Molecular Control of Activity-Dormancy Transitions in *Populus*

Madeleine Englund
Faculty of Forest Sciences
Department of Forest Genetics and Plant Physiology
Umeå

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Cover: Winter tree
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Errata

Englund, M. 2010. Molecular Control of Activity- Dormancy Transitions in *Populus*.

Page 14. Line 9. Reference Rinne & van der Schoot, 2001. Should be; Rinne et al, 2001.

Page 14. Line 18 reads *ETHYLENE TRIPLE RESPONSE1* (*ETR1*). Should be *ETHYLENE RESPONSE1* (*ETR1*)

Page 34. In section 3.1.4, in the second section, line 4 reads; Neither do the genotypes differ in their regulation of downstream pathways such as cold hardiness, the accumulation of storage proteins, or starch breakdown.
Should be; The genotypes also differ in their regulation of downstream pathways such as cold hardiness, accumulation of storage proteins, and starch breakdown.

Page 41. Section 3.3.1. First line reads; Since *FIE* in *Arabidopsis* has been reported to interact with RBR *in vivo* in maize,…
Should be; Since *FIE* in *Arabidopsis* has been reported to interact with RBR *in vivo*,…

Manuscript III, page 2. Line 4 reads; (data not shown). Should be (supplemental figure 1C).

Manuscript III, figure legend 1C reads; *PttFIE*RNAi. Should be; *PttFIE*.

Molecular Control of Activity- Dormancy transitions in *Populus*

Abstract

Perennials such as woody plants, growing in the high northern part of the earth have to cope with seasonal changes alternating between mild summer and the harsh winter periods. To survive and adapt their reproductive phase to the most favorable environmental conditions trees cycle between periods of active growth during summer and a state of growth arrest during winter, referred to as the activity- dormancy cycle.

Studies underlying this thesis are aimed to increase our understanding of how plants growing in the high northern latitudes cope with the seasonal changes at a molecular level. Analysis of the global transcription in poplar showed that progression through the different stages of the annual activity-dormancy cycle is accompanied by massive transcriptional changes. Our data shows that genes involved in cell cycle regulation, chromatin remodeling and plant hormone signaling and biosynthesis are regulated during short day induced dormancy, suggesting key roles for these genes in the regulation of dormancy. Furthermore, we show that *PttFIE*, a plant homolog of the *ESC* component of the chromatin remodeling polycomb repression complex in *Drosophila*, is a key regulator of dormancy.

We also show that variation of timing of growth cessation and other physiological processes between ecotypes during dormancy induction could results from genetic variation in components downstream of the short day signal perception rather than in the perception of the short day signal.

Keywords: Populus, meristem, dormancy, bud, hormones, chromatin remodeling, Polycomb repression complex.

Author's address: Madeleine Englund, SLU, Department of Forest Genetics and Plant Physiology, 901 83 Umeå, Sweden
E-mail: Madeleine.Englund@genfys.slu.se

Results! Why, man, I have gotten a lot of results. I know several thousand things that won't work.

-Thomas A. Edison

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 Resman L[†], Howe G[†], Jonsen D, **Englund M**, Druart N, Schrader J, Antii H, Skinner J, Moritz T, Chen T, and Bhalerao RP. Molecular analysis reveals a role for components acting downstream of the day length perception machinery in regulating differential response to short day signal in regulating growth cessation and related processes in poplar. *Submitted*

II Karlberg A[†], **Englund M**[†], Petterle A, Molnar G, Sjodin A, Bako L, and Bhalerao RP. Analysis of global changes in gene expression during activity-dormancy cycle in hybrid aspen apex. *Plant Biotechnology* 27(1); 1-16

III **Englund M**, Karlberg A, Petterle A, Bako L and Bhalerao RP. Polycomb repression complex component regulates dormancy in perennial plants. Manuscript

[†]To be considered joint first authors.

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Abbreviations

ABA	Abscisic Acid
ABI3	ABSCISIC ACID-INSENSITIVE3
AIL1	ANTEGUMENTA- LIKE1
ANT	ANTEGUMENTA
BSP	BARK STORAGE PROTEIN
CBF	C-REPEAT BINDING FACTOR
CBFL	CBF-like
CDK	CYCLIN- DEPENDENT KINASE
CLF/SWN	CURLY LEAF/ SWINGER
CMT3	CHROMOMETHYLASE 3
CO	CONSTANS
COR	COLD RESPONSIVE
CYC	Cyclin
DME	DEMETER
DML	DEMETERL-LIKE
DOG1	DELAY OF GERMINATION1
DRE	DEHYDRATION RESPONSIVE ELEMENT
DRM2	DOMAINS REARRENGED METHYLTRANSFERASE2
E(su)12	SUPPRESSOR OF ZESTE 12
E(z)	ENHANCER OF ZESTE
EMF2	EMBRYONIC FLOWER2
ESC	EXTRA SEX COMB
ETR1	ETHYLENE TRIPLE RESPONSE1
FIE	FERTLIZATION INDEPENDENT ENDOSPERM
FIS2	FERTLIZATION INDEPENDENT SEED2
FLC	FLOWERING LOCUS C
FT	FLOWERING LOCUS T
GA	Gibberellic Acid
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HMT	Histone methyltransferase
HUB1 & 2	HISTONE UBIQUITINATION 1 & 2
ICE1	INDUCER OF CBF EXPRESSION1
MEA	MEDEA
MET1	METHYLTRANSFERASE1
MSI1	MULTICOPY SUPRESSOR OF IRA1
NURF55/p55	NUCLEOSOME REMODELLING FACTOR55
PcG	Polycomb group

PHYA	PHYTOCHROME A
PHYB	PHYTOCHROME B
PRC	PcG repression complex
ROS1	REPRESSOR OF SILENCE1
SIN3	SWI-independent 3
TrxG	Trithorax group
VRN2	VERNALIZATION 2

1 Introduction

Woody plants are among the largest and longest-lived organisms on the earth. Since they may live for many decades, or even centuries, they need strategies that enable them to survive constantly changing environmental conditions. Such strategies are of particular importance to woody plants growing in high northern latitudes where they are exposed to extreme climatic changes between the mild summer seasons and harsh winter periods. Any damage caused by the winter cold may seriously affect the plant's chances of survival until the following spring. Plants have therefore evolved a mechanism that enables them to anticipate the onset of winter by terminating growth, acquire cold hardiness, and enter a state of dormancy. Similarly, in the spring when reactivation occurs, newly growing tissues are exposed to temperatures that fluctuate between warm days and cold nights. As spring approaches, these plants initiate growth only after they have experienced a sufficient number of warm days, by which they reduce the probability of suffering frost damage.

The aim of the present study is to deepen our understanding of how plants, growing in high northern latitudes, cope with seasonal changes through their adaptations at the molecular level. A better knowledge of how forest trees are able to adapt to large seasonal changes in environmental conditions is of great interest, both for basic research, as well as from an economical point of view. For example, rapidly changing climate conditions caused by global warming are likely to have serious effects on the growth habits of woody trees. A better understanding of how, at the molecular level, plants cope with climate change would therefore facilitate attempts to breed and/or engineer trees that will be capable of adapting rapidly to future climatic conditions.

1.1 Dormancy

Woody plants growing in high northern latitudes are subjected to large seasonal variations in temperatures and other climatic and environmental conditions that influence their growth. Warm temperatures and the long photoperiod during summer months provide plants with the optimal environmental conditions for growth and reproduction, while the freezing temperatures during winter can cause severe damage to actively growing plant tissues. Survival of these plants is therefore dependent on their ability to synchronize their growth and reproduction with seasonal changes in the environment. To do this plants of seasonal climates have evolved an annual growth cycle that alternates between a state of active growth during summer and a state of dormancy during winter, hereafter referred to as the active-dormant cycle.

Dormancy, in its broadest definition, can be defined as a lack of visible growth. In 1987 Lang et al. described three states of dormancy: ecodormancy, endodormancy and paradormancy. During ecodormancy, growth is temporally terminated by external factors e.g. unfavorable environmental conditions, but growth can resume if the environmental conditions become favorable. Endodormany is induced by endogenous plant factors. At this stage the tree is no longer able to resume growth even under favorable conditions, such as long days and warm temperatures. Plants, such as trees of boreal forests, need to have been exposed to low temperatures for a prolonged period of time (chilling requirement) in order to break endodormancy. During paradormancy, growth is inhibited by factors originating in parts of the plant other than the dormant bud, such as apical dominance when signals from the apical meristem prevent growth of the axillary buds as long as the apical bud remains intact (Howe et al., 1999; Lang et al., 1987).

The transition from an active to a dormant state occurs gradually and is initiated during late summer when plants cease their growth in response to shortening day-length (Figure 1). Almost simultaneously with the cessation of growth, bud formation occurs at the apex, which involves a developmental switch from leaf primordial formation to bud scale formation. The formation of the bud (generally referred to as bud set) leads to the formation of bud scales that bend over and enclose the underlying tissue – i.e. leaf primordia and the shoot meristem - thereby protecting them (Figure 2) (Rohde et al., 2002). In the early stages after bud set, the plant is in a ecodormant state, and growth can be reinitiated if plants are exposed to favorable conditions e.g. long days.

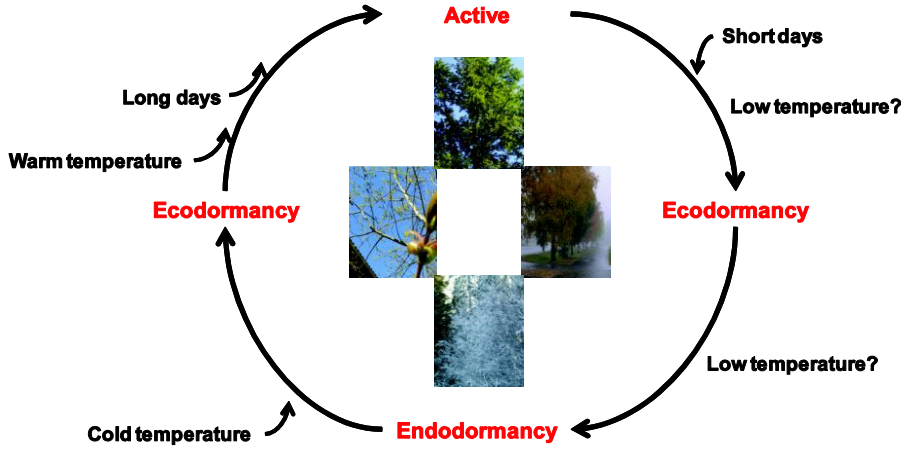

Figure 1. A schematic representation of the activity-dormancy cycle of the plant meristem throughout the seasons of the year.

As day length continues to decrease and temperature falls during autumn, a transition from ecodormancy to endodormancy takes place.

As plants are further exposed to low temperatures during winter, the chilling requirement is fulfilled and a transition from endodormancy to ecodormancy occurs. On the return of spring, plants are exposed to several days of relatively warm temperatures thereby releasing the apical bud from its ecodormant state, and the changes that occurred in the bud during autumn are gradually reversed, resulting in bud break (Figure 1).

In the present thesis, dormancy is referred to as that state of growth cessation, when plants are unable to resume growth, even under favorable growth conditions - namely long days.

In addition to the apical meristem, meristem in root and vascular cambium can also become dormant during the autumn, and many of the fundamental processes that occur during bud dormancy, also occur in these other tissues during their dormancy. However, in contrast to bud dormancy, where the establishment of dormancy is accompanied by a developmental switch towards bud scale formation, fewer developmental changes occur during the establishment of cambial dormancy.

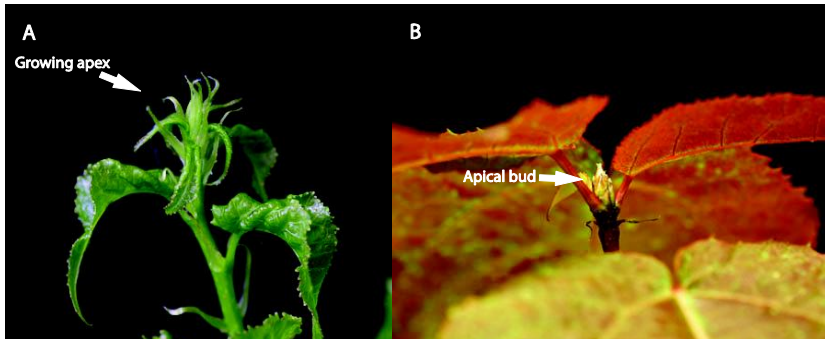

Figure 2. Short-day induced bud set in hybrid aspen (*Populus tremula* x *tremuloides*). Photograph (A) illustrates the actively growing apex of hybrid aspen under long-day conditions. Photograph (B) shows the apical bud that has formed after exposure to short days for 11 weeks.

1.2 Parallel processes occurring during dormancy

The establishment of dormancy is accompanied by several other processes that occur in the apex. One of the most important of these is cold hardiness, or a gradual increase in the ability of plants to withstand low temperatures (Welling et al., 1997). Initially after bud set, the bud becomes dehydrated and hardened, thereby reducing the probability of suffering damage that might be caused by the freezing of tissues during winter (Rohde et al., 2002; Welling et al., 2004; Welling et al., 1997). Bud set is also accompanied by processes such as changes in cellular communications in the apical meristem (Rinne & van der Schoot, 2001), and a shift in metabolism towards the accumulation of storage compounds (Rohde et al., 2007; Ruttink et al., 2007). Some of these processes are considered to be sub-processes to dormancy, and have been used as descriptors of some of the stages during the progress of dormancy. However, caution should be exercised if considering these processes as part of dormancy. For example, despite the close temporal association between bud formation and dormancy induction, they are independent processes as shown by studies in transgenic birch and poplar. In ethylene insensitive birch plants over-expressing the dominant negative version of *ETHYLENE TRIPLE RESPONSE1* (*ETR1*), and in poplar plants over-expressing the transcription factor *ABSCISIC ACID-INSENSITIVE3* (*ABI3*), bud formation was perturbed and yet these

transgenic birch and poplar plants were still able to establish dormancy (Ruonala et al., 2006; Rohde et al., 2002).

1.2.1 Induction of cold hardiness

A key process integral to the survival of perennial plants is the induction of cold hardiness. Cold hardiness is defined here as the process by which a plant increases its tolerance to damage caused by cellular dehydration and extracellular ice formation in plant tissues caused by the cold, dry climate during winter. Like dormancy, cold hardiness is induced during autumn in response, first to shortening day-length, and later to a combination of short days and low temperatures. Later, this process is further enhanced by freezing temperatures during the early winter (Weiser, 1970, Welling et al., 2004).

Although little is known about the molecular mechanisms underlying the induction of cold hardiness in trees, the process probably shares similarities with the better characterized induction of cold acclimation in model plants e.g. *Arabidopsis thaliana* (Benedict et al., 2006a). A key regulator of cold acclimation in *Arabidopsis* is the transcription factor *C- REPEAT BINDING FACTOR* (*CBF*). Upon exposure to cold, the transcription factor *INDUCER OF CBF EXPRESSION1* (*ICE1*) induces the expression of the CBF family of transcription factors (Chinnusamy et al., 2003). The CBF binds to a *cis*- acting element named *DEHYDRATION RESPONSIVE ELEMENT* (*DRE*) in the promoters of *COLD RESPONSIVE* (*COR*) genes, and activates their expression (Liu et al., 1998; Stockinger et al., 1997). *COR* genes encode proteins, such as dehydrins, that are thought to be involved in protecting cells from freezing temperatures. By over-expressing the *CBF* in *Arabidopsis*, it is possible to induce the constitutive expression of the *COR* genes and increase freezing and dehydration tolerance under non-freezing temperatures (Gilmour et al., 2000; Jaglo-Ottosen et al., 1998).

The *CBF* cold response pathway seems to be conserved among plants since *CBF* orthologues have been described in various woody trees, such as poplar (Benedict et al., 2006b), birch (Welling & Palva, 2008) and eucalyptus (El Kayal et al., 2006). Low temperatures also rapidly induce the expression of *CBFs* in birch and poplar (Welling & Palva, 2008; Benedict et al., 2006b), and the activation of *CBF* in birch activates the transcription of CORs, which are known to be regulated by *CBF* in *Arabidopsis* (Welling & Palva, 2008). Moreover, over-expression of *Arabidopsis CBF1* in poplar increases the expression of *COR*-like genes that induce cold hardiness,

suggesting conservation of the role of *CBFs* in cold hardiness between annual plants, such as *Arabidopsis*, and poplar trees (Benedict et al., 2006b).

1.3 Other plant development processes sharing common features with dormancy

The establishment and subsequent release of dormancy in buds and cambium of perennial plants shares several features in common with other physiological processes in plants e.g. seed dormancy, vernalization, and tuber dormancy in potatoes. Comparing the regulation of dormancy in perennial plants with these other processes can considerably increase our understanding of the molecular mechanism, and can help us to identify any common regulatory modules. Some of these other physiological processes are outlined briefly below.

1.3.1 Seed dormancy

Seed dormancy is generally described as a temporary failure or incapacity of a viable seed to germinate, even under conditions that normally promote germination (Bewley, 1997). Seed dormancy is initiated early during seed maturation, increases until the seed is fully developed, and is maintained after the seed is shed (Penfield & King, 2009; Hilhorst & Karssen, 1992). Dormancy is released by exposure to moist chilling (stratification), a period of dry storage (after-ripening) or by disruption of the seed coat (scarification). Once dormancy is broken, the seed regains the capacity to germinate once conditions again become favorable (Finkelstein, 2008).

Because seed and bud dormancy have many features in common, it has been assumed that there is a common basis underlying both processes (Rohde & Bhalerao, 2007; Wareing, 1956). This assumption is based on the following observations: 1) in some species, and for certain genotypes, there is a similar chilling requirement to release dormancy in seeds and buds; 2) similar influences of the plant hormones ABA and gibberellic acid (GA) on dormancy induction and release (further described in a later section); and 3) similar changes in the water content of cells and the acquisition of desiccation and freezing tolerance (Rohde et al., 2002). Together, these similarities suggest that both seed and bud dormancy could be regulated, at least in part, by similar molecular mechanisms.

1.3.2 Vernalization

Similar to perennial plants, annual plants such as *Arabidopsis thaliana,* need to adjust their development to seasonal changes in their environment. One

of the key developmental processes regulated by environmental signals, which has features similar to the release from dormancy, is the transition to flowering in certain ecotypes of *Arabidopsis*, by prolonged exposure to low temperatures – a process referred to as vernalization (He & Amasino, 2005).

A central player in the vernalization process is the MADS-box gene *FLOWERING LOCUS C* (*FLC*), which suppresses flowering. In non-vernalized plants, *FLC* is expressed at high levels and suppresses the expression of the *FLOWERING LOCUS T* (*FT*) gene, which is a promoter of flowering. When the plant has been exposed to cold for a prolonged period, the vernalization response becomes saturated, and the expression of *FLC* is repressed. Downregulation of the *FLC* gene releases repression of *FT* gene expression and the plant then becomes competent to make the transition from its vegetative phase to flowering (Hepworth et al., 2002; Michaels & Amasino, 1999).

Although vernalization in *Arabidopsis* shares no common features with the induction and maintenance of dormancy in buds of woody trees, some parallels do exist between the processes of vernalization and cold- induced breakage of bud dormancy. Transition to flowering, and release from dormancy both require a prolonged exposure to low temperatures. Chilling *per se* neither leads directly to flowering nor to the initiation of growth, but simply makes the plant responsive to further signals that do initiate flowering and reactivate growth (Rohde & Bhalerao, 2007; Sung & Amasino, 2005).

Despite the similarities between vernalization and the release from bud dormancy, important differences still can be observed. For example, the *FLC* gene is repressed during vernalization by chromatin remodeling markers. This repression is maintained at a stable level during the vegetative phase and is reset, probably during meiosis, when gametes are formed (Mylne et al., 2006). If dormancy were regulated by a mechanism similar to vernalization, this repression by chromatin remodeling would have to be reset in the meristem by some other mechanism, since no meiosis occurs in the vegetative meristem during the activity-dormancy cycle.

1.3.3 Potato tuber dormancy

At harvest, potato tubers are generally dormant and will not sprout even if tubers are placed under favorable environmental conditions. To break their dormancy, tubers require a period of storage before they will sprout. In many potato cultivars, the natural progression of dormancy occurs over a period of many months. Of the environmental factors that affect post-harvested tubers, temperature seems to be the only one that has a major influence on the length of the dormancy. Chilling at temperatures between

1°C and 3°C shortens dormancy in post-harvest tubers (Wurr & Allen, 1976), while storage at temperatures above 30 °C is stressful and results in premature termination of dormancy (Turnbull & Hanke, 1985).

Although potato tuber dormancy and bud dormancy are not strictly equivalent processes, they do seem to share some common features. For example, level of the hormone ABA increases during the establishment of both bud dormancy and tuber dormancy (Suttle & Hultstrand, 1994).

However, in contrast to bud dormancy which requires chilling for its release, simple storage is sometimes all that is needed to terminate dormancy in potato tubers. Moreover, while release from dormancy in the potato tuber can be accelerated by chilling temperatures, it is not a prerequisite, indicating a major difference between potato tuber and bud dormancy in trees.

1.4 Environmental signals controlling dormancy

Studies of dormancy induction and release in perennial plants suggest that, although dormancy is primarily regulated by cold temperatures and day length, these environmental factors operate through different signaling pathways. For example, exposure to short days alone results in fully developed endodormancy in poplar (Espinosa-Ruiz et al., 2004) and birch (Li et al., 2003), while both cold and short days are required for dormancy induction in Scottish heather (Kwolek & Woolhouse, 1982) and leafy spurge (Anderson et al., 2005), and cold temperatures alone can induce dormancy in some ecotypes of willow (Li et al., 2005).

Short-day induced growth cessation and dormancy induction are best understood in poplar, which has been used as a model plant. It has been shown that poplar senses changes in day-length by the photoreceptor phytochromes (Olsen et al., 1997b), and that the signal is transduced further through the *CONSTANS* (*CO*)/*FT* pathway (Bohlenius et al., 2006). Three genes encoding phytochromes have been identified in poplar: *PHYTOCHROME A* (*PHYA*); *PHYTOCHROME B1* (*PHYB1*); and *PHYTOCHROME B2* (*PHYB2*) (Howe et al., 1998). The important role of phytochromes in growth cessation in response to short days has been demonstrated in hybrid aspen (*Populus tremula* x *tremuloides*) where it was found that over-expression of the oat phytochrome *PHYA* led to an inability of these transgenic plants to cease growth, set buds, initiate cold hardiness, or establish dormancy following exposure to a reduction in day length (Welling et al., 2002; Olsen et al., 1997b). Similarly, a genetic association has been found between timing of growth cessation and specific

alleles of *PHYB* in genotypes of aspen that comprise a latitudinal cline (Ingvarsson et al., 2006).

In *Arabidopsis*, *PHYA* is a known regulator of the *CO* gene, which is itself known to be a positive effector of the flowering-time regulator *FT*. Analysis of the *CO/FT* pathway in *Arabidopsis* has shown that in long days, *CO* expression peaks in late afternoon and the *CO* protein is synthesized in day light (Suarez-Lopez et al., 2001). The *CO* protein then accumulates and activates the expression of *FT*, which promotes flowering. In contrast, during short days, the peak in the *CO* mRNA levels occurs during the night when the *CO* protein, being unstable in the dark, cannot activate *FT* expression (Valverde et al., 2004; Suarez-Lopez et al., 2001). *PtCO2*, *PtFT1* and *PtFT2* are homologues to *Arabidopsis CO* and *FT*, which have been identified in hybrid aspen and shown to have a similar expression pattern and function as in *Arabidopsis* (Bohlenius et al., 2006; Hsu et al., 2006). For example, *PtCO2* displays a diurnal expression pattern with expression levels peaking at the end of the day. The peak in the *PtCO2* expression was shown to correlate with the expression of *PtFT1* suggesting that the function of the *CO/FT* regulon could be conserved between poplar and *Arabidopsis* (Bohlenius et al., 2006). The role of *CO/FT* genes in growth cessation was discovered when it was shown that poplars with reduced levels of *PtCO2* and *PtFT1* were hypersensitive to a reduction in day length. Compared with the wild–type these plants underwent rapid growth cessation when exposed to short days, whereas poplars over expressing *PtFT1* made the plants insensitive to short days with respect to growth cessation (Bohlenius et al., 2006).

1.5 Hormonal control of dormancy and cold acclimation

It has been suggested that plant hormones are key regulators of seed dormancy in plants (Penfield & King, 2009). However, our knowledge concerning the effects of different plant hormones on bud dormancy is fragmentary. Given the similarities between bud dormancy in trees and seed dormancy in annual plants, and considering that the roles of many plant hormones are better understood in *Arabidopsis*, studies of the hormonal control of seed dormancy may give us important clues about the hormonal control of bud dormancy. Below I summarize the roles of the two most extensively studied plant hormones in dormancy: abscisic acid (ABA) and gibberellic acid (GA).

1.5.1 Abscisic acid

ABA is thought to be involved in dormancy because of its growth arresting effects (Johansen et al., 1986). Indeed, studies of seed dormancy in *Arabidopsis* have shown that ABA performs an important role in the induction and maintenance of seed dormancy. Mutants with a deficiency of ABA display reduced dormancy (Karssen et al., 1983), whereas the over-expression of ABA biosynthesis genes can increase ABA content in the seed and enhance seed dormancy or delay germination (Lin et al., 2007).

In trees, it has been shown that the transition to dormancy is accompanied by an increase in ABA concentration in the shoot apex of Silver birch (Li et al., 2005) and hybrid poplar (Rohde et al., 2002), and in the cambial meristem of poplar (Druart et al., 2007. It was therefore suggested that ABA may induce dormancy. This hypothesis was tested in bay willows (*Salix pentandra*) that were grown under long-day conditions and treated with exogenous ABA. The results showed that exogenous ABA was able to reduce growth; however, it did not induce apical growth cessation, which is a prerequisite for dormancy induction, suggesting that ABA alone is not sufficient to induce dormancy (Johansen et al., 1986; Junttila, 1976).

In contrast to the potential role of ABA in bud dormancy, changes in ABA levels do not seem to be important in the release from dormancy by chilling in either the cambium or the bud, since the concentrations of ABA do not decrease during reactivation (Druart et al., 2007; Rinne et al., 1994). However, it has been shown that the sensitivity of the bud to ABA does decrease upon chilling (Rinne et al., 1998), suggesting that the regulatory effect of ABA on dormancy release might be achieved through differential sensitivity of tissues to the hormone rather than through changes in its concentration.

1.5.2 Gibberellic acid

Since GA is known to control the initiation and elongation of new stem units, it is plausible that it might also be involved in the growth cessation and dormancy induced by short days. Indeed, exposure to short days leads to the downregulation of GA levels in stem of hybrid aspen; a downregulation that does not occur in plants over-expressing *PHYA* that are unable to sense changes in day length and so do not undergo growth cessation (Olsen et al., 1997b). Similarly, when bay willows (*Salix pentandra*) were exposed to short days, the levels of GA_1 in the apex decreased drastically (Olsen et al., 1995). Furthermore, exogenous GA_1 induces shoot elongation under short-day conditions in willows (Junttila & Jensen, 1988),

and hybrid aspen plants over-expressing *GA20 oxidase* have high levels of GA, and display a delay in growth cessation in response to short days (Eriksson et al., 2000).

GA is also known to have a role in the release of seeds from dormancy and in their germination (Mitchum et al., 2006; Yamauchi et al., 2004; Derkx & Karssen, 1993). The GA content in dormant seeds is relatively low, but accumulates when dormancy is released and/or germination begins.

In *Arabidopsis* seeds, release from dormancy by stratification is accompanied by an increased GA content (Yamauchi et al., 2004). This increase is preceded by elevated transcription of the GA biosynthesis genes *AtGA20ox1*, *AtGA20ox2* and *AtGA20ox3* caused by the exposure to low temperatures. At the same time, *AtGA2ox2*, which is involved in the degradation of GA, has decreased transcriptional levels at low temperatures (Yamauchi et al., 2004). Interestingly, an increase in mRNA levels of *GA20 oxidase* has also been noted in the cambium of poplar during the reactivation phase in the spring (Druart et al., 2007). Also, long-day induced bud break and growth initiation in bay willow is associated with a rapid increase in GA_1 levels in the apex of plants which have an early stage of dormancy (ecodormancy) (Olsen et al., 1997a). However, that study showed that the levels of GA_1 did not increase until dormancy was released and growth was resumed, indicating that GA does not have a role in control of dormancy release, but rather, that it is involved in growth initiation after dormancy has been released.

1.6 Regulation of the plant cell cycle during bud dormancy

The transition to dormancy is accompanied by growth cessation and termination of cell division. The two different stages of dormancy, ecodormancy and endodormancy, can be distinguished by the differential response of the cell cycle machinery to growth-promoting signals. During ecodormancy, the cell cycle can be described to be in a 'standby' state, in which cell division can be resumed as soon as cells perceive growth-promoting signals. During the transition from eco- to endodormancy, the cell cycle machinery goes from a 'standby' to an 'off-line' state, in which it is no longer able to respond to growth-promoting signals. The release from dormancy, e.g. by exposure to an extended period of cold, returns the cell cycle machinery to the 'standby' mode (Espinosa-Ruiz et al., 2004; Rinne et al., 2001).

The differences in the ability of the cell cycle to respond to growth-promoting signals during eco- and endodormancy have raised the question of how this difference is regulated. At present, little is known about the signaling pathways that block the cell cycle in these 'standby' and 'off–line' modes during dormancy in trees, but it has been suggested that regulation of the core cell cycle genes might play a major role.

Studies of cell division cycle in the apical meristem of hybrid poplar have shown that cell division is arrested after four weeks of short days (Ruttink et al., 2007). This has been corroborated by transcriptional analyses of the core cell cycle genes demonstrating that many, but not all, cell cycle regulators are downregulated in the apical meristem four weeks after the onset of short days (Ruttink et al., 2007).

It is also possible that there could be a post-transcriptional regulation of the core cell cycle genes. Dormancy induction in the cambial meristem of poplar also occurs after four weeks of short days when the cell cycle is arrested and elongation growth ceases. However, the transcript and protein levels of *PttCDKA* and *PttCDKB* remain unaffected during this period suggesting that short days induced arrest of the cell cycle does not involve reduction of the *PttCDKA* and *PttCDKB* transcript or protein levels (Espinosa-Ruiz et al., 2004). Instead, arrest of cell division appears to be caused by a substantial reduction in the kinase activity of *PttCDKA* and *PttCDKB* kinase complexes (Espinosa-Ruiz et al., 2004). What causes this reduction in *PttCDKA* and *PttCDKB* kinase activity is yet to be discovered; but it could involve a reduction in the levels of cyclin proteins and/or an increase in the levels of CDK inhibitors.

1.7 Regulation of gene expression at the level of chromatin

Transition from active growth to dormancy is accompanied by an extensive change of gene expression pattern in the bud (Ruttink et al., 2007). How this massive reprogramming of the transcriptome is achieved is not well understood but the highly coordinated regulation of transcription during dormancy suggests that chromatin remodeling plays a key role. Chromatin remodeling has already been shown to have a major role in other developmental processes that share common features with bud and meristem dormancy, such as seed dormancy and vernalization (Liu et al., 2007; Sung & Amasino, 2005). This will be described further in a later section, but will be preceded here with a brief description of chromatin and chromatin remodeling.

1.7.1 Chromatin remodeling and DNA methylation

DNA molecules are compacted into the chromatin structure by being wrapped around cores of histone proteins to form nucleosomes. One nucleosome consists of an octamer of the four core histones H2A, H2B, H3 and H4 (two molecules of each histone), and 147 base pairs of DNA wrapped around the histones (Luger et al., 1997). A fifth type of histone, H1, is bound to the linker DNA between two adjacent nucleosome cores. The linear arrays of nucleosomes are then themselves packed into more condensed chromosomal fiber structures.

Each core histone is composed of a structured domain and an unstructured amino-terminal sequence, known as the 'tail', which extends out from the nucleosome. These N-terminal tails are subjected to a variety of post-translational modifications, including acetylation, methylation, phosphorylation and ubiquitination (Jenuwein & Allis, 2001). These modifications can activate or repress expression by generating a more open or closed chromatin structure, respectively. Generally, an open structure increases the accessibility of the DNA for the transcriptional machinery, thereby activating transcription; while a closed chromatin structure represses gene transcription by preventing transcription factors from accessing the DNA sequence (figure 3).

Acetylated core histones are usually associated with transcriptionally active chromatin; deacetylated core histones are usually associated with inactive chromatin. This reversible modification of the histone tail is carried out by histone acetyltransferases (HATs), which transfer the acetyl group from acetyl-Coenzyme A to conserved lysine residues on the histone tails (Ait-Si-Ali et al., 1998). This modification is reversed by histone deacetylases (HDACs). The addition of the acetyl group on the lysine residue neutralizes the positive charge of the histone tails and decreases the affinity of the histone for DNA, which results in an open structure of the chromatin (Roth et al., 2001).

Methylation of histones can be associated with both transcriptional activation and repression, depending on the type of methylation. In *Arabidopsis*, high levels of dimethylated lysine 4 on histone H3 (H3K4me2) is found in transcriptionally active regions of the chromatin, while transcriptionally inactive regions are associated with high levels of dimethylated H3K9 (Jasencakova et al., 2003).

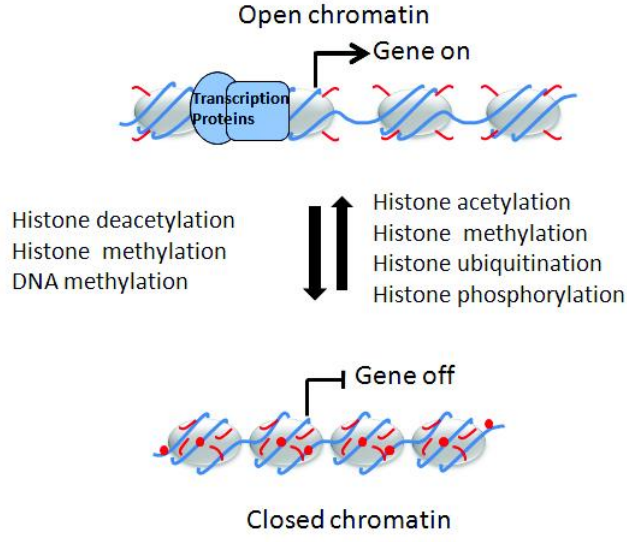

Figure 3. Chromatin modifications within the nucleosomes. A number of distinct post-translational modifications, such as acetylation and methylation, occur at the N terminal of histones H2A, H2B, H3 and H4. These modifications either repress or activate transcription, depending on the type of modification. Methylation of cytosine residues (marked as red dots) in the DNA can also contribute to repression of gene expression (Nelissen et al., 2007).

Methylation of the histone tails are catalyzed by histone methyltransferases (HMTs) that catalyze the transfer of methyl groups from the donor molecule S-adenosyl-L-methionine (SAM) to the histone.

Besides histones, the DNA itself can be subjected to modifications. Cytosines in the DNA can be methylated at the carbon-five position; this methylation is generally being associated with transcriptional silencing. In plants, the cytosine can be methylated in the CG, CNG or CNN sequence, where N represents any nucleotide but guanine (Gruenbaum et al., 1981). There are three sub-families of DNA methylases in plants: CG maintenance methyltransferases, such as *METHYLTRANSFERASE1* (*MET1*); chromomethylases, such as *CHROMOMETHYLASE3* (*CMT3*); and the de novo methyltransferases, such as the methyltransferase *DOMAINS REARRENGED METHYLTRANSFERASE2* (*DRM2*) (Henderson & Jacobsen, 2007; Henikoff & Comai, 1998). Methylations on the cytosines can be removed by DNA demethylases. Four DNA demethylases have been

reported in *Arabidopsis*: *DEMETER* (*DME*), *REPRESSOR OF SILENCE 1* (*ROS1*); *DEMETER-LIKE2* (*DML2*); and *DEMETER-LIKE3* (*DML3*) (Penterman et al., 2007; Morales-Ruiz et al., 2006).

1.7.2 The Polycomb and Trithorax group proteins

Two complexes involved in the modification of histones in mammals and plants have been extensively described in the literature: the polycomb group (PcG) proteins, and their antagonists, the trithorax group (TrxG) proteins. In general the PcG complexes are transcriptional repressors, while TrxG complexes are transcriptional activators (Lund & van Lohuizen, 2004). Both were originally discovered in the fruit fly, *Drosophila melanogaster*, but seem to be conserved in plants (Pien & Grossniklaus, 2007; Reyes & Grossniklaus, 2003). Here I will use the polycomb repression complexes from *Drosophila* and *Arabidopsis* to outline the key features of this major chromatin remodeling complexes involved in gene regulation and development. *Drosophila* has three different PcG complexes: PcG repression complex 1 (PRC1); PcG repression complex 2 (PRC2); and Pleiohomeotic repression complex (PhoRC). PRC2 is thought to be involved in the initiation of gene silencing, whereas PRC1 is involved in the stabile maintenance of gene repression. The function of PhoRC is not known. The PRC2 complex consists of four core subunits: ENHANCER OF ZESTE (E (Z)); SUPPRESSOR OF ZESTE 12 (Su(Z)12); EXTRA SEX COMBS (ESC); and NUCLEOSOME REMODELLING FACTOR 55 (NURF55), also called p55. This PcG complex acts by trimethylating H3K27 (a modification that is associated with the repression of gene transcription) via its E(Z) sub-unit, which possesses a SET domain with histone methyltransferase activity (Cao & Zhang, 2004; Muller et al., 2002).

PRC2 proteins are conserved in plants, and have been best studied in *Arabidopsis*. Unlike in *Drosophila*, all PRC2 subunits in *Arabidopsis*, except for the ESC homologue *FERTLIZATION INDEPENDENT ENDOSPERM* (*FIE*) the *NURF55* homolog *MULTICOPY SUPRESSOR OF IRA1* (*MSI1*), are encoded by more than one gene and are listed in Table 1 (Calonje & Sung, 2006).

Based on studies of mutants and protein interactions, there are at least three PRC2-like complexes in *Arabidopsis*. Each complex has specific functions during plant development, although they share common target genes. The FIS- complex, which controls seed development, consists of the subunits FERTLIZATION-INDEPENDENT SEED2 (FIS2), MEDEA (MEA), FIE, and MSI1, and have been purified from plants cells (Chanvivattana et al., 2004; Kohler et al., 2003). The VRN complex mediates the

vernalization response and consists of VERNALIZATION2 (VRN2), CURLY LEAF/SWINGER (CLF/SWN), FIE, and MSI1. The EMF complex, which represses precocious flowering and flower development, consists of EMBRYONIC FLOWER2 (EMF2), CLF/SWN, FIE, and MSI1 (Guitton & Berger, 2005; Chanvivattana et al., 2004).

Several TrxG complexes have been described in *Drosophila*, all of which differ in their subunit composition. One of these subunits is the Trithorax (TRX) protein that possesses H3K4 trimethylation activity. Trimethylated H3K4 is, in general, associated with transcriptionally active chromatin (Grimaud et al., 2006). Several *Arabidopsis Trithorax* (*ATX*) homologues have been identified in *Arabidopsis* (Alvarez-Venegas & Avramova, 2002; Alvarez-Venegas & Avramova, 2001), but only one, ATX1, has been functionally characterized so far. ATX1 has been shown to display a H3K4 methyltransferase activity, and found to be necessary in keeping the expression of the flowering homeotic genes active (Alvarez-Venegas et al., 2003).

Drosophila	***Arabidopsis***	**Note**
E(z)	CLF/ SWN MEA	H3K9 and H3K27 HMTase
ESC	FIE	WD40 repeat protein. Required for HMTase activity of E(z).
SU(z)12	EMF2 FIS2 VRN2	Zing finger protein. Required for HMTase activity of E(z).
NURF55/p55	MSI1	WD40 repeat protein. Nucleosome- binding protein.

Table 1. Summary of the PcG proteins in plants and their homologues in *Drosophila* (Modified from Calonje & Sung, 2006).

1.7.3 Role of chromatin remodeling in plant development

Chromatin remodeling has already been shown to have a major role in developmental processes that have similarities with dormancy in perennial plants. For example, downregulation of the *FLC* gene during the vernalization process is known to be regulated by chromatin remodeling (Sung & Amasino, 2005). Also, mutation in *HISTONE UBIQUITINATION 2* (*HUB2*) causes reduced seed dormancy in

Arabidopsis. *HUB2* is a homologue to *HUB1*, an E3 ligase that catalyses histone H2B monoubiquitination, a modification that is associated with actively transcribed genes. In seeds of *hub1* mutant, levels of monoubiquitinated H2B was significantly reduced compared to wild-type seeds, as was the expression of genes that influence seed dormancy, such as *DELAY OF GERMINATION1* (*DOG1*) and *CYP707A2*, suggesting that chromatin remodeling through monoubiquitination of H2B positively regulates genes necessary for seed dormancy (Liu et al., 2007).

Changes in DNA methylation levels have been observed during the induction and release of dormancy in potato tubers. The induction of dormancy was associated with an increase in levels of DNA methylation in tuber meristems, and the premature termination of dormancy results in a rapid demethylation of meristem DNA (Law & Suttle, 2003). Altered histone acetylation patterns have also been observed during the release of potato tubers from endodormancy, when the levels of acetylated histones H3 and H4 increased (Law & Suttle, 2004).

Given the similarities between potato tuber dormancy, seed dormancy, and dormancy in perennial trees, it is possible that chromatin remodeling also plays a major role in cambial meristem and bud dormancy.

2 Objectives

Proper temporal regulation of the activity-dormancy cycle is of great importance to the survival of perennial plants of boreal forests. However, our knowledge of how distinct stages of the activity-dormancy cycle in perennial plants are regulated at the molecular level is still limited.

The aim of the present thesis is to increase our understanding of the molecular processes controlling activity-dormancy cycling in buds and the cambial meristem of woody trees.

The main questions addressed are:

- What are the molecular mechanisms that lead to differences in the short-day regulated induction of growth cessation, and related processes, among different poplar ecotypes? (Paper I)
- What are the transcriptional networks underlying distinct stages of the activity-dormancy cycle in the apical buds? (Paper II)
- What role does chromatin remodeling have in the establishment, maintenance, and release of bud dormancy? (Paper III)

In order to address the questions raised above I have used poplar and the closely related aspen as experimental model trees. Although *Arabidopsis thaliana* is the plant model system of choice for studying biological processes and gene function in plants, it has limited use for analyzing processes, such as bud set and dormancy that occur in perennial plants. Of the various tree species, poplar is the most useful model for studying growth cessation and dormancy since it has a relatively small genome size of ca. 500 Mbp, and the full genome sequence has recently become available (Tuskan et al., 2006). Good genetic maps are available for poplar, and several mapping pedigrees can be used to map QTLs for the analysis of bud set (Tuskan et al., 2004;

Taylor, 2002; Frewen et al., 2000). In addition, poplar is relatively fast growing and easy to manipulate genetically, making it feasible for use when employing a functional genomics approach (Nilsson et al., 1992). For these reasons, I have chosen to use poplar, and a particular hybrid aspen clone, T89, for the experimental work in my thesis on the analysis of the activity-dormancy cycle.

3 Results and discussion

3.1 Components acting downstream of the perception of short day length are regulated differently in different ecotypes of poplar (Paper I).

Trees originating from different latitudes differ in their critical day length and timing of growth cessation. Genotypes from high northern latitudes typically undergo growth cessation earlier and have a shorter critical day length compared to more southerly genotypes, which cease their growth later during autumn. Physiological studies have shown that genotypes that differ in their timing of growth cessation also differ in their induction of other physiological processes that accompany growth cessation. For example, northern genotypes that undergo early growth cessation also induce cold acclimation earlier than more southerly genotypes (Li et al., 2002).

Although differences in the physiological processes accompanying growth cessation have been studied between different ecotypes it is still not known whether these differences are due to a differential perception of, and/or a differential response to, a short-day signal. We applied microarrays to compare the expression of genes between trees that vary in their timing of growth cessation. For this experiment we used hybrid poplar clones resulting from a cross between two F1 hybrids, which were in turn generated from a cross between poplar clones from a northern latitude (48°N) and a latitude further south (31°N) in the USA (Frewen et al., 2000). The two hybrid poplar clones used in this experiment are referred to as the early and the late clone, which undergo growth cessation after 15 days and 29 days of short-day treatment, respectively. Global changes in gene

expression after short-day treatment have been analysed in the stem tissues from the early and the late clones using cDNA microarrays. We then compared the two clones for global changes in gene expression in the stem tissues during the transition between activity and dormancy. Our analysis of microarray data revealed considerable differences in gene expression patterns between the two clones after short-day treatment. The differences in gene expression between the clones became prominent after 21 days of short-day treatment, which correlated roughly with the time when cambial cell division had ceased in the early clone, but before it had ceased in the late clone (Paper I, figure 2).

The transcriptional analysis showed that the regulation of gene expression induced by the short-day signal differs between the two clones in terms of the number of genes whose expression is significantly affected, the timing of induction or downregulation, and in the magnitude of change in transcript levels. Moreover, the clustering of gene expression patterns suggests that up-regulation of transcripts in the late clone is not simply delayed, but also appears to be slower than in the early clone (Paper I, figure 4). These results suggest that the differences in gene regulation between the two clones may not only be due to differences in their sensing of day-length, but also to differences in their speed of response to the short-day signal, the late clone being slower.

3.1.1 Early and late clones differ in their SD regulation of auxin responsive genes

We therefore investigated in detail the regulation of genes associated with key processes that differ between the two clones such as cambial cell division, cold hardiness, and metabolic pathways that are associated with short-day induced growth cessation. We first investigated differences in the regulation of auxin-responsive genes between the two clones, since auxin is known to be a key regulator of cambial cell division. It has been previously shown that hybrid aspen plants with reduced sensitivity to auxin have reduced cambial cell division activity (Nilsson et al., 2008), and that the expression of several auxin-regulated genes is downregulated in short days, as the cessation of cambial growth is induced (Schrader et al., 2004). Taken together, these data suggest that the auxin signaling pathway could be a potential target for the short-day signal when inducing growth cessation. Our comparisons of gene expression between the early and the late clone indicated that a large number of the auxin-regulated genes in our dataset were differently regulated after short-day treatment (Paper I, figure 5). The

expression levels of the auxin-regulated genes differed both in timing and magnitude, suggesting that the differences in cessation of cambial growth induced by a short-day signal, could involve a differential regulation of auxin signaling between the two clones following short-day treatment.

3.1.2 Early and late clones differ in their SD regulation of cold hardiness-related genes

While the timing of growth cessation is the most apparent and visible difference between the clones when grown in short days, our data also suggest that several other processes, such as cold acclimation and the metabolic pathways, are regulated differently in the two clones. Transcriptional analysis of cold hardiness related genes revealed that the observed delay in cold acclimation associated with delayed growth cessation could be due to differences in the regulation of cold hardiness related genes at the transcriptional level. Comparing the expression pattern of these genes between the two clones showed that fewer cold hardiness genes were expressed in the late clone, while for those genes whose expression changed significantly in both clones, the expression was either induced much later, or at a much lower level, in the late clone after short-day treatment (Paper I, figure 6A). The expression of several cold hardiness related genes is regulated by the CBF/DREB family of transcription factors. All of the transcripts that were found to be members of the CBF/DREB family on the microarray were up-regulated in both clones after growth in short-day conditions. However, this up-regulation was delayed in the late clone (Paper I, figure 6B), indicating that the delay or reduction of cold hardiness related genes in the late clone, might be due to a delayed induction of expression of the CBF/DREB family of transcription factors.

3.1.3 Genes involved in starch breakdown and accumulation of storage proteins are differently regulated between clones

We also examined the regulation of genes associated with starch degradation and the accumulation of storage proteins, both of which are important processes that occur during autumn. Our dataset shows that the regulation of genes involved in both these processes also differs between the early and the late clones. The induction of transcripts of, e.g. storage protein and β-amylase was significantly delayed in the late clone compared to the early clone; and the levels of the transcripts of enzymes involved in starch

breakdown and storage protein were not induced to the same levels in the late clone compared with the early clone (Paper I, figure 7).

3.1.4 Early and late clones differences in their perception of short day signal

Differences in the timing of growth cessation and the global regulation of gene expression between the two clones could be due to differences in their perception of the short-day signal, or their distinct response to the short day signal, or to a combination of these factors. Although the role of differences in the perception of the short-day signal in regulating differences in timing of growth cessation has been explored previously (Bohlenius et al., 2006), it is not known whether factors involved in the short-day response pathway might also contribute to differences in the timing of growth cessation and related responses. We therefore investigated whether the two clones differed in their perception of the short-day signal by analyzing the regulation of *PtFT* after short-day treatment. Downregulation of the flowering-time gene, *PtFT*, is one of the earliest markers of short-day perception. We therefore assumed that if the two clones differed in their perception of the short-day signal, they should also differ in the timing of their downregulation of *PtFT* expression. As expected, the transcript levels of *PtFT1* and *PtFT2* were downregulated in both clones upon exposure to short days, but we were unable to detect any significant difference in the timing of this downregulation between the two clones (Paper I, figure 8A). This suggests that the early and late clone do not differ in their perception of the short-day signal, but that differences between them were probably due to a differential response to the signal, arising from factors acting downstream of *PtFT*.

To test whether any difference in response to the short-day signal, rather than the perception of it, was specific to the two hybrid poplar clones, we also tested the expression of *PtFT* in four Swedish aspen genotypes that span a latitudinal cline from 56.3°N to 66.2°N (Ingvarsson et al., 2006). The results of this investigation showed that, while these plants clearly differed in the timing of their growth cessation and bud set, the downregulation of *PtFT* occurred at the same time in all clones (Paper I, figure 8B and C).

In conclusion, our results demonstrate that although the early and late poplar genotypes differ in the timing of their growth cessation and bud set, they do not differ in their timing of *PtFT* downregulation. Neither do the genotypes differ in their regulation of downstream pathways such as cold hardiness, the accumulation of storage proteins, or starch breakdown. This

indicates that the two genotypes perceive the short-day signal at the same time, but differ in their various responses to it, downstream of *PtFT*. Exactly what the differences are in the downstream pathways, is not yet understood; but our results suggest that differences in the short-day regulation of hormone signaling pathways might be involved, since both auxin-responsive genes, and genes involved in ethylene biosynthesis are regulated differently in the two genotypes.

3.2 Global analysis of changes in gene expression during the activity-dormancy cycle in the apical bud (Paper II).

Dormancy and bud set in perennial trees are developmental processes that occur at a specific time of the year in response to seasonal variations in the local climate. The timing of these processes is crucial for the survival of the tree, and is under strong genetic control. Gene expression during the activity- dormancy cycle has been studied extensively in a variety of tree species (Druart et al., 2007; Ruttink et al., 2007; Schrader et al., 2004). However, most of these studies have focused on analyzing the onset of dormancy, while only a few have been aimed at investigating the molecular mechanisms involved in the release from dormancy. Previous studies have used cDNA microarrays for which probes were obtained from libraries primarily constructed from actively growing tissues. However, coverage of such first generation poplar cDNA microarrays have been only partial thus not all the genes in the genome were represented on these arrays. To overcome some of the limitations of these previous experiments (Druart et al., 2007; Ruttink et al., 2007; Schrader et al., 2004), we have used a whole genome microarray supplied by Nimblegene (www.nimblegene.com) to analyze the transcriptional changes that occur in the apical meristem during the following processes: the induction of growth cessation; the transition to dormancy; the release from dormancy; and the reactivation of growth.

We obtained experimental material by growing the hybrid aspen clone, T89, under short-day conditions (8h light/ 16h dark) in a climate chamber for up to 11 weeks. To reactivate growth, plants were exposed to low temperatures for 4 weeks, followed by transfer to a greenhouse for growth under long-day conditions. Bud break and the reactivation of plants occurred within 2-3 weeks in the greenhouse.

We analyzed changes in gene expression during four stages of the experiment: induction of growth cessation (0 and 5 weeks of short-day treatment); establishment of dormancy (5 weeks to 11 weeks of short-day

treatment); release of dormancy (4 weeks of cold temperature treatment following 11 weeks of short-day treatment); and induction of bud burst (treatment in long days and warm temperatures following 4 weeks of cold treatment).

3.2.1 Expression pattern of cell cycle related genes during the activity-dormancy cycle.

The induction of dormancy is preceded by growth cessation and cell cycle arrest. Earlier studies have shown that short-day signals induce cell cycle arrest by modulating the expression of several core cell-cycle genes (Druart et al., 2007; Ruttink et al., 2007; Schrader et al., 2004). We therefore analyzed the expression pattern of poplar homologues of the *Arabidopsis* core cell-cycle genes (Menges et al., 2005). Of all members of the cyclin- dependent kinases (CDK) gene family, only the expression of *CDKB* was significantly changed, being downregulated during growth cessation and dormancy induction, indicating that *CDKB* expression is under strong transcriptional control (Paper II, figure 1). This observation contrasts with an earlier report by Espinosa-Ruiz et al. (2004), who showed that both *PttCDKA* and *PttCDKB* are post-transcriptionally regulated during the induction of growth cessation in the cambium.

In contrast with CDK, the expression of A- and B-type cyclins was downregulated early during growth cessation, whereas the D-type cyclins showed a more divergent expression pattern with members of this family being differentially regulated during growth cessation (Paper II, figure 1). Our current knowledge concerning the individual function of different D-type cyclins in the cell cycle is insufficient to explain this differential expression pattern. Distinct expression might imply that the D-type cyclins are having a more complex role in the regulation of the cell cycle, although these could also be regulated post-transcriptionally. Taken together, these results suggest that the downregulation of the cyclins and B-type CDKs could be a key mechanism for decreased cell cycle activity during short-day induced growth cessation in the apex.

In contrast with the downregulation of the cell-cycle genes during growth cessation and dormancy, release from dormancy by treatment with low temperatures is not correlated with an up-regulation of core cell-cycle genes. This indicates that the release form dormancy may not involve a simple reversal of downregulation of cell-cycle genes.

3.2.2 Regulation of cold hardiness- related genes

A key feature of the activity-dormancy transition is the acquisition of cold hardiness that is essential for the survival of perennial plants in high northern latitudes. Studies have shown that cold acclimation is regulated by short-day signals and low temperatures (Welling et al., 2002, Welling et al., 2004), but little is known about the downstream factors mediating the induction of cold hardiness. The most extensively studied pathway in plants controlling cold hardiness is the CBF cold response pathway. We identified four poplar homologues of the *Arabidopsis CBFs* (*PttCBF1-4*), and two genes that are considered to be *CBF-like* (*PttCBFL1-2*) genes. None of the closest homologues (*PttCBF1-4*) was significantly up-regulated during short-day treatment. *PttCBFL2* was the only *CBF* homologue that was up-regulated during the first 5 weeks of short days, but the expression was subsequently downregulated to almost the same level as held before the start of the short-day treatment (Paper II, figure 2). In contrast to the other *CBFs*, *PttCBFL1* was continuously downregulated during the entire short-day treatment. Since the function of this gene in unknown we are unable to explain the significance of its expression pattern; but one hypothesis is that it could be a repressor of cold hardiness and is therefore downregulated as cold hardiness is induced. The lack of any significantly up-regulated *CBFs* during short-days was surprising, but might indicate that, either these genes are not needed for short-day induced cold hardiness in the apex, or that *CBFs* play a role in the later stages of cold hardiness induced by exposure to low temperatures.

3.2.3 Dormancy induction and release is accompanied by transcriptional regulation of genes involved in hormone biosynthesis.

Our observations suggest that short-day and cold temperature signals might be involved in regulating the levels of the key hormones ABA and GA by modulating the expression of genes involved in their biosynthesis and degradation. Transcription of several genes encoding enzymes involved in ABA biosynthesis, such as *NCED6*, *NCED3* and *ABA2*, were up-regulated after 5 weeks of short days. At the same time, the transcription of *CYP707A*, an enzyme known to be involved in ABA degradation, was downregulated (Paper II, figure 3). These results suggest that the short-day induced increase in ABA levels in the apex, that have been reported earlier (Rohde et al., 2002), might be caused by a transcriptional up-regulation of genes involved in the biosynthesis of ABA, and a simultaneous downregulation of genes involved in the degradation of ABA. However,

this experiment does not rule out the possibility that the elevated levels of ABA in the apex are present because of other mechanisms e.g. the transport of ABA from other tissues. In contrast, release from dormancy by cold treatment had the opposite effect on many of these genes. Dormancy release and bud burst were associated with the downregulation of several genes involved in ABA biosynthesis, while the expression of *CYP707A* was up-regulated, suggesting that the concentration of ABA in the bud might decrease during dormancy release (Paper II, figure 3).

Exposure to short days has been shown to lead to decreased levels of GA in hybrid aspen (Olsen et al., 1997b). It was therefore surprising to note, therefore, that several genes encoding for enzymes involved in the early steps of GA biosynthesis were strongly up-regulated during the first 5 weeks of short-day treatment. During the same period however *GA20OX2a*, which has a key role in the production of active GA, was downregulated, and several other genes involved in the inactivation of active GA were simultaneously up-regulated. This was followed by a further downregulation of genes involved in GA biosynthesis between 5 and 11 weeks of short-day treatment (Paper II, figure 5). These results, together with the observations of decreased levels of GA during dormancy induction reported earlier, suggest that genes involved in the inactivation of GA is one of the main targets of the short-day signal. Low temperatures and the release from dormancy lead to the up-regulation of several genes encoding for enzymes for the production of active GA, and to the downregulation of several catabolic genes (Paper II, figure 6), suggesting that cold treatment could promote bud burst by stimulating the production of active GA.

3.2.4 Role of chromatin remodeling during activity- dormancy cycle

The massive and highly coordinated regulation of transcription during dormancy suggests that chromatin remodeling has a key role in the process of regulation. In support of this hypothesis, we found that several genes involved in chromatin remodeling underwent an altered expression during short-day treatment. For example, after the onset of short days, the expression of several genes of the trithorax family was downregulated, as were poplar homologues of the *Arabidopsis DME* gene. After 5 weeks of short-day treatment the expression of genes encoding poplar homologues of the *Arabidopsis* histone deacetylases (*HDA14* and *HDA08*), a lysine methyltransferase (*SUVR3*), and a histone ubiquitination (*HUB2*), were all up-regulated (Paper II, figure 7). Although the role of these chromatin remodeling factors in regulating dormancy in trees is currently unknown,

the importance of DNA methylation, histone acetylation, and *HUB2* have been described in potato tuber dormancy and *Arabidopsis* seed dormancy (Liu et al., 2007; Law & Suttle, 2004; Law & Suttle, 2003).

Collectively, our data suggest that the simultaneous repression of the trithorax and *DME* genes involved in transcriptional activation together with an induction of the *HDAC*, *SUVR3* and *HUB2* genes involved in repression of gene transcription, could lead to a globally synchronized repression of target genes during growth cessation and dormancy induction.

Since our data indicated that changes in the expression of genes involved in chromatin remodeling occurred during dormancy induction, we wanted to know if there were any detectable changes in chromatin modifications during the activity-dormancy cycle. We chose to focus on the acetylation of histone H3, since an increase in the acetylation of histone H3 is generally associated with activated gene expression, while its deacetylation is associated with repressed gene expression (Kouzarides, 2007). However, our results revealed that the levels of acetylated histone H3 did not change significantly during the cycle between active growth and dormancy (Paper II, figure 9). These results indicate that either acetylation of histone H3 is of little importance in the establishment and release of dormancy, or acetylation of histones other than H3 might be more important during dormancy. It is also possible that histone acetylation at chromatin regions that are repressed during the activity-dormancy cycle are reduced; while simultaneously, histone acetylation at regions that are activated increases, and therefore no change in the global levels of acetylated histones could be detected. This hypothesis is supported by the data from the microarray analysis showing that both poplar homologues of a histone acetyltransferase (such as the HAT gene *GCN5*), and two histone deacetylases were simultaneously up-regulated during dormancy induction.

In contrast to dormancy induction, chromatin-remodeling genes seem to play a minor role in dormancy release, since only a few such genes were significantly changed during this stage. This suggests that a simple transcriptional downregulation of chromatin-remodeling genes by cold treatment may not be the primary mechanism of dormancy release. Among the genes that exhibited a difference in transcription during cold treatment, there was a homologue of *DEMETER LIKE* (*DML*), which was induced during cold treatment (Paper II, figure 10). This *DML* homologue might therefore be involved in demethylating DNA and inducing the expression of target genes contributing to the release from dormancy. Furthermore,

two homologues of HDACs (*HDA9* and *SIN3*) were induced by cold treatment (Paper II, figure 10). These HDACs might also contribute to dormancy release by repressing the expression of genes promoting dormancy.

3.3 Role of the PcG complex in dormancy regulation (Paper III).

In Paper II several genes involved in chromatin remodeling and DNA methylation was shown to differ in their transcriptional regulation during growth cessation and dormancy induction, suggesting a role for chromatin remodeling in the regulation of the dormancy process. We therefore aimed to analyze further the role of chromatin remodeling in the regulation of dormancy. Several of the genes represented on the microarray were good candidates for this study, but since *PttFIE*, an aspen homologue of the *Arabidopsis* PcG complex member *FIE*, has previously been reported to be strongly induced during cambial dormancy induction (Druart et al., 2007; Schrader et al., 2004) we chose to study this gene.

The main reasons for studying *FIE* in dormancy regulation is because in *Arabidopsis*, *FIE* is a member of the PRC2 complex, which has been well studied and is known to have a role in important developmental processes, such as those involved in seed development, flowering time and vernalization responses (Calonje & Sung, 2006). FIE has also been reported to be able to interact with RBR, and might therefore be involved in the repression of cell-cycle genes that are regulated by the RBR/E2F pathway (Mosquna et al., 2004).

To analyze whether *FIE* has a role in the regulation of dormancy, we generated several hybrid aspen lines with reduced levels of expression of *PttFIE* (*PttFIE* RNAi lines). All lines were analysed for the expression of FIE mRNA and were shown to have a major reduction in the expression levels of *PttFIE* (Paper III, Supplementary figure 1A).

To test the ability of plants to establish full dormancy, they were first grown in short-day conditions for 11 weeks and then transferred to a greenhouse with long-day conditions without prior exposure to cold temperatures. Both wild-type and *PttFIE* RNAi lines ceased growth and set buds after 5 weeks of short-day treatment (Paper III, Supplementary Figure 1B). However, when plants were exposed to long-day conditions the *PttFIE* RNAi lines flushed their buds after 2-3 weeks, while the wild-type plants remained dormant until the experiment was terminated after 4 weeks (Paper III, figure 1A). These results indicate that *PttFIE* is not required for short-

day induced growth cessation or bud set, but that the *PttFIE* protein is required to maintain dormancy in hybrid aspen.

3.3.1 Expression of cell proliferating genes is not altered by downregulation of *PttFIE*

Since *FIE* in *Arabidopsis* has been reported to interact with RBR *in vivo* in maize, we investigated whether the failure to establish dormancy in the *PttFIE* RNAi lines could be due to altered expression of the cell-cycle genes in the mutant lines. We therefore examined the expression of the cell-cycle proliferating genes *PttCYCD3*, *PttCYCD6*, and *PttAINTEGUMENTA-LIKE1* (*PttAIL1*). CYCD-genes are key regulators of the cell cycle (Dewitte & Murray, 2003), and *AINTEGUMENTA* (*ANT*) is a known regulator of cell proliferation, which has previously been shown to be strongly reduced in the cambium and apex during growth cessation (Ruttink et al., 2007; Schrader et al., 2004). In wild-type plants, *PttCYCD3*, *PttCYCD6*, and *PttAIL1* were all downregulated after 6 weeks of short days. These genes showed similar expression patterns in the *PttFIE* RNAi lines as in the wild-type (Paper III, Figure 1B), indicating that *PttFIE* does not regulate dormancy progression by regulating the transcription of these cell-proliferation genes.

3.3.2 *Ptt*FIE does not affect dormancy related processes

We also wanted to know whether dormancy related processes, other than bud set and dormancy induction, were affected in the *PttFIE* RNAi lines. We therefore investigated the expression of three genes, *PttCOR*, *PttLTP*, and *BARK STORAGE PROTEIN* (*BSP*), whose expression is induced in hybrid aspen during short-day treatment.

Analysis of *PttCOR*, *PttLTP* and *BSP* showed them to be strongly expressed during the induction of growth cessation and the transition to dormancy, both in wild-type plants and in the *PttFIE* RNAi lines (Paper III, Figure 1C), indicating that processes such as cold hardiness induction, or storage protein induction, that typically accompany activity-dormancy transitions, are not affected in *PttFIE* RNAi lines.

We also observed that cold treatment does not suppress the transcription of *PttFIE* wild-type plants (Paper III, Supplementary Figure 1C). This observation indicates that the release from dormancy by cold temperatures induces another mechanism that counteracts *PttFIE* function at a post-

transcriptional level, probably by removing the chromatin remodeling marks that were added to the chromatin by a *PttFIE* containing complex.

Together, these results suggest that *PttFIE* and chromatin remodeling play a key role in the induction and/or maintenance of dormancy, but not in bud set or growth cessation. Other processes also seem to be unaffected when *Ptt*FIE is downregulated, suggesting that *PttFIE* specifically affects dormancy establishment and/or maintenance. In *Arabidopsis* and *Drosophila*, *FIE* is a subunit of the PRC2 complex (Calonje & Sung, 2006; Cao & Zhang, 2004). In *Drosophila*, this PRC2 complex represses genes by methylating histone H3 at the lysine H27 position (Cao & Zhang, 2004). It is therefore possible that *PttFIE* might regulate dormancy by repressing the expression of genes that could promote reactivation. However, the identity of these genes currently remains unknown; neither is it known whether the mode of action of the PRC2 complex (e.g. with respect to histone H3 methylation) is completely conserved between plants and *Drosophila*.

4 Conclusions and future perspectives

This thesis provides new insights into the molecular mechanism controlling bud and cambial meristem dormancy in woody trees.

We have compared the regulation of global gene expression in stem tissues during short day regulated induction of cambial growth cessation in two hybrid clones that differ in their timing of growth cessation in an attempt to identify the molecular mechanism responsible for the variations in timing of growth cessation (Paper I). Our results showed that the regulation of genes involved in key processes associated with growth cessation, such as cold hardiness and metabolic pathways, differ between hybrid polar clones. However, the clones did not differ in their perception of day length as shown by the simultaneously downregulation of *PtFT* in both clones. This observation strongly suggest that factors acting downstream of the early-acting components in the shortday perception machinery contribute to variations in the timing of growth cessation, rather than variations in the day length perception (Paper I).

We have also shown that progression through the different stages of the annual activity-dormancy cycle is accompanied by massive transcriptional changes, suggesting that transcriptional control could play a key role in the regulation of dormancy progression. In particular, this transcriptional control seems to play a role in the modulation of hormones, such as ABA and GA, known to be involved in the regulation of dormancy. Also, chromatin remodeling genes seems to have a role in the coordination of the gene expression changes during dormancy transition (paper II). Although we could not detect a significant change in the global level of acetylated histone H3 (paper II), downregulation of *PttFIE*, a component of the PRC2 complex, lead to lack of dormancy regulation suggesting a role for this gene in the regulation of bud dormancy (Paper III). Since the PRC2 complex in

Arabidopsis possesses HMTase activity and is known to repress gene expression during plant development we proposed a model according to which Ptt*FIE* and chromatin remodeling plays a key role in induction and/or maintenance of dormancy by stably repressing transcription of genes that promotes growth reactivation.

Our results have identified several potential target genes for future analysis of the molecular mechanism underlying dormancy processes. It would be particularly interesting to investigate further the role of chromatin remodeling in the activity-dormancy cycle, especially the roles of the PcG and TrxG genes. The inability of hybrid aspen with reduced levels of *Ptt*FIE to maintain dormancy, together with the observed transcriptional downregulation of the TrxG gene family during dormancy induction, strongly suggests that those gene families might have key roles in the activity-dormancy cycle. Since no other processes associated with short day induced growth arrest but dormancy seems to be affected in *PttFIE* mutant trees, this mutant may be very useful for study dormancy control in trees. For example, the *PttFIE* mutant can be used to test the candidate genes of dormancy regulation identified in paper II.

One future strategy would be to identify target genes for the TrxG and PcG complexes and to explore the mechanism by which they act by using transgenic plants and microarray.

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