Molecular Analysis of Growth Cessation and Dormancy in Hybrid Aspen

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Abstract

Perennial plants such as trees living in temperate zones experience extreme changes in temperature during summers and winters. In order to survive, shoot apical and cambial meristem undergo a transition from active growth to dormant state, well in advance of winter onset. The transition between an active growth and dormancy involves physiological and developmental processes such as bud formation, acquisition of cold hardiness that are underpinned by massive changes transcriptional and metabolic programs.

The studies of this thesis provide an insight into the molecular regulation underlying the photoperiodic control of growth cessation, adaptive response and acquisition of dormancy in model tree hybrid aspen. Our data show that components of flowering pathway i.e. *LAP1* and *FDL1* have evolved new functions to mediate in SD-control of growth cessation and adaptive response. Furthermore, we found that *FIE*, a component of evolutionary conserved PRC2 complex, in concert with Abscisic Acid (ABA) are key regulators of dormancy in *Populus*. We demonstrate that an interplay between these two components and yet another chromatin remodelling factor *PICKLE* is necessary for the development of dormancy.

In summary, this thesis sheds new light on molecular regulation of activity-dormancy cycle in hybrid aspen. Our better understanding of how trees regulate growth cessation, adaptive response and dormancy may be useful to devise strategies to engineer trees with altered activity-dormancy traits in order to improve their productivity under the impending climate change.

Keywords: Populus, activity-dormancy cycle, growth cessation, adaptive response, ABA, chromatin remodelling, apical bud

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Dedication

To my beloved Weronika and Pola

It is not the strongest of the species that survives, nor the most intelligent that survives. It is the one that is most adaptable to change.

Charles Darwin

Contents

List of Publications

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

I Abdul Azeez; Pál Miskolczi; Szymon Tylewicz; Rishikesh P Bhalerao (2014). A tree ortholog of APETALA1 mediates photoperiodic control of seasonal growth. *Current Biology* 24(7), 717–724

II Szymon Tylewicz, Hiroyuki Tsuji, Pál Miskolczi , Anna Petterle, Abdul Azeez, Kristoffer Jonsson, Ko Shimamoto and Rishikesh P. Bhalerao. Dual role of tree florigen activation complex component *FD* in photoperiodic growth control and adaptive response pathways (*Manuscript,* in revision).

III Szymon Tylewicz, Anna Petterle, Madeleine Englund, Lars Resman and Rishikesh P. Bhalerao. Polycomb and ABA mediate photoperiodic control of bud dormancy (*Manuscript*).

Paper I is reproduced with the permission of the publisher.

Abbreviations

All abbreviations are explained when they first appear in the text.

1 Introduction

In contrast to animals, plants are sessile and their survival depends on constant modulations of their growth and development in response to changes in their environment. An interesting example of such environmental modulation of growth is the annual growth cycle of trees (and other perennial plants) in temperate climates. They grow in summers, when environmental conditions are permissive, but well before the advent of winter they stop growing, adjust their metabolism and physiological processes in manners that protect their meristematic tissues, and acquire dormancy. All these physiological and developmental changes are adaptive responses to environmental cues that enable the plants to survive the extremely low temperatures they encounter during the winter (Cooke et al., 2012, Petterle et al., 2013, Rohde and Bhalerao, 2007, Weiser, 1970).

Knowledge of trees' growth cessation and dormancy processes is highly important for both fundamental understanding and commercial purposes, because they strongly influence plant productivity. In addition, ongoing climate change is expected to substantially change plants' growth habits, and deeper molecular level understanding of these processes might be useful for engineering trees with improved adaptive capabilities to cope with such changes and/or other stresses. Thus, the goal of the studies this thesis is based upon was to improve understanding of the molecular mechanisms underlying growth cessation and development of dormancy in the model tree hybrid aspen (*Populus tremula* x *P. tremuloides*).

1.1 Growth ceases and dormancy is established before the advent of winter

To survive the extremely low temperature during winter, trees in temperate regions must stop growing in the autumn as winter approaches. The visible

signs of growth cessation are the arrest of elongation growth and bud set, i.e. formation of bud structures at apices that enclose the leaf primordia (Nitsch, 1957; Petterle et al, 2013; Rohde et al, 2002; Ruttink et al, 2007). Subsequently, dormancy is established after growth has ceased. Once dormancy is established, prolonged exposure to low temperatures is required for release from the dormant state (Cooke et al, 2012; Espinosa-Ruiz et al, 2004; Rinne & van der Schoot, 1998).

Dormancy has been described as a temporal suspension of growth that is required for survival in unfavourable environmental conditions. It should be noted that dormancy only develops in plant tissues that are capable of growth, (e.g. shoot and cambial meristems, and leaf primordia) (Lang et al, 1987). In recent years considerable progress has been made towards understanding the development of dormancy in seeds, through intensive research driven by its importance for agricultural yields. In comparison, research into the dormancy of perennial trees' buds is still in its infancy, especially at the molecular level (Cooke et al, 2012, Rohde and Bhalerao, 2007).

In 1987 Gregory Lang and co-workers distinguished three types of dormancy: paradormancy, ecodormancy and endodormancy (Lang et al, 1987). Paradormancy refers to growth inhibition that is imposed on one tissue by another part of the plant (e.g. apical dominance, dormancy in axillary buds induced by the apex). Ecodormancy is defined as suspension of growth in response to temporarily unfavourable environmental conditions (e.g. various stress responses), and can be released when conditions become permissive. In contrast, endodormancy can be imposed by signals originating from either outside or within the tissue or organ itself and is not simply reversible by removal of these signals. For example, endodormant trees require prolonged exposure to low temperature (LT) in order to first break dormancy (Horvath et al, 2003; Lang et al, 1987; Petterle et al, 2013; Rohde & Bhalerao, 2007).

1.1.1 Trees must acquire cold hardiness to survive low temperatures during winter

In addition to ceasing growth and establishing dormancy, trees living in temperate and boreal zones need to acquire "cold hardiness" to withstand extremely low temperatures (LT) for extended periods of time during winter. This capacity involves a complex suite of biochemical and physiological adaptations that provides plants the ability to withstand the conditions. Without such adaptations the low temperatures would be highly damaging for several

reasons. First, they induce intercellular ice formation, and hence cellular dehydration. Ice is primarily formed in the intercellular matrix outside of the cells, mainly because osmotic pressure is higher (and thus the freezing point lower) inside the cells (Thomashow, 1999). Low temperatures also reduce rates of enzymatic reactions, thereby impairing plants' ability to perform essential physiological processes, e.g. photosynthesis and energy transduction. Ice formation can also cause freezing injuries that severely damage membrane systems, causing rupture of cells and eventually cell death (Uemura et al, 1996; Uemura et al, 1995; Webb & Steponkus, 1993).

Thus, plants' responses to low temperatures include changes to the lipid composition of their membranes, which protect them from freezing damage (Uemura et al, 1995). Another key feature of acquisition of cold hardiness is accumulation of various metabolites and proteins. Notably, for example, the accumulation of late embryogenesis abundant (LEA) proteins is crucial for plants' adaptation to low temperatures. They function together with sugars in dehydration tolerance, which is the main stress caused by suboptimal temperatures (Wolkers et al, 2001). Dehydrins are a group of LEA proteins that have suggested functions as cryprotectants in responses to LT (Welling et al, 2004). Furthermore, in woody plants expression levels of dehydrins display seasonal fluctuations, which correlate with acquisition and subsequent loss of cold hardiness (Wisniewski et al, 1996). Another crucial process in the development of cold hardiness is the accumulation of soluble sugars, which allows plants' cells to regulate osmolarity and maintain turgor under cold stress. Morphological changes also occur during acclimation to LT, typically including reductions in vacuoles' sizes and the disappearance of starch granules (Ruttink et al, 2007).

1.1.2 Sensing winter's advent involves day length sensing

Clearly, trees (and other perennials) have evolved mechanisms that sense the approach of winter and allow them to cease growth, establish dormancy and activate associated processes at appropriate times, thereby enabling them to survive in extremely low temperatures. These are the main phenomena addressed in this thesis. In *Populus,* the model tree used in the underlying studies (and most tree species of temperate and high-latitude regions), reductions in day length (short day signals) provide cues indicating the approach of winter (Cooke et al, 2012; Garner, 1923; Nitsch, 1957; Petterle et al, 2013; Rohde & Bhalerao, 2007). Thus, when the length of the day falls below a critical threshold (defined as the shortest day length promoting

growth), first elongation growth is inhibited, then bud set occurs, i.e. a structure called an apical bud forms that encloses the shoot apical meristem (SAM) and leaf primordia (Rohde et al, 2002). Since the timing of advent of winter varies depending on geographical location (occurring increasingly early with increases in latitude) the timing of growth cessation varies accordingly (Bohlenius et al, 2006; Pauley, 1954). Thus critical day length, which is a key determinant in timing of growth cessation, is a highly adaptive trait for forest trees and varies among tree populations depending on their geographical origins (Bohlenius et al, 2006; Pauley, 1954). It is worth noting that shortening of the period of daylight is a more robust cue than reduction in temperature and thus utilised almost exclusively by most of tree species to sense the approach of winter and initiate not only growth cessation but also other physiological processes that are critical for their survival. Given the central role of day length sensing in the growth of perennial trees, the underlying mechanism in plants is summarized below.

1.2 Photoperiodism - responses of plants to light signals

Day length is a key signal utilised in mechanisms that modulate plant development. Photoperiodism is defined as a physiological reaction of organisms that enables them to respond to seasonal changes in day length (Borthwick & Hendricks, 1960; Leopold, 1951; Mathur, 1947): a critical and complex aspect of plants' interaction with their environment. The photoperiod can be utilised to anticipate the approach of winters since winters are preceded by autumnal shortening of the day. Thus, it is not surprising that plants have evolved the ability to perceive and react to changes in day length as a crucial part of their survival strategy. This thesis focuses on the mechanisms whereby shortening of the day length triggers growth cessation and establishment of dormancy, but these are not the only physiological responses controlled by day length in plants.

Another example of photoperiodically controlled responses, and by far the best understood, is the switch from vegetative to reproductive development (Andres & Coupland, 2012). This must occur at a time that is favourable for fertilization and formation of seeds, thus ensuring maximal reproductive success. Hence, plants have also evolved mechanisms that sense changes in day length, which together with other internal (phytohormones) and external cues (particularly temperature), help them to flower at appropriate times.

Clearly, the day length plays crucial roles in plants' growth and development, thus the sensing mechanisms have been intensively researched,

considerable progress has been made towards elucidating them in *Arabidopsis*, and some progress has also been made in elucidating mechanisms of perception and transmission of day length signals in trees (Petterle et al, 2013). For example, it has been demonstrated that photoreceptors play key roles in the regulation of bud set and growth cessation in trees (Kozarewa et al, 2010; Olsen et al, 1997). In order to respond to day length signals, plants clearly need to sense when it is day and night, and secondly the duration of the day (photoperiod) and night. I briefly describe how plants perceive day length below.

1.2.1 Perception of light signals

In order to modulate their growth in response to changes in day length signals, plants need to discriminate between day and night. The photosensory systems that plants have evolved in response to selective pressures associated with this need include three major classes of photoreceptors: the phytochromes (PHY), cryptochromes (CRY), and phototropins (PHOT) (Gyula et al, 2003; Yeh & Lagarias, 1998). These families of photoreceptors provide the ability to monitor light from UV-B to the near infrared, thereby allowing plants to sense the spectral quality of light as well as the amount in their environment. Phytochromes mediate red and far red light signals, and are the best characterized family of photoreceptors in plants. In *Arabidopsis* five genes encode this family (phyA-phyE) (Nagy & Schafer, 2002). Three distinct classes of UV-A/blue light sensors have also been identified: cryptochromes (*cry1 and cry2*), phototropins (phot1 and phot2) and zeitlupes (ZTL, FKF1 and LKP2) (Demarsy & Fankhauser, 2009; Lin & Shalitin, 2003). However, here I focus mainly on phytochromes as they have well-established roles in focal processes of this thesis, i.e. growth cessation and bud set.

Photoreceptors are chromoproteins, composed of apo-proteins bound to various chromophores, which are responsible for light absorption. Phytochromes are in an inactive form, known as Pr, in darkness. Upon absorption of red light (R) (λ_{max}=670 nm), the chromophores isomerize, transforming into an active Far-red light (FR)-absorbing state (λ_{max}=730 nm) known as Pfr, whereas absorption of FR converts Pfr to inactive Pr state. The light-induced changes of chromophores lead to a transduction of light signals, via interactions with phytochrome interacting factors (PIFs), that triggers expression of downstream genes involved in various photomorphogenic processes (Castillon et al, 2007; Harper et al, 2003; Pfeifer et al, 2010).

As phytochromes serve as light signalling "antennae", it is not surprising that they have been connected with the regulation of seasonal growth control in trees (Ingvarsson et al, 2006; Junttila et al, 1997; Kozarewa et al, 2010). Interestingly, poplar trees ectopically expressing oat *PHYA* cannot respond to the shortening of days and thus are unable to cease growth and set buds (Junttila et al, 1997; Olsen et al, 1997). Moreover, downregulation of *PHYA* leads to earlier growth cessation and bud set in hybrid aspen, and affects the phasing of genes associated with the plants' internal clocks (Kozarewa et al, 2010), as discussed below. Involvement of *PHYB* has also been implicated in bud set control in *Populus*, as it has been mapped to QTLs associated with bud set and association with *PHYB* and bud set has also been reported (Frewen et al, 2000; Ingvarsson et al, 2006). It remains to be elucidated if other components of the light-sensing machinery, e. g. PIFs, also participate in short day-mediated responses in trees.

1.2.2 Circadian rhythms and the clock

Earth's rotation and revolution around the sun results in alternating day and night periods, accompanied by seasonal changes that have major consequences for all living organisms. The cyclical periods of light and darkness are reflected in marked diurnal rhythms in their physiology, metabolism and behaviour. Hence, most living organisms have evolved an innate ability to measure the time, which is essential for coordinating their responses to anticipated changes. Interestingly, even when the time input is removed, many of these diurnal rhythms still occur, indicating the existence of an endogenous biological circadian clock (McClung, 2006; McClung, 2009; Nagel & Kay, 2012).

In plants, the circadian clock consists of three sets of transcriptional feedback loops, known as the core oscillator, and input and output signalling pathways. Loops of the core oscillator can be divided into a central loop and two interlocked loops named "morning" and "evening" loop. Names of the latter two loops originate from the time of the day in which main component of the respective loop is maximally expressed (Harmer, 2009; McClung, 2006; McClung, 2009; Nagel & Kay, 2012). The core oscillator loop, also known as the central loop, has three key components: *TIMING OF CAB EXPRESSION (TOC1)* and two MYB transcription factors, *CIRCADIAN CLOCK ASSOCIATED1 (CCA1)* and *LATE ELONGATED HYPCOTYL (LHY)* (Nagel & Kay, 2012). Numerous studies with loss-of-function mutants and chromatin immunoprecipitation (ChIP) followed by deep sequencing have conclusively shown that TOC1 is a DNA-binding transcriptional repressor of *CCA1* and

LHY, while *CCA1* and *LHY* are negative regulators of *TOC1* and themselves (Alabadi et al, 2001; Gendron et al, 2012; Mizoguchi et al, 2002). This model is based entirely on transcriptional repression and was independently demonstrated by three groups (Gendron et al, 2012; Huang et al, 2012; Pokhilko et al, 2012), thereby changing the previous view (that *TOC1* was a positive regulator of transcription) of the regulatory network of the circadian clock in *Arabidopsis*. It was demonstrated that TOC1 binds to the promoter regions of *CCA1* and *LHY*, thereby inhibiting their expression. TOC1 can also bind to promoters and inhibit expression of oscillator components *PRR5*, *PRR9*, *LUX, GIGANTEA* (*GI*) and *EARLY FLOWERING 4* (*ELF4*) (see Figure 1). However, the DNA-binding motif (*cis*-element) to which TOC1 binds remains unclear since targets share low sequence similarity. This suggests that TOC1 may have the ability to recognize multiple *cis*-elements and/or function in concert with other transcription factors in regulation of the expression of downstream targets (Gendron et al, 2012; Huang et al, 2012; Nagel & Kay, 2012; Pokhilko et al, 2012).

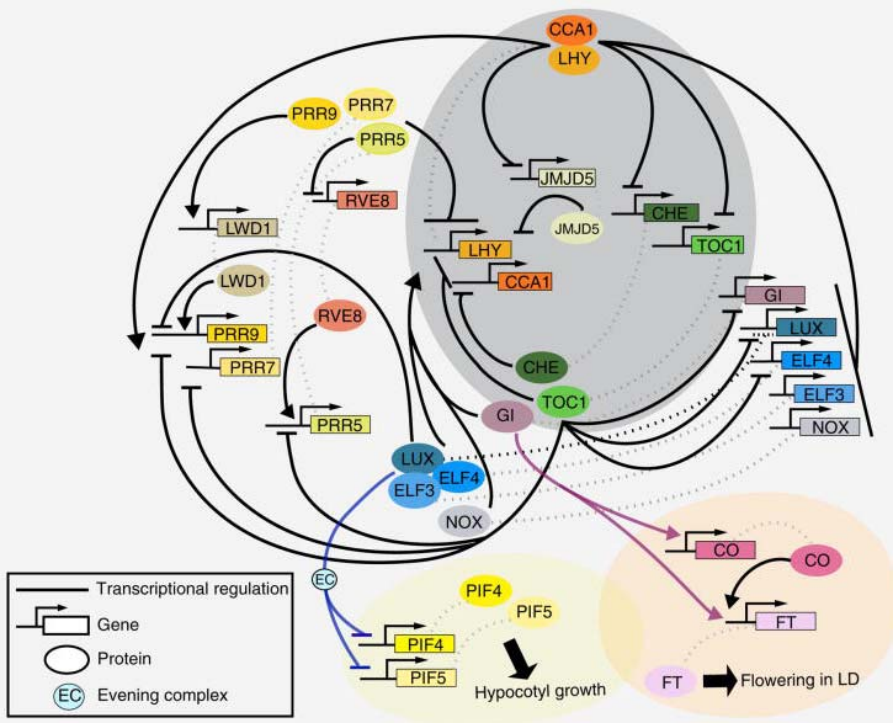

Figure 1. Schematic representation of the plant circadian clock (reprinted with permission from (Nagel & Kay, 2012)see text for details).

1.2.3 The external coincidence theory

Another key issue is the mechanism whereby plants modulate their development in response to changes in day length. Foundations of current understanding of this mechanism were established in pioneering studies in the mid-20th century by Erwin Bünning (Bunning, 1946) and subsequent molecular genetic analysis in *Arabidopsis* and other plants (Kobayashi & Weigel, 2007). Erwin Bünning in 1946 proposed a theory, named the external coincidence theory, explaining how plants integrate information about the day and night cycle to measure photoperiod. He observed that bean seedlings' leaf movements show a daily rhythm, and this periodicity is maintained even after transferring the plants to permanent darkness. Bünning accurately deduced that plants possess an internal "biological clock", which is partly independent of the daily light/dark rhythms. In accordance with his theory, 24-h days can be divided into light-sensitive and dark-sensitive phases, and diurnal changes in

them are entrained by the circadian oscillator. Hence, a plant senses whether it is exposed to a short or a long day from the presence or absence of external light signals during one of the phases (Bunning, 1946; Kobayashi & Weigel, 2007).

A well-characterized developmental process that best illustrates the external coincidence model is the induction of flowering in *Arabidopsis*. *Arabidopsis* flowers rapidly in long days whereas flowering is delayed in short days. *CONSTANS* (*CO*), which encodes a transcription factor containing a B-box zinc finger domain, is an important player in day length-mediated flowering control (Putterill et al, 1995; Suarez-Lopez et al, 2001). CO promotes flowering by positively regulating the expression of *FLOWERING LOCUS T* (*FT*), an inducer of flowering and is an important mediator between the circadian clock and the flowering pathway (Kardailsky et al, 1999; Kobayashi et al, 1999). It is important to note that *CO* mRNA shows a diurnal expression pattern, peaking close to the end of the day. On the other hand, CO protein is unstable in the dark, being degraded rapidly (Valverde et al, 2004). Therefore, when *Arabidopsis* plants are grown in short days, *CO* expression peaks in the dark but the CO protein is degraded. Conversely, when grown in long days the CO protein is made during the day and therefore is stable, enabling activation of *FT* and transition to flowering. A mechanism proposed by (Valverde et al, 2004) has also explained the involvement of photoreceptors in degradation of CO protein in the darkness by PhyB and its stabilization by PhyA and CRY, thereby allowing inputs from the photoreceptor system to participate in the clock-mediated control of flowering time.

The evolutionary conservation of the CO-FT module in day length-mediated control of flowering in other plants was subsequently shown. For example, in plants that flower upon exposure to short days, e.g. rice (*Oryza sativa*), the mechanism of floral transition is similar, except that the rice homolog of *CO*, *Heading-date 1* (Hd1), prevents flowering when LDs are perceived and promotes floral transition upon perception of SD signals (Kobayashi & Weigel, 2007; Tsuji et al, 2011).

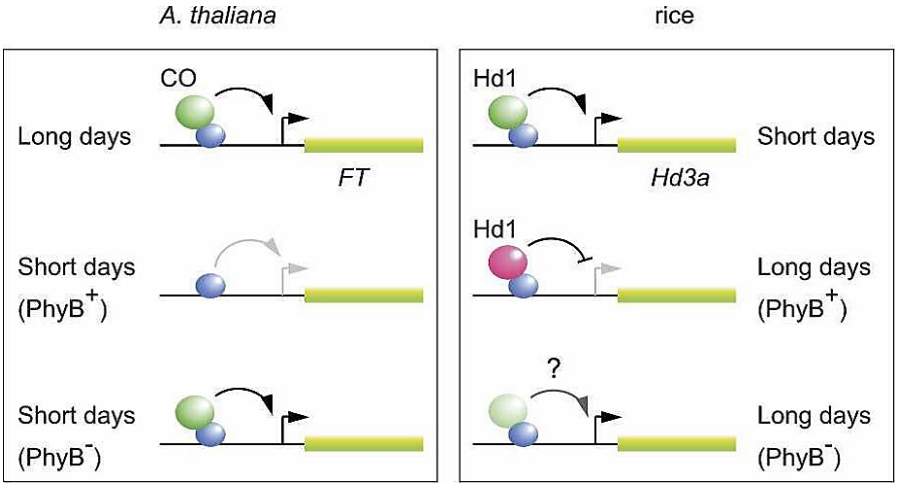

Figure 2. Flowering regulation in Arabidopsis and rice. Regulation of flowering in an LD-flowering plant by CO (*Arabidopsis*; left panel), and in an SD flowering plant by the *CO* ortholog *Hd1* (in rice, right panel). Reproduced with permission from (Kobayashi & Weigel, 2007).

1.3 Molecular analysis of growth cessation

1.3.1 Roles of photoreceptors and clock components in growth cessation

It has been demonstrated that photoreceptor phytochromes are early acting components of the photoperiodic pathway controlling growth cessation (Olsen et al., 1997; Kozareva et al., 2010). Hybrid aspen trees overexpressing the oat *PHYA* gene have a dwarf phenotype and are insensitive to photoperiods, and thus unable to cease growth in response to SD signals. Interestingly, these plants maintain a constant level of GA when subjected to SDs, in contrast to wild type plants in which GA levels are downregulated. Thus, the inability of PHYA overexpressors to cease growth in short days could be due to inability to sense short days that downregulate GA levels (Olsen et al, 1997). Support for this hypothesis comes from analysis of trees that overexpress *GA-20* oxidase with elevated levels of GA, in which the growth cessation response is significantly delayed (Eriksson et al, 2000). More detailed descriptions of the hormonal control of SD-related processes are presented in section 1.7 of this thesis.

As the circadian clock is critical for control of photoperiodic responses, unsurprisingly its components are involved in the regulation of seasonal growth cessation in *Populus* (Bohlenius et al, 2006; Hsu et al, 2011; Ibanez et

al, 2010). An elegant study by Ibanez et al (2010) showed that modulation of *PttLHY1*, *PttLHY2* and *PttTOC1* expression leads to alteration in the critical day length (CDL) requirement for growth cessation and delay of bud set. In addition, downregulation of *PttLHYs* reduces cold responses and hardening, indicating a general role in photoperiodic responses such as growth cessation and the accompanying acquisition in cold hardiness. Conversely, plants in which *PttTOC1* is downregulated have increased freezing tolerance, indicating its repressive function in this process (Ibanez et al, 2010). The cited studies clearly show that, as in flowering, clock components play a critical role in trees' annual growth cycles. Not only growth cessation, but also clock maybe involved in dormancy, since clock regulation is altered during dormancy in chestnut (Ramos et al, 2005).

1.3.2 The CO/FT regulon's role in photoperiodic control of growth in trees

As outlined above, the CO/FT regulon plays a key role in the day length-mediated control of flowering downstream of photoreceptors and the clock, and recent findings in *Populus* have shown it participates in the photoperiodic control of cessation of growth and bud set in trees (Bohlenius et al, 2006; Hsu et al, 2011). In the *Populus* genome there are two orthologs of *FLOWERING LOCUS T*, named *FT1* and *FT2*. As in *Arabidopsis*, overexpression of either of them leads to very early flowering, even at the tissue culture stage (Bohlenius et al, 2006; Hsu et al, 2011). Bohlenius et al (2006) showed that expression of the *CO* ortholog, a positive regulator of *FT2,* has a diurnal pattern, peaking close to the end of the day. Although not yet shown, CO is also probably labile in darkness in trees. Thus, in SD conditions the level of CO protein is likely to be low due to its degradation since it peaks in the darkness (Bohlenius et al, 2006). Consequently, *FT2* expression remains low and growth cessation occurs in SDs. This model is well supported by functional analysis showing that downregulation of *CO* or *FTs* leads to precocious growth cessation. In contrast, overexpression of *FT1* or *FT2* causes early flowering and abolishes proper growth cessation responses even in short days (Bohlenius et al, 2006; Hsu et al, 2011). Interestingly, poplars overexpressing *CO* have normal growth cessation responses, indicating that the diurnal expression pattern of *CO*, rather than its mRNA and protein level, is critical for photoperiodic control of growth (Hsu et al, 2011; Hsu et al, 2012). Although SD mediated regulation of *FT* leading to growth cessation is a paradigm, in spruce *FT/TFL-like* gene *PaFTL2* is actually induced during bud set and when overexpressed can cause growth cessation.

Thus, there could be difference between different tree species, in the role of *FT/TFL* genes in photoperiodic control of growth (Karlgren et al, 2013).

Studies in *Arabidopsis* and rice have shown that the transcription factor FD, belonging to a group of bZIP transcription factors, interacts with FT in shoot apical meristems (SAMs), triggering transition to flowering. Plants with a mutation in the *FD* gene exhibit a late flowering phenotype, indicating that interaction between FT and FD is crucial for flowering (Abe et al, 2005; Tsuji et al, 2013a; Tsuji et al, 2013b; Wigge et al, 2005). The C-terminal end of FD contains a phosphorylation motif, which is targeted by calcium-dependent protein kinases (CDPKs) and is necessary for interaction with 14-3-3, and indirectly with FT. It has been suggested that FD may tether the florigen activation complex (FAC) to its target promoter DNA (Taoka et al, 2011; Tsuji et al, 2013b). In *Populus*, Ruttink et al (2007) observed strong upregulation of *FD* upon SD signals, implying a role of the *FD1-like* gene in bud development. However, the function *of FD-like* genes in *Populus* is still not known. The co-authors of the appended papers and I (hereafter we) have shown that like FT, FD homologs also participate in photoperiodic responses and FDL1, the hybrid aspen homolog of FD, interacts with FT and mediates in photoperiodic growth control (Paper II). More recently, it has been shown that bulbing in onion and tuberisation in potato are also controlled by day length and involve FT homologs (Lee et al, 2013; Navarro et al, 2011). These studies demonstrate that the CO/FT module has been extensively used during a long course of evolutionary history in the control of developmental transitions mediated by day length.

1.3.3 Signalling downstream of the CO/FT module

A recent study on SD-regulated growth cessation showed that *AINTEGEUMENTA-like 1* (*AIL1*) is a new player acting downstream of the CO/FT module in hybrid aspen trees (Karlberg et al, 2011). Exposure of wild type (WT) trees to SD conditions resulted in strong downregulation of *AIL1*, suggesting it is involved in growth cessation. *AIL1* is a known positive regulator of expression of core cell-cycle genes (e.g. D-cyclins), hence its repression upon SDs triggers growth cessation. Although the data presented by (Karlberg et al, 2011) clearly indicate that *AIL1* acts downstream of the *CO/FT* regulon, they also show that *FT* does not directly control *AIL* expression, as downregulation of *AIL1* occurs significantly after *FT2* downregulation, which occurs within 5-7 days. Moreover, upregulation of *FT2* in leaves does not trigger induction of *AIL1*, further suggesting that *AIL1* is

indirectly regulated by *FT2* (Hsu et al, 2011; Karlberg et al, 2011). Results acquired in the studies this thesis is based upon have further filled gaps in understanding the regulatory pathway underlying SD-mediated growth cessation downstream of the CO/FT regulon (Azeez et al, 2014). It was shown that the aspen homolog of the *Arabidopsis* floral meristem identity gene *APETALA1* (*AP1*), named *Like-AP1* (*LAP1*), acts downstream of the CO/FT regulon and in turn controls expression of *AIL1*.

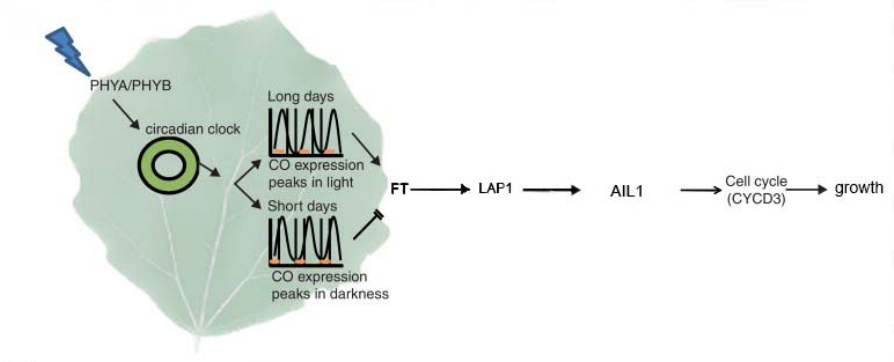

Figure 3. SD-mediated signalling pathway of growth cessation in *Populus spp.*, modified from (Petterle et al, 2013). *LAP1* has been identified as MADS box transcription factor acting between CO/FT and AIL (Azeez et al, 2014).

1.4 Molecular control of cold hardiness

As outlined above, in addition to growth cessation, SDs also induce cold hardiness. Although acquisition of cold hardiness is crucial for survival during winter, our knowledge of the molecular control of this process remains rudimentary in trees. Signalling pathways of cold acclimation are best characterized in the herbaceous plant *Arabidopsis*, and significant progress has been made in recent years in revealing its transcriptional networks regulating cold acclimation. Upon exposure to low, non-freezing temperature profound changes in the plant transcriptome are triggered that activate "cold-regulated" (*COR*) genes, which encode hydrophilic polypeptides known to promote freezing tolerance (Hajela et al, 1990). Identification of C-repeat/DRE binding factors (CBFs) has laid foundations for understanding the signalling involved in cold acclimation (Stockinger et al, 1997). These transcription factors regulate the expression of COR genes and are part of a known CBF regulon. The *Arabidopsis* genome contains six *CBF* paralogues, three of which are strongly upregulated by LT (*CBF1-3*) (Liu et al, 1998; Shinwari et al, 1998)

through binding of the MYC-type transcription factor *INDUCER OF CBF EXPRESSION (ICE1)* to their promoter region that contains cis-elements known as *DEHYDRATION RESPONSE ELEMENT/ C-REPEAT/LOW TEMPERATURE RESPONSIVE ELEMENT* (*DRE/CRT/LTE*) (Chinnusamy et al, 2003; Stockinger et al, 1997). *ICE1* and *CBF* mutants have demonstrated hypersensitivity to chilling stress (Liu et al, 1998), while overexpressors reportedly have enhanced freezing tolerance even without prior chilling treatment (Jaglo-Ottosen et al, 1998).

Similarly to *Arabidopsis*, woody plants in temperate and boreal zones undergo cold acclimation (cold hardiness) in response to LT. However, if they are to survive winters trees must anticipate the associated freezing temperatures well in advance. Thus, they develop not only dormancy via sensing day-shortening, but also cold hardiness (Li et al, 2003; Welling et al, 2004). Cold hardiness is acquired by woody plants through the sequential action of environmental stimuli, i.e. SD in combination with LT and finally exposure to freezing temperatures (Puhakainen et al, 2004; Weiser, 1970). Interestingly, it has been shown that both SD and LT signals can induce cold acclimation responses in trees, but they seem to be independently regulated by different mechanisms. Analysis of trees with defective light perception has shown that ectopic overexpression of *PHYA* impairs development of SD-induced cold hardiness, but does not affect cold acclimation responses induced by low temperature itself (Olsen et al, 1997; Welling et al, 2002). Components of the CBF regulon have been identified in various tree species, including *Populus* and *Betula*, indicating that this conserved signalling regulon might play an important role in development of cold hardiness in trees. Indeed, these studies confirmed the role of CBFs in cold acclimation in woody plants, since they were induced by LT and overexpression of *CBFs* (the *Arabidopsis* gene in poplar and birch gene in *Arabidopsis*) led to enhanced freezing tolerance and activation of a similar set of genes as in *Arabidopsis* (Benedict et al, 2006; Welling & Palva, 2008). Further insight has been gained from microarray analysis of transcriptomic changes in wild type *Populus* plants subjected to SD conditions (Karlberg et al, 2010). In the cited study, Karlberg and colleagues identified several cold hardiness-related genes e.g. *CORs, DEHYDRINS* and *LIPID TRANSFER PROTEINS* (*LTPs*) that were upregulated upon SD treatment. Interestingly, they observed two waves of expression, an early one after 5 weeks of SD and a later one at 11 weeks. These data suggest that upregulation of this set of cold hardiness-related genes represents a response to short day signals as the experiments were performed at 22˚C. Analysis of *CBF* genes, and their regulator *ICE* showed that they are not regulated by SD signals, as their expression remained unchanged after exposure to SDs

(Karlberg et al, 2010). However, it is likely that they control late stages of cold acclimation dependent on LT, as they are cold-inducible, or an additional layer of regulation is present e.g. chromatin modifications (Benedict et al, 2006; Karlberg et al, 2010). It is worth noting that our data suggest that tree orthologs of *FD*, i.e. *FDL1* and *FDL2*, could be involved in the acquisition of cold hardiness as well, since they are induced after SDs when the first stage of cold hardiness is activated and FDL1 has overlapping targets with ABI3, which controls the expression of many of the genes associated with cold hardiness (Ruttink et al, 2007) Paper II).

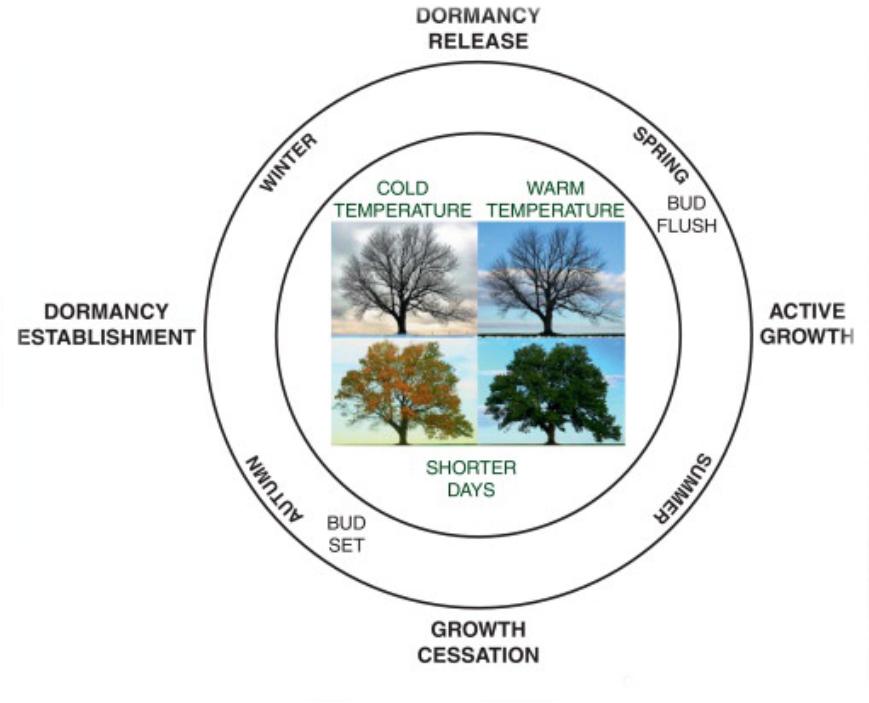

Figure 4. Annual growth cycle in *Populus* spp., reproduced with permission from (Petterle et al, 2013)

1.5 Molecular control of dormancy in trees

Dormancy in trees can be divided into following phases: entry, establishment, maintenance and release. The molecular control underlying dormancy regulation still remains poorly understood, partly due to difficulties in

analysing dormancy as dormant tissues are difficult to obtain, being as they are located inside apical buds. In hybrid aspen the same signals (short days) that regulate bud set also regulate bud dormancy (Espinosa-Ruiz et al, 2004). However, the two processes seem to be regulated by independent mechanisms, since overexpression of *ABSCISIC ACID INSENSITIVE 3* (*ABI3*) in *Populus* leads to aberrant formation of apical buds, but trees can develop dormancy (Rohde et al, 2002). Similarly, in birch ectopic expression of a dominant-negative version of *ETHYLENE TRIPLE RESPONSE 1* (*ETR1*) resulted in the delay or absence of terminal buds, and yet they developed dormancy (Ruonala et al, 2006). Previous reports imply a role of Abscisic Acid (ABA) in establishment of dormancy, as increased ABA levels have been detected following exposure to SD in SAMs and cambium of poplar trees (Druart et al, 2007; Karlberg et al, 2010; Rohde et al, 2002). Furthermore, transcriptional analyses have shown that genes involved in ABA biosynthesis are upregulated during SD treatment, supporting its involvement in the process. Changes in the responsiveness of cells to ABA may also be involved in the development of dormancy, in addition to changes in ABA levels, the genes of the signalling pathway, such as *RCAR* receptors and *PP2C* phosphatases, are also upregulated after SDs (Karlberg et al, 2010; Ruttink et al, 2007). Moreover plants with reduced responses to ABA are unable to establish dormancy (Resman et al, 2010).

An attractive hypothesis regarding dormancy regulation concerns the role of plasmodesmata, which are conduits that connect cells symplastically. A role for plasmodesmata in dormancy regulation was initially raised in reports that plasmodesmata are closed in birch and hybrid aspen during dormancy development (Rinne et al, 2001; Rinne & van der Schoot, 1998). Symplasmic connectivity plays a key role during morphogenesis, as it facilitates exchange of various molecules and substances (Simpson et al, 2009; Thomas et al, 2008). However, following exposure to SD plasmodesmata are gradually closed by deposition of 1,3 β-glucan, and concomitantly proteins and callose are transported to plasmodesmatal channels (Rinne & van der Schoot, 1998). Consequently, symplasmic connections are completely blocked. In addition, sieve tubes become occluded with dormancy-related callose (Levy & Epel, 2009), indicating that transport of substances is most likely impaired. It is known that plants that cannot cease growth properly do not suspend plasmodesmatal communication or are unable to maintain plasmodesmata in a closed state, e.g. plants overexpressing *PHYA* cannot close plasmodesmatal connections and cannot become dormant (Ruonala et al, 2008), however this raises the issue whether the closure of PDs is consequence of growth cessation. Nevertheless, to date no functional analysis of plasmodesmatal closure has

been performed and whether blockage of plasmodesmata is the cause or a consequence of dormancy establishment remains unclear. Thus, identification of "mutants" that cease growth normally but have altered dormancy would be informative for assessing the role of plasmodesmata in dormancy regulation in the future.

Most relevant research in recent years has focused on molecular controls of growth cessation and acquisition of dormancy in trees, thus these processes are better understood than release from dormancy and subsequent bud break (Azeez et al, 2014; Bohlenius et al, 2006; Hsu et al, 2011; Karlberg et al, 2011; Karlberg et al, 2010; Rohde et al, 2007). Dormancy release requires extended exposure to LT, which restores plants' ability to grow, but does not promote it (Chouard, 1960). However, the mechanism that acts via cold and the primary targets of cold signals remain to be determined (Heide, 1993; Junttila & Hanninen, 2012; Myking & Heide, 1995). Extensive transcriptional analyses have provided some insight into molecular regulators that could be involved in dormancy release and bud break (Karlberg et al, 2010). However, in the absence of any functional analysis, these results remain preliminary. Nevertheless transcriptional analysis has suggested that exposure to dormancy-breaking LT is accompanied by induction of genes involved in synthesis of gibberellins (GA), indicating that cold treatment could potentially promote the production of active GA. Elevated levels of active GA have been found in analysis of dormant cambium subjected to cold temperatures, demonstrating the involvement of GAs in release from dormancy (Druart et al, 2007). Antagonistic effects of GA and ABA may also potentially be involved in dormancy release, but (if so) the mechanisms involved remain to be established. Interestingly, plasmodesmatal connections, which are blocked during the establishment and maintenance phases of dormancy, are gradually opened during release, possibly through the action of 1,3 β-glucanases (Rinne et al, 2011). Thus, GA could hypothetically act antagonistically to dormancy-promoting ABA, and this may impinge on dynamic control of plasmodesmata in dormancy regulation.

1.5.1 Chromatin remodelling and regulation of bud dormancy

One approach to study the molecular basis of dormancy regulation is to identify changes in gene expression associated with the process. Acquisition of dormancy is associated with massive changes in the transcriptome of apical meristems in *Populus* (Karlberg et al, 2010). Thus, it seems plausible that some of the reported transcriptional changes might be associated with the regulation

of dormancy development. Massive changes in global regulation of gene expression often involve modulation of chromatin through modifications of different types of histones and/or modifications of DNA via methylation (Gentry & Hennig, 2014; He et al, 2011). Thus, since results presented in Paper III and other publications (Karlberg et al, 2010; Leida et al, 2012; Rios et al, 2014) have indicated a role for chromatin remodelling in bud dormancy regulation, I summarise chromatin remodelling and its possible role in bud dormancy regulation in trees below in view of the possible role of *FIE* in dormancy regulation as well as changes in chromatin remodelling genes during dormancy induction and release.

1.5.2 Epigenetic regulation

An epigenetic change can be defined as a heritable change of gene expression and chromatin structure that does not involve alterations in DNA sequence (Chen et al, 2010; He et al, 2011). Several mechanisms for epigenetic inheritance have been identified, including (*inter alia*) histone modifications, DNA methylation and RNA-based mechanisms (Bologna & Voinnet, 2014; Furner & Matzke, 2011; Law & Jacobsen, 2009). In recent decades, epigenetic phenomena have received great attention, leading to breakthrough discoveries such as the roles of epigenetic regulation in various human diseases (Gluckman et al, 2011) and regulation of development (Shao et al, 1999). Furthermore, in recent years plant scientists have made considerable progress in understanding how epigenetics may contribute to various aspects of plant development. It has been shown that epigenetic changes of chromatin, and thus gene expression status, are involved in vernalisation, seed development, embryo development and stress responses (Gendall et al, 2001; Leroy et al, 2007; Liu et al, 2007; Saleh et al, 2007; Yoshida et al, 2001). I briefly summarise the key components involved in epigenetic regulation of gene expression below.

1.5.3 Polycomb group repression and Trithorax complexes

Polycomb group (PcG) complexes were first discovered in fruit fly (*Drosophila melanogaster*) and found to play important roles in maintaining repressed transcriptional states of genes (Nekrasov et al, 2005). These complexes have methyltransferase activity and trimethylate H3 histones at lysine 27 (H3K27me3), which is a known transcription-silencing chromatin marker. In *Drosophila*, PcG proteins are required for the control of body

segmentation via tight regulation of the expression of homeotic (Hox) genes, but it is now clear that PcG proteins have much wider roles in development and are highly conserved among animals and plants (Hennig & Derkacheva, 2009; Kohler & Villar, 2008; Schwartz & Pirrotta, 2007). Three PcG complexes have been described in *Drosophila*: Polycomb repressive complex 1 (PRC1), Polycomb repressive complex 2 (PRC2) and Pleiohomeotic repressive complex (PhoRC). PRC2 is thought to be an initiation factor of gene silencing, whereas PRC1 is involved in maintenance of a silenced state. However, the function of PhoRC remains poorly understood (Oktaba et al, 2008).

Biochemical studies of PRC2 in Drosophila have shown that it consists of four core subunits: Enhancer of zeste [E(z)], a SET (Suvar3-9, Enhancer of zeste, trithorax) domain protein with methyltransferase activity; Suppressor of zeste 12 [Su(z)12]; and two WD40 domain proteins, Extra sex combs (Esc) and Nurf55 (Nucleosome remodelling factor 55). Every component is needed for appropriate binding and efficient methylation of nucleosomes. In mammals, three complexes similar to PRC2 have been identified, named PRC2, PRC3 and PRC4. All of them have methyltransferase activity, but with different substrate specificities (Kohler & Villar, 2008). In plants several PRC2 complexes with different developmental functions have been identified. The EMF complex, consisting of CURLY LEAF/SWINGER (CLF/SWN), EMBRYONIC FLOWER 2 (EMF2), FERTILISATION INDEPENDENT ENDOSPERM (FIE) and Multicopy Suppressor of IRA1 (MSI1), promotes vegetative development of plants and maintains cells in a differentiated state (Yoshida et al, 2001). The VERNALIZATION (VRN) complex contains CLF/SWN, VRN2, FIE and MSI1, and is responsible for epigenetic silencing of the flowering repressor *FLOWERING LOCUS C* (*FLC*), thereby enabling flowering after vernalisation (De Lucia et al, 2008). The third described PRC2 complex, FERTILISATION INDEPENDENT SEED (FIS), prevents seeds development in the absence of fertilisation. It comprises MEA, SWN, FIS2, FIE and MSI1 (Chaudhury et al, 1997; Guitton et al, 2004; Kohler et al, 2003; Luo et al, 1999). To date, very little is known about PRC1 in plants, but some components of this complex have been characterized. A chromodomain protein, LHP1, also known as TERMINAL FLOWER 2, is a plausible candidate for PRC1 function, as it is reportedly necessary for silencing of euchromatic genes targeted by PcG proteins (Derkacheva et al, 2013; Turck et al, 2007). In addition, recent studies on flowering have provided several indications that LHP1 is a H3k27me3 reader in PRC1 complexes. Its chromodomain is responsible for H3K27me3 binding specificity and it was discovered to interact with RING-domain proteins, which are potential

components of PRC1 and responsible for deposition of monoubiquitin on H2AK119 (de Napoles et al, 2004; Mylne et al, 2006; Wang et al, 2004).

Trithorax proteins (TrxG) are thought to antagonize the function of PcG complexes. They are SET-domain proteins with H3K4 methyltransferase activity (a modification that is generally associated with transcriptionally active regions), and their presence has been shown to prevent PRC2 from trimethylating target genes (Avramova, 2009). One of the described targets of TrxG proteins is *AGAMOUS* (*AG*), a floral homeotic gene involved in determination of reproductive floral organs such as stamens and carpels. Expression of AG in *atx1* (a trithorax mutant) is reduced, in accordance with a role of ATX1 in gene activation. Interestingly, AG expression is induced in a *clf* mutant background, indicating an antagonistic role of TrxG to PcG (Doyle & Amasino, 2009). Loss of function of ATX1 in a *clf* background leads to suppression of the *clf* mutant phenotype (Kohler & Hennig, 2010; Saleh et al, 2007). Recently, a group of putative ATP-dependent chromatin remodellers named PICKLE have been implicated in activation of PcG target genes. In Arabidopsis, there are two *PICKLE* (PKL) paralogues, *PKL* and *PICKLE RELATED2* (*PKR2*), both of which have similar functions to TrxG proteins, acting as transcriptional activators of PcG target genes (Aichinger et al, 2009; Ho et al, 2013). Moreover, it has been demonstrated that PcG and PICKLE are targeted to the same loci, and when both complexes are present the gene remains inactive, but when PICKLE is deposited without PcG, the gene becomes active (Aichinger et al, 2009; Kohler & Hennig, 2010).

1.5.4 Role of chromatin remodelling in plant development

Transcriptional regulation via epigenetic mechanisms has been extensively studied in plants in recent decades and implicated in many plant developmental processes, *inter alia* flowering and seed development (Graeber et al, 2012; Grossniklaus et al, 1998; Guitton et al, 2004; Mylne et al, 2006). One of the best known processes regulated by chromatin remodelling factors is transition from a vegetative to reproductive state (Zhu et al, 2014). As mentioned above, plants have evolved sophisticated mechanisms that control the seasonal timing of flowering in response to selective pressures to maximize their reproductive success. Vernalisation, the acquisition of flowering competence after extended exposure to low temperature, has provided an excellent experimental system for analyses that have led to the emergence of several key concepts in chromatin regulation of development in plants (Michaels & Amasino, 1999). It has been shown that expression of *FLC*, a potent floral repressor belonging to

the MADS-box family of transcription factors, is a target of vernalisation (He, 2012; Ietswaart et al, 2012; Kim & Sung, 2012; Romera-Branchat et al, 2014).

Before vernalization, plants maintain expression of *FLC*, via induction of *FRIGIDA* (*FRI*), which is known to activate *FLC* expression (Choi et al, 2011; Johanson et al, 2000). Genetic evidence has shown that *FRI* acts upstream of *FLC* and mutation in this gene causes an early flowering phenotype. Activity of the *FLC* locus is suppressed by long-term exposure to cold, via the deposition of repressive marks (H3k27me3) on the chromatin region in the first intron by Polycomb repression complex (Gendall et al, 2001; Michaels & Amasino, 1999). The mitotically stable repressive state is maintained by PRC2 in concert with LHP1, which is thought to be a component of a PRC1-like complex in *Arabidopsis* (Mylne et al, 2006). Interestingly, long non-coding RNAs (lncRNAs) have been demonstrated to play a crucial role as potent *cis*- and *trans*-regulators of gene activity and act as scaffolds for chromatin modifying complexes. An antisense lncRNA (known as *COOLAIR*) has postulated involvement in vernalization-mediated FLC repression, presumably recruiting the PRC2 complex to the *FLC* locus (Kim & Sung, 2012; Swiezewski et al, 2009).

Chromatin remodelling has been implicated in seed dormancy development, since mutation in the gene encoding *HISTONE UBIQUITINATION 1* (*HUB1*) leads to the development of seeds with impaired dormancy (Liu et al, 2007). It is a C3HC4 RING finger protein, which functions as the E3 ligase responsible for monoubiquitination of histone H2B, which is correlated with transcription of genes. (Liu et al, 2007) found that expression levels of genes related to seed dormancy (such as *DOG1, ATS2*, *NCED9* and *PER1*) are significantly reduced in *hub1* mutant background, implying a role of this chromatin modification in the process.

Another fascinating process regulated by epigenetic mechanism in plant development is the formation of endosperm during fertilization. Endosperm serves as a source of nutrients in the form of starch, oils and proteins, and is crucial in early stages of seed germination. The FIS-PRC2 complex has been implicated in regulation of this process, since *fis* mutants with alleles derived from the maternal line abort seeds with embryos at the late heart stage and display non-cellularized endosperm with strongly overproliferated chalazal endosperm domains (Grossniklaus et al, 1998; Leroy et al, 2007). Such differential expression of an autosomal gene that is dependent on parental origin is known as genetic imprinting and has evolved in flowering plants and mammals. With regard to epigenetics, imprinted genes are largely controlled by DNA-methylation and the Polycomb group of proteins (Kohler et al, 2012; Lafon-Placette & Kohler, 2014).

1.5.5 Regulation of bud dormancy and the role of chromatin remodelling

Due to the similarity between vernalisation and dormancy release an attractive hypothesis is that chromatin remodelling and epigenetic regulation could be involved in bud dormancy regulation. This hypothesis is further supported by a global analysis of gene expression during dormancy establishment and release presented by Karlberg et al. (2010). The transcriptional data suggest that chromatin remodelling machinery might be involved in bud dormancy regulation as transcript levels of several chromatin remodellers, e.g. histone deacetylases (HDA08 and HDA14) and histone lysine methyltransferase (SUVR3), are elevated during the transition to dormancy. Simultaneously, the expression of Trithorax family genes, which counteract repression by Polycomb repression complexes, are downregulated (Karlberg et al, 2010). More importantly data presented in Paper III and by Englund (2010) show that plants with reduced expression of *FIE*, the evolutionarily conserved component of PRC2, fail to establish dormancy. Changes in histone modifications during dormancy release have also been recently observed in several perennials, implying their involvement in activity-dormancy cycles (Karlberg et al, 2010; Leida et al, 2012; Rios et al, 2014). However, the functions of the genes targeted by these chromatin modifications during dormancy release have not been elucidated, thus it remains to be proven whether the modifications are consequences of dormancy release or play a causal role in dormancy regulation.

1.6 Activation of bud break

Even less is known about the activation of bud break than bud dormancy, partly because it is difficult to separate the regulation of dormancy release and bud break. However, some insights have been obtained from molecular data. For example, *FT1* expression is upregulated after dormancy-releasing cold treatment, as is expression of GA biosynthesis-related genes (Karlberg et al, 2010; Rinne et al, 2011). Given that both of these factors promote growth, they could both be potentially involved in bud break, although there is no functional evidence of their involvement. Furthermore, it has also been shown that *CEN-like* genes could be involved in dormancy release and/or bud break since their overexpression delays bud break whereas their downregulation advances it.

Interestingly, Yordanov et al (2014) recently identified a novel putative regulator of bud break in *Populus* named *EARLY BUD BREAK 1* (*EEB1*). Ectopic expression of *EEB1* leads to precocious bud burst (relative to WT timing), while downregulation has the opposite effect. *EBB1* encodes an AP2/ERF domain transcription factor and was shown to have primary effects on cell division, as *EBB1oe* plants displayed 80% higher cell division rates than WT counterparts. It seems that *EEB1* predominantly affects cell proliferation, thereby activating this process in SAMs and leaf primordia, which eventually leads to bud break (Yordanov et al, 2014). It remains unclear whether it acts together with one of the *PopCENs*, which are also implicated in bud break (Mohamed et al, 2010; Yordanov et al, 2014) or via an independent pathway.

1.7 Hormonal regulation of activity-dormancy transitions

Hormones play key roles in the growth and development of plants, thus unsurprisingly various hormones also have demonstrated involvement in the control of diverse aspects of trees' annual growth cycles. This section characterizes current knowledge of the hormonal regulation of SD-controlled responses in trees. I particularly focus on ABA and GA, as these are most relevant to growth cessation, adaptive response and dormancy in the apex.

1.7.1 Abscisic Acid (ABA)

ABA is a known phytohormone that plays a critical role in adaptive responses to various stresses such as salinity, cold and drought, hence it is often named the stress response hormone (Umezawa et al, 2010). It has also been implicated in various developmental processes, such as maintenance of seed dormancy and stomatal opening (Graeber et al, 2012; Kim et al, 2010; Kucera et al, 2005; Mishra et al, 2006).

Extensive investigation of the ABA signalling pathway in the herbaceous plant *Arabidopsis* has led to the discovery of the main molecular players, and a group of ABA receptors (named *PYR/PYL/RCAR*) has been recently identified, filling a gap in understanding of ABA perception and signalling in plant cells (Ma et al, 2009; Park et al, 2009). Protein phosphatases PP2C (known as *ABI1* and *ABI2*) play important role in ABA signalling (Leung et al, 1997). At low levels of ABA, PP2Cs dephosphorylate SnK2 kinases, rendering them unable

to phosphorylate downstream components, e.g. transcription factors such ABA Response Element Binding (AREB) proteins (Choi et al, 2000; Uno et al, 2000). However, in the presence of higher levels of ABA, receptors of the hormone bind to PP2Cs, rendering them unable to dephosphorylate SnRK2 kinases. Consequently, SnRK2 kinases can phosphorylate transcription factors that can bind to promoter regions of ABA-responsive genes and activate their expression (Umezawa et al, 2010).

Responses to short day signals such as acquisition of cold hardiness are similar to stress responses e.g. cold hardiness acquisition in response to low temperature. Thus there have been speculations that ABA may participate in their regulation as well in dormancy control (Karlberg et al, 2010; Li et al, 2003; Rohde et al, 2002; Ruttink et al, 2007). Accordingly, ABA levels increase in poplar following exposure to SDs (Ruttink et al, 2007). Furthermore, in their global transcription analysis Karlberg et al (2010) found that genes of the ABA biosynthetic pathway were strongly upregulated under SDs, including *NCED* genes, which catalyse the first step of ABA production, and the alcohol dehydrogenase *ABA2a*, catalysing conversion of xanthoxin to abscisic aldehyde. In contrast, ABA inactivation genes such as *CYP707A* and *UGT73B3* were strongly downregulated. Interestingly, in concert with upregulation of ABA biosynthetic genes, increased expression of genes involved in the ABA signalling pathway was observed, such as *RCAR* genes encoding ABA receptors. Clearly, this could enhance ABA responses following SDs and may mediate some of the SD-controlled responses, such as cold hardiness-related transcriptional changes.

Additional evidence for the involvement of ABA in diverse SD-controlled responses comes from analysis of ABA-insensitive hybrid aspen plants. Overexpression of the dominant-negative version of *ABI1* in hybrid aspen, which makes plants virtually insensitive to ABA, reportedly leads to deformation of apical buds, making them smaller and greener, with immature bud scales, fewer hairs and no external cuticular layer on the scales (Petterle, 2011). Interestingly, ABA-insensitive plants (named *abi1-1*) display normal growth cessation responses, but are unable to develop endodormancy. In the same study it was also shown that changes in ABA sensitivity play a crucial role in SD-induced adaptive responses (cold acclimation, dehydration and metabolic changes) since genes associated with these responses were differentially regulated between *abi1-1* and wild type plants after exposure to SDs. In summary, these data strongly indicate that ABA is an important player in SD-related responses in trees' annual growth cycles.

1.7.2 Gibberellins (GA)

Gibberellins are known regulators of stem elongation in trees and other plants (Eriksson et al, 2000; Olsen et al, 1995b), and GA levels are key targets in SD-induced growth cessation (Olsen et al, 1995a). In woody plants it has been shown that levels of GAs decline in response to SD signals and hybrid aspens that cannot downregulate GA levels after exposure to SDs are defective in growth cessation (Eriksson et al, 2000; Junttila et al, 1997; Olsen et al, 1995a). It is thought that lowering levels of active GAs is a crucial step for growth cessation, since overexpression of *GA20 oxidase* (*GA20ox*), a key enzyme in the GA biosynthetic pathway, leads to delayed growth cessation following exposure to SD (Eriksson et al, 2000). Indeed, transcript levels of *GA20ox* appear to be downregulated upon SD in wild type poplars (Eriksson et al, 2014; Eriksson et al, 2000; Karlberg et al, 2010). Additionally, the expression of genes involved in inactivation of active GAs, e.g. *GA2OX8a*, *GA2OX6*, *GA2OX2* and *GA2OX4* (Hedden & Proebsting, 1999) is strongly induced by SDs (Karlberg et al, 2010). These findings, in concert with measurements of GA levels in poplars subjected to SD, show that genes of both GA biosynthetic and catabolic pathways are primary targets of SD signalling pathways that downregulate GA levels (Karlberg et al, 2010; Olsen et al, 1997).

Recently, Eriksson et al (2014) attempted to elucidate whether sensitivity to GAs might play a role in SD-induced growth cessation. Interestingly, they found that trees ectopically expressing the GA receptor *GID1* enter growth cessation as early as WT counterparts, suggesting that growth cessation primarily depends on concentrations of bioactive GAs rather than the abundance of GID1. They also observed that in plants overexpressing *GA20ox* subjected to SD treatment *FT2* was rapidly downregulated, as in WT plants. These data suggest that the regulation of *FT2* expression by SDs is independent of GA regulation or, alternatively, that GA is regulated downstream of *FT*. Thus, it is possible that cessation of elongation growth in trees may be controlled by two parallel pathways involving GA and photoperiods (Eriksson et al, 2014). Not only growth cessation but also GAs are implicated in dormancy release and possibly bud burst. For example, it has been shown that exposure to low temperature leads to upregulation of *GA20ox* and other genes involved in GA biosynthesis, but downregulation of catabolic genes such as *GA2OX8a* and *GA2OX8b*, suggesting that cold treatment could promote production of GAs, thereby participating in bud burst (Karlberg et al, 2010). Moreover exogenous application of GAs leads to bud break accompanied by opening of plasmodesmata (Rinne et al, 2011).

2 Objectives

The overall aim of the studies this thesis is based upon was to improve understanding of the molecular level regulation of growth cessation, dormancy and other SD-related processes in hybrid aspen. More specifically, the following questions were addressed in the studies reported in the three appended papers.

- How do photoperiodic signals control seasonal growth cessation in trees? (**Paper I and Paper II**)
- What is the mechanism underlying the temporal co-ordination of growth cessation and adaptive responses to the advent of winter? (**Paper II**)
- How is bud dormancy regulated? (**Paper III**)

3 Results and discussion

In the work underlying this thesis we addressed some of the key questions regarding the molecular regulation of growth cessation (**Papers I and II**), adaptive responses to the advent of winter (**Paper II**) and dormancy (**Paper III**) in the model tree hybrid aspen. We found that tree homologs of components of the *Arabidopsis* flowering pathway (*APETALA1* and *FD*) are involved in the control of seasonal growth cessation by photoperiodic signals in *Populus* (Papers I and II).Moreover, *FD* was also found to control transcriptional programs associated with the adaptive responses (**Paper II**). We showed that *FIE*, a component of an evolutionarily conserved epigenetic protein complex (PRC2), participates in development of bud dormancy (**Paper III**). We also demonstrated that the plant hormone ABA plays a role in the acquisition of dormancy in hybrid aspen (**Paper III**).

3.1 A model for tree biology

Arabidopsis thaliana was adopted as a model research plant species for many obvious reasons, including its small genome, short generation time, ease of genetic analysis and convenience to grow (Koornneef & Meinke, 2010). Vast numbers of tools and molecular techniques are now available to study gene functions in *Arabidopsis*, making it by far the most intensively studied plant. The corresponding model in cereals and monocots is rice. Although not as well developed (as a model) as *Arabidopsis*, rice is increasingly a model of choice for those interested in monocots and cereals, and diverse tools have been developed for its analysis, rivalling those available for *Arabidopsis*.

In several respects trees are clearly very different from *Arabidopsis* and rice, particularly in their size, long life span, long generation time and

perennial growth cycle. However, there was no corresponding model for trees and other long-lived perennial plants until relatively recently, despite their commercial importance and marked biological differences from annual models such as *Arabidopsis* and rice. Thus there was a need for an experimental model for studying biological processes that occur in trees and cannot be studied in an annual plant such as *Arabidopsis*. To meet this need *Populus* has recently emerged as the main model species for trees (Jansson & Douglas, 2007). The poplar genome was sequenced in 2006 (Tuskan et al, 2006), thereby facilitating the cloning and functional studies of genes of interest, and greatly expanding possibilities to use genetic techniques like QTL mapping, DNA microarrays and next generation sequencing (NGS). As a result novel insights have been obtained into mechanisms underlying many biological processes in trees, e.g. autumnal leaf senescence, secondary wood formation and seasonal regulation of vascular cambium (Andersson et al, 2004; Baba et al, 2011; Derba-Maceluch et al, 2015; Druart et al, 2007; Tallis et al, 2010; Zhong et al, 2011). *Populus* was chosen as the first tree organism to be sequenced for several reasons. It has a relatively small genome compared to many other trees, e.g. Norway spruce and pines. Several *Populus* genotypes can be propagated in vitro and transformed by Agrobacterium-mediated techniques, facilitating analysis of gene function. Moreover it is a commercially interesting species due to its suitability for fast biomass production in short rotation forestry. Nevertheless, working with trees also has disadvantages, most of which are related to space and time constraints, but the dioecious nature of some trees, including *Populus*, also complicates genetic analyses (Jansson & Douglas, 2007).

3.2 How is seasonal growth cessation regulated? (Papers I and II)

3.2.1 LAP1 is the target of SD signals downstream of the CO/FT module in growth cessation

The regulation of timing of growth cessation in *Populus* has been extensively studied in recent years, and the results have revealed (*inter alia*) the central role of the CO/FT module in this process (Bohlenius et al, 2006; Hsu et al, 2011). A crucial step in the induction of growth cessation is rapid downregulation of *FT2*, after perception of the SD signal. Functional analysis

of *FT* genes has proven that they are required for SD-induced growth cessation responses, since their overexpression causes failure in growth cessation upon SD treatment, whereas reduction in their expression leads to earlier responses to SD signals than in wild type plants (Bohlenius et al, 2006; Hsu et al, 2011). However, these are early acting factors in photoperiodic control of growth and it was not known how downregulation of *FT2* could lead to growth cessation following SDs, particularly how genes that regulate cell cycles could be controlled by the CO/FT module. Although the signalling pathway downstream of CO/FT involved in the control of flowering time had been intensively studied, it was not thought that the acquired information could be useful for understanding growth cessation, since in this case the pathway would converge on control of cell cycle genes, which would need to be switched off after SDs following the downregulation of *FT2*. A key discovery, by my group in previous studies, was that the transcription factor *AIL1* (*AINTEGUMENTA-Like 1*) acts downstream of the CO/FT regulon. Although AIL1 and related transcription factors control cell cycle genes, providing an explanation of CO/FT-mediated control of growth cessation, it still remained unclear how CO/FT could control AIL expression since obtained data suggested that FT does not directly control *AIL1* expression (Karlberg et al, 2011). This prompted us to search for genes that could be primary targets of the CO/FT module and fill the gap in understanding of growth cessation regulation at the molecular level in *Populus*. We used a functional genomic approach to discover new players in the pathway acting between CO/FT and *AIL1*: subjecting a collection of transgenic hybrid aspen plants overexpressing transcription factors (TFs) to SDs and identifying lines with altered growth cessation responses. Our experimental setup enabled the discovery of transgenic hybrid aspen plants that displayed a severe delay in SD-induced growth cessation. These hybrid aspen plants overexpressed a MADS-box transcription factor that was closely related to *APETALA1*, which plays an important role in flowering in *Arabidopsis*. Amino acid alignments and a constructed phylogenetic tree suggested that this is an ortholog of *APETALA1* (**Paper I, Figure S2**), hence it was named *Like-APETALA1* (*LAP1*).

Studies using the annual plant *Arabidopsis* have shown that *AP1* acts downstream of the CO/FT module in the regulation of flowering time and flower development (Ferrandiz et al, 2000), therefore we investigated whether *LAP1* is a target of CO/FT. In order to test this hypothesis we used several approaches. *LAP1* is rapidly downregulated in WT plants subjected to SD, whereas in trees overexpressing FTs we observed no downregulation of *LAP1* (**Paper I, Figure 2B and C**). Interestingly, steady state levels of *LAP1* in *FToe* and *FTRNAi* plants in long days were higher and lower, respectively, than in

wild type plants (**Paper I, Figure 2 D),** suggesting positive regulation by FTs. If the assumption that *LAP1* acts downstream of CO/FT is correct, the perception of SD signal itself should not be affected (as reflected by rapid downregulation of *FT2* in WT) in plants overexpressing *LAP1*, which have attenuated growth cessation responses. Accordingly, our experimental data showed that *FT2* downregulation is not altered by SD treatment in *LAP1oe* trees, relative to WT trees, strongly suggesting that *LAP1oe* plants can perceive SD signals normally (in other words LAP1 is not involved in perception of SD stimuli) and thus acts downstream of CO/FT in the signalling pathway (**Paper I, Figure 2 E-G**). However, to conclusively prove this point we downregulated *LAP1* in FT-overexpressing plants. *FT* overexpressors fail to cease growth in response to SDs, but when *LAP1* expression is downregulated in *FT* overexpressors, the double transgenics (overexpressing FT with reduced LAP1 expression) can respond to SDs and cease growth, confirming that *LAP1* acts downstream of the CO/FT module.

3.2.2 AIL1 is the direct downstream target of LAP1

Paper I presents an extensive analysis of LAP1's role in hybrid aspen, showing that this transcription factor is needed to promote growth in long days, since its expression is downregulated upon SD treatment (**Paper I, Figure 2B and C**) and its overexpression leads to altered growth cessation responses, manifested by the production of higher than WT numbers of leaves under SD **(Paper I, Figure 1 E**). Nevertheless, these findings still did not identify the link between CO/FT, LAP1 and growth control in the manner provided by their *Arabidopsis* homologs in the transition to flowering. Thus, we also wanted to identify components acting downstream of LAP1 in the signalling pathway linked with the control of growth. A potential candidate that could provide such a link was discovered by Karlberg et al (2011), transcription factor *AIL1*. As already described, it is a target of SD signals and its misregulation leads to altered growth cessation responses (Karlberg et al, 2011). It is also known from studies in *Arabidopsis* that *AINTEGUMENTA* is a positive regulator of cell proliferation and its overexpression results in increased numbers of cells in plants (Mizukami & Fischer, 2000), strongly indicating that it is a potential target of LAP1 (since cell division needs to be arrested during growth cessation). Therefore, we next investigated the regulation of *AIL1* in LAP1 transgenics and found that *LAP1* can control *AIL1* expression since LAP1 overexpressors fail to downregulate *AIL1* expression after SDs. However, a remaining question was whether *LAP1* could directly control the expression of

AIL1 or whether there was yet another intermediary factor acting between *LAP1* and *AIL1*. We addressed this question by testing whether LAP1 binds to the promoter region of *AIL1* using EMSA assays. The results indicate that LAP1 does indeed bind to the promoter region of *AIL1* (**Paper I, Figure 4D**). Since *AIL1* and its homologs are known to positively control the expression of key cell cycle regulators such as D-type cyclins, *LAP1* control of *AIL1* provides the elusive link between the CO/FT module and regulators of growth such as *AIL* genes. Thus our observations fill a major gap in knowledge of the signalling pathway of photoperiodic control of growth cessation in hybrid aspen (**Paper I, Figure 5**).

3.2.3 Is LAP1 the only member of the family contributing to photoperiodic control of growth cessation?

As can be seen in **Fig. S2A and B** there are closely related paralogs of *LAP1* in *Populus*, as in *Arabidopsis*, where there are two closely related *AP1* paralogs, *CAULIFLOWER* (*CAL*) and *FRUITFULL* (*FUL*), which also act in flowering. Thus the question arises whether the *LAP1* paralog in *Populus* is also involved in growth cessation or may have gained a new function through evolution? Currently the answer to this question is not known. Thus it would be interesting in the future to functionally characterise the function of these paralogs of *LAP1*.

3.2.4 Is LAP1 only involved in photoperiodic control of growth in trees?

Interestingly, ectopic expression of *LAP1* in *Arabidopsis* causes an early flowering phenotype (**Paper I, Fig S3**), similarly to overexpression of *AP1* in *Arabidopsis* (Bowman et al, 1993). However, when *LAP1* is ectopically expressed in hybrid aspen it does not result in early flowering, contrary to overexpression of FTs, in both *Populus* and *Arabidopsis* (Bohlenius et al, 2006; Hsu et al, 2011; Kardailsky et al, 1999). A possible explanation for this difference is that this function may have been taken over by a paralog of *LAP1*. Alternatively, a different mechanism may regulate flowering time and flower development in trees related to the long juvenile phase, in which trees do not flower. Consequently, factors other than LAP1 may be involved in flowering in trees.

Two other interesting questions that remain open are whether *AIL1* is the only target of LAP1 and if it is at all involved in flower development in trees? For example, ChIP assays and global transcript profiling of *Arabidopsis AP1* transgenics have revealed several hundred direct targets of AP1 (Kaufmann et al, 2010; Sundstrom et al, 2006). Thus, it seems likely that *LAP1* is also involved in other pathways, although surprisingly *LAP1* overexpressors and RNAi-silenced plants do not display easily identifiable phenotypes. To address this possibility, a detailed analysis of LAP1 and its paralogues should be carried out (e.g. chromatin IP analysis followed by deep sequencing to identify downstream targets). Knowledge of the interacting partners of LAP1 and their roles in LAP1 function is also required. It is well known that AP1 interacts with other MADS box proteins, e.g. in *Arabidopsis* (Smaczniak et al, 2012), and that homologs of these interacting proteins are present in *Populus*. However, currently we do not know if LAP1 interacts with other transcription factors or cofactors and (if so) how they contribute to its function. For example, it can be hypothesised that one reason why LAP1 overexpressors do not flower early is because juvenile plants may lack LAP1 interactors that are essential for the formation of LAP1 complexes involved in flowering.

3.2.5 Evolutionary conservation of signalling pathways responding to photoperiodic signals

In recent years it has become apparent that the CO/FT module is not only involved in flowering time control but also several other pathways, e.g. pathways controlling growth cessation, bulbing in onion and tuberisation in potato (Lee et al, 2013; Navarro et al, 2011). However *AP1* orthologs in other plants such as onion or potato have not been characterised as yet, so it is not known whether *AP1/LAP1* orthologs are involved in bulbing or tuberisation. Thus it remains to be seen whether the divergence in signalling pathways in the control of bulbing, tuberisation and other processes occurs downstream of the CO/FT module at the AP1/LAP1 level or further downstream than these MADS box transcription factors.

3.3 The FD transcription factor mediates photoperiodic control of growth cessation

The identification of *FT* as the key early target of SDs in the induction of growth cessation clearly suggested that photoperiodic control of growth cessation has parallels with flowering time regulation. FT does not possess DNA binding activity, raising questions regarding the mechanism whereby FT could control the expression of downstream genes such as *LAP1* at the transcriptional level. In flowering time regulation, FT and its orthologs in other plants (e.g. rice) are known to interact with the bZIP transcription factor FD in control of the expression of its downstream targets, such as *AP1* and *OsMADs15* in *Arabidopsis* and rice, respectively (Abe et al, 2005; Taoka et al, 2011). However, in contrast with flowering time, few studies have analysed the role of *FD* so far, other than in stomatal control and leaf development in *Arabidopsis* and rice (Tsuji et al, 2013a), respectively. Thus the questions we sought to address were how does FT control the expression of downstream genes and, more importantly, does FT interact with tree FD orthologs in the photoperiodic control of growth cessation in trees as well.

3.3.1 The Populus genome contains two closely related *FD* orthologs

To address the role of hybrid aspen FD in growth cessation we identified a bZIP transcription factor that is an *Arabidopsis* ortholog of *FD* (Abe et al, 2005; Wigge et al, 2005), named *FD-like 1* (*FDL1*). Analysis of the Populus genome led to the identification of two closely related homologs of *Arabidopsis* and rice *FDs* that we named *FD-like 1* (*FDL1*) and *FD-like 2* (*FDL2*). Phylogenetic analysis demonstrated that *FDL1* and *FDL2* are counterparts of *Arabidopsis FD* and indicated that the two *FDLs* have arisen via a gene duplication event (**see Figure 5**). Sequence analysis indicated that both of these *FDLs* have the conserved motifs, such as the S/TAP motif essential for interaction with 14-3-3 protein that mediates interaction with FT. The main difference between the two *FDLs* is that the putative protein encoded by *FDL2* has more amino acids at the C-terminus, and thus a longer bZIP domain, than the protein putatively encoded by *FDL1*.

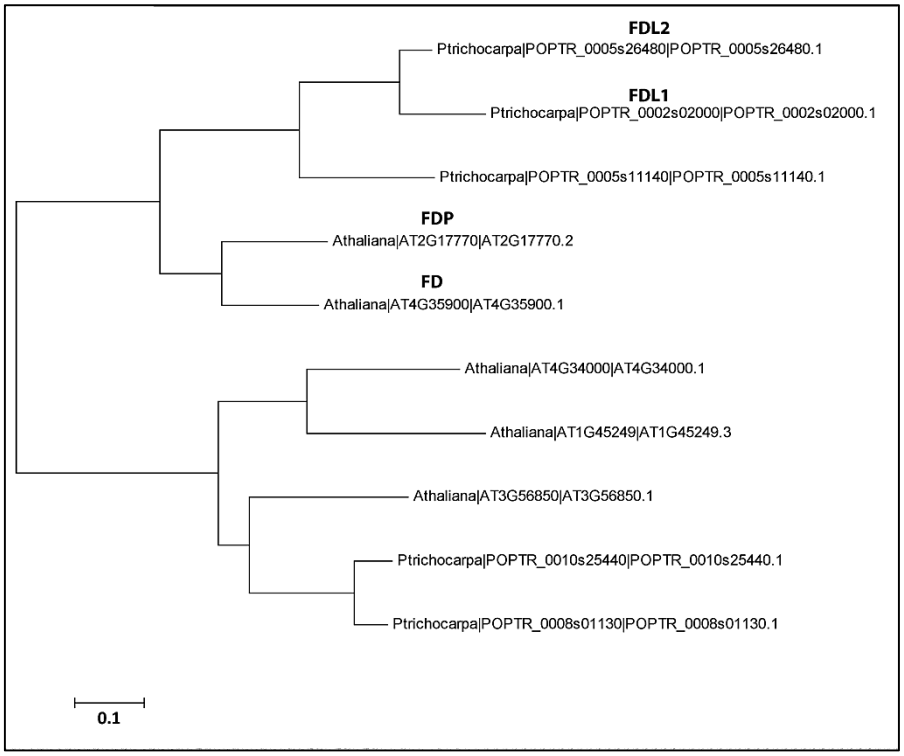

Figure 5. Phylogenetic tree of *FD* orthologs from *Arabidopsis* and *Populus*

3.3.2 *FDL1*, but not *FDL2*, mediates photoperiodic control of growth

In *Arabidopsis*, like *Populus*, *FD* has a close homolog named *FDP* and both are involved in flowering time control (Jaeger et al, 2013), raising the possibility that (analogously) both *FDL1* and *FDL2* may be involved in photoperiodic control of growth in hybrid aspen. To test this hypothesis, we generated transgenic hybrid aspen plants that overexpressed *FDL1* and *FDL2* or had reduced expression of these two genes. Detailed functional analysis revealed that overexpression of *FDL1* leads to delayed responses to SD signals, whereas RNAi downregulation of *FDL1* results in faster responses to SD than in wild type plants (**Paper II, Figure S4 A**). Interestingly, despite the high similarity between *FDL1* and *FDL2*, neither the overexpression of *FDL2* nor its downregulation resulted in altered growth cessation responses in hybrid aspen. Thus, *FDL1* but not *FDL2* is involved in photoperiodic control of growth cessation. Intriguingly, ectopic expression of *FDL2* leads to a dwarf phenotype of hybrid aspen (**Paper II, Figure S3**). These studies reveal that in

contrast to *Arabidopsis*, the functions of the two *FDLs* have diverged. However, the role of *FDL2* is still unclear.

3.3.3 FDL1 and FDL2 can interact with FT

Florigen activation complex (FAC; consisting of FT, FD and protein 14-3-3) regulates flowering in various organisms (Abe et al, 2005; Taoka et al, 2011; Wigge et al, 2005). FT does not have DNA binding activity, thus it is believed that regulation of its downstream targets occurs via an interacting partner, e.g. FD or BRANCHED1 (BRC1), which confers DNA binding specificity to FAC. Thus, some differences in FDLs' functions could possibly be due to differences in their ability to interact with FT. To address this possibility we used two approaches. Firstly, we investigated the ability of FDLs to physically interact with FT using a Bimolecular Fluorescence Complementation (BiFC) assay (Walter et al, 2004). The results confirmed that they can indeed physically interact (**Paper II, Fig. S5**). Secondly, we used a transcription read out assay in rice to probe the transcriptional activity of FDL-FT complexes. In this assay, co-transfection of FT and FD leads to activation of the rice orthologue of *AP1*, *OsMADs15*, a FT target in rice. Surprisingly, while both FDLs interact with FTs, as shown in BiFC assays, FDL1 but not FDL2 activated the expression of *OsMADS15* when co-expressed with *FTs* (**Paper II, Figure 2**). Thus, these results suggest that while both FDLs are capable of interacting with FTs, there is a clear difference between FDL1-FT and FDL2-FT transcriptional complexes. Although the reason for the difference is not clear, it may be related to the difference in structure between the FDL1 and FDL2 DNA binding domains due to a C-terminal extension in FDL2. Regardless of the reason for the differences between FDL1-FT and FDL2-FT complexes, the data obtained from the rice read-out assay corroborate the hypothesis that FDL1 and FDL2 have different functions, and provide insights into why *FDL1* is involved in SD-mediated growth cessation, but not *FDL2*.

The role of *FDL2* appears to be quite puzzling. According to our data it does not participate in SD-related growth cessation, yet it is upregulated upon exposure to SD signals in wild type plants, implying some role in SD-related responses (**Figure 6**). Its interaction with FTs indicates that it is a part of transcriptional complex with FT, but with an undiscovered role. It is possible that FTs interact with FDL2 in the activation of novel, unknown targets involved in regulatory processes other than growth cessation.

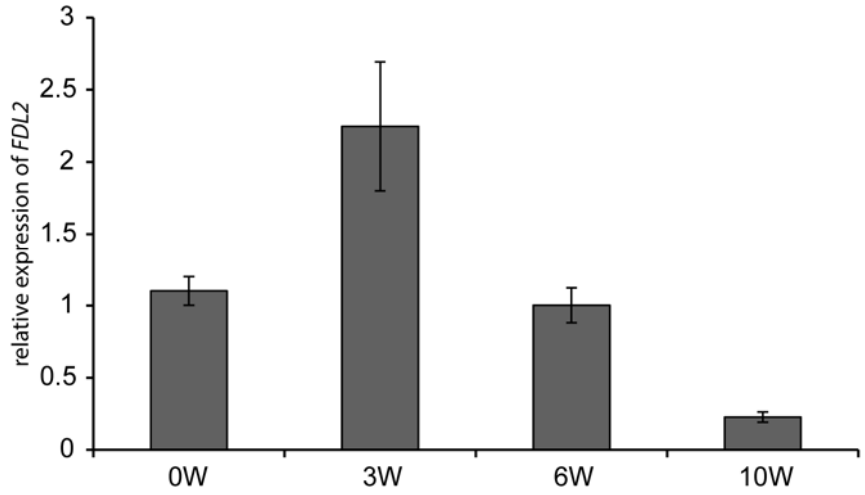

Figure 6. Relative expression of *FDL2* in hybrid aspen subjected to SD

Additionally, we discovered that *FDL2* is alternatively spliced (**Figure 7**), so it is tempting to speculate that this additional level of regulation further reflects its role in the control of hitherto undiscovered physiological responses. However, elucidating the function of *FDL2* will require further detailed and careful analysis in the future.

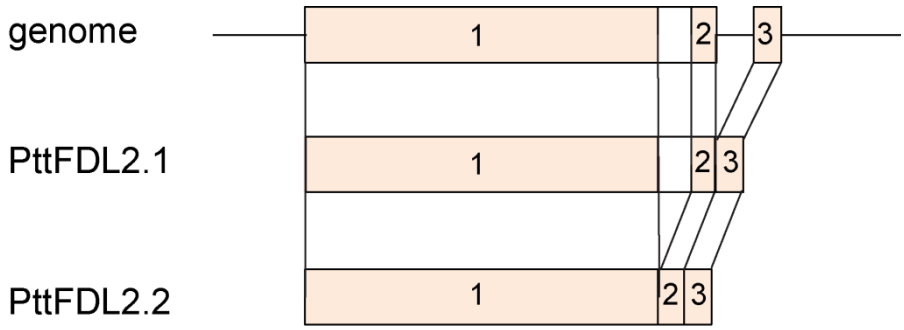

Figure 7. Schematic representation of FDL2 splice variants.

3.3.4 Are tree orthologs of negative regulators of flowering involved in growth cessation?

Tree orthologs of *CO*, *FT* and *AP1*, all of which promote flowering, act in photoperiodic control of growth. This raises the question whether the negative acting components in flowering are also involved in growth cessation. None of the (few) tree homologs of negative regulators of flowering in *Arabidopsis* that have been analysed to date appear to have major roles in growth cessation. A study of the *Arabidopsis* ortholog *TFL1* (named *CENTRORADIALIS-LIKE 1*) in *Populus* by Ruonala et al (2008) initially indicated a role of this gene in the regulation of stem elongation, but later studies with trees in which the gene was up- and down-regulated did not corroborate this hypothesis (Mohamed et al, 2010; Ruonala et al, 2008b). It has been speculated that *RAV1* and *RAV2*, which are orthologs of *Arabidopsis TEMPRANILLO*, could be involved in controlling seasonal growth cessation and bud set since *Arabidopsis* counterparts repress expression of *FT* (Castillejo & Pelaz, 2008; Moreno-Cortes et al, 2012). However, a detailed analysis of *Populus RAV1* and *RAV2* did not corroborate this hypothesis either. Another interesting candidate that has not received attention to date is the tree homolog of BRC1 (Niwa et al, 2013). In *Arabidopsis* BRC1 interacts with FT like FD, however in contrast with *FD*, *BRC1* delays flowering. Thus it would be interesting in the future to investigate the role of *BRC1* in growth cessation responses of trees.

3.3.5 Additional role of FDL1 in SD-related responses

Analysis of *FDL1* expression in WT hybrid aspen revealed that it is upregulated upon SD treatment, implying some additional role in SD-related processes (**Paper II, Figure 4**). Its upregulation coincides with bud formation, bud maturation and adaptive responses (between weeks 3 and 6), suggesting some role in these processes. Thus, we investigated whether the expression of several known marker genes e.g. *CHALCONE SYNTHASE* (*CHS*) *CINNAMATE 4-HYDROXYLASE* (*C4H*), *OSMOTIN* (*OSM*), and *LATE EMBRYOGENESIS ABUNDANT* (*LEA*) is changed in plants with altered expression of *FDL1*. Our initial assumption was verified by qPCR analysis, showing that the abovementioned genes are misregulated in transgenics with altered *FDL1* expression (Paper II, Figure 5), implying that *FDL1* plays a key role in mediating the adaptive responses controlled by SDs. Further support for a role of *FDL1* in bud maturation comes from the observation that buds of *FDL1* overexpressors are green (in accordance with their weak expression of

CHS and *C4H* genes), while those of wild type plants are normally reddish-brown due to the accumulation of phenylpropanoids.

3.3.6 FDL1 mediation in adaptive responses may involve interaction with ABI3

Findings discussed above showed that FDL1 interacts with FT in photoperiodic control of growth, but the mechanisms whereby it participates in adaptive responses and bud maturation remained to be elucidated. Both of these processes are activated following exposure to SDs, when *FT* expression is reduced. A possibility we considered is that FDL1 may interact with other transcription factors in control of the processes, potentially bZIPs as they are known to form transcription factor complexes with other transcription factors (Vinson et al, 2006). Furthermore, we had already noted that bud maturation and adaptive responses in FDL1 transgenics show similar alterations to those in ABI3 transgenics (Ruttink et al., 2007; Petterle, 2011). Notably, in ABI3 overexpressors the expression of adaptive response-related genes and bud maturation are altered, with buds being greener. Thus, *ABI3* (a transcription factor involved in ABA responses) had been shown to participate in the control of bud maturation and adaptive responses. These observations prompted us to investigate whether FDL1 and ABI3 interact. Results of two independent assays, BiFC and co-IP FDL1, corroborated this hypothesis (**Paper II, Figures 6 and S9**). These results, together with the similarity of phenotypes of FDL1 and ABI3 transgenics, and similarity of FDL1 and ABI3 effects on transcriptional control of bud maturation and adaptive response-related genes, strongly support the hypothesis that *FDL1* and *ABI3* impinge on similar downstream targets in adaptive SD-responses and the development of apical buds.

3.3.7 Temporal co-ordination of growth cessation and adaptive responses

Cessation of growth occurs simultaneously with the activation of programs including bud maturation and cold hardiness acquisition. Thus, growth cessation and activation of adaptive responses needs to be temporally coordinated so that the adaptive response program can be activated only when growth cessation is induced. How is this temporal coordination achieved? Clearly, the same photoperiodic signal is involved in control of both growth

cessation and the adaptive response program, but how does this signal coordinate two programs? The molecular mechanism underlying the temporal co-ordination of these physiological and developmental processes in response to SDs is not well understood, but our data provide indications of a possible pathway. *FT* downregulation leads to growth cessation, but simultaneously makes FDL1 available for interaction with other factors, e.g. ABI3, which are induced after SDs and may then activate the adaptive response program. Moreover, since *FT* overexpression suppresses *FDL1* activation by SDs, *FT* downregulation may further contribute to activation of the adaptive response program since, in the absence of FT, SDs can induce *FDL1* expression.

3.4 How is bud dormancy regulated?

The regulation of bud dormancy remains poorly understood. One of the obstacles hindering elucidation of its regulation is the lack of suitable mutants, which has greatly facilitated elucidation of seed dormancy regulation in *Arabidopsis* (Bentsink et al, 2010; Bentsink et al, 2006; Koornneef et al, 2002). Consequently, bud dormancy in trees has been mostly explored in correlative studies. These studies have implicated hormones, e.g. GA and ABA (Rinne et al., 2012), and cell-cell communication as key players in bud dormancy regulation, but their roles in the process remain essentially correlative (Cooke et al., 2012).

3.4.1 Identification of potential regulators of bud dormancy

In order to explore bud dormancy establishment at a molecular level we first conducted a global transcriptomic analysis, in which we identified candidate genes and pathways to elucidate their roles in the process. Previous transcriptome analysis had shown that ABA biosynthetic genes are upregulated during SD treatment (most strongly between 6-10 weeks), and ABA levels increase before the onset of dormancy (Karlberg et al., 2010; Petterle, 2011). Moreover, ABA plays a clear role in seed dormancy (Lin et al, 2007; Matakiadis et al, 2009; Raz et al, 2001). These observations led us to investigate the role of ABA in bud dormancy development. In addition to ABA we had previously noticed that genes known to be involved in chromatin remodelling are also upregulated (Druart et al, 2007; Ruttink et al, 2007). Interestingly, some of these candidates, e.g. *FERTILISATION INDEPENDENT*

ENDOSPERM (*FIE*) belonged to Polycomb Repression Complex 2 (PRC2), known to deposit repressive marks on chromatin (Karlberg et al, 2010). This prompted us to investigate whether ABA and PRC2 complexes participate in acquisition of dormancy in hybrid aspen and whether these potential players interact.

3.4.2 PRC2 and ABA are required for bud dormancy establishment

To test the roles of PRC2 and ABA in bud dormancy establishment, we constructed trees with downregulated expression of *FIE* (named FIERNAi) and trees with reduced sensitivity to the hormone ABA (by overexpressing the dominant negative version of the abi1-1 allele ABI1 from *Arabidopsis*, named henceforth abi1-1) (**Paper III**). Subsequently we tested whether downregulation of *FIE* or attenuating the ABA response affects bud dormancy establishment. In order to test for dormancy we subjected FIERNAi, abi1-1 and WT plants trees to 11 weeks of SD, which is sufficient time for WT plants to develop endodormancy. Following this dormancy induction treatment we moved the trees back to LD conditions in order to test whether dormancy was established in the WT and the two transgenics. Under these assay conditions, WT plants failed to reactivate growth and did not undergo bud burst, indicating the establishment of dormancy. In contrast, neither FIERNAi nor abi1-1 plants developed dormancy, since they were able to reactivate growth as shown by bud burst upon transfer to long days after 11 weeks of SDs (**Paper III, Figures 1 and 3**). Interestingly, both transgenics responded normally to SD signals in terms of growth cessation, indicating that ABA and *FIE* are required for the photoperiod-mediated dormancy establishment and it is not a secondary effect of altered growth cessation or perturbed perception of SD signals.

3.4.3 Photoperiodic control of bud dormancy involves crosstalk between PRC2 and ABA responses

Our data showing that PRC2 and ABA are involved in photoperiodic control of bud dormancy prompted us to investigate if these two components operate in the same pathway or parallel pathways. To address this we firstly investigated whether ABA responses were altered in the FIERNAi lines. Therefore we analysed ABA responses in FIERNAi plants and compared them with wild type responses both before and after SDs. We observed that the induction of *KIN2*, a gene whose expression is induced by ABA and is thus a marker for

ABA responses, was reduced in FIERNAi lines especially after SDs when dormancy is established, suggesting that ABA responses are attenuated in FIERNAi plants. Moreover, in agreement with attenuation of ABA responses, transcriptomic analysis of genes involved in ABA signalling *PYR/PYL/RCAR* (encoding ABA receptors) and *ABI1* (encoding a PP2C with negative activity in ABA responses) revealed that ABA receptor and *ABI1* levels were respectively higher and lower in FIERNAi plants than in wild type plants (data not shown). The simultaneous reduction in ABA receptor levels and increased *ABI* levels potentially explain why the ABA response is attenuated in FIERNAi plants. These data also suggest crosstalk between PRC2 and ABA responses, with PRC2 acting via ABA in photoperiodic control of dormancy. PRC2 acts by repressing gene expression through depositing repressive marks, e.g. at the *FLC* locus in vernalisation (De Lucia et al, 2008; Sung & Amasino, 2004), thus it will be interesting to identify the target of PRC2 in bud dormancy regulation in the future.

3.4.4 PICKLE, a negative regulator of dormancy

To further investigate how ABA could mediate in bud dormancy regulation we compared gene expression in abi1-1 and wild type plants after SDs (Petterle, 2011). Our transcriptional analysis revealed that the tree ortholog of *PICKLE* (*PKL*), a known chromatin remodelling factor of the CHD3 family, is expressed more strongly in abi1-1 than in wild type plants. This observation was particularly interesting since it has been reported that *PKL* acts antagonistically to PRC2 in *Arabidopsis* (Aichinger et al, 2009). Therefore we hypothesized that ABA (which promotes dormancy) may promote dormancy establishment by repressing the expression of *PKL*, which acts antagonistically to the dormancy-promoting PRC2. In order to test this hypothesis we generated abi1-1 trees with downregulated expression of *PKL* (named abi1-1/PKLRNAi) and investigated dormancy as outlined above. Interestingly, assessment of dormancy showed that abi1-1/PKLRNAi plants fail to reactivate growth when exposed to 11 weeks of SDs followed by LDs treatment (**Paper III, Figure 4**) in contrast with the parental abi1-1 plants, suggesting that ABA promotes establishment of dormancy via negative regulation of *PKL*.

3.4.5 Interaction of PRC2 and ABA with other potential regulators of bud dormancy

Although few studies have addressed the molecular regulation of bud dormancy, correlative data suggest that cell-cell communication is blocked during dormancy by deposition of callose in the plasmodesmata (Rinne et al., 2013). Moreover, low temperature treatment removes these blockages. Such blockage could potentially contribute to dormancy establishment by preventing the movement of growth promoters, e.g. hormones or proteins, to SAMs. However, the available data do not conclusively prove the role of plasmodesmata in dormancy since no mutants are available that lack dormancy and display a lack of plasmodesmata, and conversely it is not known whether blocking plasmodesmata would lead to establishment of dormancy. Thus, changes in plasmodesmata could simply reflect establishment of dormancy and its release. Nevertheless, the hypothesis that dormancy may be established via blockage of plasmodesmata is attractive and needs further evaluation. Importantly, the availability of "mutants" that lack dormancy could now provide means to test the role of plasmodesmata in bud dormancy regulation and whether PRC2, PKL and ABA may act via their blockage in bud dormancy regulation.

4 Summary and future perspectives

The work presented in this thesis and the appended papers furthers our knowledge and understanding of the annual growth cycle in tress, especially regulation of growth cessation (**Papers I and II**), adaptive responses to SDs (**Paper II**) and acquisition of dormancy (Paper III). We have shown that components of the flowering pathway (*LAP1* and *FDL*) have acquired new roles in trees associated with the regulation of seasonal growth cycles. We have also demonstrated roles of ABA and the chromatin remodelling complex PRC2 in the regulation and establishment of dormancy (**Paper III**).

In order to summarize the findings I would like to highlight the major discoveries presented in the appended papers. **Paper I** filled a gap in understanding of the regulation of the growth cessation pathway by showing that the tree ortholog of *AP1* acts downstream of the CO/FT module in SD-mediated cessation of growth and directly regulates expression of the *AIL1* transcription factor, which was previously shown to control core cell cycle machinery (Karlberg et al, 2011). Importantly, our results provide insights into how the photoperiodic signalling pathway has evolved to control different processes, i.e. flowering and growth cessation, in different species. In **Paper II** we showed that the component of evolutionarily conserved Flowering Activation Complex (FAC) plays an important role in regulation of SD-mediated growth cessation. Additionally, FDL1, a component of FAC, plays another role in transcriptional control of adaptive responses, which is independent of its interaction with FT in trees. FDL1 interacts with ABI3 in the control of stress-related processes in trees. Our results provide evidence for the involvement of ABA and the PRC2 component FIE in regulation of dormancy development (**Paper III**). Moreover, they reveal a negative role in dormancy establishment for *PICKLE*, which seems to be a target of ABA and counteracts the function of PRC2 complexes.

However, many interesting questions remain open and/or unanswered. For instance, it would be interesting to identify targets of *FIE* and ABA in dormancy establishment. Chromatin Immunoprecipitation (ChIP) assays could be very valuable for identification of these targets during SD treatment, and generation of plants with altered expression of the identified targets could provide further insights into their roles. Most importantly, we would like to investigate whether impaired dormancy development is a consequence of the inability of FIERNAi and abi1-1 plants to deposit callose in plasmodesmatal connections. Transmission electron microscopy observations would be highly valuable for this. It could be also interesting to see whether downregulation of genes involved in biosynthesis of callose would lead to similar phenotypes to those we obtained with FIERNAi and abi1-1, thereby confirming that plasmodesmatal blockages are essential in dormancy development.

A similar ChIP approach could be utilized to identify downstream targets of FDL1 and LAP1 in SD-mediated growth cessation and adaptive responses. Plants overexpressing tagged versions of FDL1 and LAP1 proteins should be analysed by ChIP followed by sequencing. Another very interesting aspect that remains unclear is the role of other paralogues of AP1 and FD in *Populus*. It seems that *FDL2* is not involved in SD-mediated growth cessation in hybrid aspen, but its ability to interact with FT suggests that it may have evolved new, unknown functions. Detailed analysis could provide further insights into possible roles. Interestingly, *FDL2* is alternatively spliced and it would also be tempting to examine roles of different splice variants in hybrid aspen.

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