasmodesmata where it promotes bud break (Rinne et al., 2011). However, the positive regulator of the strong induction of *FT1* is still to be uncovered.

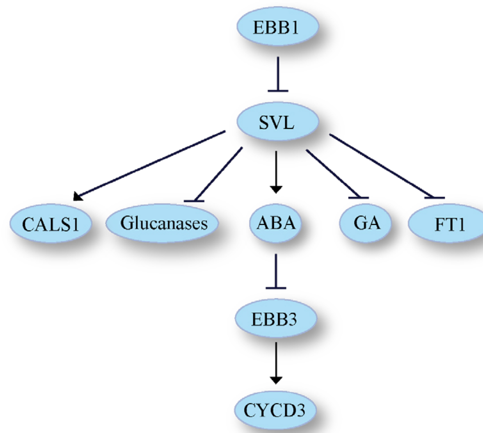

Figure 11. Regulation of bud flush.

Schematic representation of the main regulatory genes in bud flush. Arrows and T-ends indicate positive or negative regulation, respectively.

1.6.4 Flowering

Woody perennial species, such as poplar, display a long juvenile phase that can last for many years, before they reach sexual maturation and start flowering. A prolonged juvenile phase allows trees to allocate energy and nutrients towards vegetative growth, to reach a competitive size in order to outcompete other plants for the access to light. The timing of the transition from the juvenile to the mature phase is a trade-off between reproduction and growth to optimise fitness and reproductive success over the tree's lifespan. Upon maturation, vegetative and reproductive phases coexist in an annual cycle regulated by environmental cues.

Populus are dioecious, which means that male and female flowers are carried on separate trees. In addition, shoot architecture is related to tree maturation and consists of long and short shoots. Long shoots are responsible for the tree size and normally decrease in number with aging. Short shoots instead, predominately carry flowers, have a pre-determined growth and set bud early during the season (Dickmann, 2001). In both long and short shoots, the apical bud always remains vegetative (Yuceer et al., 2003).

The stages from floral initiation and flower development to floral bud flush occur in several seasons. In an enclosed terminal bud, a shoot with early preformed leaves develops; after vegetative bud break the newly emerged

shoot elongates and leaves expand. During this season the axillary meristems are determined as vegetative or reproductive and new buds are formed throughout summer and autumn. The new shoot can be divided in three zones, identified by the nature of the axils (Figure 12). The first zone (Vegetative Zone I) is the most proximal to the shoot apex, and contains vegetative buds, below the Floral Zone includes floral buds and at last a second vegetative zone is present (Vegetative Zone II) (Yuceer et al., 2003). After determination of the inflorescence meristems, floral organs will develop during the consecutive months inside the floral buds, which will flush concurrently with warm temperatures in spring in the following year. From floral buds, drooping catkins (pendulous inflorescences) emerge before vegetative bud burst to facilitate wind pollination (Yuceer et al., 2003).

Studying the molecular pathways which lead to flowering in *Populus* is rather complex. Many aspects of *Populus* life strategies makes experiment settings complicated, such as the long juvenile phase. Moreover, flowering induction occurs in trees without any clear phenology or positioning, thus when floral buds are distinguishable to the vegetative ones due to a bigger and rounder shape, it is too late to investigate this mechanism because meristems are already determined, and floral organs are developing.

To investigate the genes and regulatory pathways which control flowering in *Populus*, reverse genetics approaches can be employed to study gene function and compare these mechanisms to well-characterised flowering pathways in other species, such as *Arabidopsis thaliana*.

In arabidopsis, members of the PEBP family *FT* and *TFL1* are floral integrators of several flowering inducing pathways. In poplar, *FT* and *TFL1* homologues have duplicated and are present in two copies: *FT1* and *FT2* and *CENL1* and *CENL2* (Böhlenius et al., 2006; Mohamed et al., 2010). Over the past years, it has been demonstrated that these genes have adapted to control seasonal growth, even though they also appear to be involved in flowering.

Both *FT1* and *FT2* when overexpressed under constitutive promoters can induce early flowering similar to what was observed in arabidopsis (Böhlenius et al., 2006; Hsu et al., 2006). *TFL1* homologues in poplar are called *CEN*-like genes, due to the snapdragon homologue, *CENTRORADIALIS* (*CEN*), whose mutation leads to the generation of terminal flowers, same as for *tfl1* mutants in arabidopsis (Bradley et al., 1996; Shannon & Meeks-Wagner, 1991). *CENL1* is expressed in the rib meristem of the shoot apex (Ruonala et al., 2008) and when knocked out

plants display a strong early flowering phenotype already in tissue culture, opposite to *cenl2* mutants which showed only vegetative growth (Sheng et al., 2023). Despite the data accumulated on flowering mutants in *Populus*, the specific functions of *FT*- and *TFL1*-like genes need to be elucidated. Furthermore, downstream targets of these genes and their role in flowering needs to be confirmed. Orthologues genes from arabidopsis have been investigated but their function is yet to be clarified. For instance, *LAP1* in poplar has been associated to vegetative growth as a downstream target of *FT2,* but its overexpression is not enough to induce flowering as in the case of arabidopsis *AP1* (Azeez et al., 2014). A similar result was observed for overexpression lines of the *LFY* homologue, *PTLF*, for which only a few lines displayed precocious flowering (Rottmann et al., 2000).

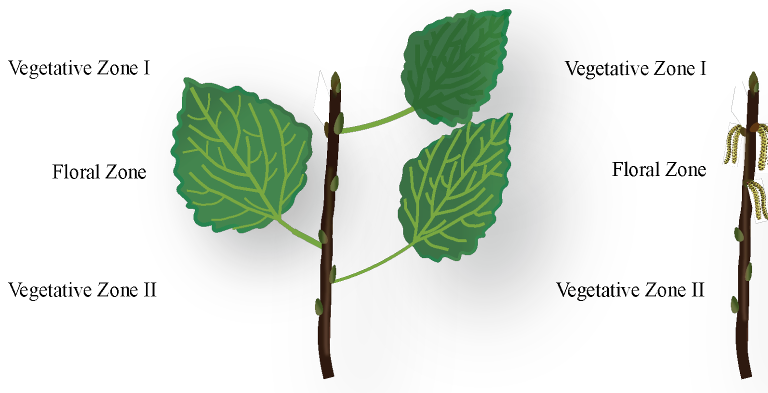

Figure 12. Flower development in *Populus.*

Representation of the newly formed shoot after bud flush in summer and position of the vegetative and floral buds, which release catkins on the consecutive season after a period of cold exposure in winter.

2. Objectives

The aim of my project is to understand the roles of the *Populus* homologues of *AtFT*, *FT1* and *FT2*, as well as the putatively *FT*-antagonistic genes *CENL1* and *CENL2* (homologues of arabidopsis *TFL1*), in relation to the control of the annual growth cycle and flowering time in trees.
In addition to inducing premature flowering, like in the arabidopsis model, overexpression of either *FT* gene abolishes the short-day response (Böhlenius et al., 2006, Hsu et al., 2011). Initially both genes were thought to be redundant in both controlling flowering and the timing of growth cessation and bud set (Böhlenius et al., 2006, Hsu et al., 2006). However, it was later shown that the two *FT* paralogues have completely different expression patterns, with *FT1* being expressed at the end of winter and *FT2* being expressed during the growing season (Hsu et al., 2011). It was then speculated that *FT1* controls flowering while *FT2* regulates growth (Hsu et al., 2011). The role of *CENL* genes is still unclear regarding the regulation of growth. Although more data has been collected for *CENL1*, which seems to be an important factor in flowering repression, the function of *CENL2* is unknown, in part due to the low expression in most tissues (Mohamed et al., 2010; Sheng et al., 2023).

More specifically in this thesis I explore:

- The sub-functionalization of *FT1* and *FT2* in *Populus* trees, and the role of their upstream regulators in controlling the annual growth cycle.
- The interplay between the *FT*-like genes and the *TFL1*-like genes (*CENL1* and *CENL2*) in *Populus* trees in the control of phenology and flowering time.
- The genetic mechanisms controlling bud set, bud break and flowering by co-expression analysis throughout the annual growth cycle of *Populus* trees.

3. Material and Methods

This section includes a general description of plant material, growth conditions and experimental techniques. For more detailed information, refer to the individual articles and manuscripts.

3.1 Plant material and growth conditions

Experiments were performed in hybrid aspen clone T89, a cross between the European aspen (*Populus tremula*) and the American aspen (*Populus tremuloides*). The clone, originating from the Czech Republic, has a high efficiency of transformation and it can be regenerated through stem cuttings, which makes *in vitro* culture more practicable than using other tree species (Nilsson et al., 1992). Plantlets are grown *in vitro* on MS medium (Murashige & Skoog, 1962) in sealed jars until transferred to soil. During the first two weeks the plants are covered with plastic bags to facilitate acclimatation to the new environment.
The growth chambers conditions simulate the seasonal changes during the annual growth cycle of aspen (Figure 13). After potting, trees are subjected to long day conditions (LD), and fertilisation which allows the plants to grow taller and thicker. LD growing conditions consists of 18h of light and 6h of darkness at ~20°C. To induce growth cessation and simulate autumn, trees are shifted to short day conditions (SD), consisting of a cycle of 14h light/ 10h dark. The critical day length of T89 which triggers growth cessation is around 15.5 hours (Olsen et al., 1997), therefore, when trees are subjected to 14 hours of light the induction of growth cessation and bud set is rather slow, allowing the detection of even small differences between the growing lines. When plants are shifted to SD, fertilisation is stopped, and the treatment lasts up to 15 weeks, to ensure that every tree has established dormancy and set bud. It is known that to release dormancy trees need to be exposed to cold, so plants are shifted to a cold treatment between 4°C and 6°C, and an even shorter photoperiod of 8 hours of light for 8 to 10 weeks. To resume growth and observe bud flush, trees are subjected to LD conditions again, completing the growth cycle.

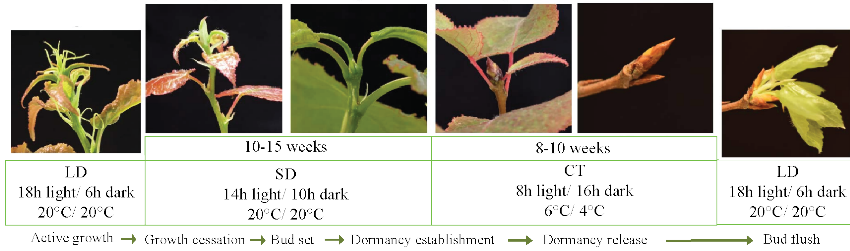

Figure 13. Growth conditions mimicking the change of seasons.

3.2 Phenotyping

A few weeks after potting the plants, we start the phenotyping by measuring height and number of leaves to assess if transgenic lines grow differently to wild types. In SD, trees stop growing and producing new leaves, and after a few weeks they set buds. To assess this process, we score the plants according to a system described in Ibáñez et al. (2010). The stages range from 3 to 0 (Figure 14), where stage 3 corresponds to an active growing apex with generation of new leaves; stage 2 defines growth cessation; stage 1 corresponds to the beginning of bud formation; stage 0 represents a fully closed bud with hardened and darker stipules. Measuring the number of leaves, the height and bud set, gives an accurate assessment of how trees respond to SD, and allows us to identify differences between lines.

At the end of SD, trees are subjected to cold treatment during which leaves fall; however, no other visible changes occur throughout this period.

When trees are shifted back to LD, warmer temperatures trigger bud break. To score bud flush, we follow again the system described in Ibáñez et al. (2010). The score ranges from 0 to 5 (Figure 14). Stage 0 identifies buds at the end of cold treatment when they are still completely enveloped by their scales; stage 1 corresponds to buds which are swelling and becoming greener; at stage 2 leaf tips appear between the scales; at stage 3 the buds are open; stage 4 corresponds to leaves which are still emerging despite being completely unfolded; and finally, at stage 5, the newly formed shoot elongates. We score both apical and lateral buds. However, since lateral buds are subjected to apical dominance, the score from apical buds is considered more meaningful (Singh et al., 2017).

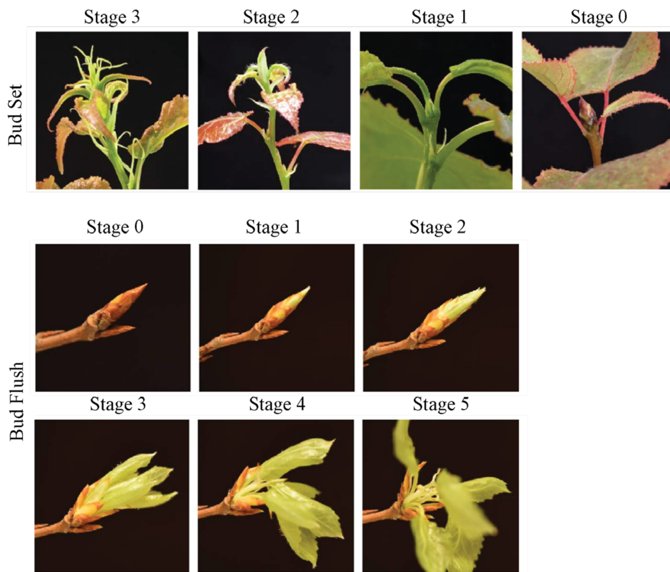

Figure 14. Bud set and bud flush stages in *Populus*.

3.3 Generation of transgenic lines

Generating transgenic lines is fundamental in reverse genetics. Manipulation of one or multiple genes gives the possibility to study gene function observing the effect on the phenotype and the molecular pathways. Several approaches can be applied to overexpress, knock down or knock out a gene of interest.

RNA interference (RNAi) is a technique that allows us to silence specific genes by targeting messenger RNA (mRNA) molecules for degradation or translational repression. This mechanism is mediated by small interfering RNAs (siRNAs) that, when incorporated in a protein complex, target complementary mRNA sequences (Fire et al., 1998). This process allows us to design specific siRNAs which recognise the sequence of the gene of interest and promote its downregulation. Although being a powerful tool, RNAi often leads to a partial knockdown of the gene expression rather than a complete knockout, which makes it complex to study the mutants' phenotype. In addition, especially in case of multiple paralogues like in *Populus*, there might be an off-target effect on similar mRNA sequences (Davidson & McCray Jr, 2011). Because of these limitations, nowadays

CRISPR/Cas9 is the preferred choice for gene editing. Compared to RNAi, CRISPR/Cas9 is more specific, efficient and versatile.

The CRISPR/Cas system is a defence mechanism described in prokaryotes, in which DNA molecules, integrated in the host genome upon previous infections, are transcribed into RNA sequences, and together with CRISPR associated (Cas) proteins target and cleave the correspondent viral DNA. To make sure that the system only targets viral DNA sequences, a sequence of three nucleotides NGG, called Protospacer Adjacent Motif (PAM), must be present in proximity of the cleavage site (Hille et al., 2018). More than ten years ago this system was genetically engineered to precisely knock out genes of interest in many organisms, including plants. The RNA molecules which recognise the target sequences were simplified to a single guide RNA (sgRNA), and together with the endonuclease Cas9, bind the genome to induce a double strand break (Jinek et al., 2012).

Because the natural DNA repair system is prone to errors, when repairing the break produced by the Cas9, it is likely that an insertion, a deletion or a single nucleotide polymorphism (SNP) will be generated. If the sgRNAs are designed within the coding regions, even a single mutation can translate to a stop codon and disrupt the frameshift, leading to a truncated non-functional protein. The requirement of a PAM sequence limits the design of sgRNAs to specific regions of DNA, especially in coding regions which are highly conserved in case of paralogous genes. However, this can be overcome by designing several sgRNAs which can lead to a bigger genomic deletion.

The opposite approach to produce knock outs, is to induce overexpression of genes of interest. However, the lack of knowledge of promoter regions in *Populus* forces researchers to use constitutive promoters, such as the *35S* promoter from the plant pathogen Cauliflower Mosaic Virus (CaMV). When using a constitutive promoter, gene expression is induced continuously in all tissues at high levels, obscuring the natural regulation and function of the gene. Moreover, gene overexpression can lead to artificial phenotypes due to the gene being expressed in a tissue where it is normally not.

Despite these weaknesses, overexpressing lines with constitutive promoters remain a valuable option. In fact, they provide a simple, effective and quite fast method to create transgenic lines to study gene function. It is also possible to use constitutive promoters to trigger the expression of tagged genes, where the gene of interest is fused to a reporter or epitope tag, such as

Green Fluorescent Protein (GFP) or Myc. Tagged lines simplify the analysis of the regulation of proteins and downstream targets.

3.4 Sequence and phylogenetic analysis

High-quality genome sequences are essential for sequence analysis as they provide the information to study gene structure and genetic variations. Reliable genome sequences also allow the identification of homologous genes across species, constructing phylogenetic relationships. Moreover, they are crucial in approaches of reverse genetics to design specific primers or single guide RNAs (sgRNAs). Although *Populus tremula* x *tremuloides* clone T89 is a hybrid between two parental lines, both haplotypes of the T89 genome sequences are accessible in Plantgenie (https://plantgenie.org/) thanks to the new technologies that makes it possible to distinguish the haplotypes through the sequencing of long reads.
When identifying homologous genes from other species, one can use the BLAST tool on the Plantgenie website. BLAST, which stands for "Basic Local Alignment Search Tool", is an algorithm for comparing biological sequence information. To compare sequences from different species it is also important to understand the phylogenetic relationship between genes. To perform phylogenetic and sequence analyses, we use QIAGEN CLC Main Workbench. Among its many tools, it enables users to construct phylogenetic trees using the clustering method known as neighbour joining (Saitou & Nei, 1987).

3.5 Cloning systems

Cloning is widely used in molecular biology to assemble recombinant DNA molecules into a vector which is transferred into a host organism to promote replication of multiple copies of DNA (Watson, 2007). It is for instance, employed to assemble constructs for gene editing and protein expression.
The DNA fragments, intended to be cloned are normally obtained by PCR amplification. Through digestion with restriction enzymes of both the insert and the vector backbone, and the action of ligases, the DNA fragment is covalently linked to form the final vector. To produce multiple copies, the

recombinant vector is then transformed into a host cell, such as *E. coli* or *A. tumefaciens* by electroporation or heat shock.
During the years, companies have been implemented several cloning methods, both the Gateway and GreenGate systems are commonly used in many laboratories.

Gateway cloning method

The Gateway cloning method (Hartley et al., 2000) has been commercialised by Invitrogen since early 2000s. This system uses an adaptation of bacteriophage lambda and bacteria attP and attB sites respectively, recognised by enzyme mixes called BP Clonase and LR Clonase. When the phage integrates into the bacterial genome trough recombination of attP and attB, two new recombination sites are produced: attLeft (attL) and attRight (attR). In the Gateway system, two steps are required. The first reaction allows the insertion of the DNA fragment, which is flanked by attB, into an entry vector with attP sites. The recombination generates attL sites on the sides of the inserted DNA. The second reaction finalise the cloning into the destination vector which contains attR.
The availability of large archives of Gateway Entry clones, provides a quick way to transfer genes of interests into predetermined cassettes.

GreenGate cloning method

GreenGate is a rapid system to assemble transformation constructs. It only uses one type of IIS restriction endonuclease to insert several expression cassettes into one destination vector (Lampropoulos et al., 2013) (Figure 15). GreenGate is based on another cloning system, the Golden Gate. The method uses restriction endonucleases to isolate DNA fragments and insert them into specific positions in the target vector. All inserts need to be flanked by the recognition sites of specific restriction enzymes, which define the orientation and the order in which the cassettes are assembled in the final construct. With GreenGate, the overhangs flanking the DNA fragments are non-palindromic to avoid ligation of inverted fragments. In addition, GreenGate uses only one enzyme, BsaI, which reduces the probability of targeting naturally occurring restriction sites in inserts.

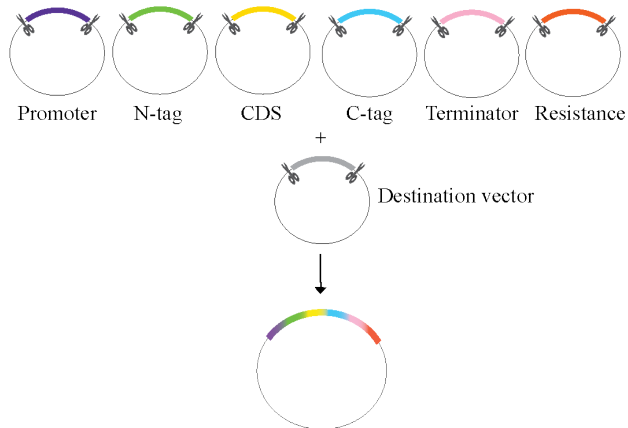

Figure 15. Green Gate cloning system.

Schematic representation of the GreenGate system. Several entry vectors carrying different expression cassettes can be inserted in one step into a destination vector.

3.6 Gene expression and transcriptomic analysis

To study gene expression, a critical step is the quantification of mRNA transcripts, to understand gene activity under different developmental and environmental conditions.

Total RNA is extracted by grinding tissues with the CTAB extraction buffer (Chang et al., 1993) and purification with the RNeasy kit (Qiagen). The DNase treatment is performed on-column (Qiagen) during purification. After quantification, the RNA is reverse transcribed into double stranded complementary DNA (cDNA) with poly(T) primers which recognise the polyA tail of mRNA molecules. cDNA is the starting material to study gene expression.

To investigate gene expression of a few candidate genes, quantitative PCR (qPCR) is a suitable technique. It is accurate and highly sensitive and allows the amplification of low levels of transcript using fluorescence-based detection methods, such as SYBR Green (Heid et al., 1996).

To analyse large-scale gene expression profiling instead, the most common choice nowadays is to perform RNA sequencing (RNA-seq). The high-throughput sequencing of RNA provides a comprehensive view of the entire transcriptome to analyse gene expression. The cDNA is ligated to adapter sequences to generate libraries compatible with the Illumina sequencing

platforms. The raw data is pre-processed through quality assessment protocols which filter reads from organelle and ribosomal RNA and trim the adapters. To define expression levels, reads are mapped to a reference genome and normalised to compare among samples. To be able to compare the results, it is important to verify that the number of expressed genes does not vary among samples and very low expressed genes are not considered in the analysis. Expression profiles of the data can be plotted as single graphs or heatmaps.

When analysing big datasets with many samples, it is possible to identify clusters of genes with similar expression patterns generating co-expression networks. They are constructed by correlation of expression profiles and generate hubs of genes which are likely to interact together in the same pathways or processes.

If co-expression analysis finds similar patterns of gene expression, differential expression (DE) analysis identifies genes which have different gene expression in determined samples. DE is performed to compare treatments or mutants with control lines to identify up- and down-regulated genes under specific conditions.

3.7 *In Situ* Hybridization (ISH)

Quantitative PCR analyses gene expression levels. However, it is difficult to localise the signal since the grinded samples include several different cell types. This is particularly true for buds, that despite their small size are formed by many tissues.

In situ hybridization (ISH) is a molecular technique which detects either DNA or RNA molecules within fixed tissues. It uses complementary probes which hybridise to the target sequences. The samples are fixed and cut into thin slices which are incubated with the probe and then visualised under the light microscope, in case of chromogenic labels. This technique provides a spatial resolution that cannot be reached with qPCR or RNA-seq.

3.8 Chromatin Immunoprecipitation (ChIP)

Understanding when and where a gene is expressed, provides valuable information on its function. Additionally, the determination of downstream

targets can also give insights in the biological processes and molecular pathways in which the gene is involved.
Chromatin Immunoprecipitation (ChIP) is a technique which allows to study where on the DNA a specific protein binds, identifying its targets in living cells or tissues. The first step consists of cross-linking the proteins in a covalent way to the DNA to preserve their interactions. Then the cells are lysed and the nuclei isolated to extract the chromatin. Chromatin is fragmentated into shorter molecules through sonication. The next step is the immunoprecipitation by using an antibody which recognises the protein of interest. The antibody is bound to magnetic or agarose beads which facilitate the pull-down of the protein-DNA complex.
Since there are not antibodies present for all proteins, tagged lines can be used to overcome this limitation. A well-characterised tag is fused to the protein of interest and recognised by a specific antibody available on the market. Once that the complex has been isolated, the cross-link is reverted and the DNA purified. The obtained DNA fragments represent the regions bound by the protein of interest and can be analysed by qPCR to investigate specific regions using known primers or by high-throughput sequencing to identify protein-binding sites genome-wide (ChIP-Seq). If the protein of interest is a co-transcription factor, and not a transcription factor with the ability to bind DNA, it is possible to study downstream targets using specific fixatives, such as Epoxy succinimide (EPS). EPS forms covalent bonds between amine groups on proteins and nucleic acids and links the protein of interest to other proteins in the complex which bind the DNA.

3.9 Protein-protein interactions (PPIs)

ChIP investigates the binding region of a protein of interest, but it is rarely the case that a protein acts singularly. Often proteins interact with each other, forming complexes to perform their roles in different biological processes. There are several techniques to investigate protein-protein interactions (PPIs), each with its strengths and limitations.

Yeast Two-Hybrid (Y2H) assay

Y2H is a technique used to study protein-protein interactions in yeast cells. Y2H tests pair-wise interactions of specific proteins which are respectively called, bait and prey (Figure 16). The bait is fused to the binding

domain of a reporter protein, while the prey is fused to the activation domain of the same reporter. The constructs carrying the bait and the prey are then co-transformed into yeast cells. If bait and prey interact with each other, the binding and activation domain combine to form an active transcription factor which induces the expression of a reporter gene. The activation of the reporter indicates a positive interaction and is detectable by growth on selective media (lacking histidine) or by a colour change (β-galactosidase assay).

Y2H is a simple and relatively cheap and fast assay to perform; it allows a direct visualisation of the results without the need of other instruments as in the case of the Bimolecular Fluorescence Complementation (BiFC) assay, which require the use of a fluorescent microscope to detect the interaction.

However, the limitation of Y2H depends on the fact that it relies on a heterologous system. The interaction of the bait and the prey might depend on other factors that might not be present in yeast, as well as mechanisms of post translational modifications such as phosphorylation or glycosylation that will not take place in yeast.

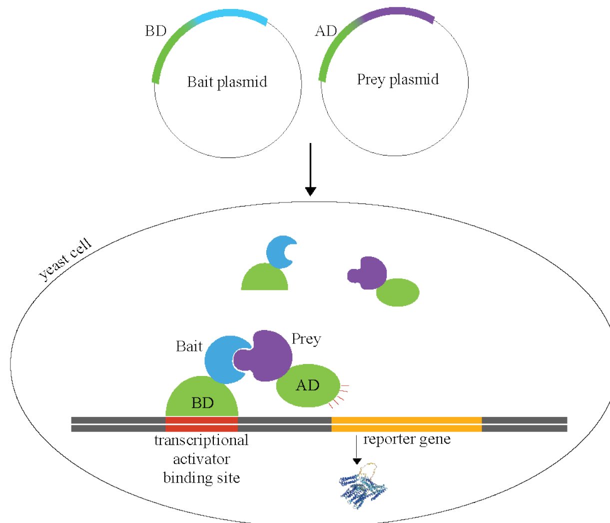

Figure 16. Yeast Two-Hybrid system.

Schematic representation of the yeast two hybrid system. Two vectors carrying the bait and the prey fused to the binding (BD) and activation domain (AD) respectively, are co-transformed in yeast. If the bait and the prey interact, the BD and AD induce the expression of the reporter gene.

4. Results and Discussion

4.1 Paper I

FLOWERING LOCUS T paralogs control the annual growth cycle in *Populus* trees

In Paper I we characterise the three *Populus FLOWERING LOCUS T* (*FT*) paralogues and describe their function in the regulation of vegetative growth.

Populus FT1 and *FT2* were identified many years ago (Böhlenius et al., 2006; Hsu et al., 2006) and are the result of a whole-genome duplication event occurring in the salicoid clade of the *Salicaceae* family (Tuskan et al., 2006). In addition, recently, an *FT2* duplication was described in *P. tremula* (Wang et al., 2018). The previously characterised *FT2* gene corresponds to *FT2a*, while the duplicated gene is referred to as *FT2b* (André et al., 2022). The two *FT2* paralogues are located in a 500 kb introgression, which is strongly associated to SNPs involved in local adaptation (Rendón-Anaya et al., 2021). Both *FT2a* and *FT2b* are present in several *Populus* species such as *P. tremuloides* and *P. trichocarpa,* even though in the latter, *FT2b* is truncated (Figure 1A and S1A), raising the question about its function in *P. tremula*.

First, we analysed the expression of all three *FT* genes both in indoor-grown and field-grown trees. *FT2a* and *FT2b* have a similar expression pattern, which peaks at the end of the day in leaves (Figure 1B-D). Moreover, in the samples collected in growth chambers, *FT2b* is expressed at higher levels compared to *FT2a*. Due to the similarity in expression patterns, it might be that the two paralogues share common regulatory elements. *FT1* instead, is expressed in buds in winter (Figure 1C-D). *In situ* hybridisation showed that the expression is specifically located to vasculature tissues and embryonic leaves (Figure S1D).

Like for *AtFT*, overexpression of *FT1* and *FT2a* leads to early flowering (Böhlenius et al., 2006). We reported a similar phenotype for *FT2b* overexpressing lines (Figure 1SC). Because of the high similarity of the *FT* sequences, the individual downregulation of these genes by RNAi failed in the past. In this paper, we generated knock-out mutants employing CRISPR/Cas9, which made it possible to specifically target the individual

paralogues without off-targets mutations. *FT2a* mutants had no differences in growth, or the timing of growth cessation compared to the wild type (Figure S2C). On the other hand, *FT2b* knockouts set bud after 8 weeks in growth-promoting LD conditions (Figure S2D). The double mutants of *FT2a* and *FT2b* displayed a more severe phenotype with dwarf plants, setting buds soon after potting (Figure 2A). Despite the strong alterations in growth cessation and bud set, *FT2* double mutants flushed buds after cold treatment at the same time as wild type plants; however, they set bud again shortly afterwards (Figure 2D). The mutant phenotypes demonstrate that both *FT2a* and *FT2b* are important in the control of growth cessation and bud set, working synergistically.
Regarding *FT1* knock outs, plants were able to grow and did not show any difference in response to SD compared to WT. After cold treatment though, trees were unable to flush their buds (Figure 2A-D). To confirm that the phenotype was due to *FT1* downregulation, we performed a viability staining test which proved that the closed buds were alive and metabolically active (Figure S4A). Thus, we demonstrated that *FT1* is crucial to resume growth after winter.
In arabidopsis, *FT* is expressed in the leaves and the protein is later transported to the shoot apical meristem where it performs its function (Jaeger & Wigge, 2007). We confirmed through grafting experiments the mobility of FT2 proteins, as already reported in Miskolczi et al. (2019). In fact, when grafting *ft2* shoots on a WT rootstock, growth is resumed (Figure S3A). In contrast, in *ft1* grafts on wildtype plants, buds do not flush (Figure S3B). These results suggest that *FT1* is expressed locally in the buds where it fulfils its role.
The sole study of *FT1* mutant phenotypes is insufficient to determine its specific function in either dormancy release, bud flush or both since dormancy release is a prerequisite for bud flush. However, *ft1* trees were able to flush buds and resume growth when transferred back to LD conditions before dormancy was established (Figure S4B), which suggests that *FT1* is required for dormancy release rather than bud flush *per se*. Since dormancy release occurs in trees during cold with no apparent anatomical differences, we designed an RNA sequencing experiment collecting buds at different stages of cold treatment for *ft1* and WT trees. After 4 weeks of cold exposure, the transcriptome of both mutants and WT underwent remarkable changes. However, after 8 weeks, the *ft1* transcriptome displayed a similar profile

without significant changes from the previous timepoint, while WT plants showed major transcriptional rearrangements (Figure 4A-B). These transcriptomic changes coincide with dormancy release and therefore reinforces the hypothesis that *FT1* is involved in dormancy release. A very large number of genes were differentially expressed between *ft1* and WT at 8 weeks of cold treatment (Figure 3C), indicating that *FT1* is important in the release of the dormant state through a general chromatin remodelling which makes gene accessible for subsequent inductive signals.

A deeper investigation allowed us to identify enrichment of genes associated with the gibberellin metabolic pathway and reception (Figure 3D), and in particular *GIBBERELLIN-INSENSITIVE DWARF* (*GID*) genes, that act as GA receptors (Nakajima et al., 2006). These genes are downregulated in *ft1* mutants compared to WT, supporting a possible role of gibberellins in dormancy release. A similar pattern of downregulation was observed for *PICKLE* (*PKL*), an antagonist of the polycomb repression complex 2. *PKL* repression has been reported to mediate ABA-induced plasmodesmata closure through deposition of callose plugs to establish dormancy in *Populus* trees (Tylewicz et al., 2018).

To assess if *FT1* has any role in the removal of callose plugs from plasmodesmata concomitantly with dormancy release, we collected apical bud samples 12 weeks after short-day induced induction of dormancy as well as after cold treatment to release dormancy from both *ft1* and WT trees.

In both genotypes, electron-dense plasmodesmata callose plugs were clearly visible after the SD treatment (Figure 4A, C). After cold treatment, WT trees, contrary to *ft1* mutants, had released dormancy, nevertheless no callose plugs were detected in neither WT nor *ft1* (Figure 4B, D). These findings indicate that *FT1* has no role in the removal of dormancy sphincters, and it assumedly acts downstream or in parallel to this process. They also show that the degradation of the callose plugs is not sufficient to release dormancy. It is still unclear to what extent dormancy release is dependent on the callose plugs removal.

Taken together, these data show that *FT* paralogues have evolved to cover different roles in the control of the annual grow cycle of *Populus* trees, being expressed at different times and in different tissues. Both *FT2* genes are involved in the regulation of SD-induced growth cessation, while *FT1* is required for dormancy release. Both *FT1* and *FT2* can interact with FD-like proteins to induce downstream targets like *LAP1* to promote growth

(Tylewicz et al., 2015). It might then be speculated that *FT* paralogues in *Populus* act on the same downstream targets through similar pathways. A deeper understanding of direct targets of the *FT* genes is required to better comprehend the mechanisms for growth cessation, dormancy release and bud flush.

4.2 Paper II

The interplay between *FT* and *TFL1*-like genes in the control of phenology and flowering time in *Populus* trees.

In Paper II we investigate the function of *CENL* genes in the regulation of growth and flowering time and their interplay with the *FT* paralogues. *CENL* are the orthologous genes of arabidopsis *TFL1*, which is responsible for maintaining the inflorescence meristem identity (Bradley et al., 1997) and acts as a repressor of flowering (Shannon & Meeks-Wagner, 1991). As for other members of the PEBP family, the *CENL* paralogues are the result of a whole genome duplication in the salicoid clade of the *Salicaceae* family (Tuskan et al., 2006). Two genes, *CENL1* and *CENL2* have been characterised in several *Populus* species (Mohamed et al., 2010; Ruonala et al., 2008). CENL1 and CENL2 amino acid sequences are highly conserved, they differ for only one amino acid in length and share up to 90% similarity (Figure 1B). Despite the conservation of sequence identity, phylogenetic analyses highlight the specific clustering patterns between aspens and poplars (Figure 1A). Species belonging to the *Populus* section of the *Populus* genus, such as *P. tremula*, *P. alba* and *P. tremuloides* group closely together, while *P. nigra*, *P. trichocarpa* and *P. deltoides* form another cluster and belong to the section *Tacamahaca* (Wang et al., 2022). *P. euphratica* is the most distantly related species and is part of the section *Turanga*. The *Populus* species belonging to the different sections have a wide habitat distribution covering all the Northern Hemisphere, thus, the conservation of CENL proteins reflects the evolutionary distances within the genus more than environmental adaptations.
Even though *CENL1* and *CENL2* share similar protein sequences, they are expressed at very different levels and at different times in buds. Therefore, it might be speculated that what determines *CENL* function is the regulation at the transcriptional level. *CENL1* is expressed for a short time window in the apical meristem of newly formed shoots after bud break, while *CENL2* expression peaks before the opening of the buds (Figure 2A, C). *In situ* hybridization specifically localises *CENL1* transcript to the upper part of the rib meristem with a bell-shaped expression domain (Figure 2D). In addition to being expressed in buds, both *CENL1* and *CENL2* expression is detected in other tissues as well. *CENL1* is expressed in the inflorescence, the petiole and in the roots (Figure 2E). Roots is also the tissue where *CENL2* is

expressed the highest (Figure 2E). Expression of other *TFL1* homologues from different species such as tomato, maize, rice and apple, have been detected in the root systems as well (Carmel-Goren et al., 2003; Danilevskaya et al., 2010; Mimida et al., 2009; Nakagawa et al., 2002). *CENL1* and *CENL2* expression in multiple organs might reflect a specific functional specialization of *CENL* genes in growth and development that need to be elucidated.

The generation of single mutants for *CENL1* and *CENL2* using CRISPR/Cas9 allowed us to investigate their role in aspen (Figure S1). *cenl1* knock out mutants display early flowering in tissue culture; plants remain dwarf due to the transition of the apical and the axillary meristems into inflorescence-like structures, characterised by a cluster of stamens and small rounded leaves (Figure 3A). On the other hand, *cenl2* mutants display normal vegetative growth (Figure 3B) suggesting that *CENL2* has no major role in controlling the flowering transition. The phenotype of *cenl1* plants resembles that of *tfl1* mutants in arabidopsis (Shannon & Meeks-Wagner, 1991) indicating that *CENL1* in *Populus* has a conserved function as a repressor of flowering. Because *CENL1* and *TFL1* are both expressed in the rib meristem (RM) (Figure 2D), it might be speculated that *CENL1* has retained the *TFL1* mode of action, where it moves from the RM into the SAM to specify indeterminacy (Conti & Bradley, 2007; Goretti et al., 2020). Despite the apparent conservation of function there are also important differences between *TFL1* and *CENL1*. In arabidopsis, *TFL1* is strongly induced in the SAM after floral induction (Baumann et al., 2015) and its mutant flowers only a few days earlier than wild type (Alvarez et al., 1992; Shannon & Meeks-Wagner, 1991). Therefore, *TFL1* has a minor role in the regulation of flowering time compared to *CENL1* in *Populus*, which when mutated instead leads to floral induction after a few weeks (Figure 3A) (Sheng et al., 2023), instead of the naturally occurring flowering after 15-20 years.

While *CENL1* represses flowering, *FT*-like genes in *Populus* have conserved the flowering promotion ability of *AtFT*. Overexpression of any of the *FT* paralogues in transgenic *Populus* trees leads to precocious flowering (André et al., 2022; Böhlenius et al., 2006; Hsu et al., 2006). The early flowering phenotype of *cenl1* plants made us wonder to what extent the *FT1* or *FT2* genes are important for this precocious floral induction. FT1 has previously been suggested to regulate flowering, while *FT2* would control vegetative growth (Hsu et al., 2011; Rinne et al., 2011).However, recently *FT2* was

proposed to be the gene responsible for flowering (Sheng et al., 2023). To investigate the interplay between *CENL1* and *FT*-like genes, we generated double and triple knockouts in *ft1* and *ft2a ft2b* mutant backgrounds. While *cenl1 ft1* mutants retained the early flowering phenotype (Figure 3C), *cenl1 ft2a ft2b* mutants did not display any floral formation and were able to grow normally (Figure 3D). These results suggest that *FT2* is responsible for controlling flowering in time. However, after exposure to cold and the subsequent dormancy release, *cenl1 ft2a ft2b* lines were able to produce flowers (Figure 4E). So, natural expression of *FT2* in the leaves is sufficient to induce early flowering, when *CENL1* is knocked out, during the first growing season. However, when *cenl1 ft2a ft2b* plants follow a regular growth cycle going through bud set and dormancy, followed by bud flush after cold exposure, they flower during the second growing season. Although the induction of flowering in these mutant lines occurs very early compared to wild type trees, the fact that *cenl1 ft2a ft2b* can complete the growth cycle, mimicking natural conditions, seems more physiologically relevant. Therefore, the hypothesis of *FT2* being the main promoter of flowering needs to be redefined, as our results suggest that it is more likely to be *FT1* expression in buds which triggers floral induction after a period of dormancy, as previously suggested by Hsu et al (2011). Supporting this hypothesis, we showed that *FT1* is upregulated in unopened buds both during cold and the first week of LD, during which the determination of floral meristems might occur (Figure 4E, S1C). At the same timepoints we also detected upregulation of *LAP1* and *LFY* (Figure 4E)*,* orthologues of the arabidopsis floral meristem identity genes *AP1* and *LFY,* whose mutants do not form flower meristems and develop shoots instead (Mandel et al., 1992; Weigel et al., 1992). *LAP1* and *LFY* are then good candidates for early stages of floral initiation.

Altogether, these data support the idea that it is the balance between *CENL1* and *FT1* expression that is of importance for determining flowering time in *Populus* trees. Indeed, down-regulation of CENL1 expression is sufficient to trigger flowering. However, formally, we can still not definitely conclude that the *FT*-like genes have a role in *Populus* flowering under natural conditions.

Unfortunately, it will be difficult to answer if *FT1* is required for normal flowering since *ftl1* plants fail to release dormancy and flush their buds

(André et al., 2022). A better understanding of FT1 downstream targets prior to floral induction might shed light on this unsolved question.
Likewise, the strong flowering phenotype of *cenl1* and *cenl1 ft1* mutants obscures any possible effect of *CENL1* in the regulation of seasonal growth. However, it is clear that CENL1 has a role in the regulation of growth cessation and bud set in the *cenl1 ft2a ft2b* lines which are able to sustain vegetative growth and can set bud. Contrary to wild type, they do set bud in LD conditions after 8 weeks of growth (Figure 3E), but this is a much milder phenotype than that of *ft2* double mutants that are dwarf plants which set bud already in *in vitro* culture (André et al., 2022). The role of *CENL1* as a repressor of vegetative growth is confirmed by the premature bud set phenotype of overexpressing lines (Figure 3F). The same phenotype was observed for overexpressing lines of *CENL2*, while *cenl2* mutants showed a slightly delayed bud set (Figure 3F), suggesting a minor redundant role of *CENL2* in repressing vegetative growth. As already observed by Mohamed et al. (2010), when CENL1 and CENL2 are overexpressed, trees delay bud flush (Figure 4C, D); while we show here that *cenl2* and *cenl1 ft2a ft2b* lines flushed earlier than WT (Figure 4A-D).
Since the only visible result of dormancy release is represented by bud flush, it is rather complex to uncouple the two processes and define the specific role of *CENL1*. However, it can be speculated that *CENL1* downregulation and the concomitant *FT1* induction triggers dormancy release. When this balance is disrupted, as in *CENL* overexpressing lines, CENL might compete for the same targets as FT1, such as *LAP1* (Azeez et al., 2014), and dormancy release is slowed down resulting in delayed bud flush.
A conserved downstream pathway might be conserved in summer as well, when CENL1 competes with FT2 in regulating vegetative growth in the rib meristem (RM) where CENL1 is expressed (Ruonala et al., 2008). Shoot elongation is regulated in the RM (Sachs, 1965), to where FT2 is transported to regulate the GA metabolism (André et al., 2022; Corbesier et al., 2007; Gómez-Soto et al., 2021). While CENL1, at first, competes for the same targets with FT2 repressing growth, it is later transported to the SAM to maintain meristem indeterminacy.

FT and CENL proteins might share conserved target genes, and in the same way compete for the same co-factors. In arabidopsis, both FT and TFL1 interact with the transcription factor FD through 14-3-3 proteins (Abe et al.,

2005; Collani et al., 2019; Goretti et al., 2020; Hanano & Goto, 2011). Previous studies have shown the interaction of FT with two FDL proteins in poplar as well. However, more recently three *FDL* genes have been described (Parmentier-Line & Coleman, 2016; Sheng et al., 2022). In this paper, we identified three *FDL* paralogues in *P. tremula* which are highly conserved with *FDL* homologues of *P. deltoides* (Figure S3A-B). All protein motifs are conserved among the two species and arabidopsis FD and FDP, specifically the SAP motif which is responsible for FD interaction with 14-3-3 proteins (Taoka et al., 2011). *FDL* genes in *Populus*, as for the *FT* and the *CENL* paralogues, have differentiated and display different expression profiles (Sheng et al., 2022). Our RNA-Seq data of samples collected from outdoor and indoor grown aspens indicates that all three *FDL* genes are highly expressed during the entire growth cycle of trees, despite showing distinct seasonal expression profiles (Figure 5) (Paper IV). Since the *FDL* genes are expressed in different tissues at different time, it can be speculated that FDL proteins interact with different co-factors. I performed yeast two hybrid assays to test the interaction between FDL and the FT-like proteins, FT1, FT2a and FT2b, as well as the TFL1-like proteins, CENL1 and CENL2. Each FDL paralogue displayed specific interaction profiles with both the FT and the CENL proteins (Figure 6, S4). FDL1 strongly interacted with both FT2 proteins. All three genes are expressed in leaves during summer and *FDL1* is also expressed in apical buds (Figure 5), where the protein might interact with FT2 after being transported to the SAM. Additionally, it has been previously shown that *FDL1* overexpressing lines, similarly to *FT2* overexpressing plants, display a delayed SD-induced growth cessation response (Böhlenius et al., 2006; Tylewicz et al., 2015). Because of their co-expression in leaves it cannot be excluded that FT2 and FDL1 interact in this tissue as well. In arabidopsis, both *FT* and *FD* are expressed in the guard cells which suggest a role of the FT/FD complex in the regulation of stomatal opening (Kinoshita et al., 2011). When overexpressed, *FDL2* triggers flowering already in tissue culture (Sheng et al., 2022; Tylewicz et al., 2015). Our results show how FDL2 interacts with both FT2b and CENL1 (Figure 6), which in this paper have been shown to be crucial in the regulation of flowering. Even though *FDL2* and *CENL1* expression peaks do not coincide, *FDL2* expression levels are still considerably high throughout all year (Figure 2A, 5) allowing potential protein interaction.

The specific interaction with FT2b but not FT2a could be explained by amino acid variations among the two FT paralogues (Figure S3C), that despite the high similarity might cause a difference in the protein conformations. At last, FDL3 interacts with both CENL proteins and partially with FT1. FDL3 has been previously suggested to be involved in the promotion of apical growth (Sheng et al., 2022). However, its interaction with CENL proteins might indicate the formation of a complex with repressing function. While CENL1 interacts to different extent with all FD paralogues, CENL2 only shows a strong interaction for FDL3 (Figure 6). Moreover, co-expression of *FDL3* and *CENL2* in April (Figure 2A, 5), might suggest that both genes are involved in the regulation of bud flush.

Our results highlight functional divergence among FD paralogues in relation to seasonal growth and flowering and provide insights into the specific interactions between FDL proteins, FT, and CENL in *P. tremula*. However, the domains responsible for this specificity remain to be identified.

4.3 Paper III

Populus SVL **Acts in Leaves to Modulate the Timing of Growth Cessation and Bud Set.**

In paper III, we describe the role of *SVL* in the regulation of SD-induced growth cessation and bud set. Poplar *SVL* is the homologue of *AtSVP* and is related to the *DORMANCY ASSOCIATED MADS* (*DAM*) genes described in peach (Bielenberg et al., 2008) (Figure S1A). In arabidopsis, SVP is a repressor of flowering involved in the downregulation of *FT* and *svp* mutants show an early flowering phenotype (Lee et al., 2007). When overexpressing *SVL* in arabidopsis *svp* mutants, the mutant phenotype is rescued, leading to later flowering in both the mutant and WT background (Figure S2). This suggests that the function of *AtSVP* and *SVL* is conserved.

SVL has previously been shown to be expressed in buds after exposure to SD, leading to dormancy establishment (Singh et al., 2019). In this paper we focus on the *SVL* expression in leaves. In outdoor trees, *SVL* expression peaks at the end of summer and early autumn (Figure 1A), which, when trees are grown in controlled SD conditions, corresponds to a morning peak of expression (Figure 1B). In addition, we demonstrated that protein levels accumulate in response to both SD and lower temperatures (Figure 1C, D), indicating an integration of the photoperiod and ambient temperature pathways.

To study the role of *SVL* in growth cessation, we generated both *SVL* overexpressing and RNAi lines (Figure S4). All transgenic lines grew similarly to WT in LD conditions (Figure 2A), but when transferred to SD, SVL RNAi lines displayed a subtle delay in growth cessation (Figure 2B). In contrast, *SVL* overexpressing trees set bud significantly earlier than WT (Figure 2C). The phenotype of the transgenic lines demonstrates that *SVL* is a repressor of vegetative growth and promotes SD-induced growth cessation and bud set in *Populus* trees. Since *SVL* is expressed in both the shoot apex and in leaves, we tested through grafting experiments in which organ *SVL* fulfil its function. We compared the timing of growth cessation and bud set between reciprocal graftings of *SVL* RNAi and WT, with shoots grafted to their own rootstock. Independently of in which part of the tree (scion or stock) *SVL* was downregulated, the plants displayed the same phenotype as in *SVL* RNAi homografts (Figure 2D). These results suggest that *SVL* acts both in the shoot apex and in leaves to regulate growth cessation and bud set.

We then focused on the downstream targets of *SVL*, to investigate its mode of action. In arabidopsis, *FT* is a direct target of SVP (Li et al., 2008) and in poplar, *FT2* has a major role in the control of growth cessation in leaves (André et al., 2022). *FT2* is expressed in leaves during the growing season, but toward the end of summer the expression level drops (Figure S3). It can be speculated that *SVL* induction at the end of summer represses *FT2* expression in leaves to induce growth cessation. After two weeks in SD, there was no detectable expression of *FT2* in WT and *SVL* overexpressing trees, while it was highly expressed in *SVL* RNAi lines (Figure 3A). Additionally, *LAP1*, a downstream target of *FT2*, was also upregulated in *SVL* RNAi lines (Figure S5B).
It is known that in arabidopsis, SVP, in addition to acting on *FT*, affects the gibberellin metabolic pathway (Andrés et al., 2014), and so is SVL in poplar (Singh et al., 2019). We, therefore examined the expression profile of a key biosynthetic gene in the gibberellin metabolism, *GA20 oxidase2* (*GA20ox2*). *GA20ox2* expression was downregulated in *SVL* overexpressing lines and upregulated in *SVL* RNAi lines (Figure 3B). Because many factors modulate the gibberellin metabolism, we measured the levels of active GA in leaves of both *SVL* RNAi and WT trees, to make sure that no feedback regulation restored the gibberellin levels. However, GA_1 was higher in *SVL* RNAi leaves compared to WT both in LD and SD (Figure S5). To understand if SVL, being a transcription factor, acts directly on *FT2* and *GA20ox2*, we performed chromatin immunoprecipitation (ChIP) assays of SVL fused to a Myc tag.
Six enrichment regions were identified by qPCR upstream and downstream of the *FT2* transcriptional start site (Figure 3C), while four fragments were found at the *GA20 oxidase2* promoter (Figure 3D). ChIP experiments from shoot apex samples did not detect *GA20ox2* as a target of SVL (Singh et al., 2019); suggesting that MADS-box proteins and co-transcription factors acting in complex with SVP-like proteins differ between tissues affecting SVL binding.
Taken together, these results show that SVL affects the timing of growth cessation and bud set through direct repression of *FT2* and the gibberellin biosynthetic gene, *GA20ox2.*
To get a wider overview of the SVL targets in leaves during SD conditions, we performed RNA sequencing of both *SVL* RNAi lines and WT. Samples were collected in LD and after one, two, three and ten weeks in SD. Upon

the shift from LD to SD, transcriptomes of both WT and transgenic lines changed dramatically with more than 12,000 differentially expressed genes (DEGs) (Figure 4A, S6). However, after the first three weeks of SD no significant transcriptome rearrangements occurred (Figure 4A). This is reflected as well in the low number of DEGs between RNAi lines and WT for each timepoint in SD (Figure 4B) and the gene ontology analysis which did not detect the enrichment of any specific terms.
The minor influence of *SVL* on the leaf transcriptome might indicate that its role is limited to a restricted set of genes.
In conclusion, *SVL* has a dual role as promoter of SD-induced growth cessation in leaves by repressing *FT2* and GA biosynthesis and as an inducer of dormancy establishment in the shoot apex by repressing *FT1* and GAs (Singh et al., 2019).

4.4 Paper IV

A transcriptional roadmap of the yearly growth cycle in *Populus* trees

In paper IV we provide a transcriptional atlas of the annual growth cycle of aspen. We performed RNA sequencing on 207 samples collected from outdoor grown trees and from trees grown indoors in controlled conditions. Outdoor samples were collected monthly from mature and juvenile *P. tremula* trees growing in Umeå, Sweden (63.8°N). Indoor samples were harvested from young plants under different controlled conditions which mimic seasonal changes in day-length and temperature (Figure 1A). This created a complete transcriptional roadmap, allowing us to investigate the molecular responses which regulate essential developmental processes during the growth cycle in trees.

First, we analysed the overall dataset variation by principal component analysis (PCA). The first component, explaining 43% of the variation, cluster the samples based on the environmental conditions, while the second, explaining the 20%, cluster the samples based on the tissue type (Figure 1C). In most cases outdoor and indoor samples display a similar seasonal distribution, with the indoor settings of light and temperature matching the outdoor environmental conditions. However, outdoor samples collected in winter cluster poorly with samples from indoor conditions, maybe because of the more extreme temperatures to which outdoor trees are subjected during that period. Instead, indoor samples harvested during cold treatment cluster with samples from March. Also, the samples from September and October do not group with any indoor samples; in fact, samples collected in SD light conditions (SDW15 and CTW2) cluster with samples collected in April. In April, the temperature resembles what trees experience during cold treatment (4°C), while the natural daylength is closer to the 14 hours of light used in SD. The latter stages of bud bread (BBW2 and BBW3) cluster with samples from May when bud break and initial leaf expansion occur. In turn, May samples group in between bud and leaf samples collected under LD conditions. LD leaves cluster separately from outdoor leaf samples, indicating that the tissue developmental stage have a larger effect on the transcriptome than the environmental conditions under which the samples were collected. Leaves collected in June, July and August cluster together, although displaying a gradual transition which reflects the transcriptome

rearrangements of the shift from leaf expansion to growth cessation at the end of summer. Accordingly, the indoor leaves collected in the first two weeks of SD (SDW1 and SDW2), which are still in a stage of active growth, group with June; while SDW3 and SDW10 samples cluster with July and August.

To identify seasonal patterns of gene expression, we performed co-expression analysis on the entire dataset. The analysis generated 46 modules (Figure 2A), of which 36 show a seasonal profile. The remaining ten, are defined by either age or location. Of the 36 modules with a seasonal profile, we identified 12 of them which together cover the entire year (Figure 2B). Genes in these modules have a time-restricted expression, which is high in outdoor and indoor samples in similar conditions. For these 12 modules we performed Gene Ontology (GO) and gene network analysis, highlighting the actors involved in some crucial developmental changes during the growth cycle of aspen trees.

As daylength shortens and temperatures drop, trees begin preparing to endure the approaching winter, protecting their tissues from frost damage. In addition to phenological changes such as bud formation and leaf senescence, trees implement several molecular changes to modify both the cell wall and membrane composition. Module A1 and A2 includes genes expressed during autumn (September and October) and in SD and beginning of CT. GO analysis produced terms such as: "cell wall polysaccharide metabolic process", "membrane lipid metabolic process" and "cutin biosynthetic process" (Figure 4A). Among the genes associated to the most enriched GO terms, displayed in a gene network, we identified genes that are part of the biosynthetic pathway of suberin, a lipophilic biopolymer composed of long-chain fatty acids and glycerol (Figure 4B); esterases/lipases which are involved in the polymerization of the cuticle (Shen et al., 2022) and lipid transfer proteins, which facilitate the movement of lipids between membranes and to the cuticle (Debono et al., 2009). Both suberin and cutin form a protective barrier increasing cell wall thickness and hydrophobicity.

Module W1 and W2 include genes expressed during winter and cold treatment. In the extreme temperature conditions and the few hours of light to which trees are exposed, they enter a dormant state during which they focus on coping with the harsh environment and the scares resources. For these modules we identified GO terms referring to response to cold and abscisic acid, a hormone involved in abiotic stress responses but also in

dormancy (Yordanov et al., 2014). The genes belonging to these modules are enriched for AP2-EREBP (ethylene-responsive element binding proteins) transcription factors, including C-repeat/DREB binding factors 2 and 3 (*CBFs*) (Figure 4D), which regulate the expression of cold-regulated (*COR*) genes (Shi et al., 2018). In the same network we identified *EBB1* and *FT1*, both known to regulate dormancy release (André et al., 2022; Yordanov et al., 2014), and *FD*-like genes, which can interact with *FT*-like genes (Tylewicz et al., 2018). As neighbour of *FD*-like genes we identified *PKL*, which has been proven to mediate ABA-induced plasmodesmata closure through callose deposition (Singh et al., 2017). Accordingly, in module W1 we found the GO term “callose deposition in cell wall”.
When temperatures raise at the beginning of spring, environmental molecular responses trigger expression of genes activating transcription and translation. Modules SP1 and SP2 include genes expressed under these warmer conditions and associated to RNA processing and ribosome biogenesis (Figure 5A). Many genes in the modules are part of the WD40-like family and regulate several functions, from signal transduction and transcription regulation to cell cycle control (Van Nocker & Ludwig, 2003). Other genes in the joined SP1 and SP2 network, are putative transcription termination factors and genes involved in the initiation of translation.
In late spring, buds eventually flush, and the newly formed shoots start elongating. Genes clustering in module SP3 and SP4 are expressed in May and at different stages of bud flush in LD indoor samples. During these stages tissues undergo major temporal and spatial changes, defined by a strong mitotic activity (Kwiatkowska, 2008). We identified GO terms such as “SAM development” and “cell fate specification” (Figure 5C). The generated network showcases the cell cycle regulator *AtCYCD3* and the meristem identity gene *AtWOX4* as crucial players of the developmental processes defined in these two modules (Figure 5D). In addition, we identified *CENL1*, a homologue of *AtTFL1*. In arabidopsis, *TFL1* determines meristem identity and plays an antagonistic role to *FT/TSF* in the regulation of flowering time (Conti & Bradley, 2007). As neighbours of *CENL1* we identified *AtCLF,* involved in cell fate determination (Zhang et al., 2022), as well as *AtLOF1* and *AtLOF2* that are expressed in organ boundaries (Lee et al., 2009).
In summer, trees experience a period of vegetative growth, which is reflected by the induction of many molecular pathways. Module S1 and S2 include

genes whose expression peaks in flushed buds and LD leaves. GO terms enriched in these modules describe an active tree where photosynthetic processes and the production of carbohydrates are predominant (Figure 6A). Many members of the *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL)* family are present in module S1 in the network (Figure 6B). *SPL3a*, *SPL5a* and *SPL5b* have been described in regulation of vegetative growth, acting in the promotion of growth cessation (Liao et al., 2023). In module S2 we identified instead members of the *PIF* family such as *PIF1*, *PIF8a*, *PIF8b* and *PIF4*. Both *PIF4* and *PIF8* regulate shoot elongation. However, only PIF8 has a major role in regulating seasonal growth (Ding et al., 2021). PIFs contain a phytochrome binding (APB) domain to mediate interaction with PhyB (Pham et al., 2018). Accordingly, in the module we also found *PhyB2*, a suppressor of SD-induced growth cessation (Ding et al., 2021).

At the end of summer, SD conditions trigger growth cessation and leaf senescence. Module S3 and S4 include genes expressed in leaves collected in August and at the end of SD. GO terms such as "leaf senescence" and "positive regulation of proteolysis" are enriched in these modules (Figure 6C). Senescence-associated proteolysis is crucial for mobilisation of nutrients from old or stressed tissues to sink organs (Diaz-Mendoza et al., 2016). Centrally located in the network, we identified *CDF3* which is involved in the stress tolerance response (Corrales et al., 2017). *CDF3* overexpressing lines in poplar display instead an early growth cessation phenotype (Ding et al., 2018). First neighbour of *CDF3* is the homologue of *AtWRKY47*, which in arabidopsis controls leaf senescence through the regulation of programmed cell death (PCD)-associated genes (Cui et al., 2024). Other genes in the network are also associated to PCD.

The modules generated with co-expression analysis provide a transcriptional roadmap of the annual growth cycle of aspen and allowed us to identify hubs of genes related to seasonal phenological and developmental changes. To make the data easily accessible we developed POPUL-R, an app which allows users to visualize gene expression data and create interactive networks.

Because the dataset includes both samples collected outdoors and in growth chambers, we wondered how accurately the controlled conditions replicate the natural environment and what are the transcriptomic differences between indoor- and outdoor-grown trees. The PCA analysis showed a considerable

similarity of the profiles of outdoor and indoor samples; however, some of the samples collected in controlled conditions do not match the outdoor samples as expected. To investigate the differences in these conditions we performed differential gene expression analysis and GO enrichment for each of the indoor settings: LD, SD and CT.

In the first contrast we compared LD leaves and outdoor leaves collected in summer. In the PCA, leaves harvested indoor cluster closer to May samples, even though the hours of light set in the growth chambers in LD (18 h) match approximately the natural daylength of the summer months. This is probably due to the developmental stage of the tissue which is young and possibly more similar to the emerging leaves of May than leaves in June. GO terms associated to upregulated genes in indoor leaves indicate an intense mitotic activity, “cell development” and “plant organ morphogenesis” (Figure 8A). Among the most highly differentiated genes we identified cyclins and cyclin interacting proteins and several members of the actin family. In contrast, GO terms associated to outdoor trees are related to the jasmonic (JA) and salicylic acid (SA) pathways in response to both abiotic and biotic stress. Additionally, we identified GO terms such as “Response to UV-B”, “response to far-red light” and other terms related to light stimulation. Previous studies investigated the role of JA in response to UV radiation (X. Liu et al., 2012). These terms underline the differences in the light spectrum between natural light and the lamps used in controlled settings.

We then compared buds collected at the end of SD and beginning of CT, with autumnal buds from September and October. The indoor buds collected under these conditions group in the PCA with samples collected in April, during which the temperature is closer to what trees experience in CT (4°C), and the natural daylength is similar to the 14 hours of light used in SD. During autumn instead, daylength varies from 11 to 9 hours of light. Like for the previous contrast, the GO analysis shows that in SD indoor trees grow more actively than the outdoor ones. We identified terms like: “shoot meristem development”, “plant organ formation” and “cellular response to brassinosteroids” (BRs). BRs are plant hormones involved in development and growth (Bishop & Yokota, 2001). Upregulated genes associated to their response are members of the serine/threonine kinase family. GO terms associated to highly differentiated genes in outdoor trees are related to osmotic and oxidative stress. Compared to indoor plants, which are watered regularly, trees growing outdoor experience water shortage triggering a

drought stress response which involves osmotic and oxidative pathways. DE genes are peroxidases and a superoxide dismutase.
The last contrast is performed between samples collected at the end of cold treatment and winter samples. The extreme environment that trees experience in the North, makes the transcriptome profiles of winter and CT samples very different. In indoor samples, we identified GO terms related to photosynthesis and its regulation and production of flavonoids. Flavonoids have antioxidant properties which protect plants against high light exposure (Ferreyra et al., 2021). Among the most upregulated genes, we identified members of their biosynthetic pathways. This suggest that trees in growth chambers experience light stress. The highly differentiated genes in outdoor trees are involved in the response to cold and many are AP2-EREBP transcription factors such as the *CBF* genes. Other DE genes are U-box domain containing proteins and E3 enzyme involved in ubiquitination. These genes are associated to GO terms such as: “apoptotic process”, “cell death” and “protein ubiquitination”. Moreover, we identified terms related to the catabolic pathways of both carbohydrates and proteins, whose degradation allows trees to exploit the stored resources to survive winter.
To summarise, the main differences between indoor and outdoor trees are related to milder conditions and higher availability of nutrients in growth chambers, while in nature trees are exposed to more adverse climatic conditions.

5. Conclusions

During my PhD, I have investigated the molecular mechanisms regulating flowering time and the annual growth cycle of *Populus* trees, focusing on the genetic regulation of *FLOWERING LOCUS T* (*FT*) and *TERMINAL FLOWER 1* (*TFL1*)-like genes.
Three *FT*-like genes have been identified and have functionally diverged to regulate different phases of the growth cycle (Paper I). Two *FT2* paralogues, *FT2a* and *FT2b*, are expressed in leaves during summer and act as promoters of growth in long day conditions. In fact, *ft2a ft2b* double mutants, remain dwarf because the plants enter growth cessation and set bud already during *in vitro* culture. *FT2* downregulation naturally occurs at the end of summer and beginning of autumn when the hours of light reach below the critical day length. In paper III, we show the direct repression of *FT2* by *SHORT VEGETATIVE PHASE-LIKE* (*SVL*). *SVL* is induced in the leaves in SD and in addition to *FT2*, it also represses the biosynthesis of gibberellins, well known to promote growth. While photoperiod is the main environmental cue regulating growth cessation, we demonstrated that SVL protein is more stable at cold temperatures, indicating an integration of the photoperiod and ambient temperature pathways.
The last of the *FT*-like genes in aspen, *FT1*, is instead expressed in winter in buds (Paper I). Screening of *ft1* mutants and transcriptomic data demonstrate the crucial role of *FT1* in dormancy release and bud flush. *ft1* mutants, contrary to *ft2* mutants, are able to grow and respond normally to SD-induced growth cessation. However, when the trees are moved back to LD condition after prolonged cold exposure, they do not flush their buds. Moreover, RNA-Seq data indicate major transcriptional rearrangements in wild type plants after 8 weeks of cold treatment, during dormancy release. In contrast, the transcriptome of *ft1* mutants only show minor changes during this time frame, suggesting that these mutants remain in a dormant state. Among the thousands of genes which were downregulated in *ft1* plants, we identified genes involved in gibberellin signalling, which suggest that *FT* paralogues in *Populus* act on the same downstream targets through similar pathways to directly or indirectly promote growth.
In paper II we describe two *TFL1*-like paralogues: *CENL1* and *CENL2* acting antagonistically to the *FT* genes. Both genes are mainly expressed in buds, but the expression of *CENL1* in considerably higher. *CENL1* is a strong floral

repressor as *cenl1* mutants display extreme precocious flowering from both the apical and all axillary meristems. Screening of double and triple mutants of *CENL1* and *FTs* demonstrated the essential interplay between these genes to ensure the proper control of flowering time. While *FT2* expression is enough to promote early flowering in *cenl1* plants, *FT1* expression during cold treatment induces flowering in a more physiological relevant phase of the growth cycle of trees, which suggest that it is the antagonistic expression of *CENL1* and *FT1* that controls floral induction. In addition, both *CENL1* and *CENL2* are involved in the regulation of bud set and bud flush acting as repressor of growth, even though *CENL2* seems to play a minor role.
Finally, in paper IV, we provide a complete transcriptional atlas of the annual growth cycle of aspen, performing RNA sequencing on samples collected from trees growing outdoors as well as from indoor grown trees grown under controlled conditions mimicking seasonal changes. This has allowed me to investigate the molecular responses which regulate essential developmental processes of the growth cycle in trees such as dormancy, bud break and growth cessation. Co-expression networks uncovered hubs of genes specifically expressed in determined stages of the annual growth cycle of trees. Among these, *FT* and *CENL* were identified and were strongly linked to key factors involved in either dormancy release or meristem identity. Moreover, while the controlled settings successfully mimic many aspects of natural conditions, some transcriptomic differences highlight the extreme conditions and the multiple environmental factors to which trees are exposed naturally and are difficult to replicate in indoor experiments. Because the transcriptomic atlas we generated is of valuable importance to enhance the understanding of seasonal regulation in perennial plants, we developed POPUL-R, an app which allows users to visualize gene expression data and create interactive networks.
Taken together, my work provides a better understanding of the intricate molecular mechanisms which trees employ to synchronize growth with seasonal environmental cues. The improved comprehension of this regulation has broad implications for both forest management and tree breeding, particularly in the context of climate change, where extreme and unpredictable environmental conditions may disrupt natural growth cycles.

References

Abe, M., Kaya, H., Watanabe-Taneda, A., Shibuta, M., Yamaguchi, A., Sakamoto, T., Kurata, T., Ausín, I., Araki, T., & Alonso-Blanco, C. (2015). FE, a phloem-specific Myb-related protein, promotes flowering through transcriptional activation of FLOWERING LOCUS T and FLOWERING LOCUS T INTERACTING PROTEIN 1. *The Plant Journal*, *83*(6), 1059-1068.

Abe, M., Kobayashi, Y., Yamamoto, S., Daimon, Y., Yamaguchi, A., Ikeda, Y., Ichinoki, H., Notaguchi, M., Goto, K., & Araki, T. (2005). FD, a bZIP protein mediating signals from the floral pathway integrator FT at the shoot apex. *science*, *309*(5737), 1052-1056.

Adams, S., Manfield, I., Stockley, P., & Carré, I. A. (2015). Revised morning loops of the Arabidopsis circadian clock based on analyses of direct regulatory interactions. *PLoS One*, *10*(12), e0143943.

Aguilar-Martínez, J. A., Poza-Carrion, C., & Cubas, P. (2007). Arabidopsis BRANCHED1 acts as an integrator of branching signals within axillary buds. *The Plant Cell*, *19*(2), 458-472.

Ahn, J. H., Miller, D., Winter, V. J., Banfield, M. J., Lee, J. H., Yoo, S. Y., Henz, S. R., Brady, R. L., & Weigel, D. (2006). A divergent external loop confers antagonistic activity on floral regulators FT and TFL1. *The EMBO journal*, *25*(3), 605-614.

Al-Sady, B., Ni, W., Kircher, S., Schäfer, E., & Quail, P. H. (2006). Photoactivated phytochrome induces rapid PIF3 phosphorylation prior to proteasome-mediated degradation. *Molecular cell*, *23*(3), 439-446.

Alabadí, D., Oyama, T., Yanovsky, M. J., Harmon, F. G., Más, P., & Kay, S. A. (2001). Reciprocal regulation between TOC1 and LHY/CCA1 within the Arabidopsis circadian clock. *science*, *293*(5531), 880-883.

Alvarez, J., Guli, C. L., Yu, X. H., & Smyth, D. R. (1992). terminal flower: a gene affecting inflorescence development in Arabidopsis thaliana. *The Plant Journal*, *2*(1), 103-116.

André, D., Marcon, A., Lee, K. C., Goretti, D., Zhang, B., Delhomme, N., Schmid, M., & Nilsson, O. (2022). FLOWERING LOCUS T paralogs control the annual growth cycle in Populus trees. *Current Biology*, *32*(13), 2988-2996.e2984. https://doi.org/10.1016/j.cub.2022.05.023

Andrés, F., & Coupland, G. (2012). The genetic basis of flowering responses to seasonal cues. *Nature Reviews Genetics*, *13*(9), 627-639.

Andrés, F., Porri, A., Torti, S., Mateos, J., Romera-Branchat, M., García-Martínez, J. L., Fornara, F., Gregis, V., Kater, M. M., & Coupland, G. (2014). SHORT VEGETATIVE PHASE reduces gibberellin biosynthesis at the Arabidopsis shoot apex to regulate the floral transition. *Proceedings of the National Academy of Sciences*, *111*(26), E2760-E2769.

Azeez, A., Miskolczi, P., Tylewicz, S., & Bhalerao, R. P. (2014). A tree ortholog of APETALA1 mediates photoperiodic control of seasonal growth. *Current Biology*, *24*(7), 717-724.

Bagnall, D. J., King, R. W., Whitelam, G. C., Boylan, M. T., Wagner, D., & Quail, P. H. (1995). Flowering responses to altered expression of phytochrome in mutants and transgenic lines of Arabidopsis thaliana (L.) Heynh. *Plant Physiology*, *108*(4), 1495-1503.

Balasubramanian, S., & Weigel, D. (2006). Temperature induced flowering in Arabidopsis thaliana. *Plant signaling & behavior*, *1*(5), 227-228.

Baumann, K., Venail, J., Berbel, A., Domenech, M. J., Money, T., Conti, L., Hanzawa, Y., Madueno, F., & Bradley, D. (2015). Changing the spatial pattern of TFL1 expression reveals its key role in the shoot meristem in controlling Arabidopsis flowering architecture. *Journal of Experimental Botany*, *66*(15), 4769-4780.

Bell, C. D., Soltis, D. E., & Soltis, P. S. (2005). The age of the angiosperms: a molecular timescale without a clock. *Evolution*, *59*(6), 1245-1258.

Bergonzi, S., & Albani, M. C. (2011). Reproductive competence from an annual and a perennial perspective. *Journal of Experimental Botany*, *62*(13), 4415-4422.

Bielenberg, D. G., Wang, Y., Li, Z., Zhebentyayeva, T., Fan, S., Reighard, G. L., Scorza, R., & Abbott, A. G. (2008). Sequencing and annotation of the evergrowing locus in peach [Prunus persica (L.) Batsch] reveals a cluster of six MADS-box transcription factors as candidate genes for regulation of terminal bud formation. *Tree Genetics & Genomes*, *4*(3), 495-507.

Bishop, G. J., & Yokota, T. (2001). Plants Steroid Hormones, Brassinosteroids: Current Highlights of Molecular Aspects on their Synthesis/Metabolism, Transport, Perception and Response. *Plant and Cell Physiology*, *42*(2), 114-120. https://doi.org/10.1093/pcp/pce018

Böhlenius, H., Huang, T., Charbonnel-Campaa, L., Brunner, A. M., Jansson, S., Strauss, S. H., & Nilsson, O. (2006). CO/FT regulatory module controls timing of flowering and seasonal growth cessation in trees. *science*, *312*(5776), 1040-1043.

Bowman, J. L., Alvarez, J., Weigel, D., Meyerowitz, E. M., & Smyth, D. R. (1993). Control of flower development in Arabidopsis thaliana by APETALA1 and interacting genes. *Development*, *119*(3), 721-743.

Bradley, D., Carpenter, R., Copsey, L., Vincent, C., Rothstein, S., & Coen, E. (1996). Control of inflorescence architecture in Antirrhinum. *Nature*, *379*(6568), 791-797.

Bradley, D., Ratcliffe, O., Vincent, C., Carpenter, R., & Coen, E. (1997). Inflorescence commitment and architecture in Arabidopsis. *science*, *275*(5296), 80-83.

Brunner, A. M., & Nilsson, O. (2004). Revisiting tree maturation and floral initiation in the poplar functional genomics era. *New Phytologist*, *164*(1), 43-51.

Chang, S., Puryear, J., & Cairney, J. (1993). A simple and efficient method for isolating RNA from pine trees.

Chardon, F., & Damerval, C. (2005). Phylogenomic analysis of the PEBP gene family in cereals. *Journal of molecular evolution*, *61*, 579-590.

Chen, D., Yan, W., Fu, L.-Y., & Kaufmann, K. (2018). Architecture of gene regulatory networks controlling flower development in Arabidopsis thaliana. *Nature Communications*, *9*(1), 4534.

Christenhusz, M. J., & Byng, J. W. (2016). The number of known plants species in the world and its annual increase. *Phytotaxa*, *261*(3), 201–217-201–217.

Clough, S. J., & Bent, A. F. (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. *The Plant Journal*, *16*(6), 735-743.

Collani, S., Neumann, M., Yant, L., & Schmid, M. (2019). FT modulates genome-wide DNA-binding of the bZIP transcription factor FD. *Plant Physiology*, *180*(1), 367-380.

Conti, L., & Bradley, D. (2007). TERMINAL FLOWER1 is a mobile signal controlling Arabidopsis architecture. *The Plant Cell*, *19*(3), 767-778.

Cooke, J. E., Eriksson, M. E., & Junttila, O. (2012). The dynamic nature of bud dormancy in trees: environmental control and molecular mechanisms. *Plant, cell & environment*, *35*(10), 1707-1728.

Corbesier, L., Vincent, C., Jang, S., Fornara, F., Fan, Q., Searle, I., Giakountis, A., Farrona, S., Gissot, L., & Turnbull, C. (2007). FT protein movement contributes to long-distance signaling in floral induction of Arabidopsis. *science*, *316*(5827), 1030-1033.

Corrales, A. R., Carrillo, L., Lasierra, P., Nebauer, S. G., Dominguez-Figueroa, J., Renau-Morata, B., Pollmann, S., Granell, A., Molina, R. V., Vicente-Carbajosa, J., & Medina, J. (2017). Multifaceted role of cycling DOF factor 3 (CDF3) in the regulation of flowering time and abiotic stress responses in <i>Arabidopsis</i>. *Plant, Cell & Environment*, *40*(5), 748-764. https://doi.org/10.1111/pce.12894

Cui, X., Fan, X., Xu, S., Wang, S., Niu, F., Zhao, P., Yang, B., Liu, W., Guo, X., & Jiang, Y.-Q. (2024). WRKY47 transcription factor modulates leaf senescence through regulating PCD-associated genes in Arabidopsis. *Plant Physiology and Biochemistry*, *213*, 108805. https://doi.org/https://doi.org/10.1016/j.plaphy.2024.108805

Davidson, B. L., & McCray Jr, P. B. (2011). Current prospects for RNA interference-based therapies. *Nature Reviews Genetics*, *12*(5), 329-340.

de, A. G. I. g. t. o. g. g. (2000). Analysis of the genome sequence of the flowering plant Arabidopsis thaliana. *Nature*, *408*(6814), 796-815.

Debono, A., Yeats, T. H., Rose, J. K. C., Bird, D., Jetter, R., Kunst, L., & Samuels, L. (2009). <i>Arabidopsis</i> LTPG Is a Glycosylphosphatidylinositol-Anchored Lipid Transfer Protein Required for Export of Lipids to the Plant Surface. *The Plant Cell*, *21*(4), 1230-1238. https://doi.org/10.1105/tpc.108.064451

Diaz-Mendoza, M., Velasco-Arroyo, B., Santamaria, M. E., González-Melendi, P., Martinez, M., & Diaz, I. (2016). Plant senescence and proteolysis: two processes with one destiny. *Genetics and Molecular Biology*, *39*(3), 329-338. https://doi.org/10.1590/1678-4685-gmb-2016-0015

Dickmann, D. (2001). *Poplar culture in north America*. NRC Research Press.

Ding, J., Böhlenius, H., Rühl, M. G., Chen, P., Sane, S., Zambrano, J. A., Zheng, B., Eriksson, M. E., & Nilsson, O. (2018). <i>GIGANTEA-</i>like genes control seasonal growth cessation in <i>Populus</i>. *New Phytologist*, *218*(4), 1491-1503. https://doi.org/10.1111/nph.15087

Ding, J., Zhang, B., Li, Y., André, D., & Nilsson, O. (2021). Phytochrome B and PHYTOCHROME INTERACTING FACTOR8 modulate seasonal growth in trees. *New Phytologist*, *232*(6), 2339-2352. https://doi.org/10.1111/nph.17350

Eckenwalder, J. E. (1996). Systematics and evolution of Populus. *Biology of Populus and its implications for management and conservation*, Chapter 1.

Ferreyra, M. L. F., Serra, P., & Casati, P. (2021). Recent advances on the roles of flavonoids as plant protective molecules after <scp>UV</scp> and high light exposure. *Physiologia Plantarum*, *173*(3), 736-749. https://doi.org/10.1111/ppl.13543

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., & Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature*, *391*(6669), 806-811.

Fornara, F., de Montaigu, A., & Coupland, G. (2010). SnapShot: control of flowering in Arabidopsis. *Cell*, *141*(3), 550-550. e552.

Garner, W. W., & Allard, H. A. (1922). Photoperiodism, the response of the plant to relative length of day and night. *science*, *55*(1431), 582-583.

Gómez-Soto, D., Ramos-Sánchez, J. M., Alique, D., Conde, D., Triozzi, P. M., Perales, M., & Allona, I. (2021). Overexpression of a SOC1-Related Gene Promotes Bud Break in Ecodormant Poplars. *Frontiers in Plant Science*, *12*. https://doi.org/10.3389/fpls.2021.670497

Goretti, D., Silvestre, M., Collani, S., Langenecker, T., Méndez, C., Madueño, F., & Schmid, M. (2020). TERMINAL FLOWER1 functions as a mobile transcriptional cofactor in the shoot apical meristem. *Plant Physiology*, *182*(4), 2081-2095.

Gu, X., Le, C., Wang, Y., Li, Z., Jiang, D., Wang, Y., & He, Y. (2013). Arabidopsis FLC clade members form flowering-repressor complexes coordinating responses to endogenous and environmental cues. *Nature Communications*, *4*(1), 1947.

Hanano, S., & Goto, K. (2011). Arabidopsis TERMINAL FLOWER1 is involved in the regulation of flowering time and inflorescence development through transcriptional repression. *The Plant Cell*, *23*(9), 3172-3184.

Hanzawa, Y., Money, T., & Bradley, D. (2005). A single amino acid converts a repressor to an activator of flowering. *Proceedings of the National Academy of Sciences*, *102*(21), 7748-7753.

Hartley, J. L., Temple, G. F., & Brasch, M. A. (2000). DNA cloning using in vitro site-specific recombination. *Genome research*, *10*(11), 1788-1795.

Hazen, S. P., Schultz, T. F., Pruneda-Paz, J. L., Borevitz, J. O., Ecker, J. R., & Kay, S. A. (2005). LUX ARRHYTHMO encodes a Myb domain protein essential for circadian rhythms. *Proceedings of the National Academy of Sciences*, *102*(29), 10387-10392.

Hedman, H., Källman, T., & Lagercrantz, U. (2009). Early evolution of the MFT-like gene family in plants. *Plant molecular biology*, *70*, 359-369.

Heid, C. A., Stevens, J., Livak, K. J., & Williams, P. M. (1996). Real time quantitative PCR. *Genome research*, *6*(10), 986-994.

Hernando, C. E., Romanowski, A., & Yanovsky, M. J. (2017). Transcriptional and post-transcriptional control of the plant circadian gene regulatory network. *Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms*, *1860*(1), 84-94.

Hille, F., Richter, H., Wong, S. P., Bratovič, M., Ressel, S., & Charpentier, E. (2018). The biology of CRISPR-Cas: backward and forward. *Cell*, *172*(6), 1239-1259.

Hoffmann, M. H. (2002). Biogeography of Arabidopsis thaliana (l.) heynh.(Brassicaceae). *Journal of Biogeography*, *29*(1), 125-134.

Howe, G. T., Bucciaglia, P. A., Hackett, W. P., Furnier, G. R., Cordonnier-Pratt, M.-M., & Gardner, G. (1998). Evidence that the phytochrome gene family in black cottonwood has one PHYA locus and two PHYB loci but lacks members of the PHYC/F and PHYE subfamilies. *Molecular Biology and Evolution*, *15*(2), 160-175.

Hsu, C.-Y., Adams, J. P., Kim, H., No, K., Ma, C., Strauss, S. H., Drnevich, J., Vandervelde, L., Ellis, J. D., & Rice, B. M. (2011). FLOWERING LOCUS T duplication coordinates reproductive and vegetative growth in perennial poplar. *Proceedings of the National Academy of Sciences*, *108*(26), 10756-10761.

Hsu, C.-Y., Adams, J. P., No, K., Liang, H., Meilan, R., Pechanova, O., Barakat, A., Carlson, J. E., Page, G. P., & Yuceer, C. (2012). Overexpression of CONSTANS homologs CO1 and CO2 fails to alter normal reproductive onset and fall bud set in woody perennial poplar.

Hsu, C.-Y., Liu, Y., Luthe, D. S., & Yuceer, C. (2006). Poplar FT2 shortens the juvenile phase and promotes seasonal flowering. *The Plant Cell*, *18*(8), 1846-1861.

Hwang, D. Y., Park, S., Lee, S., Lee, S. S., Imaizumi, T., & Song, Y. H. (2019). GIGANTEA regulates the timing stabilization of CONSTANS by altering the interaction between FKF1 and ZEITLUPE. *Molecules and cells*, *42*(10), 693-701.

Hyun, Y., Richter, R., & Coupland, G. (2017). Competence to flower: age-controlled sensitivity to environmental cues. *Plant Physiology*, *173*(1), 36-46.

Ibáñez, C., Kozarewa, I., Johansson, M., Ögren, E., Rohde, A., & Eriksson, M. E. (2010). Circadian clock components regulate entry and affect exit of

seasonal dormancy as well as winter hardiness in Populus trees. *Plant Physiology*, *153*(4), 1823-1833.
Jaeger, K. E., & Wigge, P. A. (2007). FT protein acts as a long-range signal in Arabidopsis. *Current Biology*, *17*(12), 1050-1054.
Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., & Charpentier, E. (2012). A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity. *science*, *337*(6096), 816-821.
Kamioka, M., Takao, S., Suzuki, T., Taki, K., Higashiyama, T., Kinoshita, T., & Nakamichi, N. (2016). Direct repression of evening genes by CIRCADIAN CLOCK-ASSOCIATED1 in the Arabidopsis circadian clock. *The Plant Cell*, *28*(3), 696-711.
Karlberg, A., Bako, L., & Bhalerao, R. P. (2011). Short day–mediated cessation of growth requires the downregulation of AINTEGUMENTALIKE1 transcription factor in hybrid aspen. *PLOS Genetics*, *7*(11), e1002361.
Karlberg, A., Englund, M., Petterle, A., Molnar, G., Sjödin, A., Bako, L., & Bhalerao, R. P. (2010). Analysis of global changes in gene expression during activity-dormancy cycle in hybrid aspen apex. *Plant Biotechnology*, *27*(1), 1-16.
Karlgren, A., Gyllenstrand, N., Källman, T., Sundström, J. F., Moore, D., Lascoux, M., & Lagercrantz, U. (2011). Evolution of the PEBP gene family in plants: functional diversification in seed plant evolution. *Plant Physiology*, *156*(4), 1967-1977.
Kinoshita, T., Ono, N., Hayashi, Y., Morimoto, S., Nakamura, S., Soda, M., Kato, Y., Ohnishi, M., Nakano, T., & Inoue, S.-i. (2011). FLOWERING LOCUS T regulates stomatal opening. *Current Biology*, *21*(14), 1232-1238.
Kivinen, S., Koivisto, E., Keski-Saari, S., Poikolainen, L., Tanhuanpää, T., Kuzmin, A., Viinikka, A., Heikkinen, R. K., Pykälä, J., & Virkkala, R. (2020). A keystone species, European aspen (Populus tremula L.), in boreal forests: Ecological role, knowledge needs and mapping using remote sensing. *Forest Ecology and Management*, *462*, 118008.
Kobayashi, Y., Kaya, H., Goto, K., Iwabuchi, M., & Araki, T. (1999). A pair of related genes with antagonistic roles in mediating flowering signals. *science*, *286*(5446), 1960-1962.
Kobayashi, Y., & Weigel, D. (2007). Move on up, it's time for change—mobile signals controlling photoperiod-dependent flowering. *Genes & development*, *21*(19), 2371-2384.
Koornneef, M., Hanhart, C., & Van der Veen, J. (1991). A genetic and physiological analysis of late flowering mutants in Arabidopsis thaliana. *Molecular and General Genetics MGG*, *229*, 57-66.
Kozarewa, I., Ibáñez, C., Johansson, M., Ögren, E., Mozley, D., Nylander, E., Chono, M., Moritz, T., & Eriksson, M. E. (2010). Alteration of PHYA expression change circadian rhythms and timing of bud set in Populus. *Plant molecular biology*, *73*, 143-156.

Kumar, S. V., Lucyshyn, D., Jaeger, K. E., Alós, E., Alvey, E., Harberd, N. P., & Wigge, P. A. (2012). Transcription factor PIF4 controls the thermosensory activation of flowering. *Nature*, *484*(7393), 242-245.

Kumimoto, R. W., Zhang, Y., Siefers, N., & Holt III, B. F. (2010). NF–YC3, NF–YC4 and NF–YC9 are required for CONSTANS-mediated, photoperiod-dependent flowering in Arabidopsis thaliana. *The Plant Journal*, *63*(3), 379-391.

Kwiatkowska, D. (2008). Flowering and apical meristem growth dynamics. *Journal of Experimental Botany*, *59*(2), 187-201.

Lampropoulos, A., Sutikovic, Z., Wenzl, C., Maegele, I., Lohmann, J. U., & Forner, J. (2013). GreenGate-a novel, versatile, and efficient cloning system for plant transgenesis. *PLoS One*, *8*(12), e83043.

Lang, G. A. (1987). Dormancy: a new universal terminology.

Lazaro, A., Valverde, F., Piñeiro, M., & Jarillo, J. A. (2012). The Arabidopsis E3 ubiquitin ligase HOS1 negatively regulates CONSTANS abundance in the photoperiodic control of flowering. *The Plant Cell*, *24*(3), 982-999.

Lee, D.-K., Geisler, M., & Springer, P. S. (2009). LATERAL ORGAN FUSION1 and LATERAL ORGAN FUSION2 function in lateral organ separation and axillary meristem formation in Arabidopsis.

Lee, J., Oh, M., Park, H., & Lee, I. (2008). SOC1 translocated to the nucleus by interaction with AGL24 directly regulates LEAFY. *The Plant Journal*, *55*(5), 832-843.

Lee, J. H., Park, S. H., Lee, J. S., & Ahn, J. H. (2007). A conserved role of SHORT VEGETATIVE PHASE (SVP) in controlling flowering time of Brassica plants. *Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression*, *1769*(7-8), 455-461.

Lee, J. H., Ryu, H.-S., Chung, K. S., Posé, D., Kim, S., Schmid, M., & Ahn, J. H. (2013). Regulation of temperature-responsive flowering by MADS-box transcription factor repressors. *science*, *342*(6158), 628-632.

Lemus, R., & Lal, R. (2005). Bioenergy crops and carbon sequestration. *Critical Reviews in Plant Sciences*, *24*(1), 1-21.

Li, D., Liu, C., Shen, L., Wu, Y., Chen, H., Robertson, M., Helliwell, C. A., Ito, T., Meyerowitz, E., & Yu, H. (2008). A repressor complex governs the integration of flowering signals in Arabidopsis. *Developmental cell*, *15*(1), 110-120.

Liljegren, S. J., Gustafson-Brown, C., Pinyopich, A., Ditta, G. S., & Yanofsky, M. F. (1999). Interactions among APETALA1, LEAFY, and TERMINAL FLOWER1 specify meristem fate. *The Plant Cell*, *11*(6), 1007-1018.

Lin, C. (2000). Photoreceptors and regulation of flowering time. *Plant Physiology*, *123*(1), 39-50.

Liu, B., Zuo, Z., Liu, H., Liu, X., & Lin, C. (2011). Arabidopsis cryptochrome 1 interacts with SPA1 to suppress COP1 activity in response to blue light. *Genes & development*, *25*(10), 1029-1034.

Liu, C., Chen, H., Er, H. L., Soo, H. M., Kumar, P. P., Han, J.-H., Liou, Y. C., & Yu, H. (2008). Direct interaction of AGL24 and SOC1 integrates flowering signals in Arabidopsis.

Liu, L.-J., Zhang, Y.-C., Li, Q.-H., Sang, Y., Mao, J., Lian, H.-L., Wang, L., & Yang, H.-Q. (2008). COP1-mediated ubiquitination of CONSTANS is implicated in cryptochrome regulation of flowering in Arabidopsis. *The Plant Cell*, *20*(2), 292-306.

Liu, L., Liu, C., Hou, X., Xi, W., Shen, L., Tao, Z., Wang, Y., & Yu, H. (2012). FTIP1 is an essential regulator required for florigen transport. *PLoS biology*, *10*(4), e1001313.

Liu, L., Zhang, Y., & Yu, H. (2020). Florigen trafficking integrates photoperiod and temperature signals in Arabidopsis. *Journal of Integrative Plant Biology*, *62*(9), 1385-1398.

Liu, X., Chi, H., Yue, M., Zhang, X., Li, W., & Jia, E. (2012). The regulation of exogenous jasmonic acid on UV-B stress tolerance in wheat. *Journal of Plant Growth Regulation*, *31*, 436-447.

Locke, J. C., Kozma-Bognár, L., Gould, P. D., Fehér, B., Kevei, E., Nagy, F., Turner, M. S., Hall, A., & Millar, A. J. (2006). Experimental validation of a predicted feedback loop in the multi-oscillator clock of Arabidopsis thaliana. *Molecular systems biology*, *2*(1), 59.

MacKenzie, N. (2010). Ecology, conservation and management of aspen: a literature review.

Mandel, M. A., Gustafson-Brown, C., Savidge, B. et al. Molecular characterization of the Arabidopsis floral homeotic gene APETALA1. Nature 360, 273–277 (1992). https://doi.org/10.1038/360273a0

Mandel, M. A., & Yanofsky, M. F. (1995). A gene triggering flower formation in Arabidopsis. *Nature*, *377*(6549), 522-524.

Marks, R. A., Amézquita, E. J., Percival, S., Rougon-Cardoso, A., Chibici-Revneanu, C., Tebele, S. M., Farrant, J. M., Chitwood, D. H., & Vanburen, R. (2023). A critical analysis of plant science literature reveals ongoing inequities. *Proceedings of the National Academy of Sciences*, *120*(10). https://doi.org/10.1073/pnas.2217564120

Mathieu, J., Warthmann, N., Küttner, F., & Schmid, M. (2007). Export of FT protein from phloem companion cells is sufficient for floral induction in Arabidopsis. *Current Biology*, *17*(12), 1055-1060.

Maurya, J. P., Singh, R. K., Miskolczi, P. C., Prasad, A. N., Jonsson, K., Wu, F., & Bhalerao, R. P. (2020). Branching regulator BRC1 mediates photoperiodic control of seasonal growth in hybrid aspen. *Current Biology*, *30*(1), 122-126. e122.

McWatters, H. G., Kolmos, E., Hall, A., Doyle, M. R., Amasino, R. M., Gyula, P., Nagy, F., Millar, A. J., & Davis, S. J. (2007). ELF4 is required for oscillatory properties of the circadian clock. *Plant Physiology*, *144*(1), 391-401.

Melzer, S., Lens, F., Gennen, J., Vanneste, S., Rohde, A., & Beeckman, T. (2008). Flowering-time genes modulate meristem determinacy and growth form in Arabidopsis thaliana. *Nature genetics*, *40*(12), 1489-1492.

Mimida, N., Goto, K., Kobayashi, Y., Araki, T., Ahn, J. H., Weigel, D., Murata, M., Motoyoshi, F., & Sakamoto, W. (2001). Functional divergence of the TFL1-like gene family in Arabidopsis revealed by characterization of a novel homologue. *Genes to Cells*, *6*(4), 327-336.

Miskolczi, P., Singh, R. K., Tylewicz, S., Azeez, A., Maurya, J. P., Tarkowská, D., Novák, O., Jonsson, K., & Bhalerao, R. P. (2019). Long-range mobile signals mediate seasonal control of shoot growth. *Proceedings of the National Academy of Sciences*, *116*(22), 10852-10857.

Mizuno, T., Nomoto, Y., Oka, H., Kitayama, M., Takeuchi, A., Tsubouchi, M., & Yamashino, T. (2014). Ambient temperature signal feeds into the circadian clock transcriptional circuitry through the EC night-time repressor in Arabidopsis thaliana. *Plant and Cell Physiology*, *55*(5), 958-976.

Mohamed, R., Wang, C.-T., Ma, C., Shevchenko, O., Dye, S. J., Puzey, J. R., Etherington, E., Sheng, X., Meilan, R., Strauss, S. H., & Brunner, A. M. (2010). Populus CEN/TFL1 regulates first onset of flowering, axillary meristem identity and dormancy release in Populus. *The Plant Journal*, *62*(4), 674-688. https://doi.org/10.1111/j.1365-313x.2010.04185.x

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum*, *15*(3).

Nakajima, M., Shimada, A., Takashi, Y., Kim, Y. C., Park, S. H., Ueguchi-Tanaka, M., Suzuki, H., Katoh, E., Iuchi, S., & Kobayashi, M. (2006). Identification and characterization of Arabidopsis gibberellin receptors. *The Plant Journal*, *46*(5), 880-889.

Nilsson, O., Aldén, T., Sitbon, F., Anthony Little, C., Chalupa, V., Sandberg, G., & Olsson, O. (1992). Spatial pattern of cauliflower mosaic virus 35S promoter-luciferase expression in transgenic hybrid aspen trees monitored by enzymatic assay and non-destructive imaging. *Transgenic Research*, *1*, 209-220.

Nohales, M. A., & Kay, S. A. (2016). Molecular mechanisms at the core of the plant circadian oscillator. *Nature structural & molecular biology*, *23*(12), 1061-1069.

Nusinow, D. A., Helfer, A., Hamilton, E. E., King, J. J., Imaizumi, T., Schultz, T. F., Farré, E. M., & Kay, S. A. (2011). The ELF4–ELF3–LUX complex links the circadian clock to diurnal control of hypocotyl growth. *Nature*, *475*(7356), 398-402.

Olsen, J. E., Jensen, J. B., Molmann, J. A., Ernstsen, A., & Junttila, O. (2004). Photoperiodic Regulationof Apical Growth Cessation in Northern Tree Species: The Role of Phytochrome and Gibberellin. *Journal of crop improvement*, *10*(1-2), 77-112.

Olsen, J. E., Junttila, O., Nilsen, J., Eriksson, M. E., Martinussen, I., Olsson, O., Sandberg, G., & Moritz, T. (1997). Ectopic expression of oat phytochrome

A in hybrid aspen changes critical daylength for growth and prevents cold acclimatization. *The Plant Journal*, *12*(6), 1339-1350.

Osnato, M., Castillejo, C., Matías-Hernández, L., & Pelaz, S. (2012). TEMPRANILLO genes link photoperiod and gibberellin pathways to control flowering in Arabidopsis. *Nature Communications*, *3*(1), 808.

Parmentier-Line, C. M., & Coleman, G. D. (2016). Constitutive expression of the Poplar FD-like basic leucine zipper transcription factor alters growth and bud development. *Plant Biotechnology Journal*, *14*(1), 260-270. https://doi.org/10.1111/pbi.12380

Penfield, S., & King, J. (2009). Towards a systems biology approach to understanding seed dormancy and germination. *Proceedings of the Royal Society B: Biological Sciences*, *276*(1673), 3561-3569.

Pham, V. N., Kathare, P. K., & Huq, E. (2018). Phytochromes and phytochrome interacting factors. *Plant Physiology*, *176*(2), 1025-1038.

Pin, P., & Nilsson, O. (2012). The multifaceted roles of FLOWERING LOCUS T in plant development. *Plant, cell & environment*, *35*(10), 1742-1755.

Posé, D., Verhage, L., Ott, F., Yant, L., Mathieu, J., Angenent, G. C., Immink, R. G., & Schmid, M. (2013). Temperature-dependent regulation of flowering by antagonistic FLM variants. *Nature*, *503*(7476), 414-417.

Quail, P. H., Boylan, M. T., Parks, B. M., Short, T. W., Xu, Y., & Wagner, D. (1995). Phytochromes: photosensory perception and signal transduction. *science*, *268*(5211), 675-680.

Rendón-Anaya, M., Wilson, J., Sveinsson, S., Fedorkov, A., Cottrell, J., Bailey, M. E., Ruņģis, D., Lexer, C., Jansson, S., & Robinson, K. M. (2021). Adaptive introgression facilitates adaptation to high latitudes in European aspen (Populus tremula L.). *Molecular Biology and Evolution*, *38*(11), 5034-5050.

Rinne, P. L. H., Welling, A., Vahala, J., Ripel, L., Ruonala, R., Kangasjärvi, J., & Van Der Schoot, C. (2011). Chilling of Dormant Buds Hyperinduces<i>FLOWERING LOCUS T</i>and Recruits GA-Inducible 1,3-β-Glucanases to Reopen Signal Conduits and Release Dormancy in<i>Populus</i>. *The Plant Cell*, *23*(1), 130-146. https://doi.org/10.1105/tpc.110.081307

Rottmann, W. H., Meilan, R., Sheppard, L. A., Brunner, A. M., Skinner, J. S., Ma, C., Cheng, S., Jouanin, L., Pilate, G., & Strauss, S. H. (2000). Diverse effects of overexpression of LEAFY and PTLF, a poplar (Populus) homolog of LEAFY/FLORICAULA, in transgenic poplar and Arabidopsis. *The Plant Journal*, *22*(3), 235-245.

Ruonala, R., Rinne, P. I. L. H., KangasjäRvi, J., & Van Der Schoot, C. (2008). <i>CENL1</i>Expression in the Rib Meristem Affects Stem Elongation and the Transition to Dormancy in<i>Populus</i>. *The Plant Cell*, *20*(1), 59-74. https://doi.org/10.1105/tpc.107.056721

Sachs, R. M. (1965). Stem elongation. *Annual Review of Plant Physiology*, *16*(1), 73-96.

Saitou, N., & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, *4*(4), 406-425.

Savill, P. (2013). *The silviculture of trees used in British forestry*.

Schultz, E. A., & Haughn, G. W. (1991). LEAFY, a homeotic gene that regulates inflorescence development in Arabidopsis. *The Plant Cell*, *3*(8), 771-781.

Searle, I., He, Y., Turck, F., Vincent, C., Fornara, F., Kröber, S., Amasino, R. A., & Coupland, G. (2006). The transcription factor FLC confers a flowering response to vernalization by repressing meristem competence and systemic signaling in Arabidopsis. *Genes & development*, *20*(7), 898-912.

Shannon, S., & Meeks-Wagner, D. R. (1991). A mutation in the Arabidopsis TFL1 gene affects inflorescence meristem development. *The Plant Cell*, *3*(9), 877-892.

Shen, G., Sun, W., Chen, Z., Shi, L., Hong, J., & Shi, J. (2022). Plant GDSL Esterases/Lipases: Evolutionary, Physiological and Molecular Functions in Plant Development. *Plants*, *11*(4), 468. https://doi.org/10.3390/plants11040468

Sheng, X., Hsu, C.-Y., Ma, C., & Brunner, A. M. (2022). Functional Diversification of Populus FLOWERING LOCUS D-LIKE3 Transcription Factor and Two Paralogs in Shoot Ontogeny, Flowering, and Vegetative Phenology. *Frontiers in Plant Science*, *13*. https://doi.org/10.3389/fpls.2022.805101

Sheng, X., Mahendra, R. A., Wang, C.-T., & Brunner, A. M. (2023). CRISPR/Cas9 mutants delineate roles of <i>Populus FT</i> and <i>TFL1/CEN/BFT</i> family members in growth, dormancy release and flowering. *Tree Physiology*, *43*(6), 1042-1054. https://doi.org/10.1093/treephys/tpad027

Shi, Y., Ding, Y., & Yang, S. (2018). Molecular Regulation of CBF Signaling in Cold Acclimation. *Trends in Plant Science*, *23*(7), 623-637. https://doi.org/10.1016/j.tplants.2018.04.002

Singh, R. K., Maurya, J. P., Azeez, A., Miskolczi, P., Tylewicz, S., Stojkovič, K., Delhomme, N., Busov, V., & Bhalerao, R. P. (2018). A genetic network mediating the control of bud break in hybrid aspen. *Nature Communications*, *9*(1), 4173.

Singh, R. K., Miskolczi, P., Maurya, J. P., & Bhalerao, R. P. (2019). A tree ortholog of SHORT VEGETATIVE PHASE floral repressor mediates photoperiodic control of bud dormancy. *Current Biology*, *29*(1), 128-133. e122.

Singh, R. K., Svystun, T., AlDahmash, B., Jönsson, A. M., & Bhalerao, R. P. (2017). Photoperiod-and temperature-mediated control of phenology in trees–a molecular perspective. *New Phytologist*, *213*(2), 511-524.

Slavov, G. T., & Zhelev, P. (2010). Salient biological features, systematics, and genetic variation of Populus. *Genetics and genomics of Populus*, 15-38.

Somers, D. E., Devlin, P. F., & Kay, S. A. (1998). Phytochromes and cryptochromes in the entrainment of the Arabidopsis circadian clock. *science*, *282*(5393), 1488-1490.

Song, Y. H., Estrada, D. A., Johnson, R. S., Kim, S. K., Lee, S. Y., MacCoss, M. J., & Imaizumi, T. (2014). Distinct roles of FKF1, GIGANTEA, and ZEITLUPE proteins in the regulation of CONSTANS stability in Arabidopsis photoperiodic flowering. *Proceedings of the National Academy of Sciences*, *111*(49), 17672-17677.

Srikanth, A., & Schmid, M. (2011). Regulation of flowering time: all roads lead to Rome. *Cellular and molecular life sciences*, *68*, 2013-2037.

Tao, Z., Shen, L., Liu, C., Liu, L., Yan, Y., & Yu, H. (2012). Genome-wide identification of SOC1 and SVP targets during the floral transition in Arabidopsis. *The Plant Journal*, *70*(4), 549-561.

Taoka, K.-I., Ohki, I., Tsuji, H., Furuita, K., Hayashi, K., Yanase, T., Yamaguchi, M., Nakashima, C., Purwestri, Y. A., Tamaki, S., Ogaki, Y., Shimada, C., Nakagawa, A., Kojima, C., & Shimamoto, K. (2011). 14-3-3 proteins act as intracellular receptors for rice Hd3a florigen. *Nature*, *476*(7360), 332-335. https://doi.org/10.1038/nature10272

Thines, B. C., Youn, Y., Duarte, M. I., & Harmon, F. G. (2014). The time of day effects of warm temperature on flowering time involve PIF4 and PIF5. *Journal of Experimental Botany*, *65*(4), 1141-1151.

Tiwari, S. B., Shen, Y., Chang, H. C., Hou, Y., Harris, A., Ma, S. F., McPartland, M., Hymus, G. J., Adam, L., & Marion, C. (2010). The flowering time regulator CONSTANS is recruited to the FLOWERING LOCUS T promoter via a unique cis-element. *New Phytologist*, *187*(1), 57-66.

Tuskan, G. A., Difazio, S., Jansson, S., Bohlmann, J., Grigoriev, I., Hellsten, U., Putnam, N., Ralph, S., Rombauts, S., & Salamov, A. (2006). The genome of black cottonwood, Populus trichocarpa (Torr. & Gray). *science*, *313*(5793), 1596-1604.

Tylewicz, S., Petterle, A., Marttila, S., Miskolczi, P., Azeez, A., Singh, R. K., Immanen, J., Mähler, N., Hvidsten, T. R., & Eklund, D. M. (2018). Photoperiodic control of seasonal growth is mediated by ABA acting on cell-cell communication. *science*, *360*(6385), 212-215.

Tylewicz, S., Tsuji, H., Miskolczi, P., Petterle, A., Azeez, A., Jonsson, K., Shimamoto, K., & Bhalerao, R. P. (2015). Dual role of tree florigen activation complex component <i>FD</i> in photoperiodic growth control and adaptive response pathways. *Proceedings of the National Academy of Sciences*, *112*(10), 3140-3145. https://doi.org/10.1073/pnas.1423440112

Valverde, F. (2011). CONSTANS and the evolutionary origin of photoperiodic timing of flowering. *Journal of Experimental Botany*, *62*(8), 2453-2463.

Valverde, F., Mouradov, A., Soppe, W., Ravenscroft, D., Samach, A., & Coupland, G. (2004). Photoreceptor regulation of CONSTANS protein in photoperiodic flowering. *science*, *303*(5660), 1003-1006.

Van Nocker, S., & Ludwig, P. (2003). The WD-repeat protein superfamily in Arabidopsis: conservation and divergence in structure and function. *BMC genomics*, *4*, 1-11.

Wang, J.-W., Czech, B., & Weigel, D. (2009). miR156-regulated SPL transcription factors define an endogenous flowering pathway in Arabidopsis thaliana. *Cell*, *138*(4), 738-749.

Wang, J., Ding, J., Tan, B., Robinson, K. M., Michelson, I. H., Johansson, A., Nystedt, B., Scofield, D. G., Nilsson, O., & Jansson, S. (2018). A major locus controls local adaptation and adaptive life history variation in a perennial plant. *Genome biology*, *19*, 1-17.

Wang, Y., Huang, J., Li, E., Xu, S., Zhan, Z., Zhang, X., Yang, Z., Guo, F., Liu, K., & Liu, D. (2022). Phylogenomics and biogeography of Populus based on comprehensive sampling reveal deep-level relationships and multiple intercontinental dispersals. *Frontiers in Plant Science*, *13*, 813177.

Watson, J. D. (2007). *Recombinant DNA: genes and genomes: a short course*. Macmillan.

Weigel, D., Alvarez, J., Smyth, D. R., Yanofsky, M. F., & Meyerowitz, E. M. (1992). LEAFY controls floral meristem identity in Arabidopsis. *Cell*, *69*(5), 843-859.

Weigel, D., & Nilsson, O. (1995). A developmental switch sufficient for flower initiation in diverse plants. *Nature*, *377*(6549), 495-500.

Wigge, P. A., Kim, M. C., Jaeger, K. E., Busch, W., Schmid, M., Lohmann, J. U., & Weigel, D. (2005). Integration of spatial and temporal information during floral induction in Arabidopsis. *science*, *309*(5737), 1056-1059.

Wühlisch, G. v. (2009). Eurasian aspen (Populus tremula). *EUFORGEN Technical Guidelines for Genetic Conservation and Use*.

Xi, W., Liu, C., Hou, X., & Yu, H. (2010). MOTHER OF FT AND TFL1 regulates seed germination through a negative feedback loop modulating ABA signaling in Arabidopsis. *The Plant Cell*, *22*(6), 1733-1748.

Yamaguchi, A., Kobayashi, Y., Goto, K., Abe, M., & Araki, T. (2005). TWIN SISTER OF FT (TSF) acts as a floral pathway integrator redundantly with FT. *Plant and Cell Physiology*, *46*(8), 1175-1189.

Yoo, S. J., Chung, K. S., Jung, S. H., Yoo, S. Y., Lee, J. S., & Ahn, J. H. (2010). BROTHER OF FT AND TFL1 (BFT) has TFL1-like activity and functions redundantly with TFL1 in inflorescence meristem development in Arabidopsis. *The Plant Journal*, *63*(2), 241-253.

Yoo, S. Y., Kardailsky, I., Lee, J. S., Weigel, D., & Ahn, J. H. (2004). Acceleration of flowering by overexpression of MFT (Mother of FT and TFL1). *Molecules and cells*, *17*(1), 95-101.

Yordanov, Y. S., Ma, C., Strauss, S. H., & Busov, V. B. (2014). EARLY BUD-BREAK 1 (<i>EBB1</i>) is a regulator of release from seasonal dormancy in poplar trees. *Proceedings of the National Academy of Sciences*, *111*(27), 10001-10006. https://doi.org/10.1073/pnas.1405621111

Yuceer, C., Land Jr, S. B., Kubiske, M. E., & Harkess, R. L. (2003). Shoot morphogenesis associated with flowering in Populus deltoides (Salicaceae). *American Journal of Botany*, *90*(2), 196-206.

Zhang, X., Li, W., Liu, Y., Li, Y., Li, Y., Yang, W., Chen, X., Pi, L., & Yang, H. (2022). Replication protein RPA2A regulates floral transition by cooperating with PRC2 in Arabidopsis. *New Phytologist*, *235*(6), 2439-2453.

Zhu, D., Maier, A., Lee, J.-H., Laubinger, S., Saijo, Y., Wang, H., Qu, L.-J., Hoecker, U., & Deng, X. W. (2008). Biochemical characterization of Arabidopsis complexes containing CONSTITUTIVELY PHOTOMORPHOGENIC1 and SUPPRESSOR OF PHYA proteins in light control of plant development. *The Plant Cell*, *20*(9), 2307-2323.

Popular science summary

Since most plants are firmly rooted to the ground, and therefore unable to move, they must continuously adapt to their environment to survive. This is especially true in temperate and boreal regions, where seasonal conditions change extremely. Plants adapt by anticipating seasonal transitions through a "sensing" of environmental cues such as temperature and daylength. In contrast to temperature, which can fluctuate significantly between years, daylength is a more constant predictor of the time of year. Trees rely on the length of daylight to detect the end of summer, when days begin to shorten, and stop growing. Growth cessation is induced when daylight drops below a critical threshold. After growth cessation, trees form terminal buds to protect the meristematic tissues from the approaching winter conditions. Before winter arrival, trees need to achieve cold hardiness, which enhance their resistance to frost damage. Among the many strategies which trees implement to reach this status, trees modify cell walls to increase thickness and plasticity and accumulate antifreeze proteins to prevent ice crystal formation within the tissues. To survive winter, trees enter a dormant state during which metabolic activity is slowed down. When dormancy is established, trees are unreceptive to growth-promoting signals to ensure that buds do not flush prematurely in case of, for instance, temperature fluctuations. A prolonged exposure to cold releases dormancy, even though trees do not resume growth immediately because this typically occurs in early winter. When spring arrives bringing warmer temperatures, buds flush. It has been proven that increased daylength *per se* it is not important for the timing of bud flush, and what matters is the combination of the number of days at warmer temperatures and the extent of these higher temperatures. If spring is particularly warm trees will flush earlier.

New shoots and leaves emerge from the open buds and trees experience an intense period of vegetative growth. In summer, long days promote photosynthesis and the accumulation of resources. At the end of summer when resources decrease in availability and photosynthesis is halted deciduous trees as poplars drop their leaves as strategy to prevent water loss through transpiration and conserve nutrients. Summer ends and the cycle repeats itself.

In this thesis, I have investigated the genetic and molecular mechanisms regulating these seasonal adaptations, and how environmental cues such as

daylength and temperature are perceived by trees and translated to a molecular response triggering major physiological changes. Understanding how these processes are controlled at the molecular level provides valuable insights that can be applied to improve plant breeding and forestry practices, leading to trees better adapted to a future climate.

Populärvetenskaplig sammanfattning

Eftersom de är fast rotade i marken, och därför orörliga, så måste växter anpassa sig till en mängd olika miljöförhållanden. Detta gäller särskilt i tempererade och boreala regioner, där säsongsförhållandena förändras extremt. Växterna har anpassat sig genom att ”förutse” övergången mellan säsongerna genom att "känna av" miljösignaler som temperatur och dagslängd. I motsats till temperaturen, som kan fluktuera avsevärt från år till år, så är dagslängden en mer konstant signal. Träd är beroende av dagsljusets längd för att upptäcka slutet av sommaren när dagarna börjar bli kortare, vilket leder till att träden slutar att växa. Tillväxtstoppet induceras när dagsljuset sjunker under en kritisk tröskel, den s.k. ”kritiska dagslängden”. Strax efter att tillväxten upphört så bildar träden terminala knoppar för att skydda de meristematiska vävnaderna från de annalkande vinterförhållandena. Före vinterns ankomst måste träd uppnå köldhärdighet, vilket förbättrar deras motståndskraft mot frostskador. Detta uppnås bl.a. genom att träden modifierar sina cellväggar för att öka tjockleken och plasticiteten och genom att ackumulera frostskyddsproteiner för att förhindra iskristallbildning i vävnaderna. För att överleva vintern går träd in i ett vilande tillstånd under vilket den metaboliska aktiviteten bromsas. När vinterdvalan är etablerad är träden okänsliga för tillväxtfrämjande signaler för att säkerställa att knopparna inte bryter i förtid, vid t.ex. temperaturfluktuationer. En långvarig exponering för kyla frigör viloläget, även om träden inte återupptar tillväxten omedelbart eftersom detta vanligtvis inträffar tidigt på vintern. När våren kommer med varmare temperaturer bryter knopparna. Det har bevisats att ökad dagslängd i sig inte är viktig för tidpunkten för knoppbrytning, och det som spelar roll är kombinationen av antalet dagar vid varmare temperaturer och omfattningen av dessa högre temperaturer. Om våren är särskilt varm kommer träden att bryta sina knoppar tidigare.

Nya skott och löv slår ut från de öppna knopparna och träden går in i en intensiv period av vegetativ tillväxt. På sommaren befrämjar de långa dagarna fotosyntes och ackumulering av resurser. I slutet av sommaren när resurserna minskar i tillgänglighet och fotosyntesen avtar, fäller lövträden sina löv som en strategi för att förhindra vattenförlust genom transpiration och bevara näringsämnen. Sommaren tar slut och cykeln upprepar sig.

I min avhandling har jag undersökt de genetiska och molekylära mekanismer som reglerar dessa säsongsanpassningar, och hur miljösignaler som dagslängd och temperatur uppfattas av träd och översätts i ett molekylärt svar som utlöser stora fysiologiska förändringar. Att förstå hur dessa processer kontrolleras på molekylär nivå ger värdefulla insikter som kan användas för att förbättra växtförädling och skogsbruksmetoder för att få fram träd som är bättre anpassade till ett framtida klimat.

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Current Biology

Report

FLOWERING LOCUS T paralogs control the annual growth cycle in *Populus* trees

Highlights

- *Populus* trees contain several paralogs of *FLOWERING LOCUS T* (*FT*) genes
- The *FT* paralogs regulate different aspects of the tree's yearly growth cycle
- The *FT2* paralogs control summer growth
- The *FT1* paralog controls the release of winter dormancy

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In brief

André et al. show that in *Populus tremula* trees, a pair of *FLOWERING LOCUS T* (*FT*) paralogs are required for vegetative growth during spring and summer and regulate the entry into winter dormancy. Another paralog is required for bud flush in spring. This function is linked to the release of dormancy rather than to the regulation of bud flush per se.

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Current Biology

Report

FLOWERING LOCUS T paralogs control the annual growth cycle in *Populus* trees

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SUMMARY

In temperate and boreal regions, perennials adapt their annual growth cycle to the change of seasons. These adaptations ensure survival in harsh environmental conditions, allowing growth at different latitudes and altitudes, and are therefore tightly regulated. *Populus* tree species cease growth and form terminal buds in autumn when photoperiod falls below a certain threshold.[1] This is followed by establishment of dormancy and cold hardiness over the winter. At the center of the photoperiodic pathway in *Populus* is the gene *FLOWERING LOCUS T2* (*FT2*), which is expressed during summer and harbors significant SNPs in its locus associated with timing of bud set.[1–4] The paralogous gene *FT1*, on the other hand, is hyper-induced in chilling buds during winter.[3,5] Even though its function is so far unknown, it has been suggested to be involved in the regulation of flowering and the release of winter dormancy.[3,5] In this study, we employ CRISPR-Cas9-mediated gene editing to individually study the function of the *FT*-like genes in *Populus* trees. We show that while *FT2* is required for vegetative growth during spring and summer and regulates the entry into dormancy, expression of *FT1* is absolutely required for bud flush in spring. Gene expression profiling suggests that this function of *FT1* is linked to the release of winter dormancy rather than to the regulation of bud flush per se. These data show how *FT* duplication and sub-functionalization have allowed *Populus* trees to regulate two completely different and major developmental control points during the yearly growth cycle.

RESULTS AND DISCUSSION

Populus species have several *FT* genes

The *Populus FT1* and *FT2* paralogs are the result of the salicoid whole-genome duplication event.[6] A more local *FT2* duplication event has been described in European aspen (*Populus tremula*),[4] but the functional relevance is so far unknown. From here on, we will refer to the duplicated *FT2* genes as *FT2a* and *FT2b*, where *FT2a* corresponds to the previously characterized *FT2* gene. A 500 kb introgression event in the genome region harboring *FT2a* and *FT2b* was recently shown to be strongly associated with local adaptation.[4,7] Phylogenetic analysis revealed that *FT2a* and *FT2b* are present in *Populus tremula*, *P. tremuloides*, and *P. trichocarpa*, indicating that the duplication took place before the species separated (Figures 1A and S1A). We then compared the gene synteny, the genomic regions surrounding the *FT*-like genes in Arabidopsis, *P. tremula*, and *P. trichocarpa* (Figure 1A). Orthologous genes have a similar arrangement in all three species. The duplication around *FT2* seems to have included the orthologous gene of *FASCIATA1* (*FAS1*). However, in *P. trichocarpa* both *FT2b* and *FAS1* are truncated, while both genes are full length in *P. tremula* (Figure 1A).

Expression patterns of *FT2* genes are similar but different from *FT1*

The expression patterns of *FT1* and *FT2a* have previously been established,[3] but nothing was known about *FT2b*. Thus, we analyzed the expression of all three *FT* genes in our model species T89 (*Populus tremula* × *tremuloides*) as well as in field-grown mature *Populus tremula* (Figures 1B–1D). In our samples, *FT2a* and *FT2b* expression was limited to leaves in long days. *FT2b* was significantly more highly expressed than *FT2a* (Figures 1B and 1C), but both followed a circadian rhythm with a peak at the end of the light period (Figure 1B), similar to Arabidopsis *FT*.[8] *FT2b* overexpression also induced a dramatic early flowering phenotype (Figures S1B and S1C) similar to what has been reported for *FT1* and *FT2a*.[1,2] *FT1*, on the other hand, was exclusively expressed in buds exposed to cold temperatures (Figures 1C and 1D). In these buds, *in situ* hybridization revealed that the transcript is broadly present in shoot apex, embryonic leaves, and vasculature in February, but is undetectable in May (Figure S1D).

*FT*s are required for vegetative growth

Previous attempts to study the role of individual *FT* genes in *Populus* trees have been hampered by the fact that due to high levels

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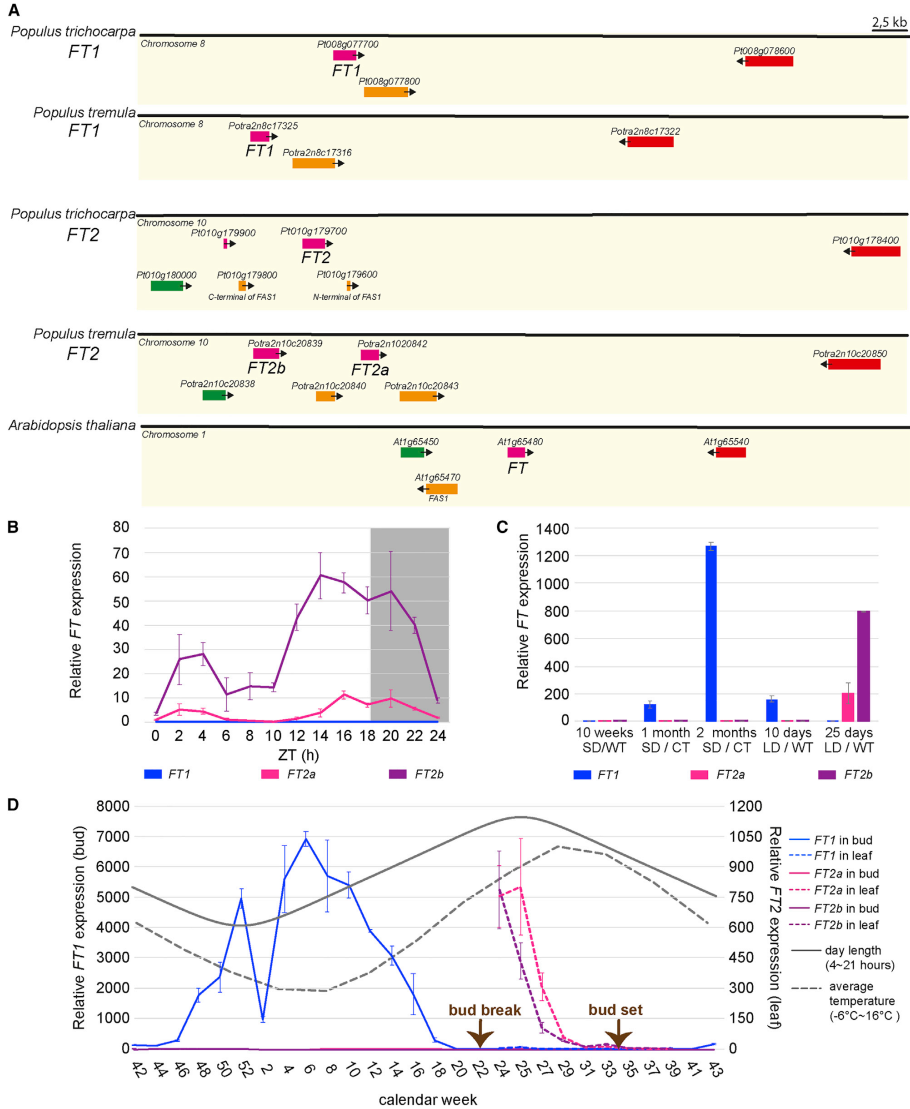

Figure 1. *Populus FT* genes are the result of both a whole-genome and a local duplication event and are expressed at different times in different tissues

(A) Genome organization of *FT* genes in *Arabidopsis thaliana*, *Populus trichocarpa*, and *Populus tremula*. A first salicoid whole-genome duplication created the paralogs *FT1* and *FT2*. A second local duplication created *FT2a* and *FT2b*, the latter being truncated in *P. trichocarpa*. Orthologous genes are indicated by the same colors.

(legend continued on next page)

of homology it has been impossible to generate gene-specific knockdowns using RNAi or artificial microRNAs. To understand their individual roles, we generated specific knockout mutants for *FT1* and *FT2* using CRISPR-Cas9 (Figures S2A and S2B) and subjected the mutant plants to a simulation of the changing seasons to examine their phenotypes (Figure 2). *FT2* has previously been identified as an important regulator of the timing of bud set since RNAi-mediated downregulation of *FT* expression leads to earlier bud set while *FT* overexpression prevents growth cessation and bud set.[1] SNPs at the *FT2a* locus are also very strongly associated with timing of bud set in *Populus tremula*.[4] However, surprisingly, knockout of *FT2a* expression had no visible effect on vegetative growth or timing of growth cessation/bud set (Figure S2C). In contrast, knockout of *FT2b* expression had a clear effect leading to bud set already in long day conditions (Figure S2D). However, a much more dramatic phenotype was seen in *FT2a FT2b* double knockouts, which displayed a severely dwarfed phenotype because of bud set even in tissue culture under 23-h-long days, and immediately after transplanting to soil (Figures 2A and 2E), suggesting that the *FT2* genes are necessary to maintain vegetative growth. While *FT2b* appears to have the most important function, presumably linked to higher levels of expression compared to *FT2a*, the genes are partially redundant. A similarly extreme phenotype has been shown for *GIGANTEA* (*GI*) RNAi plants, which display very low *FT2* expression.[9] However, in contrast to *GI* RNAi trees, *FT2* double-knockout plants were not impaired in their bud flush (Figure 2D), suggesting that the *FT2* genes have specific roles in the maintenance of vegetative growth and in the regulation of growth cessation and bud set. Recently, it was shown that CRISPR knockouts of *FT2a* lead to early growth cessation and inhibition of elongation growth in *P. tremula* × *alba*.[10] Since the presence of the active *P. tremula* gene *FT2b* reported here was not known at that time, the retention of this active gene likely explains the relatively weaker phenotypes reported in these CRISPR lines compared to our double *ft2a ft2b* lines.

While RNAi-mediated downregulation of *FT2* expression had already hinted at their function,[1] only preliminary data regarding the phenotypes of *ft1* and *ft1 ft2* trees have been described.[11] In our experiments, disruption of *FT1* function using CRISPR-Cas9 had no visible effect on vegetative growth or SD-induced growth cessation (Figure 2), confirming that the gene has no function during these processes when it is not expressed (Figures 1B–1D). However, after cold treatment to break dormancy and reactivation at warmer temperatures, *ft1* plants were unable to flush their buds and only some plants flushed a few buds several months later (Figures 2B–2D and 2F). This is a similar but stronger phenotype than the previously described preliminary data in *Populus tremula* × *alba*.[11] To exclude the possibility of *ft1* buds simply having died during the cold treatment, we performed a viability staining, which showed that buds were indeed still alive (Figure S4A). This shows that *FT1* is required to resume vegetative growth after winter. Together, these results show that both *FT1* and *FT2* are required for vegetative growth: *FT1* is required for bud flush and *FT2* is required to allow vegetative growth and prevent growth cessation and bud set during summer.

FT2 is graft transmissible while FT1 function is restricted to its place of production

We also investigated whether grafting on T89 could rescue the growth defect of FT CRISPR plants (Figure S3). FT is a mobile graft-transmissible protein in Arabidopsis[12–14] and has recently been shown to also be a long-ranged signal in poplar.[15] We grafted both *ft1* and *ft2a ft2b* scions onto wild-type (WT) rootstocks, as well as WT scions on mutant rootstocks. The early growth cessation of *ft2a ft2b* plants could be temporarily rescued (Figure S3A) by a WT rootstock. However, as the shoot grew the WT rootstock could no longer support the growth of the *ft2a ft2b* scion, and it went into growth cessation again. Conversely, WT scions initially grew slowly on *ft2a ft2b* rootstock but then started to grow normally, presumably because they were now able to produce enough *FT2* themselves, which in Arabidopsis typically occurs when leaves turn from photosynthate sinks to sources.[16]

Grafting of *ft1*, however, did not rescue the delayed bud flush phenotype (Figure S3B). WT parts of the grafts flushed simultaneously as the WT control regardless of their position. *ft1* scions, rootstocks, and controls did not flush during the entirety of the experiment. These results indicate that *FT1* is acting locally within individual buds. Since *FT1* is expressed in embryonic leaves and vasculature (Figure S1D) and has also been shown to be mobile, it is still possible that FT1 travels locally to the embryonic shoot apex, as suggested earlier.[5]

FT1 is required for dormancy release

Since dormancy release is a prerequisite for bud flush, it is very difficult to know in which of these interconnected processes *FT1* has a role, especially since there are no well-established molecular markers for dormancy release.[17] We therefore performed transcript profiling on WT and *ft1* buds at different time points during an artificial growth cycle (Figure 3) to see at what point in time lack of *FT1* expression affected the transcriptome. The analysis showed that the transcriptomes of *ft1* mutant plants look like those of WT controls up until 4 weeks of cold treatment (CTW4) (Figure 3A). After 8 weeks (CTW8), when WT endodormancy is released, the WT had drastically changed its transcriptomic profile, while *ft1* seemed to remain in the same stage as at

(B) Relative gene expression of *FT1*, *FT2a*, and *FT2b* in leaves of WT trees grown for 2 months in a greenhouse under 18 h light, 6 h dark regime. Error bars indicate SEM of six biological replicates (ramets).

(C) Relative gene expression of *FT1*, *FT2a*, and *FT2b* in buds as well as in leaves of newly flushing buds (25 days LD/WT) of WT grown in growth chamber. Lateral buds were harvested at ZT6. Plants were grown for 6 weeks in LD conditions before being subjected to the indicated treatments. SD/WT, short day and warm temperature treatment (20°C, 8 h light/16 h dark); SD/CT, short day and cold temperature treatment (4°C, 8 h light/16 h dark); LD/WT, long day and warm temperature treatment (20°C, 18 h light/6 h dark). Error bars indicate SEM of three biological replicates (ramets).

(D) Expression of *FT1*, *FT2a*, and *FT2b* over the course of 1 year in field-grown *P. tremula* in Umeå, Sweden. *FT1* expression peaked during winter, when temperatures were lowest. Both *FT2* genes were expressed in leaves during summer months. Solid lines indicate expression in apical buds; dashed line expression in leaves. All samples were taken at 2 p.m. In each panel, gene expression is normalized against the lowest detectable *FT1* and *FT2a* expression in buds and leaves, respectively. Error bars indicate SEM of three biological replicates.

See also Figure S1.

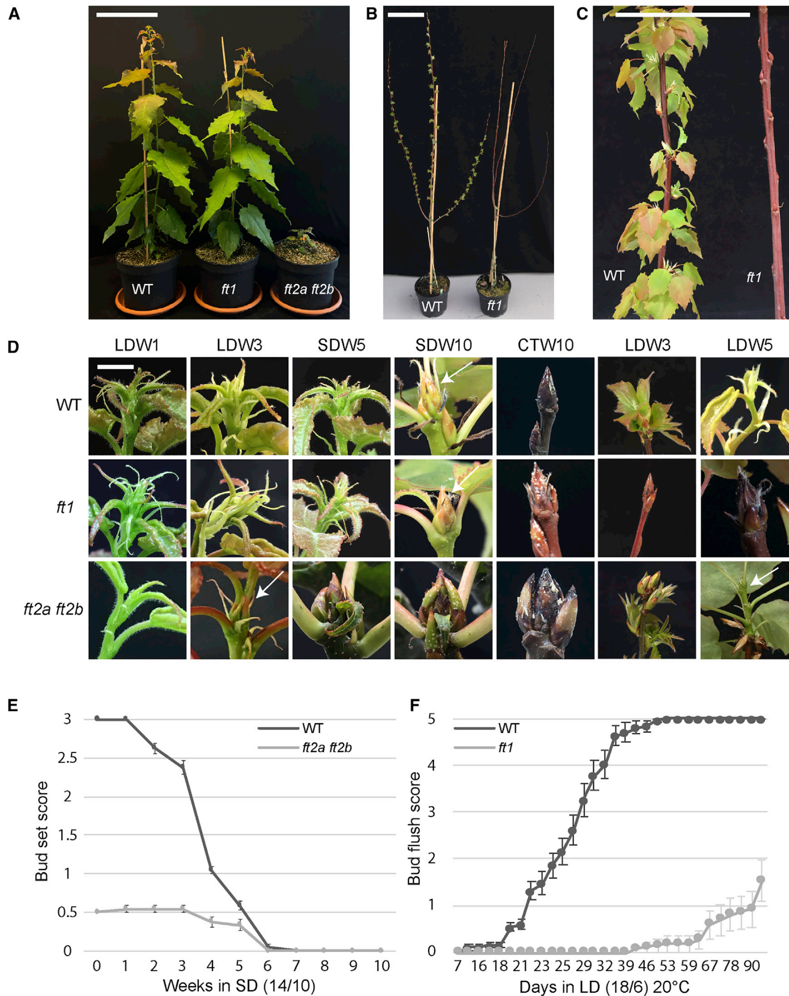

Figure 2. *FT1* and *FT2* are required for growth at different times

(A) WT, *ft1*, and *ft2a ft2b* mutants after 4 weeks in LD. *ft2a ft2b* mutants are severely dwarfed.

(B) WT and *ft1* trees after 3 weeks in warm LD following 10 weeks of cold treatment at 4°C.

(C) Close-up of WT and *ft1* shoots after 4 weeks in warm LD following 10 weeks of cold treatment at 4°C.

(D) Shoot apices over the course of one growth cycle of WT, *ft1*, and *ft2a ft2b* trees. Knockout of *FT1* had no effect on bud set, while *ft2a ft2b* double mutant lines set bud already in LD. White arrows indicate bud formation stages. Bud flush after cold was not impaired in *ft2a ft2b*, but severely delayed (if occurring at all) in *ft1* trees. LDW, weeks in LD (18/6 h light/dark, 20°C/20°C); SDW, weeks in SD (14/10 h light/dark, 20°C/20°C); CTW, weeks in cold treatment (8/16 h light/dark, 4°C/4°C).

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CTW4 (Figures 3A and 3B; Data S1A–S1F). This is also represented in the number of differentially expressed genes between WT and mutant trees at the different time points (Figure 3C; Data S1G–S1I). After 7 days of warm temperature (LDD7; just before any visible signs of bud flush), the transcriptomes were again more similar, presumably due to a similar response to the temperature increase and the fact that bud flush had not yet started. The most affected Gene Ontology (GO) terms were ''catalytic activity'' for genes downregulated in WT between CTW4 and CTW8, and that remained high in *ft1* versus WT at CTW8, and ''binding'' and metabolic process for genes with the opposite pattern of expression (Data S1G–S1I). A closer examination of the expression of genes previously suggested to be associated with the regulation of dormancy or bud set/bud break showed the most consistent changes between WT and *ft1* at CTW8 in genes associated with GA metabolism and reception (Figure 3D). In particular, the GA receptor *GIBBERELLIN-INSENSITIVE DWARF* (*GID*) genes are upregulated in WT at CTW8 while they are maintained at lower expression levels in *ft1*, suggesting a possible role for GA reception in the release of dormancy. Also, *PICKLE* (*PKL*), an antagonist of polycomb repression complex 2 whose downregulation has been shown to mediate the ABA-induced plasmodesmata closure and establishment of dormancy in *Populus* trees,[18] is induced in WT at CTW8 while it remains low in *ft1*, suggesting a possible involvement in dormancy release (Figure 3D). It can be speculated that the very large number of genes changing in expression during dormancy release could be indicative of a more general chromatin remodeling releasing a repressed state of a large number of genes. The role of FT1 would then be to release the repressed state and make the genes accessible for later inductive signals.

Our data suggest that *FT1* function is required for dormancy release rather than bud flush per se. This was further supported by moving WT and *ft1* trees with non-dormant buds back to long days before dormancy was established. Under these conditions, both WT and *ft1* trees flushed their buds normally (Figure S4B), showing that *FT1* is only required for bud flush after dormancy release. However, we cannot exclude that *FT1* also has a role in post-dormancy-specific bud flush.

Since dormancy release occurs at a similar time as removal of plasmodesmata callose plugs,[5] we wondered how FT1 influences this process. After 12 weeks of short days, both WT and *ft1* trees were dormant and had developed frequent electron-dense plasmodesmata callose plugs, or dormancy sphincters, in apices (Figures 4A and 4C), as shown before.[18] After a further 12 weeks of cold treatment, when WT dormancy has been released while *ft1* trees are still dormant, no plasmodesmata callose plugs could be found in either WT or *ft1* (Figures 4B and 4D). This shows that FT1 has no role in the removal of the callose plugs but is rather acting downstream of or parallel to this process. It also shows that it is not the removal of the callose plugs per se that determines the dormant versus non-dormant state. One possibility is that a local movement of FT1 to target cells in the shoot apex is absolutely required to release dormancy, and that the dormancy sphincters are needed to prevent this movement. However, it is still unclear to what extent opening of the dormancy sphincters is required for dormancy release after cold treatment, or if it is just a consequence of that release. Also, at the peak of its expression FT1 displays a broad expression in the shoot apex, in vasculature, and in the young leaf primordia (Figure S1), making it unclear if a restriction of FT1 movement is relevant. A better understanding of direct FT1 targets is required to better understand the mechanism for dormancy release. Taken together, these data show that FT1 is required for dormancy release and the concomitant bud flush, even though its specific mode of action remains unknown.

Taken together, our data show that *FLOWERING LOCUS T* genes are indispensable for the correct regulation of the annual growth cycle, being critical both for the growth arrest and bud set in the fall and for bud flush in the spring, i.e., the start and stop of the growing season. They evolved from a whole-genome duplication, but despite their sub-functionalization, they still share a common role as growth promoters and are able to induce a shared set of genes when ectopically expressed.[3] As a consequence, both *FT1* and *FT2* ectopic overexpression leads to early flowering and prevention of growth cessation.[1–3] This suggests that the sub-functionalization is primarily driven by changes in the regulatory elements of the two genes, leading to completely contrasting expression patterns. This is reminiscent of the situation in sugar beet where two *FT* paralogs have evolved complementary expression patterns to regulate the yearly growth cycle. However, in this case there is also a neo-functionalization leading to one of the paralogs acting as a repressor instead of an activator of flowering.[19,20] Despite the previous focus on *FT2a*, it seems that in *Populus tremula* × *tremuloides*, *FT2b* can act redundantly in promoting growth and is of even greater relative importance, since knockout of any *FT2* alone led to no (*ft2a*) or a slight (*ft2b*) growth phenotype (Figures S2C and S2D) while double knockout almost completely prevented growth by an immediate trigger of the SD response (Figure 2).

There are now several examples of how sub-functionalized *FT* paralogs have evolved within different species to regulate completely different aspects of plant development and growth, usually in response to photoperiodic cues. This includes, for instance, the regulation of flowering and tuberization in potato,[21] bulb formation in onion,[22] and short-day vernalization providing competence to flower in *Brachypodium*.[23] Besides the regulation of flowering, *FT* homologs also appear to act as general growth regulators in tomato[24] and maize.[25]

Interestingly, recent work has shown that *Gentiana trifolia*, a herbaceous perennial, has two *FT* genes where one gene is expressed during the growing season to regulate flowering while the other is expressed in underground overwintering buds to release them from dormancy.[26] CRISPR knockouts of this latter gene lead to a reduced frequency of, and delay in, bud break. This is a very similar situation to what we have shown in *Populus*

(E) Bud set score of WT and *ft2a ft2b* trees in SD. Scores describe the transition from active growth (3) to a fully developed bud (0). Error bars indicate SEM of 15 biological replicates.

(F) Bud flush score of WT and *ft1* trees after cold treatment. Scores describe the transition from hard, closed buds (0) to fully opened buds and actively growing apices (5). Error bars indicate SEM of ten biological replicates.

See also Figures S2 and S3.

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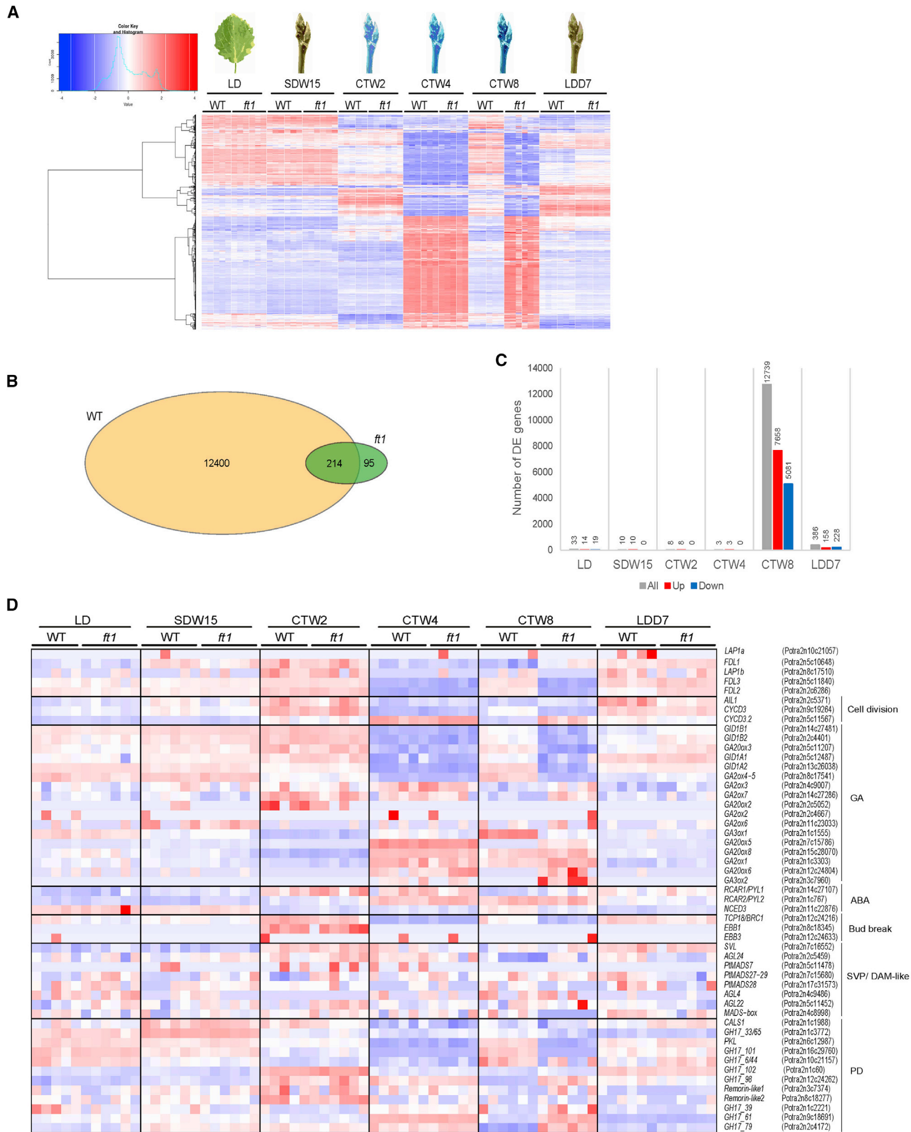

Figure 3. Differential gene expression in *ft1* trees during a growth and dormancy cycle

(A) Heatmap for all time points of WT and *ft1* samples from six biological replicates.

(B) Venn diagram comparing the number of differentially expressed (DE) genes between CTW4 and CTW8 in WT and *ft1*.

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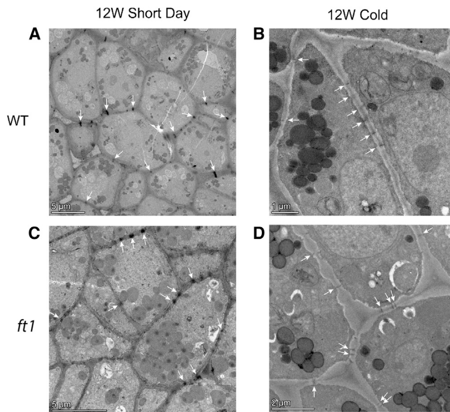

Figure 4. *ft1* shoot apices lack plasmodesmata callose plugs after cold treatment

(A and B) TEM micrographs of shoot apices in terminal buds of WT trees after 12 weeks in SD (A) followed by 12 weeks of chilling (B).

(C and D) TEM micrographs of shoot apices in terminal buds of *ft1* trees after 12 weeks in SD (C) followed by 12 weeks of chilling (D).

Both WT and *ft1* apices display frequent electron-dense plasmodesmata callose plugs in dormancy sphincters (arrows) after 12 weeks in short day (A and C), while no callose plugs are found in plasmodesmata (arrows) after cold treatment (B and D).

See also Figures S3 and S4.

trees and suggests that this type of *FT* sub-functionalization and control of dormancy release might be a more general feature of perennial plants.

Although *FT1* has previously been suggested to be the *FT* paralog controlling flowering,[3] the situation in poplar is clearly different from the one in Arabidopsis. *FT1* is not expressed in leaves and seems to be under no circadian control. Furthermore, it is specifically induced by low temperatures, which repress *FT* expression in Arabidopsis.[27] It is not known which transcription factors activate *FT1* expression. SHORT VEGETATIVE PHASE-LIKE (SVL) has been shown to attenuate its induction,[28] but simple downregulation of a repressor seems insufficient to explain the *FT1* hyper-induction. Our data clearly show that *FT1* has another function, besides potentially flowering, in promoting vegetative growth after winter. To what extent *FT1* and *FT2* are actually needed to control flowering remains an open question.

Taken together, we show here that the *FT* paralogs in *Populus* trees have sub-functionalized to control major developmental transitions during the annual growth cycle, being required to prevent premature growth cessation and bud set in the fall and to induce bud flush in the spring, most likely by releasing the trees from winter dormancy. These are all critical aspects in a tree's ability to adapt to growth in different climates such as those experienced at different latitudes and altitudes, or as a result of climate change.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2022.05.023.

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AUTHOR CONTRIBUTIONS

D.A., M.S., and O.N. planned the research. D.A., K.C.L., D.G., A.M., B.Z., and N.D. performed the experiments and analyzed the data. D.A. and O.N. wrote the manuscript. All authors reviewed and approved its final version.

(C) Number of DE genes between WT and *ft1* trees at the individual time points.

(D) Heatmap for all time points of WT and *ft1* samples of genes previously suggested to be associated with the regulation of dormancy or bud set/bud break. GA, genes involved in gibberellin metabolism and reception; ABA, genes involved in ABA metabolism and reception; PD, genes suggested to be associated with callose synthesis and degradation during plasmodesmata callose plug formation and removal.

LD, long day; SDW15, 15 weeks in SD (14 h light/10 h dark); CTW, weeks in cold treatment (8/16 h light/dark, 4°C/4°C); LDD7, 7 days after transfer back to warm temperatures (18/6 h light/dark, 20°C/20°C). LD samples were leaves taken at ZT17.5; SDW15-LDD7 were lateral buds taken at ZT6. See also Data S1 and Figure S3.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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REFERENCES

1. Böhlenius, H., Huang, T., Charbonnel-Campaa, L., Brunner, A.M., Jansson, S., Strauss, S.H., and Nilsson, O. (2006). CO/FT regulatory module controls timing of flowering and seasonal growth cessation in trees. Science *312*, 1040–1043. https://doi.org/10.1126/science.1126038.
2. Hsu, C.-Y., Liu, Y., Luthe, D.S., and Yuceer, C. (2006). Poplar FT2 shortens the juvenile phase and promotes seasonal flowering. Plant Cell *18*, 1846–1861. https://doi.org/10.1105/tpc.106.041038.
3. Hsu, C.-Y., Adams, J.P., Kim, H., No, K., Ma, C., Strauss, S.H., Drnevich, J., Vandervelde, L., Ellis, J.D., Rice, B.M., et al. (2011). FLOWERING LOCUS T duplication coordinates reproductive and vegetative growth in perennial poplar. Proc. Natl. Acad. Sci. USA *108*, 10756–10761. https://doi.org/10.1073/pnas.1104713108.
4. Wang, J., Ding, J., Tan, B., Robinson, K.M., Michelson, I.H., Johansson, A., Nystedt, B., Scofield, D.G., Nilsson, O., Jansson, S., et al. (2018). A major locus controls local adaptation and adaptive life history variation in a perennial plant. Genome Biol. *19*, 1–17. https://doi.org/10.1186/s13059-018-1444-y.
5. Rinne, P.L., Welling, A., Vahala, J., Ripel, L., Ruonala, R., Kangasjärvi, J., and van der Schoot, C. (2011). Chilling of dormant buds hyperinduces FLOWERING LOCUS T and recruits GA-inducible 1, 3-β-glucanases to reopen signal conduits and release dormancy in Populus. Plant Cell *23*, 130–146. https://doi.org/10.1105/tpc.110.081307.
6. Tuskan, G.A., DiFazio, S., Jansson, S., Bohlmann, J., Grigoriev, I., Hellsten, U., Putnam, N., Ralph, S., Rombauts, S., Salamov, A., et al. (2006). The genome of black cottonwood, Populus trichocarpa (Torr. & Gray). Science *313*, 1596–1604. https://doi.org/10.1126/science.1128691.
7. Rendón-Anaya, M., Wilson, J., Sveinsson, S., Fedorkov, A., Cottrell, J., Bailey, M.E.S., Ruņis, D., Lexer, C., Jansson, S., Robinson, K.M., et al. (2021). Adaptive introgression facilitates adaptation to high latitudes in European aspen (*Populus tremula* L.). Mol. Biol. Evol. *38*, 5034–5050. https://doi.org/10.1093/molbev/msab229.
8. Suárez-López, P., Wheatley, K., Robson, F., Onouchi, H., Valverde, F., and Coupland, G. (2001). CONSTANS mediates between the circadian clock and the control of flowering in Arabidopsis. Nature *410*, 1116–1120. https://doi.org/10.1038/35074138.
9. Ding, J., Böhlenius, H., Rühl, M.G., Chen, P., Sane, S., Zambrano, J.A., Zheng, B., Eriksson, M.E., and Nilsson, O. (2018). GIGANTEA-like genes control seasonal growth cessation in populus. New Phytol. *218*, 1491–1503. https://doi.org/10.1111/nph.15087.
10. Gómez-Soto, D., Allona, I., and Perales, M. (2022). FLOWERING LOCUS T2 promotes shoot apex development and restricts internode elongation *via* the 13-hydroxylation gibberellin biosynthesis pathway in Poplar. Front. Plant Sci. *12*, 814195. https://doi.org/10.3389/fpls.2021.814195.
11. Sheng, X. (2018). Flowering gene homologs regulate seasonal growth changes in poplar. PhD thesis (Virginia Polytechnic Institute and State University).
12. Corbesier, L., Vincent, C., Jang, S., Fornara, F., Fan, Q., Searle, I., Giakountis, A., Farrona, S., Gissot, L., Turnbull, C., and Coupland, G. (2007). FT protein movement contributes to long-distance signaling in floral induction of Arabidopsis. Science *316*, 1030–1033. https://doi.org/10.1126/science.1141752.
13. Jaeger, K.E., and Wigge, P.A. (2007). FT protein acts as a long-range signal in Arabidopsis. Curr. Biol. *17*, 1050–1054. https://doi.org/10.1016/j.cub.2007.05.008.
14. Mathieu, J., Warthmann, N., Küttner, F., and Schmid, M. (2007). Export of FT protein from phloem companion cells is sufficient for floral induction in Arabidopsis. Curr. Biol. *17*, 1055–1060. https://doi.org/10.1016/j.cub.2007.05.009.
15. Miskolczi, P., Singh, R.K., Tylewicz, S., Azeez, A., Maurya, J.P., Tarkowská, D., Novák, O., Jonsson, K., and Bhalerao, R.P. (2019). Long-range mobile signals mediate seasonal control of shoot growth. Proc. Natl. Acad. Sci. USA *116*, 10852–10857. https://doi.org/10.1073/pnas.1902199116.
16. Takada, S., and Goto, K. (1993). TERMINAL FLOWER2, an Arabidopsis homolog of HETEROCHROMATIN PROTEIN1, counteracts the activation of *FLOWERING LOCUS T* by CONSTANS in the vascular tissues of leaves to regulate flowering time. Plant Cell *15*, 2856–2865. https://doi.org/10.1105/tpc.016345.
17. Singh, R.K., Svystun, T., AlDahmash, B., Jönsson, A.M., and Bhalerao, R.P. (2016). Photoperiod and temperature mediated control of phenology in trees–a molecular perspective. New Phytol. *213*, 511–524. https://doi.org/10.1111/nph.14346.
18. Tylewicz, S., Petterle, A., Marttila, S., Miskolczi, P., Azeez, A., Singh, R.K., Immanen, J., Mähler, N., Hvidsten, T.R., Eklund, D.M., et al. (2018). Photoperiodic control of seasonal growth is mediated by ABA acting on cell-cell communication. Science *360*, 212–215. https://doi.org/10.1126/science.aan8576.
19. Pin, P.A., Benlloch, R., Bonnet, D., Wremerth-Weich, E., Kraft, T., Gielen, J.J.L., and Nilsson, O. (2010). An antagonistic pair of FT homologs mediates the control of flowering time in sugar beet. Science *330*, 1397–1400. https://doi.org/10.1126/science.1197004.
20. Pin, P., and Nilsson, O. (2012). The multifaceted roles of FLOWERING LOCUS T in plant development. Plant Cell Environ. *35*, 1742–1755. https://doi.org/10.1111/j.1365-3040.2012.02558.x.
21. Navarro, C., Abelenda, J.A., Cruz-Oró, E., Cuéllar, C.A., Tamaki, S., Silva, J., Shimamoto, K., and Prat, S. (2011). Control of flowering and storage organ formation in potato by FLOWERING LOCUS T. Nature *478*, 119–122. https://doi.org/10.1038/nature10431.
22. Lee, R., Baldwin, S., Kenel, F., McCallum, J., and Macknight, R. (2013). FLOWERING LOCUS T genes control onion bulb formation and flowering. Nat. Commun. *4*, 1–9. https://doi.org/10.1038/ncomms3884.
23. Woods, D., Dong, Y., Bouche, F., Bednarek, R., Rowe, M., Ream, T., and Amasino, R. (2019). A florigen paralog is required for short-day vernalization in a pooid grass. eLife *8*, e42153. https://doi.org/10.7554/eLife.42153.
24. Lifschitz, E., Eviatar, T., Rozman, A., Shalit, A., Goldshmidt, A., Amsellem, Z., Alvarez, J.P., and Eshed, Y. (2006). The tomato FT ortholog triggers systemic signals that regulate growth and flowering and substitute for diverse environmental stimuli. Proc. Natl. Acad. Sci. USA *103*, 6398–6403. https://doi.org/10.1073/pnas.0601620103.
25. Danilevskaya, O.N., Meng, X., McGonigle, B., and Muszynski, M.G. (2011). Beyond flowering time: pleiotropic function of the maize flowering hormone florigen. Plant Signal. Behav. *6*, 1267–1270. https://doi.org/10.4161/psb.6.9.16423.
26. Takahashi, H., Nishihara, M., Yoshida, C., and Itoh, K. (2022). Gentian FLOWERING LOCUS T orthologs regulate phase transitions: floral induction and endodormancy release. Plant Physiol. *188*, 1887–1899. https://doi.org/10.1093/plphys/kiac007.
27. Marín-González, E., Matías-Hernández, L., Aguilar-Jaramillo, A.E., Lee, J.H., Ahn, J.H., Suárez-López, P., and Pelaz, S. (2015). SHORT VEGETATIVE PHASE up-regulates TEMPRANILLO2 floral repressor at low ambient temperatures. Plant Physiol. *169*, 1214–1224. https://doi.org/10.1104/pp.15.00570.
28. Singh, R.K., Maurya, J.P., Azeez, A., Miskolczi, P., Tylewicz, S., Stojkovič, K., Delhomme, N., Busov, V., and Bhalerao, R.P. (2018). A genetic network mediating the control of bud break in hybrid aspen. Nat. Commun. *9*, 1–10. https://doi.org/10.1038/s41467-018-06696-y.
29. Koncz, C., and Schell, J. (1986). The promoter of T_L-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type

of Agrobacterium binary vector. Mol. Gen. Genet. *204*, 383–396. https://doi.org/10.1007/bf00331014.

30. Karimi, M., Depicker, A., and Hilson, P. (2007). Recombinational cloning with plant gateway vectors. Plant Physiol. *145*, 1144–1154. https://doi.org/10.1104/pp.107.106989.

31. Kumar, S., Stecher, G., Li, M., Knyaz, C., and Tamura, K. (2018). MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol. *35*, 1547–1549. https://doi.org/10.1093/molbev/msy096.

32. Ibáñez, C., Kozarewa, I., Johansson, M., Ögren, E., Rohde, A., and Eriksson, M.E. (2010). Circadian clock components regulate entry and affect exit of seasonal dormancy as well as winter hardiness in populus trees. Plant Physiol. *153*, 1823–1833. https://doi.org/10.1104/pp.110.158220.

33. Lampropoulos, A., Sutikovic, Z., Wenzl, C., Maegele, I., Lohmann, J.U., and Forner, J. (2013). GreenGate-a novel, versatile, and efficient cloning system for plant transgenesis. PLoS One *8*, e83043. https://doi.org/10.1371/journal.pone.0083043.

34. Nilsson, O., Aldén, T., Sitbon, F., Little, C.H.A., Chalupa, V., Sandberg, G., and Olsson, O. (1992). Spatial pattern of cauliflower mosaic virus 35S promoter-luciferase expression in transgenic hybrid aspen trees monitored by enzymatic assay and non-destructive imaging. Transgenic Res. *1*, 209–220.

35. Chang, S., Puryear, J., and Cairney, J. (1993). A simple and efficient method for isolating RNA from pine trees. Plant Mol. Biol. Rep. *11*, 113–116. https://doi.org/10.1007/bf02670468.

36. Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. Methods *25*, 402–408. https://doi.org/10.1006/meth.2001.1262.

37. Kopylova, E., Noé, L., and Touzet, H. (2012). SortMeRNA: fast and accurate filtering of ribosomal RNAs in metatranscriptomic data. Bioinformatics *28*, 3211–3217. https://doi.org/10.1093/bioinformatics/bts611.

38. Bolger, A.M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics *30*, 2114–2120. https://doi.org/10.1093/bioinformatics/btu170.

39. Sundell, D., Mannapperuma, C., Netotea, S., Delhomme, N., Lin, Y.-C., Sjödin, A., Van de Peer, Y., Jansson, S., Hvidsten, T.R., and Street, N.R. (2015). The Plant Genome Integrative Explorer Resource: PlantGenIE.org. New Phytol. *208*, 1149–1156. https://doi.org/10.1111/nph.13557.

40. Patro, R., Duggal, G., Love, M.I., Irizarry, R.A., and Kingsford, C. (2017). Salmon provides fast and bias-aware quantification of transcript expression. Nat. Methods *14*, 417–419. https://doi.org/10.1038/nmeth.4197.

41. Huber, W., Carey, V.J., Gentleman, R., Anders, S., Carlson, M., Carvalho, B.S., Bravo, H.C., Davis, S., Gatto, L., Girke, T., et al. (2015). Orchestrating high-throughput genomic analysis with Bioconductor. Nat. Methods *12*, 115–121. https://doi.org/10.1038/nmeth.3252.

42. Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. *15*, 550. https://doi.org/10.1186/s13059-014-0550-8.

43. Ewels, P., Magnusson, M., Lundin, S., and Käller, M. (2016). MultiQC: summarize analysis results for multiple tools and samples in a single report. Bioinformatics *32*, 3047–3048. https://doi.org/10.1093/bioinformatics/btw354.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
E. coli strain: DH5a	N/A	N/A
Agrobacterium strain: GV3101	Koncz et al.[29]	N/A
Chemicals, peptides, and recombinant proteins		
MS basal salts	Duchefa Biochemie	Cat#M0221.0050
T4 DNA ligase	ThermoFisher Scientific	Cat#EL0011
Eco31I	ThermoFisher Scientific	Cat#ER0291
Hexadecyltrimethylammonium bromide (CTAB)	MERCK	Cat#52367
Fluorescein diacetate (FDA)	Sigma-Aldrich	Cat# F7378
Critical commercial assays		
RNeasy mini kit	QIAGEN	Cat# 74106
iScript cDNA Synthesis Kit	Bio-Rad	Cat#170-8891
SsoAdvanced Universal SYBR Green Supermix	Bio-Rad	Cat#1725270
SuperScript II Reverse Transcriptase	ThermoFisher Scientific	Cat#18064014
AMPure XP beads	Beckman-Coulter	Cat#A63882
Deposited data		
Raw and analyzed data	ENA: https://ebi.ac.uk/ena	PRJEB51399
Experimental models: Organisms/strains		
Hybrid aspen (*Populus tremula x tremuloides*) clone T89 (wild-type/WT)	N/A	N/A
Populus tremula	Umeå, Sweden	N/A
Oligonucleotides		
See Table S1	N/A	N/A
Recombinant DNA		
pK2GW7	Karimi et al.[30]	N/A
pK7GWIWG2 (I)	Karimi et al.[30]	N/A
pGreenGateFT1 CRISPR-CAS9	In this paper	N/A
pGreenGateFT2a CRISPR-CAS9	In this paper	N/A
pGreenGateFT2b CRISPR-CAS9	In this paper	N/A
pGreenGateFT2ab CRISPR-CAS9	In this paper	N/A
Software and algorithms		
MEGAX software	Kumar et al.[31]	https://www.megasoftware.net/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ove Nilsson (ove.nilsson@slu.se).

Materials availability

All unique/stable reagents generated in this study are available from the lead contact with a completed Materials Transfer Agreement.

Data and code availability

- RNA-seq data have been deposited at the European Nucleotide Archive (ENA: https://ebi.ac.uk/ena) and are publicly available as of the date of publication. Accession numbers are listed in the key resources table.

- Custom R scripts used for analysis of RNA-seq data are available from https://github.com/nicolasDelhomme/PoplarFT. The raw data is available from the European Nucleotide Archive (ENA: https://ebi.ac.uk/ena) under the accession number PRJEB51399
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Hybrid aspen (*Populus tremula x tremuloides*) clone T89 was used as experimental model.

METHOD DETAILS

Plant material and growth conditions

Hybrid aspen (*Populus tremula x tremuloides*) clone T89 was used as WT control and all genetic modifications were done in this background. Plants were cultivated on ½ Murashige and Skoog medium under sterile conditions for 4 weeks or until they had rooted (max. 8 weeks). After transfer to soil, plants were grown in growth chambers in LD (18h light, 20°C/ 6h dark, 18°C) and with weekly fertilization (10 mL NPK-Rika S/plant). Illumination was from 'Powerstar' lamps (HQI-T 400W/D BT E40, Osram, Germany) giving an R/FR ratio of 2.9 and a light intensity of 150-200 μmol m^{-2} s^{-1}. To induce growth cessation, plants were moved to SD (14h light, 20°C/ 10h dark, 18°C) for up to 15 weeks and fertilization was stopped. For dormancy release, plants were treated with cold (8h light, 4°C/ 16h dark, 6°C) for 8-10 weeks and then transferred back to LD for bud flush. In both SD and LD, previously published bud scores[32] were used to assess effects on bud development (set/flush). For year-around gene expression analysis, a ca. 40-year-old local (Umeå, Sweden) aspen tree was sampled once a month around midday (May to August leaves, buds from September to April).

Design and cloning of CRISPR constructs

Escherichia coli strain DH5α was used for amplification of all plasmids, which were then confirmed by sequencing (Eurofins). GreenGate entry and destination vectors[33] were acquired from Addgene. Potential sgRNAs for target genes were identified with E-CRISP (http://www.e-crisp.org/E-CRISP/). They were introduced into entry vectors by site-directed mutagenesis PCR. The final vector (containing promoter, Cas9 CDS, terminator, two sgRNAs and resistance cassette) was assembled by GreenGate reaction (150 ng of each component, 1.5 μL FastDigest buffer, 1.5 μL of 10 mM ATP, 1 μL 30U/μL T4 ligase and 1 μL Eco31I in a 15 μL reaction) in 50 cycles of 5 min restriction/ligation at 37°C and 16°C, respectively, followed by 5 min 50°C and 5 min 80°C. All reagents were purchased from Thermo Scientific.

Design and cloning of *$35S_{pro}$:FT2b–GFP*

To create the *$35S_{pro}$:FT2b–GFP* fusion gene, the coding region (CDS) of *FT2b* was amplified from cDNA and cloned into the pGREEN-IIS destination vector[33] to C-terminally fuse it in frame to *GFP* under control of the *35S* promoter. The final construct was transformed by electroporation into *Agrobacterium tumefaciens* and into *ft2a ft2b* mutant hybrid aspen trees. All plasmids were propagated using the *Escherichia coli* strain DH5α and verified by sequencing. GreenGate entry and destination vectors were obtained from Addgene. Primers used for plasmid construction are listed in Table S1.

Generation of CRISPR-Cas9 lines

Vectors with different combinations of guide RNAs (Table S1) were transformed into Hybrid aspen using a standard protocol.[34] At least 30 individual transgenic lines from each transformation were screened for target gene deletions using PCR (Table S1; Figure S2A). For each gene (*FT1*, *FT2a* and *FT2b*) at least two independent lines with homozygous, biallelic deletions were initially characterized for growth alterations before selecting one line for deeper analysis. All deletions were confirmed to occur at, or within a few nucleotides of, the expected PAM sites. Except for the *ft2a ft2b* double mutant lines, where both genes were confirmed to be homozygously deleted, target sites in *FT2b* were sequenced in *FT2a* CRISPR constructs and vice versa to exclude "off target" effects.

Grafting experiments

Scions of soil-grown plants were grafted onto rootstocks after 4 weeks (*ft2a ft2b*) or 5 weeks (*ft1*) in the greenhouse (18h light, 20°C/ 6h dark, 18°C). Scions were between 5 and 10 cm long and had no developed leaves, while the rootstock was decapitated ca 10 cm below the apex and kept its leaves. *ft2a ft2b* grafts were kept in these conditions until the end of the experiment, while *ft1* grafts were transferred to SD (8h light, 20°C/ 16h dark, 18°C) after 5 weeks. After 10 weeks of SD treatment, plants were subjected to cold treatment as described above and returned to warm temperatures after 2 months. 5-8 plants per graft combination was used and 4 self-grafted control plants per mutant line and wild type.

RNA extraction and quality assessment

Poplar leaves were ground to fine powder, of which 100 mg were used for RNA extraction with CTAB extraction buffer[35] (2% CTAB, 100 mM Tris-HCl (pH 8.0), 25 mM EDTA, 2M NaCl, 2% PVP). The samples were incubated at 65°C for 2 min and extracted twice with

an equal volume of chloroform-isoamyl alcohol (24:1). Nucleic acids were precipitated at -20°C for 3 hours with ¼ volumes 10 M LiCl. Precipitate was collected by centrifugation (13000 rpm, 4°C, 20 min) and purified with RNeasy kit (Qiagen). DNase treatment was performed on-column (Qiagen). RNA integrity was confirmed either by agarose gel (for downstream qPCR) or by Bioanalyzer (Agilent) for subsequent RNA sequencing.

Quantitative real-time PCR

1000 ng RNA were used for cDNA synthesis with iScript cDNA Synthesis Kit (Biorad). The cDNA was diluted 50 times for downstream applications. Quantitative real-time PCR (qPCR) was run on a LightCycler 480 with SYBR Green I Master (Roche). All kits and machines were used according to the manufacturer's instructions. The reaction protocol started with 5 min pre-incubation at 95°C, followed by 50 cycles of amplification consisting of 10 s denaturation at 95°C, 15 s annealing at 60°C and 20 s elongation at 72°C. For the acquisition of a melting curve fluorescence was measured during the stepwise increase in temperature from 65°C to 97°C. Relative expression levels were obtained using the $2^{-\Delta\Delta Cq}$ method.[36] GeNorm identified *UBQ* and *18S* as the most stable reference genes. All used primers had an efficiency of >1.8 and their correct product was confirmed by sequencing. A complete list of primer sequences can be found in Table S1.

RNA sequencing analysis

For RNA sequencing experiments RNA was isolated as described above and purified with RNeasy kit (Qiagen) according to the manufacturer's instructions. DNAse treatment was performed on column (Qiagen). Concentration and quality of RNA were assessed using Qubit RNA BR Assay Kit (Invitrogen) and Bioanalyzer (Agilent), respectively. 3 μg total RNA with RIN ≧8 was sent for sequencing to SciLife Lab, Stockholm. Library preparation was carried out with an Agilent NGS Bravo workstation in 96-well plates with TruSeq Stranded mRNA kit (Illumina) according to the manufacturer's instructions. mRNA was purified through selective binding to poly dT-coated beads and fragmented using divalent cations under elevated temperature. cDNA was synthesized using SuperScript II Reverse Transcriptase (ThermoFisher Scientific), cleaned with AMPure XP solution (ThermoFisher Scientific), 3' adenylated and ligated to adapters. Fragments were cleaned with AMPure XP beads (ThermoFisher Scientific), amplified by PCR and purified with AMPure XP beads (ThermoFisher Scientific). After washing with 80% ethanol, they were eluted in EB (Qiagen). The quality and concentration of the adapter-ligated libraries were checked on the LabChip GX/HT DNA high sensitivity kit and by Quant-iT, respectively. The libraries were then sequenced using the Illumina NovaSeq-6000 platform, generating from 20 to 110 million paired-end reads (2 × 150 bp) per sample.

Pre-processing of RNA-Seq data and differential expression analyses

The data pre-processing was performed as described here: http://franklin.upsc.se:3000/materials/materials/Guidelines-for-RNA-Seq-data-analysis.pdf. The quality of the raw sequence data was assessed using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Residual ribosomal RNA (rRNA) contamination was assessed and filtered using SortMeRNA (v2.1[37]; settings–log –paired_in –fastx–sam –num_alignments 1) using the rRNA sequences provided with SortMeRNA (rfam-5s-database-id98.fasta, rfam-5.8s-database-id98.fasta, silva-arc-16s-database-id95.fasta, silva-bac-16s-database-id85.fasta, silva-euk-18s-database-id95.fasta, silva-arc-23s-database-id98.fasta, silva-bac-23s-database-id98.fasta and silva-euk-28s-database-id98.fasta). Data were then filtered to remove adapters and trimmed for quality using Trimmomatic (v0.39[38]; settings TruSeq3-PE-2.fa:2:30:10 LEADING:3 SLIDINGWINDOW:5:20 MINLEN:50). After both filtering steps, FastQC was run again to ensure that no technical artefacts were introduced. Filtered reads were pseudo-aligned to v1.1 of the *P. tremula* transcripts (retrieved from the PopGenIE resource[39]) using salmon (v1.1.0,[40] with non-default parameters –gcBias–seqBias –validateMappings) against an index containing the *P. tremula* v1.1 genome sequence as decoy. Statistical analysis of single-gene differential expression between conditions was performed in R (v4.0.0; R Core Team 2020) using the Bioconductor (v3.10[41]) DESeq2 package (v1.28.1[42]). FDR adjusted p values were used to assess significance; a common threshold of 1% was used throughout. For the data quality assessment (QA) and visualization, the read counts were normalized using a variance stabilizing transformation (VST) as implemented in DESeq2. VST data are expression counts normalized for the difference in sequencing depth, adjusted to be homoscedastic (their variance is mean independent) and set on an approximate log2 scale (VST is a heuristic that penalizes very low, i.e. uninformative, counts most). The biological relevance of the data - e.g. biological replicates similarity - was assessed by Principal Component Analysis (PCA) and other visualizations (e.g. heatmaps), using custom R scripts, available from https://github.com/nicolasDelhomme/PoplarFT. In this repository a technical overview of the data, in the form of a MultiQC report,[43] including raw and post-QC read counts and alignment rates is also available. The raw data is available from the European Nucleotide Archive (ENA: https://ebi.ac.uk/ena) under the accession number PRJEB51399.

In situ hybridization

Apical buds from mature *Populus tremula* trees grown in Umeå (63.8°N, 20.2°E) where collected in February 2020 and May 2020. Immediately after the removing of some external scales, tissues were fixed according to the protocol available at https://www.its.caltech.edu/%7Eplantlab/protocols/insitu.pdf. Tissues were embedded in paraffine. For the probe preparation, the CDS from *FT1* of P. tremula was cloned into pSP72 by using *XhoI* and *EcoRV* restriction sites. The T7 promoter was used to transcribe the antisense probe and the *SP6* promoter to transcribe the sense probe. Both probes were hydrolyzed to a length of about 250bp. All the steps

necessary to make the probes are described in the following protocol https://kramerlab.oeb.harvard.edu/files/kramerlab/files/in_situ_protocol_corrected-2.pdf?m=1430323911. The same protocol was also used as reference for the proper ISH experiment, with some minor changes. The hybridization temperature was set at 40°C, and the washes were performed at 50°C; for the tissue permeabilization we used 10 mg/mL of Proteinase K acting for 30 min. Sections (8 μm thick) were mounted on glycerol and visualized at Leica DMi8.

Viability staining

ft1 buds were taken 15 weeks after the end of cold treatment, stained with 3.6 μM fluorescein diacetate solution (Sigma-Aldrich) and photographed under a stereomicroscope (Leica DMi8). *ft1* buds from before cold treatment served as positive control, while the negative control was buds kept at -80°C for 3 days prior to staining.

Transmission electron microscopy

Both WT and *ft1* apical buds were collected after growth in 12 weeks of short photoperiod and subsequently 12 weeks of cold treatment. Apical bud samples from three biological replicates were then fixed overnight in 4% paraformaldehyde and 2.5% glutaraldehyde in 1 M Caccodylate buffer (pH 7.2); post-fixed for 2h in 1% OsO_4 in water, dehydrated, infiltrated and embedded in Spurr's resin (TAAB Laboratories Equipment Ltd, England). Ultra-thin sections of 70nm thickness were stained with uranyl acetate and lead citrate and examined with the Thermo Scientific Talos L120C transmission electron microscope.

QUANTIFICATION AND STATISTICAL ANALYSIS

The statistical details of experiments can be found in the corresponding Figure legends.

Current Biology, Volume *32*

Supplemental Information

FLOWERING LOCUS T paralogs control the annual growth cycle in *Populus* trees

Domenique André, Alice Marcon, Keh Chien Lee, Daniela Goretti, Bo Zhang, Nicolas Delhomme, Markus Schmid, and Ove Nilsson

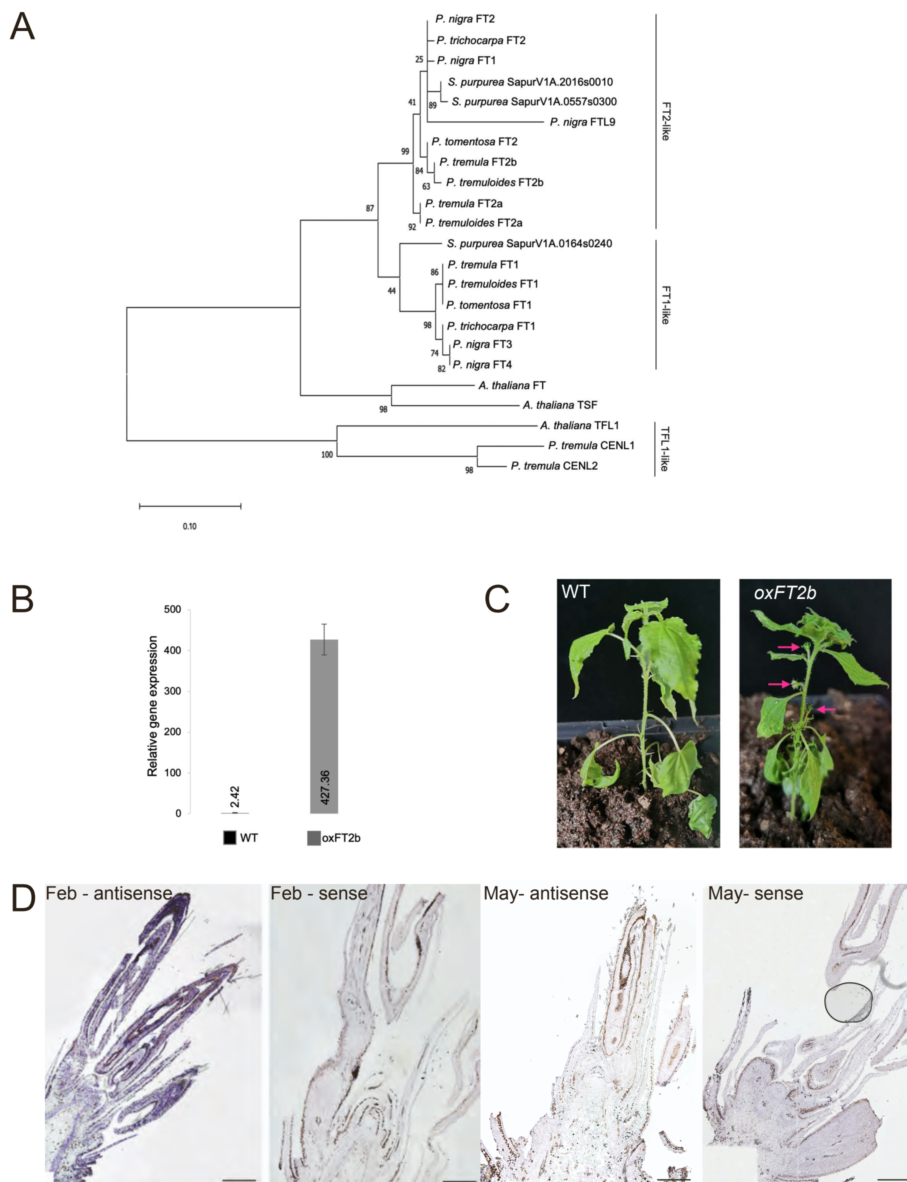
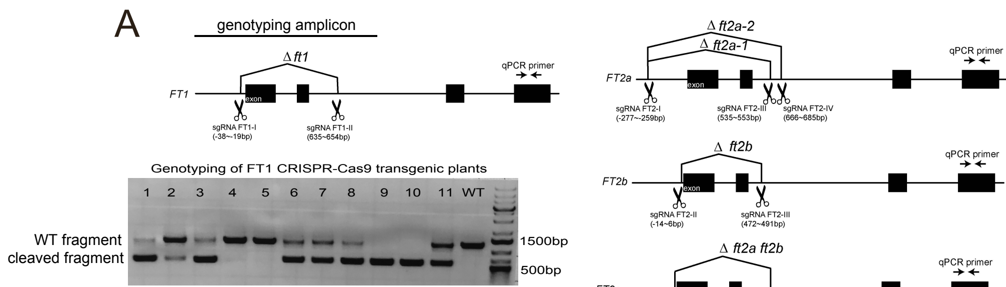
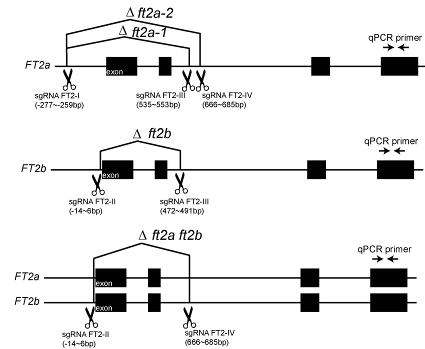


Figure S1: *FT1* and *FT2* paralogs are expressed in different tissues at different times. Related to Figure 1.
A) Maximum likelihood Phylogeny tree (Bootstrap 1000) of FT-like proteins of *Arabidopsis thaliana*, *Populus trichocarpa*, *Populus tremula*, *Populus tremuloides*, *Populus tomentosa*, *Populus nigra* and *Salix purpurea*. TFL1-like proteins of *A. thaliana* and *P. tremula* are shown for comparison. The tree was constructed using MEGAX software [44]. Scale bar indicates the average number of substitutions per site. B) Relative gene expression of endogenous *FT2b* and *FT2b:GFP* in WT and a *FT2b* overexpressing line (*oxFT2b*). Expression values are normalized against *UBQ*. Error bars indicate SEM of two biological replicates. C) Photographs of WT (left) and *oxFT2b* (right) plants two weeks after potting. Pink arrows indicate flowers. D) *In situ* hybridization of *FT1* mRNA showing localization in the embryonic leaves and vasculature within a bud. Field-grown *P. tremula* buds were sampled in February (week 6) at peak *FT1* expression and in May (week 20) after *FT1* expression ceased and hybridized with either an antisense probe or a sense probe. Scale bars represent 400 µm.


A
genotyping amplicon
Δ ft1
FT1
exon
qPCR primer
sgRNA FT1-I (-38~-19bp)
sgRNA FT1-II (635~654bp)
Genotyping of FT1 CRISPR-Cas9 transgenic plants
1 2 3 4 5 6 7 8 9 10 11 WT
WT fragment
cleaved fragment
1500bp
500bp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FT2a
exon
qPCR primer
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sgRNA FT2-III (535~553bp)
sgRNA FT2-IV (666~685bp)
Δ ft2b
FT2b
sgRNA FT2-II (-14~6bp)
sgRNA FT2-III (472~491bp)
Δ ft2a ft2b
FT2a
FT2b
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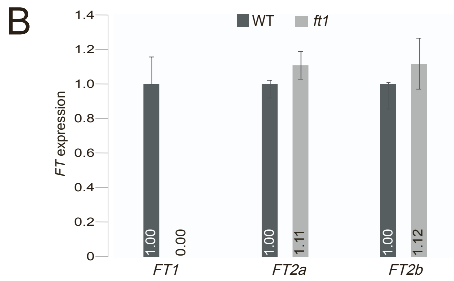
sgRNA FT2-IV (666~685bp)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FT expression
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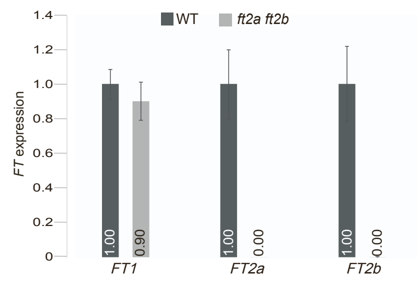
1.00 1.11
1.00 1.12
FT1
FT2a
FT2b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ft2a ft2b
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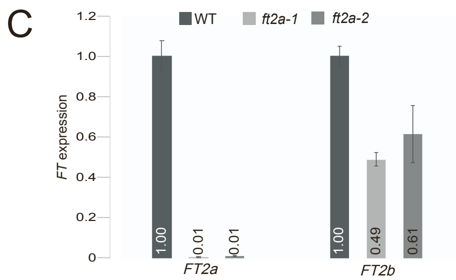
FT1
FT2a
FT2b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WT
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ft2a-2
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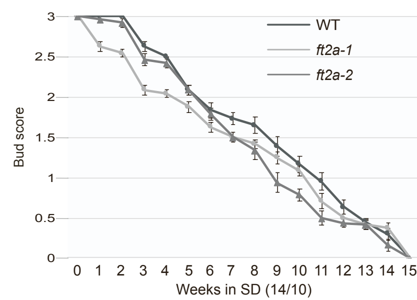
FT2a
FT2b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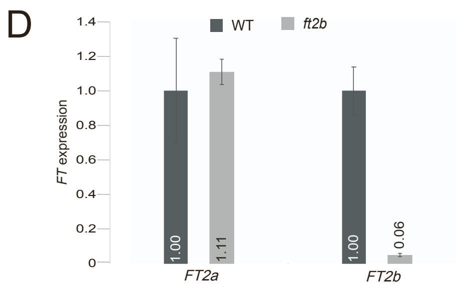
ft2a-1
ft2a-2
Bud score
Weeks in SD (14/10)


D
WT
ft2b
FT expression
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FT2a
FT2b

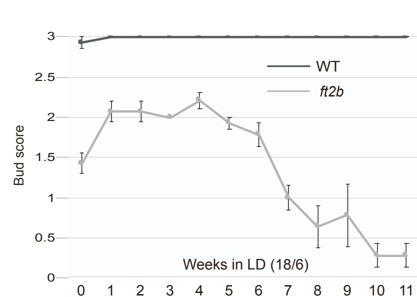

WT
ft2b
Bud score
Weeks in LD (18/6)

Figure S2: FT expression in *ft* mutant trees. Related to Figure 2.

A) Position of guide RNAs (Scissors) used to induce deletions in *FT1*, *FT2a* and *FT2b*. The 'Δ' indicates the target fragment cleaved by CRISPR-Cas9 in each mutant. The gel image shows the genotyping result of *FT1* CRISPR/Cas9 transgenic plants, which serve as an example of CRISPR-Cas9 transgenic plants screening. B) *FT* expression in *ft1* (left) and *ft2a ft2b* trees (right). All expression levels were normalized against the expression in their respective WT controls. *FT1* expression was measured in lateral buds after 8 weeks of cold treatment (8/16 h light/dark, 4°C/4°C), *FT2* was measured in the first fully expanded leaves of LD grown plants. Error bars indicate SEM of three biological replicates. C) Knock-out of *FT2a* alone (left) did not have a strong influence on bud set (right) in SD (14h light /10h dark). Error bars indicate SEM of 12 biological replicates. D) Knock-out of *FT2b* alone (left) lead to bud set (right) in LD (18h light /6h dark). Error bars indicate SEM of seven biological replicates.

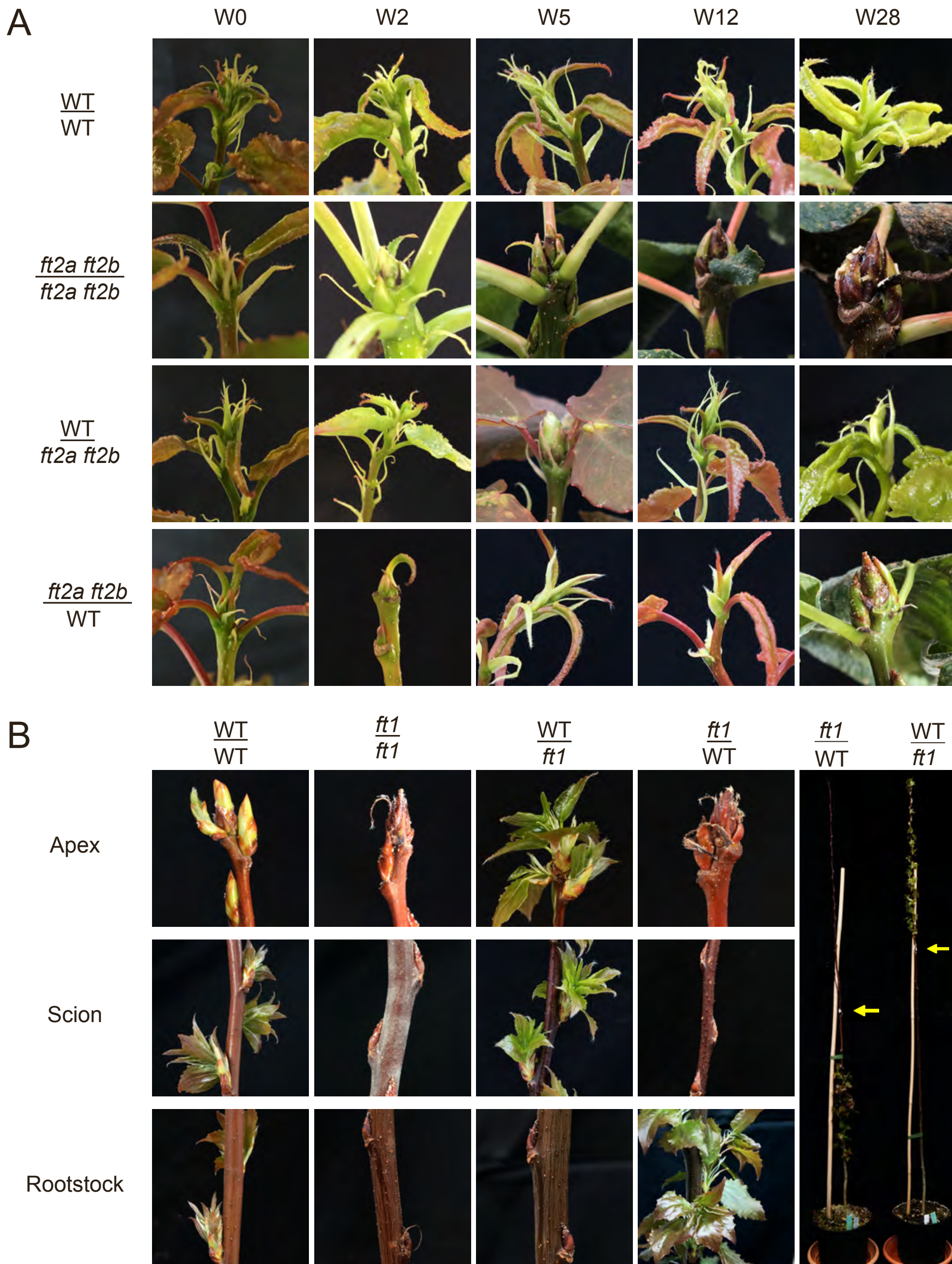
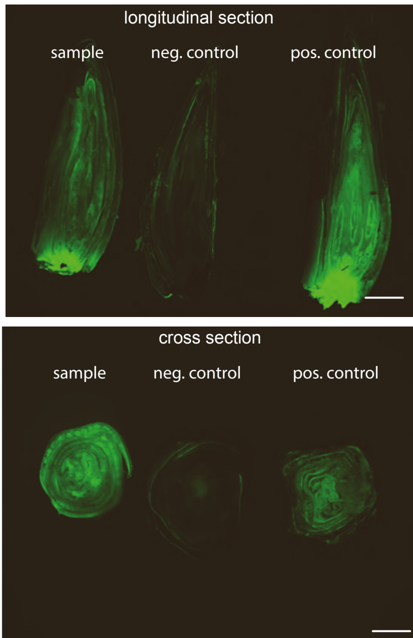


Figure S3: Grafting can temporarily rescue growth defects in *ft2a ft2b* but not *ft1* trees. Related to Figure 2 and 4.
A) WT control plants and WT scions grafted onto *ft2a ft2b* rootstocks grew continually in LD (18h light /6h dark). *ft2a ft2b* plants stopped growth very early, but bud set could be delayed by grafting onto a WT rootstock. W = week after grafting in LD.
B) Bud flush only occurred in the WT part of grafted plants and was severely delayed in ft1 parts irrespective of position. Pictures of representative grafts were taken after 5 weeks in LD (18/6, 20°C) after cold treatment. 5-8 plants per graft combination was used and 4 self-grafted control plants per mutant line and wild type. All grafts behaved in a similar way.



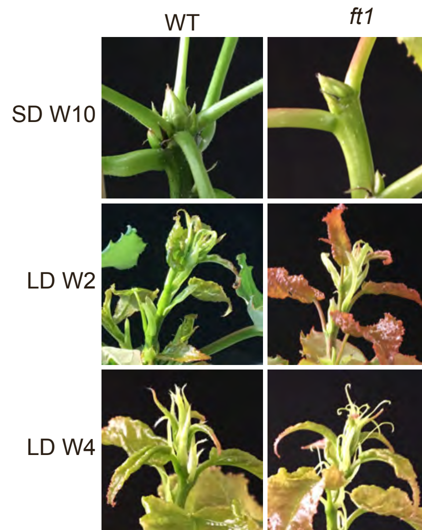


Figure . S4: Buds on *ft1* trees are viable and are not impaired in bud flush *per se.* Related to Figure 4. A) Longitudinal section (top) and cross section (bottem) of *ft1* lateral buds were stained with FDA. Samples were taken 15 weeks after the end of cold treatment, positive control was a dormant non-cold treated bud, negative control was a bud kept at -80°C for 3 days prior to staining. Scale bars represent 1mm. B) Non-dormant WT and *ft1* trees were transferred back to LD without chilling and could reflush their buds. SDW10 = 10 weeks of SD (14h light/ 10h dark) treatment; LD W = weeks in LD (18h light/ 6h dark) after transfer from SD without chilling in between.

Cloning primers		
FT2b-CDS	F	aacaGGTCTCaggctATGCCTAGGGATAGAG
	R	aacaGGTCTCtctgaCGACCTCCTTCCACC
qPCR primers		
18S	F	TCAACTTTCGATGGTAGGATAGAG
	R	CCGTGTCAGGATTGGGTAATTT
UBQ	F	GTTGATTTTTGCTGGGAAGC
	R	GATCTTGGCCTTCACGTTGT
FT1	F	GCAAGCTTTGGCCATGAAAC
	R	GGATATCTTCCTGTTATCGC
FT1 deletion	F	CGTGTTATAGGGGACGTGCT
	R	CCTTAGATCTTCCCCGCCAA
FT2a	F	AGCCCAAGGCCTACAGCAGGAA
	R	GGGAATCTTTCTCTCATGAT
FT2b	F	AGCCCAAGGCCGACAGCGGGAA
	R	GGGAATCTTTCTCTCACGAC
in situ probes		
FT1	PotraFT1_FW_XhoI	ATATCTCGAGATGTCAAGGGATAGAGATCC
	PotraFT1_REV_EcoRV	TATAGATATCTCACTATTATCGCCTCCTACCACC
sgRNA		
FT1	FT1_I	AGACTGTACCTGCTTAGGAG
	FT1_II	CATATGTACGTAGATTTACG
FT2	FT2_I	TTATTCTACTTGGACTCCGA
	FT2_II	AGTGCTGTGCAAGAATGCCT
	FT2_III	AGAATACATGTATTGGTCAC
	FT2_IV	GATTCCCCTGACATGATACT
sgRNA combinations		
FT1 CRISPR		FT1_I + FT1_II
FT2a CRISPR-1		FT2_I + FT2_III
FT2a CRISPR-2		FT2_I + FT2_IV
FT2b CRISPR		FT2_II + FT2_III
FT2 double CRISPR		FT2_II + FT2_IV

Table S1. List of used sequences . Related to Figure 1 and STAR Methods.

III

frontiers in Plant Science

ORIGINAL RESEARCH
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Populus SVL Acts in Leaves to Modulate the Timing of Growth Cessation and Bud Set

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SHORT VEGETATIVE PHASE (SVP) is an important regulator of *FLOWERING LOCUS T (FT)* in the thermosensory pathway of Arabidopsis. It is a negative regulator of flowering and represses *FT* transcription. In poplar trees, *FT2* is central for the photoperiodic control of growth cessation, which also requires the decrease of bioactive gibberellins (GAs). In angiosperm trees, genes similar to *SVP*, sometimes named *DORMANCY-ASSOCIATED MADS-BOX* genes, control temperature-mediated bud dormancy. Here we show that *SVL*, an *SVP* ortholog in aspen trees, besides its role in controlling dormancy through its expression in buds, is also contributing to the regulation of short day induced growth cessation and bud set through its expression in leaves. *SVL* is upregulated during short days in leaves and binds to the *FT2* promoter to repress its transcription. It furthermore decreases the amount of active GAs, whose downregulation is essential for growth cessation, by repressing the transcription of *GA20 oxidase*. Finally, the SVL protein is more stable in colder temperatures, thus integrating the temperature signal into the response. We conclude that the molecular function of *SVL* in the photoperiodic pathway has been conserved between Arabidopsis and poplar trees, albeit the physiological process it controls has changed. SVL is thus both involved in regulating the photoperiod response in leaves, modulating the timing of growth cessation and bud set, and in the subsequent temperature regulation of dormancy in the buds.

Keywords: poplar, FLOWERING LOCUS T, phenology, dormancy, SHORT VEGETATIVE PHASE

INTRODUCTION

Photoperiod is an important environmental cue that controls diverse developmental processes in plants, for example, flowering in Arabidopsis and timing of growth cessation in *Populus* trees (Pin and Nilsson, 2012). At the center of the mechanism, with which plants sense day length, is the *CONSTANS/FLOWERING LOCUS T* module. This module is partially conserved between *Populus* and Arabidopsis, but best understood in the latter. In Arabidopsis, *FT* expression is tightly regulated by many factors and becomes a hub for the integration of different signals, which fine-tunes the response. In addition to photoperiod (Kobayashi et al., 1999), it is regulated by age (Wang, 2014), vernalization (Searle et al., 2006), and ambient temperature (Lee et al., 2007). *SHORT VEGETATIVE PHASE* (*SVP*) is part of the latter pathway and represses *FT*

expression by binding to its promoter (Lee et al., 2007). In *Populus* two *FT* orthologs have been identified, called *FT1* and *FT2*. Only *FT2* has a comparable expression pattern to the Arabidopsis *FT*, being expressed in leaves under long photoperiods, while *FT1* is only expressed in buds during winter (Hsu et al., 2011).

For trees in boreal forests, fine-tuning of the photoperiod response is critical for survival; they need to adapt to the rapidly changing seasons. Especially during the autumn months, temperature and day length are decreasing quickly. Once the day length falls under the critical day length, a threshold for growth permitting conditions, the trees stop their growth and set terminal buds, which protect the enclosed leaf primordia and shoot apical meristems from the subsequent low temperatures (Rohde and Bhalerao, 2007). These short days (SDs) are a reliable signal, with which the trees can anticipate the onset of winter. The signal is transmitted through *FT2*, which is downregulated within a few days after shifts to SDs (Böhlenius et al., 2006; Hsu et al., 2006). Trees failing to downregulate *FT2* are unable to respond to the SD signal and continue growth indefinitely, while plants with reduced *FT2* expression respond more quickly (Böhlenius et al., 2006), leading to early growth cessation and bud set.

CO and *GI* have been identified as positive regulators of *FT2* in long days (LDs). However, their expression profiles do not dramatically change upon shift to SDs (Ding et al., 2018). Arabidopsis CO is rapidly degraded in the dark, thus unable to induce *FT* in SDs (Valverde et al., 2004), and it is so far unclear if the same is true for poplar CO. However, the lack of induction by CO is not enough to explain the rapid downregulation of *FT2* in SDs, especially since *GI* is still expressed and of higher relative importance for *FT2* expression (Ding et al., 2018). GI might contribute to the release of repressive activity on *FT2* expression, as has been shown for poplar CYCLING DOF FACORS (Ding et al., 2018). Such repressors might therefore contribute to the downregulation of *FT2* expression in response to shorter photoperiods. Another possible candidate for such a repressor would be *SVP*, a MADS domain-containing gene and a strong repressor of *FT* expression in Arabidopsis (Hartmann et al., 2000).

SVP homologs have been found in other tree species. For example, in peach trees, six *DAM* (dormancy-associated MADS-box) genes have been associated with the non-dormant phenotype of the *evergrowing* mutant (Bielenberg et al., 2008). *DAM1* and *DAM4* peak in their expression at the end of summer and are hypothesized to be involved in the regulation of growth cessation (Li et al., 2009). Also, in apple, *DAM*- and *SVP*-like genes have been suggested to control bud set and dormancy (Wu et al., 2017, 2021; Falavigna et al., 2019, 2021; Moser et al., 2020). Recently, a *Populus SVP* ortholog named *SVL* has been shown to be expressed in buds where it is involved in dormancy establishment and maintenance (Singh et al., 2018, 2019). However, all analysis so far has been focused on the role of *SVP*/*DAM* genes in the buds, and their role in regulating the photoperiodic response in leaves is still unclear.

Besides *FT2*, another important factor of the short-day response is gibberellins (GAs, Eriksson et al., 2000). GAs are growth-promoting hormones and work both through and independently of *FT2* (Eriksson et al., 2015). A decrease in the levels of active GAs is essential for growth cessation and bud set (Eriksson et al., 2000). So far it is poorly understood how the levels of active GAs are regulated upon shift to SD. In short-day grown Arabidopsis, SVP represses the expression of *GA20 oxidase*, a gene encoding a rate-limiting enzyme in the GA biosynthesis pathway, thereby keeping the amount of bioactive GAs low (Rieu et al., 2008; Andrés et al., 2014). If this function of *SVP* was conserved in trees, it could be another mechanism through which *SVL* could potentially control growth cessation.

The ability of *SVP* to control *FT* expression and GA biosynthesis in Arabidopsis as well as the involvement of *MADS* genes in the phenology of other tree species prompted us to investigate the role of *SVL* in the regulation of growth cessation and bud set in *Populus*. Our data show that *SVL* expression in the leaves modulates the timing of SD-induced growth cessation and is able to repress both *FT2* and *GA20 oxidase* by binding to their promoters. Thus, *SVPs* mode of action has been conserved between Arabidopsis and *Populus*, even though the biological process it is involved in has changed.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Hybrid aspen (*Populus tremula × tremuloides*) clone T89 was used as WT control and all genetic modifications were done in this background. Plants were cultivated on ½ Murashige and Skoog medium under sterile conditions for 4 weeks or until they had rooted (max. 8 weeks). After transfer to soil, plants were grown in growth chambers in LD (18 h light, 20°C/6 h dark, 18°C) and with weekly fertilization (10 ml NPK-Rika S/plant). To induce growth cessation, plants were moved to SD (14-h light, 20°C/ 10-h dark, 18°C) and fertilization was stopped. For dormancy release, plants were treated with cold (8 h light, 6°C/16 h dark, 6°C). In both SD and LD, previously published bud scores (Ibáñez et al., 2010) were used to assess effects on bud development (set/flush). For year-around gene expression analysis, a *ca.* 40-year-old local (Umeå, Sweden) aspen tree was sampled once a month around midday (May to August leaves, buds from September to April).

Phylogenetic Analysis

Protein sequences of SVP homologues were aligned in CLC main workbench (Qiagen) and a Maximum Likelihood Phylogeny was constructed with neighbor-joining method and 1,000 bootstrap replicates.

Cloning of Plasmids

To generate *SVL* RNAi plants, the RNAi fragment was amplified by PCR using PtSVLRNAiF and PtSVLRNAiR primers, which contain attB1 and attB2 sites, respectively. The fragment was

introduced into the pDONOR 201 vector (Invitrogen) by BP recombination. The PttSVLRNAi fragment was then transferred to the final destination vector pK7GWIGWI (Karimi et al., 2002) with Invitrogen LR recombinase, creating a double-stranded RNAi molecule driven by the constitutive Cauliflower Mosaic Virus *35S* promoter. For construction of PttSVLoe—(*35S::PttSVL:Myc*), full-length *PttSVL* CDS was amplified from hybrid aspen mRNA with oxPttSVLF and oxPttSVLR primers and cloned into pDONOR 201 with BP clonase (Invitrogen). The fragment was then transferred to the destination vector pGWB18 (Karimi et al., 2002). Primers used for construct generation are listed in **Supplementary Table S1**. All cloning reactions were performed according to the manufacturer's instructions. Hybrid aspen was transformed as previously described (Nilsson et al., 1992). *Arabidopsis thaliana* was transformed by using the floral dip method (Clough and Bent, 1998). The *svp-32* (Salk_072930) mutant seeds were ordered from Nottingham Arabidopsis Stock Centre (NASC).

Analysis of *SVP*-Overexpressing Arabidopsis Plants

Arabidopsis thaliana WT Col-0, *svp-32,* and PttSVLoe plants were grown on soil in LD (16 h light/8 h dark, 22°C). To measure flowering time, rosette leaves and cauline leaves of 10 plants per line were counted until first flowers were visible.

RNA Extraction and Quantitative Real-Time PCR

Poplar leaves (youngest fully expanded leaves) were ground to fine powder, of which 100 mg were used for RNA extraction with CTAB extraction buffer (Chang et al., 1993; 2% CTAB, 100 mM Tris-HCl (pH 8.0), 25 mM EDTA, 2 M NaCl, 2% PVP). The samples were incubated at 65°C for 2 min and extracted twice with an equal volume of chloroform–isoamyl alcohol (24:1). Nucleic acids were precipitated at −20°C for 3 h with ¼ volumes 10 M LiCl. Precipitate was collected by centrifugation (13,000 rpm, 4°C, 20 min) and washed with 70% EtOH. After drying it was dissolved in 60 µl H2O (DEPC treated). Contamination of genomic DNA was removed from 2,5 µg total nucleic acid by DNase treatment (TURBO DNA-free™ Kit, Ambion®), and 1,000 ng RNA were used for cDNA synthesis with iScript™ cDNA Synthesis Kit (Bio-Rad). The cDNA was diluted 50 times for downstream applications. Quantitative real-time PCR (qPCR) was run on a LightCycler® 480 with SYBR Green I Master (Roche). All kits and machines were used according to the manufacturer's instructions. The reaction protocol started with 5 min pre-incubation at 95°C, followed by 50 cycles of amplification consisting of 10 s denaturation at 95°C, 15 s annealing at 60°C and 20 s elongation at 72°C. For the acquisition of a melting curve, fluorescence was measured during the step-wise increase in temperature from 65°C to 97°C. Relative expression levels were obtained using the $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen, 2001). GeNorm (Vandesompele et al., 2002) identified UBQ and 18S as most stable reference genes. All used primers had an efficiency of >1,8 and their correct product was confirmed by sequencing. A complete list of primer sequences can be found in **Supplementary Table S1**.

GA Quantification

Material (about 150 mg fresh weight of the youngest fully expanded leaves) was suspended in 80% methanol–1% acetic acid containing internal standards and mixed by shaking during 1 hour at 4°C. The extract was kept a − 20°C overnight and then centrifuged and the supernatant dried in a vacuum evaporator. The dry residue was dissolved in 1% acetic acid and passed through a Oasis HLB (reverse-phase) column as described in (Seo et al., 2011). The dried eluate was dissolved in 5% acetonitrile–1% acetic acid, and the GAs were separated using an autosampler and reverse-phase UHPLC chromatography (2.6 µm Accucore RP-MS column, 100 mm length x 2.1 mm i.d.; Thermo Fisher Scientific) with a 5 to 50% acetonitrile gradient containing 0.05% acetic acid, at 400 µl/min over 21 min.

The hormones were analyzed with a Q-Exactive mass spectrometer (Orbitrap detector; Thermo Fisher Scientific) by targeted Selected Ion Monitoring. The concentrations of GAs in the extracts were determined using embedded calibration curves and the Xcalibur 4.0 and TraceFinder 4.1 SP1 programs. The internal standards for quantification were the deuterium-labeled hormones.

RNA Sequencing Analysis

For RNA sequencing experiments RNA was isolated as described above and purified with RNeasy kit (Qiagen) according to the manufacturer's instructions. DNAse treatment was performed on column (Qiagen). Concentration and quality of RNA were assessed using Qubit™ RNA BR Assay Kit (Invitrogen) and Bioanalyzer (Agilent), respectively. 3 *µ*g total RNA with RIN ≧8 were sent for sequencing to SciLife Lab, Stockholm. Library preparation was carried out with an Agilent NGS Bravo workstation in 96-well plates with TruSeq Stranded mRNA kit (Illumina) according to the manufacturer's instructions. mRNA was purified through selective binding to poly dT-coated beads and fragmented using divalent cations under elevated temperature. cDNA was synthesized using SuperScript II Reverse Transcriptase (Thermo Fisher Scientific), cleaned with AMPure XP solution (Thermo Fisher Scientific), 3′ adenylated, and ligated to adapters. Fragments were cleaned with AMPure XP beads (Thermo Fisher Scientific), amplified by PCR, and purified with AMPure XP beads (Thermo Fisher Scientific). After washing with 80% ethanol, they were eluted in EB (Qiagen). The quality and concentration of the adapter-ligated libraries were checked on the LabChip GX/HT DNA high sensitivity kit and by Quant-iT, respectively. The libraries were then sequenced using the Illumina NovaSeq-6,000 platform, generating from 20 to 110 million paired-end reads (2 × 150 bp) per sample.

Pre-processing of RNA-Seq Data and Differential Expression Analyses

The data pre-processing was performed as described here: http://www.epigenesys.eu/en/protocols/bio-informatics/1283-guidelines-for-rna-seq-data-analysis. The quality of the raw sequence data

was assessed using FastQC.[1] Residual ribosomal RNA (rRNA) contamination was assessed and filtered using SortMeRNA [v2.1 (Kopylova et al., 2012); settings--log --paired_in --fastx--sam --num_alignments 1] using the rRNA sequences provided with SortMeRNA (rfam-5 s-database-id98.fasta, rfam-5.8 s-database-id98.fasta, silva-arc-16 s-database-id95.fasta, silva-bac-16 s-database-id85.fasta, silva-euk-18 s-database-id95.fasta, silva-arc-23 s-database-id98.fasta, silva-bac-23 s-database-id98.fasta and silva-euk-28 s-database-id98.fasta). Data were then filtered to remove adapters and trimmed for quality using Trimmomatic [v0.39 (Bolger et al., 2014); settings TruSeq3-PE-2.fa:2:30:10 LEADING:3 SLIDINGWINDOW:5:20 MINLEN:50]. After both filtering steps, FastQC was run again to ensure that no technical artefacts were introduced. Filtered reads were pseudo-aligned to v1.1 of the *P. tremula* transcripts {retrieved from the PopGenIE resource (Sundell et al., 2015) using salmon [v1.1.0 (Patro et al., 2017)], with non-default parameters --gcBias--seqBias --validateMappings} against an index containing the *P. tremula* v1.1 genome sequence as decoy. Statistical analysis of single-gene differential expression between conditions was performed in R (v4.0.0; R Core Team 2020) using the Bioconductor [v3.10 (Huber et al., 2015)] DESeq2 package [v1.28.1 (Love et al., 2014)]. FDR adjusted values of p were used to assess significance; a common threshold of 1% was used throughout. For the data quality assessment (QA) and visualization, the read counts were normalized using a variance stabilizing transformation as implemented in DESeq2. The biological relevance of the data—for example, biological replicates similarity—was assessed by principal component analysis and other visualizations (e.g., heatmaps), using custom R scripts, available from https://github.com/DomeniqueA/SVL. The raw data are available from the European Nucleotide Archive[2] under the accession number PRJEB46749.

Protein Stability Assay

WT and SVLoe plants were grown in LD (18 h light, 20°C/6 h dark, 18°C) for 4 weeks before the experiment started. Upon shift to SD, half of the plants were transferred to regular SD (14 h light, 20°C/ 10 h dark, 18°C), while the other half were transferred to cold SD (14 h light, 15°C/10 h dark, 10°C). Proteins were isolated and visualized on a Western blot using anti-myc antibodies (Agrisera).

Chromatin Immunoprecipitation Analysis

WT and SVPoe plants were grown in LD. Per genotype, one fully expanded leaf was harvested from each of four biological replicates at ZT 18 and cut into small pieces. These were cross-linked in 50 ml PBS buffer +1% formaldehyde and vacuum (4 times 5 min). The reaction was stopped with addition of glycine to a final concentration of 100 mM. The pieces of leaves were frozen in liquid N_2 and ground to fine powder. Nuclei were extracted, lysed in nuclei isolation buffer [50 mM HEPES pH 7.4, 5 mM $MgCl_2$, 25 mM NaCl, 5% sucrose, 30% glycerol, 0.25% Triton X-100, 0.1% ß-mercaptoethanol, 0.1% proteinase inhibitor cocktail (Sigma)] and sonicated, resulting in DNA fragments of 500–1,000 base pair length. For immunoprecipitation, 300 µl of the nuclear extract were homogenized with 200 µl IP buffer (80 mM Tris-HCl pH 7.4, 230 mM NaCl, 1.7% NP40, 0.17% DOC) followed by 1 µl 1 M DTT, 1 µl protease inhibitor cocktail, 1 µl 10 mg/ml RNase A and 5 µl of a monoclonal myc antibody (ab32, Abcam). The mixture was incubated under soft agitation at 4°C over night and centrifuged at full speed for 15 min at 4°C. 40 µl Protein A beads were added into the supernatant and incubation was continued for another 2 hours with soft agitation at 4°C. Protein beads were first washed two times with ice-cold low salt buffer (20 mM Tris-HCl pH 8, 150 mM NaCl, 0.1%SDS, 1% Triton X-100, and 2 mM EDTA), and two times with high salt buffer (20 mM Tris-HCl pH 8, 500 mM NaCl, 0.1%SDS, 1% Triton X-100, and 2 mM EDTA). Then, beads were washed two times with ice-cold LiCl buffer (10 mM Tris-HCl pH 8, 250 mM LiCl, 1% Igepal Ca-630, 1% DOC, and 1 mM EDTA). Chromatins were eluted from the beads with elution buffer (100 mM $NaHCO_3$, 1% SDS) at 65°C for 20 min. To de-crosslink the extract, it was incubated with proteinase K (10 ng/ml) for 1 hour at 55°C. Afterward, DNA was extracted by Chromatin immunoprecipitation (ChIP) DNA Clean & Concentrator Kits (Biosite D5205). Quantities of immunoprecipitations were quantified using SYBR green (Roche) and the iQ5 light cycler (Bio-Rad). A similarly treated extract from WT without tagged protein was used as control. Primers used for amplification of genomic fragments are listed in **Supplementary Table S1**.

RESULTS

SVL Is Functionally Similar to *AtSVP*

Populus has one orthologous gene to Arabidopsis *SVP* called *SVL* (Singh et al., 2018; **Supplementary Figure S1A**). *AtSVP* and *PtSVL* share 66% identity on the amino acid level, making *SVL* the only likely *SVP* ortholog compared to other MADS domain-containing genes in *Populus* (**Supplementary Figure S1A**; Singh et al., 2018). Because of the high similarity to *SVP* (**Supplementary Figure S1B**), we hypothesized that *SVL* could act like *SVP* in Arabidopsis by having a function in the photoperiodic response in leaves. The *svp* mutant is early flowering (Hartmann et al., 2000) and we tested whether *SVL* could rescue this phenotype. For this we expressed *SVL* cDNA under the control of the *35S* promoter in *svp-32* plants. Flowering time was determined by counting rosette and cauline leaves. These plants produced significantly more leaves than *svp-32* mutants and wild-type (WT) Arabidopsis plants before developing the first flowers (**Supplementary Figure S2**). These results imply that the functionality of has been conserved between Arabidopsis *SVP* and *Populus SVL*.

Expression of *Populus SVL* Is Induced in Leaves During Short Days

Populus SVL function has previously been described in the shoot apex in relationship to the regulation of bud dormancy (Singh et al., 2018, 2019). We wanted to investigate to what extent

[1]http://www.bioinformatics.babraham.ac.uk/projects/fastqc/
[2]https://www.ebi.ac.uk/ena/

leaf-expressed SVL also contributes to the regulation of growth cessation and bud set. Investigation of the *SVL* annual expression pattern in local adult aspen trees (Umeå, Sweden) sampled in the middle of the day showed that it is highly expressed in leaves during the short days (SDs) of late summer and early autumn (**Figure 1A**), after *FT2* expression declined (**Supplementary Figure S3**), and to higher levels than what can be detected in buds. To test whether this expression pattern is consistent in juvenile trees grown in controlled growth conditions, we checked the diurnal expression pattern of *SVL* in leaves first in long days (LD) and after 2 weeks of SD treatment (**Figure 1B**). In these conditions, one of the first genes to respond is *FT2* which shows a clear downregulation after 2 weeks in 14 h SD (Ding et al., 2018). In long days, *SVL* displayed a minor peak of expression at ZT 6–8 (**Figure 1B**). The *SVL* expression increased after shift to SD and showed a prominent morning peak at around 4 hours after dawn, suggesting a role for *SVL* in the photoperiodic response in leaves. We then also wanted to know if a decrease in ambient temperature could increase the stability of the SVL protein, as has been shown for Arabidopsis SVP

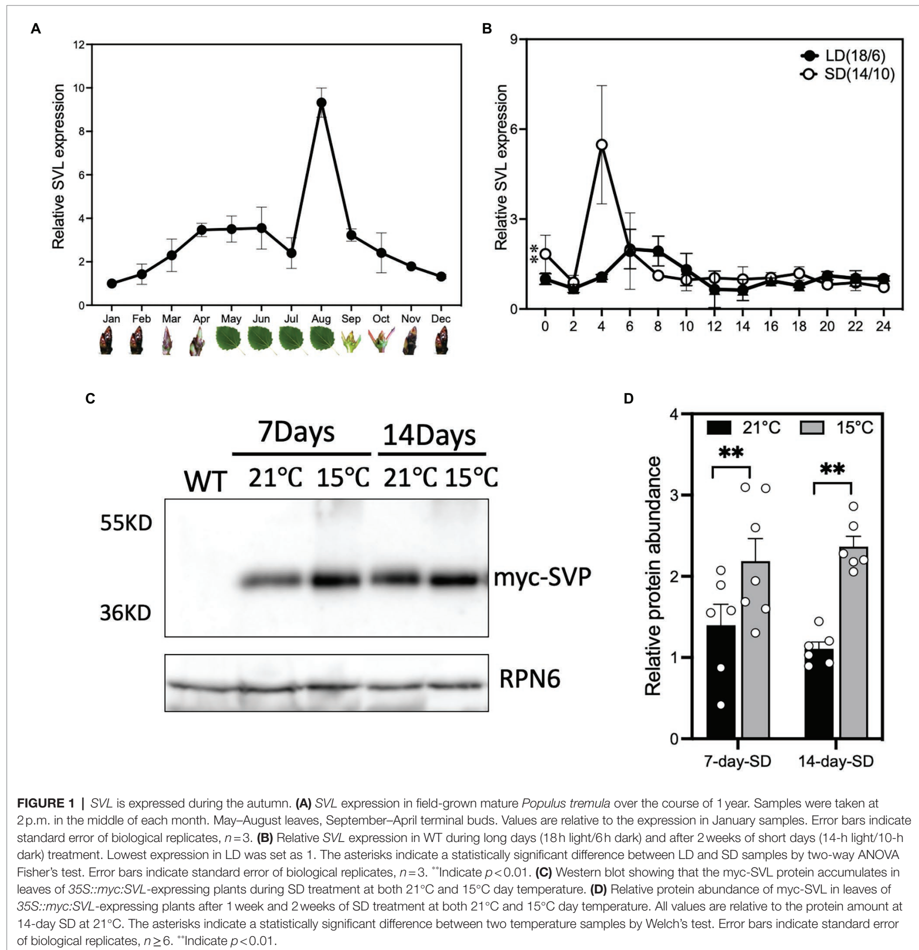

FIGURE 1 | *SVL* is expressed during the autumn. **(A)** *SVL* expression in field-grown mature *Populus tremula* over the course of 1 year. Samples were taken at 2 p.m. in the middle of each month. May–August leaves, September–April terminal buds. Values are relative to the expression in January samples. Error bars indicate standard error of biological replicates, $n = 3$. **(B)** Relative *SVL* expression in WT during long days (18 h light/6 h dark) and after 2 weeks of short days (14-h light/10-h dark) treatment. Lowest expression in LD was set as 1. The asterisks indicate a statistically significant difference between LD and SD samples by two-way ANOVA Fisher's test. Error bars indicate standard error of biological replicates, $n = 3$. **Indicate $p < 0.01$. **(C)** Western blot showing that the myc-SVL protein accumulates in leaves of *35S::myc:SVL*-expressing plants during SD treatment at both 21°C and 15°C day temperature. **(D)** Relative protein abundance of myc-SVL in leaves of *35S::myc:SVL*-expressing plants after 1 week and 2 weeks of SD treatment at both 21°C and 15°C day temperature. All values are relative to the protein amount at 14-day SD at 21°C. The asterisks indicate a statistically significant difference between two temperature samples by Welch's test. Error bars indicate standard error of biological replicates, $n \geq 6$. **Indicate $p < 0.01$.

(Lee et al., 2013). When plants expressing myc-tagged SVL from a constitutive promoter were exposed to lower temperatures than our standard SD treatment, the accumulation of SVL protein was increased (**Figures 1C,D**). This indicates that there could be a role for leaf-expressed *SVL* in response to both short photoperiods and lower temperatures.

SVL Is Promoting SD Induced Growth Cessation

To test the role of *SVL*, we generated *SVL* RNAi and *SVL* over-expressing (*SVLoe*) trees. Downregulation was up to 80% effective, while overexpression resulted in a six-fold increase of *SVL* expression at ZT17 compared to wild-type T89 (WT; **Supplementary Figure S4**). Neither downregulation nor overexpression of *SVL* had a striking effect on vegetative growth; all transgenic lines were indistinguishable from WT controls after 3 weeks in LD (**Figure 2A**). After shift to SD, poplars respond with growth cessation and bud set. We used previously described bud scores (Ibáñez et al., 2010) to test the speed of SD response in three independent transgenic lines per construct. *SVL* RNAi plants showed a small but consistent delay of bud set compared to WT (**Figure 2B**). Both growth cessation (score 2) and bud set (score 1) were delayed by *ca.* 1 week. *SVL*oe plants on the other hand ceased growth several weeks earlier than WT (**Figure 2C**). This indicates that *SVL* is a repressor of vegetative growth and promoter of SD-induced growth cessation.

SVL Acts in Both Leaf and Shoot Apex to Promote SD-Induced Growth Cessation

Expression of both *FT2* and *GA20oxidase* in rootstocks of grafted trees is sufficient to significantly delay growth cessation and bud set (Miskolczi et al., 2019). We then asked if the role of SVL in modulating the timing of growth cessation is due to SVL activity in the leaf or shoot apex or both. To investigate this we performed reciprocal graftings of *SVL* RNAi and wild-type trees and compared the timing of growth cessation to trees where scions had been grafted to their own stock. In both types of heterografts growth cessation was delayed to the same extent as in SVL RNAi homografts suggesting that SVL modulates the timing of growth cessation trough activity both in the leaf and in the shoot apex (**Figure 2D**).

SVL Regulates Growth Cessation Through Repression of *FT2* Expression and Gibberellin Biosynthesis

We then tested whether the different speeds of response in transgenic lines were due to altered expression of *FT2*. After 2 weeks of SD treatment, *FT2* expression had ceased in WT and *SVLoe*, while it was still strongly expressed in the *SVL* RNAi lines (**Figure 3A**). In addition to *FT2*, gibberellins are also known to affect growth cessation and bud set. We therefore analyzed the expression of a *GA20 oxidase2*, a key enzyme in gibberellin biosynthesis and found that it was increased in the leaves of *SVL* RNAi lines, while being reduced in SVLoe (**Figure 3B**). We focused on *GA20 oxidase2* because we have found that it is the predominantly expressed *GA20 oxidase* gene in leaves (not shown). Consequently, the amount of the active gibberellin GA_1 was increased in leaves of *SVL* RNAi lines in both LD and SD (**Supplementary Figure S5**) compared to wild type. This suggests that SVL can influence the timing of growth cessation through a repression of both the expression of *FT2* and the biosynthesis of gibberellins.

SVL Binds to the Promoters of Its Downstream Targets

We then asked if *FT2* and *GA20 oxidase2* are direct targets of SVL. Since *SVL* is a MADS-box transcription factor, we tested the ability of the SVL protein to bind to the promoter region of these genes. For that we performed a chromatin immunoprecipitation assay in leaves of WT and our myc-tagged SVLoe lines. Quantification by qPCR showed significant enrichments of six fragments surrounding the *FT2* transcriptional start site, up to 2.5 kb upstream and 1 kb downstream (**Figure 3C**). Enrichment at the *GA20 oxidase2* promoter was significant for four of six fragments (**Figure 3D**). No enrichment could be detected at a control locus (**Figures 3C,D**). These results show that SVL can associate with the promoters of *FT2* and *GA20 oxidase2* and potentially repress their expression through direct binding.

SVL Has a Minor Influence on the Leaf Transcriptome

To better understand what role SVL plays in leaves during SD treatment, we performed RNA sequencing analysis on leaves of wild-type and SVL RNAi plants. Samples were harvested at ZT17 during LD and after one, two, three and 10 weeks of SD treatment, respectively. Major transcriptional changes happened after the shift from LD to SD in both WT and *SVL* RNAi lines with more than 10,000 genes being differentially expressed between the two time points (**Figure 4A; Supplementary Figure S6**). However, no further significant changes were detectable after two and 3 weeks of SD. At the individual time points, only a small number of differentially expressed genes (DEG) could be detected between the two lines (**Figure 4B; Supplementary Figure S6**) and gene ontology (GO) enrichment resulted in no specific terms. These results indicate that the role of SVL in leaves might be limited to the regulation of a very limited set of genes.

DISCUSSION

AtSVP and *PtSVL* Are Functionally Conserved and Repress *FT*

AtSVP is a known floral repressor (Hartmann et al., 2000), acting on the *FT* promoter (Lee et al., 2007). Here we show that *PtSVL* (hereafter *SVL*) is functionally conserved as was recently shown for apple (Falavigna et al., 2021) and previously also, for instance, Kiwi *SVP*-like genes, (Wu et al., 2012). Both SVP and SVL proteins share 66% identity and *SVL* overexpression was sufficient to complement the Arabidopsis *svp-32* mutant phenotype. ChIP analysis showed that SVL could bind to both *FT2* and *GA20 oxidase* promoters, repressing their transcription.

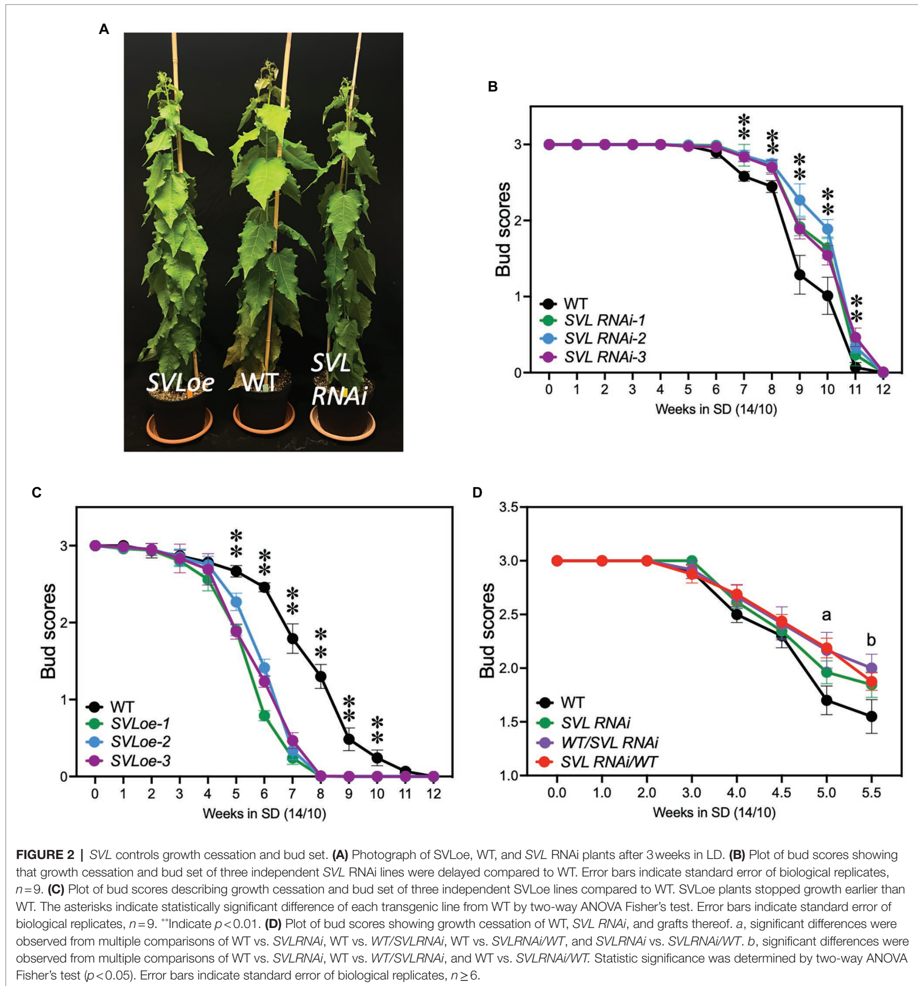

FIGURE 2 | *SVL* controls growth cessation and bud set. **(A)** Photograph of SVLoe, WT, and *SVL* RNAi plants after 3 weeks in LD. **(B)** Plot of bud scores showing that growth cessation and bud set of three independent *SVL* RNAi lines were delayed compared to WT. Error bars indicate standard error of biological replicates, $n=9$. **(C)** Plot of bud scores describing growth cessation and bud set of three independent SVLoe lines compared to WT. SVLoe plants stopped growth earlier than WT. The asterisks indicate statistically significant difference of each transgenic line from WT by two-way ANOVA Fisher's test. Error bars indicate standard error of biological replicates, $n=9$. **Indicate $p<0.01$. **(D)** Plot of bud scores showing growth cessation of WT, *SVL RNAi*, and grafts thereof. *a*, significant differences were observed from multiple comparisons of WT vs. *SVLRNAi*, WT vs. *WT/SVLRNAi*, WT vs. *SVLRNAi/WT*, and *SVLRNAi* vs. *SVLRNAi/WT*. *b*, significant differences were observed from multiple comparisons of WT vs. *SVLRNAi*, WT vs. *WT/SVLRNAi*, and WT vs. *SVLRNAi/WT.* Statistic significance was determined by two-way ANOVA Fisher's test ($p<0.05$). Error bars indicate standard error of biological replicates, $n\geq6$.

This suggests that *SVL* expression in the leaf can fulfil a similar role in poplar as in Arabidopsis, modulating the photoperiodic regulation of *FT* and *GA20 oxidase* expression. To repress downstream targets, AtSVP forms heterodimers with MADS-box proteins like FLM and FLC (Lee et al., 2007, 2013; Posé et al., 2013). In apple, it has recently been shown that the SVP-like protein MdSVPa can form transcriptional complexes with various MADS-box proteins that are expressed in buds at different dormancy-specific phases (Falavigna et al., 2021). It remains to be shown if the SVL activity in the *Populus* leaf is also dependent on one or more MADS-box proteins.

Cold Temperatures Promote SVL Activity

In Arabidopsis, *SVP* is part of the thermosensory pathway, which inhibits flowering in cold conditions (Lee et al., 2007), partially mediated by a stabilization of the SVP protein at colder temperatures

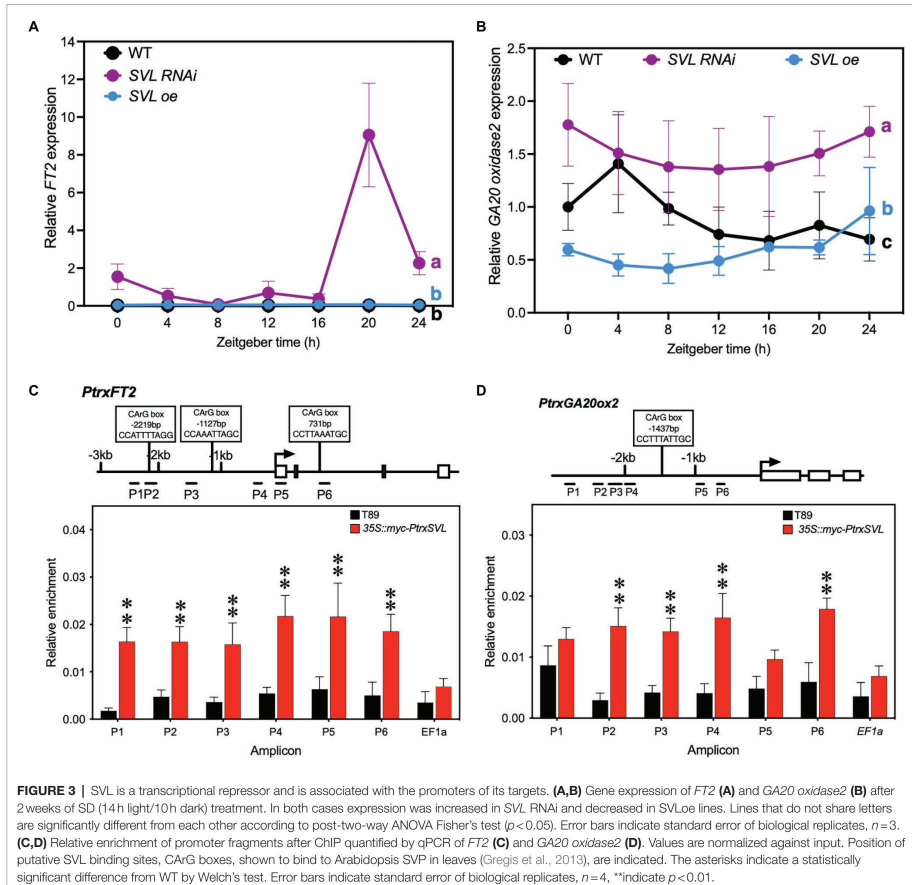

FIGURE 3 | SVL is a transcriptional repressor and is associated with the promoters of its targets. **(A,B)** Gene expression of *FT2* **(A)** and *GA20 oxidase2* **(B)** after 2 weeks of SD (14 h light/10 h dark) treatment. In both cases expression was increased in *SVL* RNAi and decreased in SVLoe lines. Lines that do not share letters are significantly different from each other according to post-two-way ANOVA Fisher's test ($p < 0.05$). Error bars indicate standard error of biological replicates, $n = 3$. **(C,D)** Relative enrichment of promoter fragments after ChIP quantified by qPCR of *FT2* **(C)** and *GA20 oxidase2* **(D)**. Values are normalized against input. Position of putative SVL binding sites, CArG boxes, shown to bind to Arabidopsis SVP in leaves (Gregis et al., 2013), are indicated. The asterisks indicate a statistically significant difference from WT by Welch's test. Error bars indicate standard error of biological replicates, $n = 4$, **indicate $p < 0.01$.

(Lee et al., 2013). Temperatures drop during autumn and we could show that SVL protein was more abundant in a combination of short-day treatment and cold (**Figure 1C**), mimicking autumn conditions. While the timing of growth cessation is mainly regulated by photoperiod, integration of temperature signals through SVL could give trees more flexibility to fine-tune their SD response, it could also be an important factor contributing to SVLs function in buds during the induction of dormancy.

SVL Regulates Growth Cessation Through *FT2* and GAs

There is no strict correlation between the downregulation of *FT2* and the upregulation of *SVL* in the year-around samples (**Figure 1A; Supplementary Figure S3**) confirming that *SVL* is not the primary regulator of the photoperiodic response and the regulation of *FT2*. However, alteration of *SVL* expression levels influenced the timing of growth cessation and bud set upon short-day (SD) treatment. We could show that this was caused by an effect on *FT2* expression and levels of GAs. Consistently, *SVL* RNAi lines had increased *FT2* expression and a delayed growth cessation, while SVLoe plants had strongly reduced *FT2* expression and early growth cessation. Furthermore, levels of the bioactive gibberellin GA_1 were increased in *SVL* RNAi plants. This suggests that the role of *SVL* is to modulate the timing of the photoperiodic response.

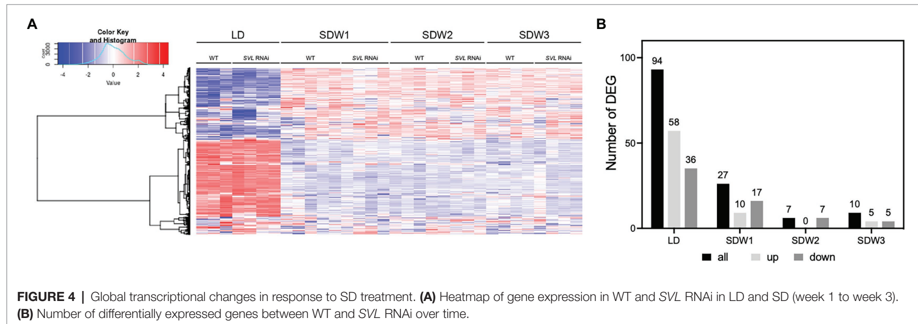

FIGURE 4 | Global transcriptional changes in response to SD treatment. **(A)** Heatmap of gene expression in WT and *SVL* RNAi in LD and SD (week 1 to week 3). **(B)** Number of differentially expressed genes between WT and *SVL* RNAi over time.

The interest for the role of *SVP*-like genes in the regulation of dormancy originally stemmed from studies of the *evergrowing* mutant (*evg*) in peach (*Prunus persica*), which fails to form terminal vegetative buds and continues to grow indeterminately under dormancy-inducing conditions. The *evg* locus was mapped and found to contain a large deletion of six tandemly repeated *SVP*-like genes called *DAM* genes (Bielenberg et al., 2008). The simultaneous downregulation of the expression of three SVP/DAM genes in apple through RNAi also caused an evergrowing phenotype (Wu et al., 2021). Because of the focus on the expression and function of these genes during dormancy-induction in the bud, to our knowledge, there appears to be no characterization of the photoperiodic regulation of expression of *FT*-like genes in leaves of the evergrowing mutants. It is an interesting possibility that the loss of *SVP/DAM*-like expression in the leaves of peach and apple could contribute to an inability to keep *FT* expression down in response to the short-day signal. Since downregulation of *FT* expression appears to be a prerequisite for growth cessation and bud set (Böhlenius et al., 2006), this could at least partially explain the fact that the evergrowing mutants are not only failing to establish dormancy, but are also not able to enter into growth cessation and bud set—which is also a prerequisite for the entry into dormancy.

There are several other *SVP*-like and *DAM*-like genes in *Populus* (**Supplementary Figure S1**). In order to fully understand the role of the individual genes, it will be important to know if complete knockouts of *SVL* expression through CRISPR-Cas9-mediated gene editing, or combinations of the deactivation of several *SVP/DAM*-like genes, also leads to an evergrowing phenotype in poplar trees.

SVL Regulates Growth Cessation and Bud Set in Both Leaves and Shoot Apices

Previous studies showed the role of *SVL* in the shoot apex, both during the establishment of dormancy during SD (Singh et al., 2019) and its release during winter (Singh et al., 2018). Both pathways work through differential regulation of gibberellin biosynthesis with *SVL* inducing *GA2 oxidase* in the developing bud, presumably in order to reduce the amount of active GAs that can reach the shoot apex, while, at the same time, repressing the expression of *GA20 oxidase* to downregulate GA biosynthesis. We show that *SVL* functions through a similar pathway in leaves. Grafting studies in *Populus* have shown that not only *FT2* but also *GA20 oxidase* expression in leaves, clearly affect growth cessation and bud set (Miskolczi et al., 2019), suggesting that both FT2 and GAs are moving from leaf to shoot apex in order to modulate this process. Our data show that SVL affects the expression of both *FT2* and *GA20 oxidase* in leaves and that SVL associates with both the *FT2* and *GA20 oxidase2* loci that both contain putative SVL binding sites in the form of CArG boxes shown to bind to Arabidopsis SVP (Gregis et al., 2013). However, in contrast to our findings here, Singh et al. (2019) could not detect an interaction between SVL and the *GA20 oxidase* loci. This discrepancy could be attributed to the use of different protocols and primers for the ChIP assay, but could also be related to the fact that we looked for interactions in leaf samples rather than in shoot apex samples as used by Singh et al. (2019). SVP-like proteins acts in complexes with other MADS-box proteins and co-transcription factors. These other factors are likely to differ between leaf and shoot apex samples and might affect the SVL binding. They might also contribute to an indirect binding to the *GA20 oxidase* locus in the ChIP assay.

One of the direct targets of *FT2* in the shoot apex is the gene *Like-AP1* (*LAP1*) which in turn mediates the regulation of cell cycle-related genes that are downregulated during growth cessation. (Azeez et al., 2014). Is it possible that *LAP1* is also a SVL target? Although our grafting experiments did not allow us to collect enough material to analyze the expression of target genes in the shoot apex, we did check for *LAP1* expression in leaves of *SVL* RNAi plants. *LAP1* is normally expressed to very low levels in leaves, but is dramatically upregulated in leaves of *SVL* RNAi trees (**Supplementary Figure S7**). The *LAP1* locus also contain several potential SVL binding sites (**Supplementary Figure S7**). Although the relevance of the *LAP1* regulation in the leaf is unclear, it shows a potential for SVL to also control growth cessation through a repression of *LAP1* expression in the shoot apex that could be part of

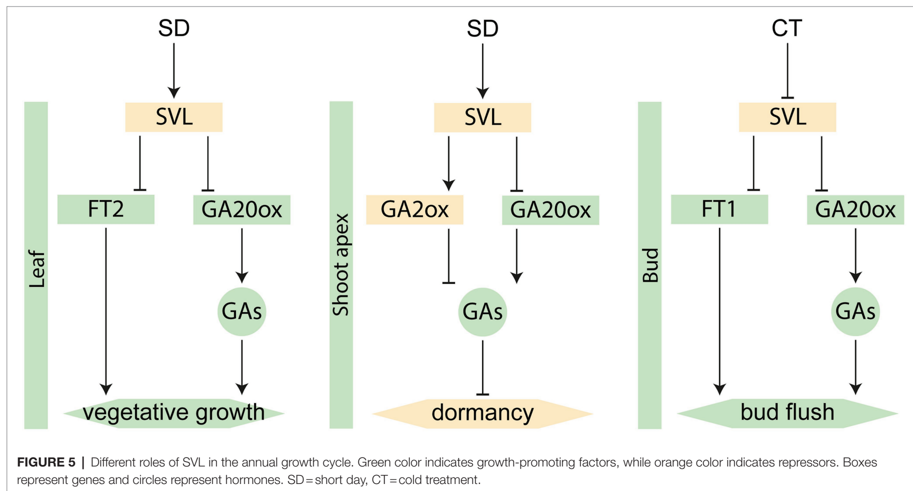

FIGURE 5 | Different roles of SVL in the annual growth cycle. Green color indicates growth-promoting factors, while orange color indicates repressors. Boxes represent genes and circles represent hormones. SD = short day, CT = cold treatment.

the explanation to the contribution of SVL expression for growth cessation in both leaf and shoot apex (**Figure 2D**).

Our data suggest that SVL has only minor effects on the leaf transcriptome, suggesting that it might have relative few targets in the leaf compared to in the shoot apex. When the dataset was specifically quired both *FT2* and *GA20 oxidase2* were not found to be higher expressed in the SVL RNAi dataset compared to wild type after the shift to SD (not shown). The reason for these genes not appearing in the DEG list is probably due to relatively low expression levels and/or the fact that this was samples from a single time-point at the end of the day when expression was not significantly different.

Additionally, *SVL* has been shown to affect *FT1* expression in buds. Thus, *SVL* is involved in three similar pathways regulating different aspects of the annual growth cycle of *Populus* trees (**Figure 5**); First through a regulation of the photoperiod response in leaves, contributing to the downregulation of *FT2* and *GA20 oxidase*, leading to growth cessation and bud set (this study); Then as an inducer of *GA2 oxidase* and *CALLOSE SYNTHASE 1* in the buds to prevent growth-inducing signals to reach the shoot apex to establish dormancy (Singh et al., 2019); And finally, its expression is reduced in response to low temperatures in winter, leading to a relieved repression of *FT1* and reduced expression of *TCP18/BRC1*, hypothesized to lead to bud break (Singh et al., 2018). Consequently, SVL serves as an important regulator of both the beginning and end of the growing season as well as the establishment of winter dormancy. Interestingly, these three different phases of *SVL* regulation corresponds to three different clusters of expression profiles for *DAM* and *SVP*-like genes from different fruit tree species (Falavigna et al., 2019, 2021). In Rosaceae, *DAM* and *SVP*-like genes have evolved into different clades. In apple, the SVP-like protein SVPa provides DNA-binding activity to different complexes with DAM proteins that are specifically expressed during different phases of the dormancy cycle (Falavigna et al., 2021). Our data from growth cessation together with the previous data from bud set and bud break (Singh et al., 2018, 2019) suggest that this could also be true in *Populus* trees, with SVL serving as the central DNA-binding hub.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ebi.ac.uk/ena, PRJEB46749.

AUTHOR CONTRIBUTIONS

DA, JZ, and ON planned the research. DA, JZ, BZ, MR, KL, and AM performed experiments and analyzed data. DA and ON wrote the manuscript and all authors reviewed and approved its final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.823019/full#supplementary-material

REFERENCES

Andrés, F., Porri, A., Torti, S., Mateos, J., Romera-Branchat, M., García-Martínez, J. L., et al. (2014). SHORT VEGETATIVE PHASE reduces gibberellin biosynthesis at the Arabidopsis shoot apex to regulate the floral transition. *Proc. Natl. Acad. Sci. U. S. A.* 111, E2760–E2769. doi: 10.1073/pnas.1409567111

Azeez, A., Miskolczi, P., Tylewicz, S., and Bhalerao, R. P. (2014). A tree ortholog of APETALA1 mediates photoperiodic control of seasonal growth. *Curr. Biol.* 24, 717–724. doi: 10.1016/j.cub.2014.02.037

Bielenberg, D. G., Wang, Y. E., Li, Z., Zhebentyayeva, T., Fan, S., Reighard, G. L., et al. (2008). Sequencing and annotation of the evergrowing locus in peach [Prunus persica (L.) Batsch] reveals a cluster of six MADS-box transcription factors as candidate genes for regulation of terminal bud formation. *Tree Genet. Genom.* 4, 495–507. doi: 10.1007/s11295-007-0126-9

Böhlenius, H., Huang, T., Charbonnel-Campaa, L., Brunner, A. M., Jansson, S., Strauss, S. H., et al. (2006). CO/FT regulatory module controls timing of flowering and seasonal growth cessation in trees. *Science* 312, 1040–1043. doi: 10.1126/science.1126038

Bolger, A. M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30, 2114–2120. doi: 10.1093/bioinformatics/btu170

Chang, S., Puryear, J., and Cairney, J. (1993). A simple and efficient method for isolating RNA from pine trees. *Plant Mol. Biol. Report.* 11, 113–116. doi: 10.1007/BF02670468

Clough, S. J., and Bent, A. F. (1998). Floral dip: a simplified method for agrobacterium-mediated transformation of *Arabidopsis thaliana*. *Plant J.* 16, 735–743. doi: 10.1046/j.1365-313x.1998.00343.x

Ding, J., Böhlenius, H., Rühl, M. G., Chen, P., Sane, S., Zambrano, J. A., et al. (2018). GIGANTEA-like genes control seasonal growth cessation in Populus. *New Phytol.* 218, 1491–1503. doi: 10.1111/nph.15087

Eriksson, M. E., Hoffman, D., Kaduk, M., Mauriat, M., and Moritz, T. (2015). Transgenic hybrid aspen trees with increased gibberellin (GA) concentrations suggest that GA acts in parallel with FLOWERING LOCUS T 2 to control shoot elongation. *New Phytol.* 205, 1288–1295. doi: 10.1111/nph.13144

Eriksson, M. E., Israelsson, M., Olsson, O., and Moritz, T. (2000). Increased gibberellin biosynthesis in transgenic trees promotes growth, biomass production and xylem fiber length. *Nat. Biotechnol.* 18, 784–788. doi: 10.1038/77355

Falavigna, V. D. S., Guitton, B., Costes, E., and Andrés, F. (2019). I want to (bud) break free: the potential role of DAM and SVP-like genes in regulating dormancy cycle in temperate fruit trees. *Front. Plant Sci.* 9:1990. doi: 10.3389/fpls.2018.01990

Falavigna, V. D. S., Severing, E., Lai, X., Estevan, J., Farrera, I., Hugouvieux, V., et al. (2021). Unraveling the role of MADS transcription factor complexes in apple tree dormancy. *New Phytol.* 232, 2071–2088. doi: 10.1111/nph.17710

Gregis, V., Andrés, F., Sessa, A., Guerra, R. F., Simonini, S., Mateos, J. L., et al. (2013). Identification of pathways directly regulated by SHORT VEGETATIVE PHASE during vegetative and reproductive development in Arabidopsis. *Genome Biol.* 14:R56. doi: 10.1186/gb-2013-14-6-r56

Hartmann, U., Höhmann, S., Nettesheim, K., Wisman, E., Saedler, H., and Huijser, P. (2000). Molecular cloning of SVP: a negative regulator of the floral transition in Arabidopsis. *Plant J.* 21, 351–360. doi: 10.1046/j.1365-313x.2000.00682.x

Hsu, C.-Y., Adams, J. P., Kim, H., No, K., Ma, C., Strauss, S. H., et al. (2011). FLOWERING LOCUS T duplication coordinates reproductive and vegetative growth in perennial poplar. *Proc. Natl. Acad. Sci. U. S. A.* 108, 10756–10761. doi: 10.1073/pnas.1104713108

Hsu, C.-Y., Liu, Y., Luthe, D. S., and Yuceer, C. (2006). Poplar FT2 shortens the juvenile phase and promotes seasonal flowering. *Plant Cell* 18, 1846–1861. doi: 10.1105/tpc.106.041038

Huber, W., Carey, V. J., Gentleman, R., Anders, S., Carlson, M., Carvalho, B. S., et al. (2015). Orchestrating high-throughput genomic analysis with bioconductor. *Nat. Methods* 12, 115–121. doi: 10.1038/nmeth.3252

Ibáñez, C., Kozarewa, I., Johansson, M., Ögren, E., Rohde, A., and Eriksson, M. E. (2010). Circadian clock components regulate entry and affect exit of seasonal dormancy as well as winter hardiness in Populus trees. *Plant Physiol.* 153, 1823–1833. doi: 10.1104/pp.110.158220

Karimi, M., Inzé, D., and Depicker, A. (2002). GATEWAY™ vectors for agrobacterium-mediated plant transformation. *Trends Plant Sci.* 7, 193–195. doi: 10.1016/S1360-1385(02)02251-3

Kobayashi, Y., Kaya, H., Goto, K., Iwabuchi, M., and Araki, T. (1999). A pair of related genes with antagonistic roles in mediating flowering signals. *Science* 286, 1960–1962. doi: 10.1126/science.286.5446.1960

Kopylova, E., Noé, L., and Touzet, H. (2012). SortMeRNA: fast and accurate filtering of ribosomal RNAs in metatranscriptomic data. *Bioinformatics* 28, 3211–3217. doi: 10.1093/bioinformatics/bts611

Lee, J. H., Ryu, H.-S., Chung, K. S., Posé, D., Kim, S., Schmid, M., et al. (2013). Regulation of temperature-responsive flowering by MADS-box transcription factor repressors. *Science* 342, 628–632. doi: 10.1126/science.1241097

Lee, J. H., Yoo, S. J., Park, S. H., Hwang, I., Lee, J. S., and Ahn, J. H. (2007). Role of SVP in the control of flowering time by ambient temperature in Arabidopsis. *Genes Dev.* 21, 397–402. doi: 10.1101/gad.1518407

Li, Z., Reighard, G. L., Abbott, A. G., and Bielenberg, D. G. (2009). Dormancy-associated MADS genes from the EVG locus of peach [Prunus persica (L.) Batsch] have distinct seasonal and photoperiodic expression patterns. *J. Exp. Bot.* 60, 3521–3530. doi: 10.1093/jxb/erp195

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 25, 402–408. doi: 10.1006/meth.2001.1262

Love, M. I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15:550. doi: 10.1186/s13059-014-0550-8

Miskolczi, P., Singh, R. K., Tylewicz, S., Azeez, A., Maurya, J. P., Tarkowská, D., et al. (2019). Long-range mobile signals mediate seasonal control of shoot growth. *Proc. Natl. Acad. Sci. U. S. A.* 116, 10852–10857. doi: 10.1073/pnas.1902199116

Moser, M., Asquini, E., Miolli, G. V., Weigl, K., Hanke, M.-V., Flachowsky, H., et al. (2020). The MADS-box gene MdDAM1 controls growth cessation and bud dormancy in apple. *Front. Plant Sci.* 11:1003. doi: 10.3389/fpls.2020.01003

Nilsson, O., Aldén, T., Sitbon, F., Little, C. A., Chalupa, V., Sandberg, G., et al. (1992). Spatial pattern of cauliflower mosaic virus 35S promoter-luciferase expression in transgenic hybrid aspen trees monitored by enzymatic assay and non-destructive imaging. *Transgenic Res.* 1, 209–220. doi: 10.1007/BF02524751

Patro, R., Duggal, G., Love, M. I., Irizarry, R. A., and Kingsford, C. (2017). Salmon provides fast and bias-aware quantification of transcript expression. *Nat. Methods* 14, 417–419. doi: 10.1038/nmeth.4197

Pin, P., and Nilsson, O. (2012). The multifaceted roles of FLOWERING LOCUS T in plant development. *Plant Cell Environ.* 35, 1742–1755. doi: 10.1111/j.1365-3040.2012.02558.x

Posé, D., Verhage, L., Ott, F., Yant, L., Mathieu, J., Angenent, G. C., et al. (2013). Temperature-dependent regulation of flowering by antagonistic FLM variants. *Nature* 503, 414–417. doi: 10.1038/nature12633

Rieu, I., Ruiz-Rivero, O., Fernandez-Garcia, N., Griffiths, J., Powers, S. J., Gong, F., et al. (2008). The gibberellin biosynthetic genes AtGA20ox1 and AtGA20ox2 act, partially redundantly, to promote growth and development throughout the Arabidopsis life cycle. *Plant J.* 53, 488–504. doi: 10.1111/j.1365-313X.2007.03356.x

Rohde, A., and Bhalerao, R. P. (2007). Plant dormancy in the perennial context. *Trends Plant Sci.* 12, 217–223. doi: 10.1016/j.tplants.2007.03.012

Searle, I., He, Y., Turck, F., Vincent, C., Fornara, F., Kröber, S., et al. (2006). The transcription factor FLC confers a flowering response to vernalization by repressing meristem competence and systemic signaling in Arabidopsis. *Genes Dev.* 20, 898–912. doi: 10.1101/gad.373506

Seo, M., Jikumaru, Y., and Kamiya, Y. (2011). Profiling of hormones and related metabolites in seed dormancy and germination studies. *Methods Mol. Biol.* 773, 99–111. doi: 10.1007/978-1-61779-231-1_7

Singh, R. K., Maurya, J. P., Azeez, A., Miskolczi, P., Tylewicz, S., Stojkovič, K., et al. (2018). A genetic network mediating the control of bud break in hybrid aspen. *Nat. Commun.* 9, 1–10. doi: 10.1038/s41467-018-06696-y

Singh, R. K., Miskolczi, P., Maurya, J. P., and Bhalerao, R. P. (2019). A tree ortholog of SHORT VEGETATIVE PHASE floral repressor mediates photoperiodic control of bud dormancy. *Curr. Biol.* 29, 128–133. doi: 10.1016/j.cub.2018.11.006

Sundell, D., Mannapperuma, C., Netotea, S., Delhomme, N., Lin, Y.-C., Sjödin, A., et al. (2015). The plant genome integrative explorer resource: PlantGenIE.Org. *New Phytol.* 208, 1149–1156. doi: 10.1111/nph.13557

Valverde, F., Mouradov, A., Soppe, W., Ravenscroft, D., Samach, A., and Coupland, G. (2004). Photoreceptor regulation of CONSTANS protein in photoperiodic flowering. *Science* 303, 1003–1006. doi: 10.1126/science.1091761

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., et al. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 3, 1–12. doi: 10.1186/gb-2002-3-7-research0034

Wang, J.-W. (2014). Regulation of flowering time by the miR156-mediated age pathway. *J. Exp. Bot.* 65, 4723–4730. doi: 10.1093/jxb/eru246

Wu, R., Cooney, J., Tomes, S., Rebstock, R., Karunairetnam, S., Allan, A. C., et al. (2021). RNAi-mediated repression of dormancy-related genes results in evergrowing apple trees. *Tree Physiol.* 41, 1510–1523. doi: 10.1093/treephys/tpab007

Wu, R., Tomes, S., Karunairetnam, S., Tustin, S. D., Hellens, R. P., Allan, A. C., et al. (2017). SVP-like MADS box genes control dormancy and budbreak in apple. *Front. Plant Sci.* 8:477. doi: 10.3389/fpls.2017.00477

Wu, R.-M., Walton, E. F., Richardson, A. C., Wood, M., Hellens, R. P., and Varkonyi-Gasic, E. (2012). Conservation and divergence of four kiwifruit SVP-like MADS-box genes suggest distinct roles in kiwifruit bud dormancy and flowering. *J. Exp. Bot.* 63, 797–807. doi: 10.1093/jxb/err304

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Table S1: List of used primers

ChIP	
PtraGA20ox_P1f	AAGGGAGATTGAAGCATGTT
PtraGA20ox_P1r	CCTATGATCTCCCTCTCTAC
PtraGA20ox_P2f	TCGGAAGTTGGAGAAACCT
PtraGA20ox_P2r	TAATTCAAGCCATGGGAGT
PtraGA20ox_P3f	CTCGGCATCATCACCTATAT
PtraGA20ox_P3r	CTGGTTTTGTTGATTGTGGT
PtraGA20ox_P4f	TCAACAAAACCAGTCCCAAA
PtraGA20ox_P4r	GGGCAGGGACATTTTATTCT
PtraGA20ox_P5f	TGAAAAGCAACCGCAATGAT
PtraGA20ox_P5r	TCCATATACCGAATGCCTAA
PtraGA20ox_P6f	GTCCCTCAAAACAGATTTCT
PtraGA20ox_P6r	AACCCATAACTTCCTGTTCT
FT2chipF1	CCATATATCTTCGAGCGTTGCA
FT2chipR1	AAGCTGGGTTCGAGTAAAG
FT2chipF2	GTATGCCGAGATGGAGACT
FT2chipR2	TCATAAAGCATGCATGGACC
FT2chipF3	GATTCGTAAGTGTACACTCG
FT2chipR3	CTCACCATAATAGTCCTATC
FT2chipF4	GGAAAACGTGAATCTGGCTC
FT2chipR4	GGTGATGCCTCGAGGCTCA
FT2chipF5	ATGCCTAGGGATAGAGACCCT
FT2chipR5	AGAAGGTCCTTAGATCTTC
FT2chipF6	GGTGATGGTGAGTCCTTGGA
FT2chipR6	CGTCGACGTACAGGTGAAGT
qPCR	
18SF	TCAACTTTCGATGGTAGGATAGAG
18SR	CCGTGTCAGGATTGGGTAATTT
UBQF	GTTGATTTTTGCTGGGAAGC
UBQR	GATCTTGGCCTTCACGTTGT
FT2F	AGCCCAAGGCCTACAGCAGGAA
FT2R	GGGAATCTTTCTCTCATGAT
SVLF	ATGAGAGACTCAAACAGCAAGTGG
SVLR	ACTGCCCTTCCTCGTAACCAAC
GA20oxF	GGTGACACCTTCATGGCTCTATCG
GA20oxR	GTGTTTGGCTGTTCACCACTG
Cloning	
SVLoeF	ATGGCAAGAGAGAGGATTCAGA
SVLoeR	TCAAACAGCAGACAAACACA
SVLRNAiF	GGGGACAAGTTTGTACAAAAAAGCAGGCTAGGCAAATGAGAGGGGAAGA
SVLRNAiR	GGGGACCACTTTGTACAAGAAAGCTGGGTCGGCCATTAGATATCTCCAC

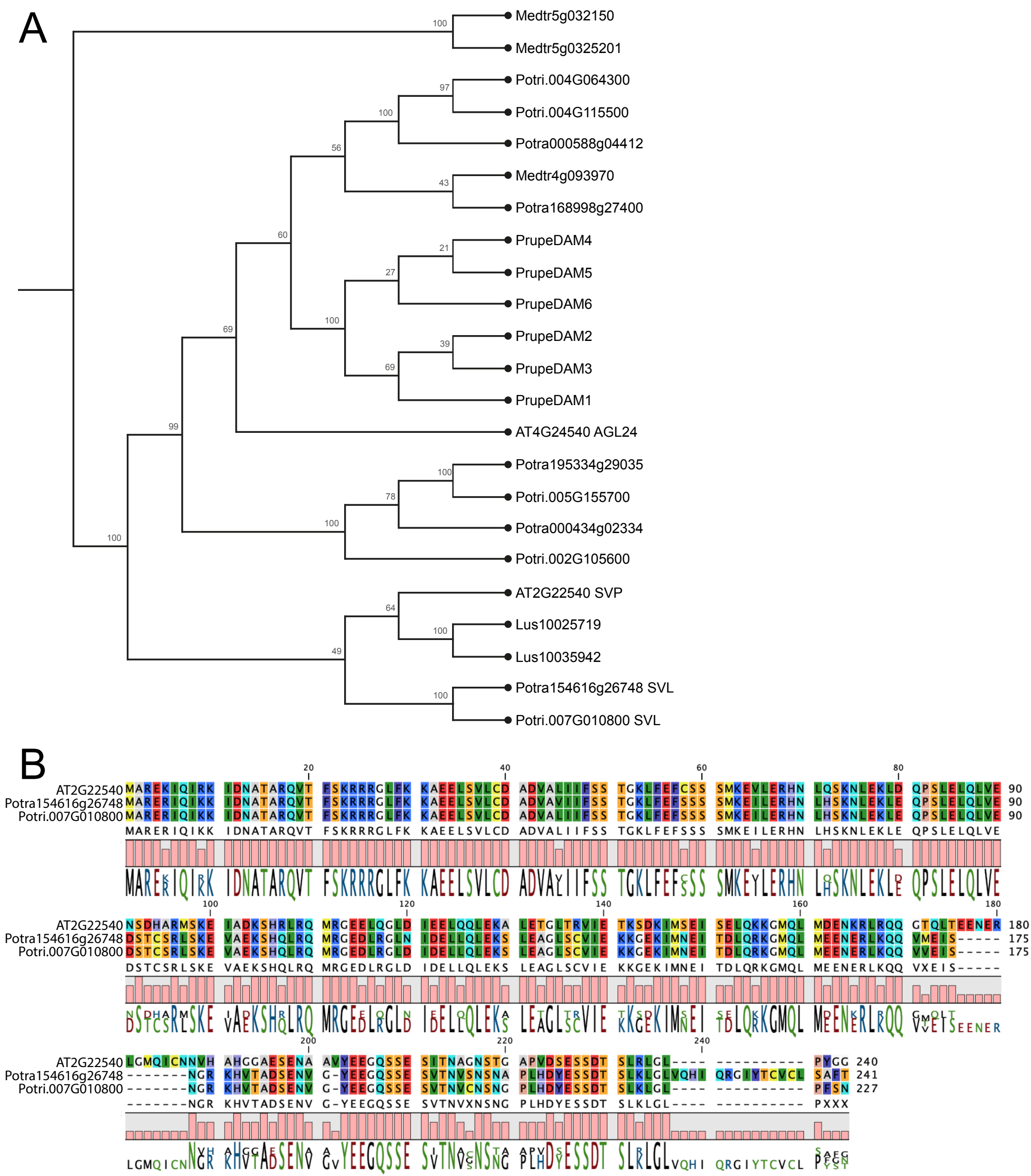


Figure S1:
A) Phylogenetic tree of MADS box containing proteins of *Populus trichocarpa* (Potri), *Populus tremula* (Potra), *Arabidopsis thaliana* (AT), *Medicago truncatula* (Medtr), *Prunus persica* (Prupe) and *Linum usitatissimum* (Lus).
B) SVL protein alignment of *Arabidopsis thaliana*, *Populus trichocarpa* and *Populus tremula*.

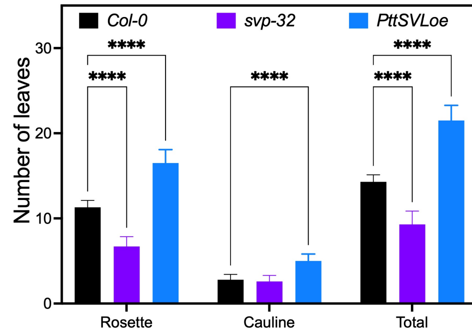


Figure S2:
Populus SVL is functionally conserved with Arabidopsis SVP and can rescue the svp-32 mutant phenotypes. Rosette and cauline eaves of 10 plants per genotype were counted until the formation of the first flowers to determine flowering time. The asterisks indicate a statistically significant difference of each genotype from WT by Welch's test. Error bars indicate standard error, n = 10. **** indicate $p < 0.0001$

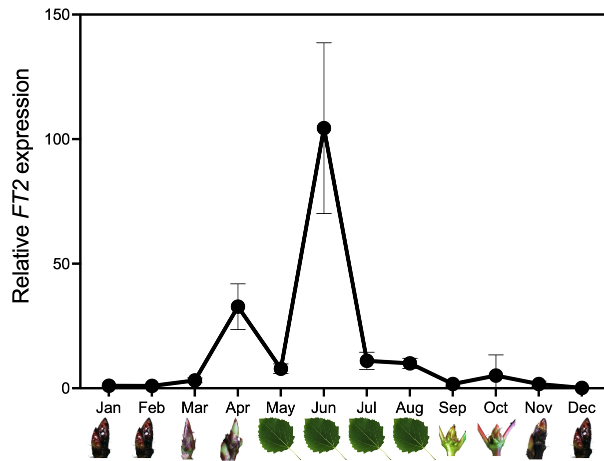
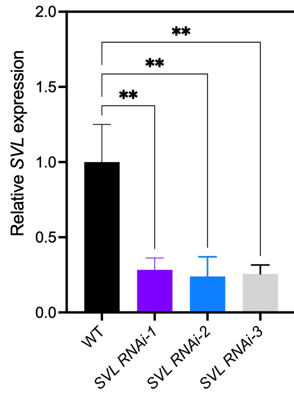


Figure S3:
Expression of *FT2* in field-grown mature *Populus tremula* over the course of one year. Samples were taken at 2 pm in the middle of each month. May-August leaves, September-April terminal buds. Error bars indicate standard error, n =3.



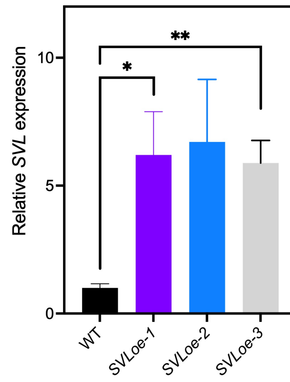


Figure S4:

A) SVL expression is reduced to less that 30% in *SVL* RNAi lines.Samples were taken at ZT4.
B) SVL expression is overexpressed in *SVLoe* lines. Samples were taken at ZT17.
The asterisks indicate a statistically significant difference of each line from WT by Welch's test. Error bars indicate standard error, n = 3. * indicate $p < 0.05$, ** indicate $p < 0.01$.

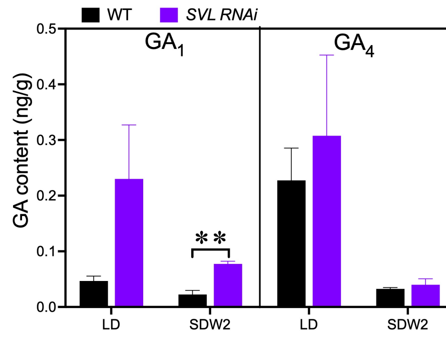


Figure S5:
GA_1 and GA_4 content in leaves of WT and *SVL* RNAi line in LD and after two weeks of SD treatment. The asterisks indicate a statistically significant difference of *SVL* RNAi line from WT by Welch's test. Error bars indicate standard error, n = 4. ** indicate $p < 0.01$.

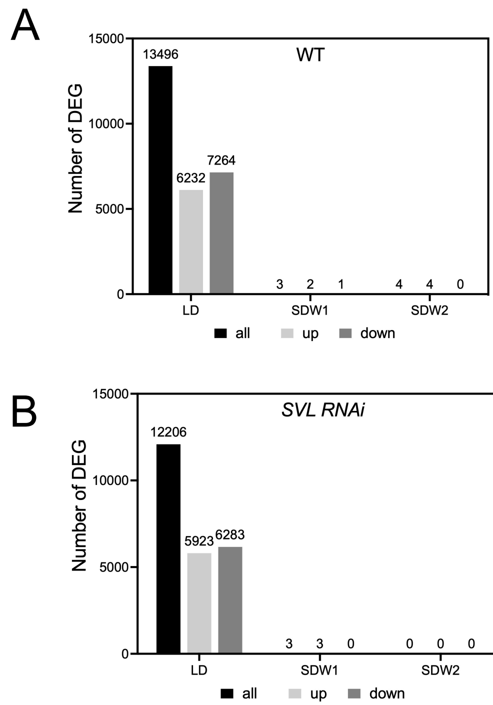


Figure S6:
A) Number of differentially expressed genes (DEG) in WT over the time course of the experiment.
B) Number of differentially expressed genes (DEG) in *SVL* RNAi over the time course of the experiment.

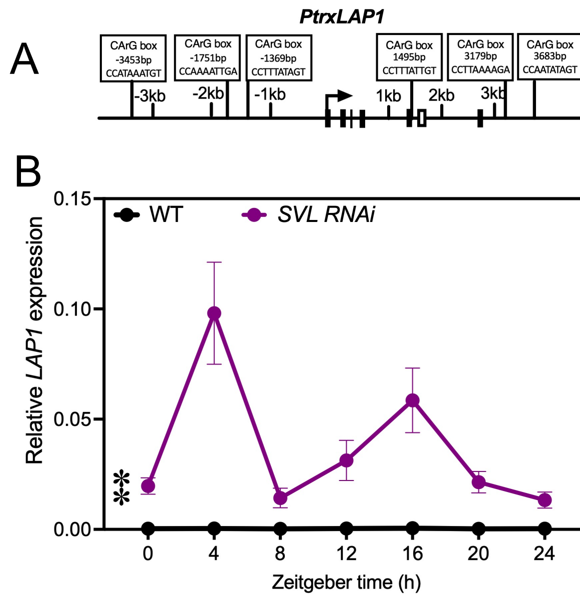


Figure S7:

A) Genomic structure of *PtrxLAP1*. Six potential SVL-binding sites, CArG boxes (NC[A/T]6GN) shown to bind to Arabidopsis SVP (Gregis et al., 2013), are indicated.
B) Gene expression of *LAP1* after two weeks of SD (14h light/ 10h dark) treatment. The asterisks indicate a statistically significant difference between SVL RNAi and WT samples by two-way ANOVA Fisher's test. Error bars indicate standard error, n = 3. ** indicate $p < 0.01$

Acta Universitatis Agriculturae Sueciae

Doctoral Thesis No. 2025:11

The growth of perennial plants such as poplar trees is dictated by the change of seasons. Previous studies of the phenology regulation in *Populus* have revealed interesting parallels to the photoperiodic flowering pathway in *Arabidopsis thaliana*. This thesis describes the role of *FLOWERING LOCUS T (FT)* and *TERMINAL FLOWER 1 (TFL1)*-like genes, in the control of flowering time and phenology in the annual growth cycle of *Populus* trees.

Alice Marcon received her graduate education at the Department of Forest Genetics and Plant Physiology at the Swedish University of Agricultural Sciences. She completed a Master programme in Plant Biotechnology at the University of Turin, Italy.

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