New Insights into Growth Cessation and Dormancy in Trees

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Abstract

Since trees are sessile they have evolved elaborate mechanisms that anticipate oncoming winter and allow the trees to withstand extreme changes in their environment. Trees need to stop growth and initiate cold hardiness well before cold temperatures become damaging, consequently the timings of growth cessation and dormancy are important adaptive traits for their ability to survive in harsh winter conditions. If the trees fail to anticipate the onset of winter they will suffer severe damage, whereas early growth cessation will reduce the growing season and their productivity, thereby causing loss of fitness. Thus, the optimal timing of growth cessation is the point at which these risks are balanced. Hence, plants from different latitudes have different critical daylengths, and cease growth at different times.

In the work this thesis is based upon, my colleagues and I examined changes in the transcription of genes involved in various processes that are co-induced by shorter than critical daylengths, e.g. cold hardiness and the production of storage proteins. In addition, we showed that differences in growth cessation timing in trees also depends on other factors acting downstream of daylength sensing. Abscisic acid (ABA) is a plant hormone that is known to play an important role in seed dormancy and also has proposed involvement in bud dormancy. It is known that ABA levels increase after growth cessation, concurrently with the establishment of dormancy, suggesting that it may play a role in dormancy induction. To further investigate this possibility we constructed trees with reduced sensitivity to ABA. These trees were unable to enter dormancy.

In summary, this thesis describes molecular-level investigations of growth cessation and the transition to dormancy, in which microarrays, real-time polymerase chain reaction assays and transgenic hybrid aspen were used. Through better understanding of how trees perceive light signals and subsequent events, in the future it may be possible to engineer trees with altered activity-dormancy transition traits to improve their productivity.

Keywords: *Populus*, dormancy, abscisic acid, bud
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Dedication

To my wonderful family

The great tragedy of Science is the slaying of a beautiful hypothesis by an ugly fact.

Thomas H. Huxley

Contents

Abbreviations

ABA	Abscisic Acid
ABI1	ABSCISIC ACID-INSENSITIVE1
ABI3	ABSCISIC ACID-INSENSITIVE3
bHLH	basic helix-loop-helix
CBF	C-REPEAT BINDING FACTOR
CCA	CIRCADIAN CLOCK-ASSOSIATED
CO	CONSTANS
COR	COLD RESPONSIVE
CRY	CRYPTOCHROME
DNA	DeoxyriboNucleic Acid
DRE	DEHYDRATION RESPONSIVE
ETR1	ETHYLENE RESPONSE1
FLC	FLOWERING LOCUS C
FD	FLOWERING LOCUS D
FT	FLOWERING LOCUS T
GA	Gibberellin
GAI	GA-INSENSITIVE
GC-MS	Gas Chromatography-Mass Spectrometry
ICE1	INDUCER OF CBF EXPRESSION1
LD	Long Day
LHY	LATE HYPOCOTYL ELONGATED
PIF	PHYTOCHROME INTERACTING FACTOR
PHYA	PHYTOCHROME A
PHYB	PHYTOCHROME B
Pfr	Phytochrome far red
Pr	Phytochrome red
QTL	Quantitative Trait Locus
RGA	REPRESSOR OF ga1-3

RCAR	REGULATORY ELEMENT OF ABA RECEPTOR
RNA	Ribonucleic Acid
SEX1	STARCH EXCESS 1
SD	Short day
SNP	Single Nucleotide Polymorphism
TOC1	TIMING OF GROWTH CESSATION
WT	Wild Type
ZTL	ZEITLUPE

List of Publications

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

I Druart, N., Johansson, A., Baba, K., Schrader, Jarmo., Sjödin A., Bhalerao, Rupali R., **Resman, L.,** Trygg, J., Moritz, T., Bhalerao, R. Environmental and hormonal regulation of the activity-dormancy cycle in the cambial meristem involves stage-specific modulation of transcriptional and metabolic networks**.** *The Plant Journal* 2007 50: 557-573

II **Lars Resman**†, Glenn Howe†, David Jonsen, Madeleine Englund, Nathalie Druart; Jarmo Schrader, Henrik Antii, Jeff Skinner, Tony Chen and Rishikesh P. Bhalerao, Components acting downstream of SD perception regulate differential cessation of cambial activity in early and late clones of hybrid poplar. *Submitted Plant Physiology*

III Anna Petterle†, **Lars Resman**†, Bhalerao R.P. Hybrid aspen with reduced sensitivity to ABA display impaired bud dormancy *(manuscript)*

†To be considered joint first authors.

1 Biological background

1.1 Dormancy

Dormancy in trees has been defined as absence of visible growth (Lang et al., 1987). This definition is problematic because many factors and environmental stresses can cause temporary cessation of growth, and even if there is no growth cells may be metabolically active. Therefore another definition of dormancy is required.

Lang recognized three classes of dormancy: paradormancy, meaning inhibition of growth by distal organs; ecodormancy, the inhibition of growth due to unfavorable environmental conditions; and endodormancy, the inhibition of growth due to cell signaling within the dormant organ. This classification is also somewhat problematic, since inhibition of growth is an ambiguous term because dormancy is synonymous with the inability to resume growth (in the absence of growth-initiating stimuli).

This problem was addressed recently by Rhode and Bhalerao (Rohde & Bhalerao, 2007), who suggested that dormancy in trees should be defined as the inability to resume growth from meristems or other organs and cells with the capacity to resume growth under favorable conditions. In this thesis I will mainly describe bud and cambial dormancy, since my experiments specifically focused on these processes. Trees in temporal regions display cyclic growth patterns that are well synchronized with changes in their environment. The long and light summers provide excellent conditions for plant growth. In contrast, conditions are harsh in the winters, and there have been severe pressures on trees to evolve mechanisms that enable them to survive during these periods. Furthermore,

trees need to anticipate the oncoming winter well in advance, otherwise there will be insufficient time for them to stop growing and initiate cold hardiness programs before temperatures become damaging for sensitive, unhardened tissues.

The transition from active growth to growth cessation in plants occurs gradually (Champagnat, 1983), starting in late summer when the plants sense daylength reductions. Shortening of the daylength also triggers a developmental switch in the apical meristem that initiates the production of bud scales, dehydration and hardening of the buds, and the accumulation of freeze-tolerance proteins and sugars inside them, which increases their cold tolerance after growth cessation. At this stage the trees can still resume growth if environmental conditions become favorable, e.g. if they are exposed to long days. Hence, this state is referred to as ecodormancy. However, in later stages plants can no longer respond to growth-promoting signals, and a prolonged period of chilling temperatures is required to break their dormancy. This state is referred to as endodormancy (Fig. 1). In the hybrid aspen clone T89, used in the studies this thesis is based upon, ecodormancy and endodormancy are established after ca. 5-6 weeks and 8-10 weeks of short days, respectively. When their chilling requirement is met, they re-enter an ecodormant state until temperatures rise, then the buds swell and growth is reinitiated.

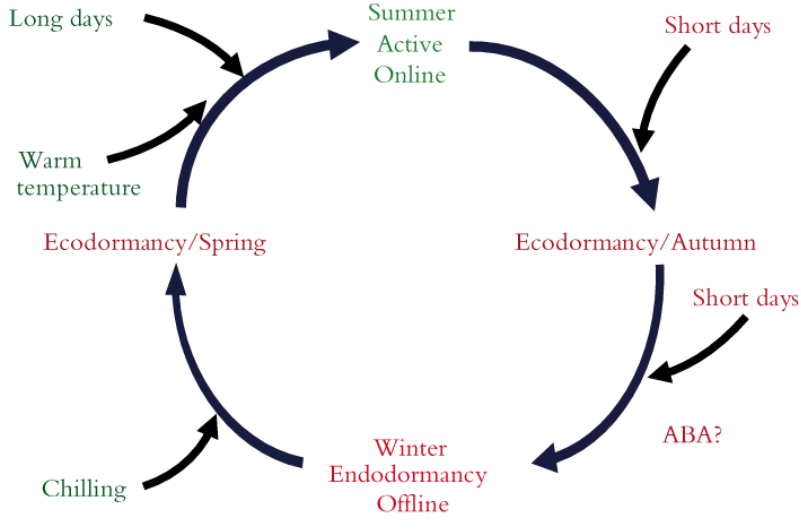

Figure 1. Illustration of the annual activity-dormancy cycle in the plant meristem.

In this thesis, dormancy is hereafter referred to as a state of inability to grow even under favorable conditions, which for *Populus* means long days and warm temperatures. There have been several suggestions regarding the mechanisms whereby dormancy may be maintained. One is that changes in cellular communication are involved. Notably, the plasmodesmata are plugged by callose during growth cessation, and this process partially overlaps with the induction of dormancy. Hence it may participate in the maintenance of dormancy (Rinne & van der Schoot, 2003), for instance by preventing the transportation of gibberellin (GA), a known growth-promoting hormone. In addition, factors like hormone sensitivity and chromatin remodeling might play important roles in dormancy induction. These processes are described later in this thesis.

1.2 Photoperiodism – How plants respond to daylength signals.

Photoperiodism can be defined as a response to daylength that enables living organism to adapt to seasonal changes and is an important and complex aspect of the interactions between plants and their environment (Vince-Prue, 1975). While many environmental signals, e.g. temperature, vary substantially from year to year, the reduction in daylength in the autumn is a reliable indicator of the approach of winter. Hence, in many tree genera, including poplars (*Populus* spp.), this reduction in daylength is used as a signal to stop growth and enter dormancy (Sylven, 1940b). Day length signal regulates several responses in addition to growth cessation and dormancy. Cold hardiness in several tree species including poplars is induced by shortening day length and its interaction with low temperatures (Molmann *et al.*, 2005; Howe *et al.*, 2003; Weiser, 1970). The timing of growth cessation and induction of cold hardiness and other responses regulated by photoperiod are thus critical to the survival of forest trees in temperate areas. It has been known for a long time that the photoperiod is an important trigger of growth cessation in many tree species (Nitsch, 1957; Sylven, 1940b), but the mechanisms whereby they sense the short day signals that induce growth cessation and dormancy are still not fully understood. The ability to perceive changes in day length and respond to these changes is not only important for survival of the plants as indicated above but underlie the ability of plants to occupy an ecological niche in space and time. An evidence of the importance of photoperiodic regulation in adaptation of plants to environment is provided by the analysis of clinal

variation in timing of growth cessation and bud set. Investigation of these processes shows that in northern latitudes the ecotypes are adapted to a longer critical photoperiod (i.e. shorter night) than at southern latitudes (Habjorg, 1972; Sylven, 1940a).

A key question in the study of photoperiodic regulation is what is the mechanism underlying the ability of plants to perceive changes in day length and respond to these changes? It is clear today that photoperiodic timekeeping is based on a circadian oscillator, which is coupled to rhythms in light sensitivity to form daylength signaling mechanisms (Mas, 2005). Below I discuss the key components of circadian clock relevant to the regulation of growth cessation, dormancy and other associated responses.

1.2.1 Circadian clock

As an adaption to different seasons, most living organisms, including plants, have evolved an internal timing mechanism that is responsible for perceiving and disseminating daylength signals. This regulatory network is called the circadian clock. In recent years circadian clock has been thoroughly studied in *Arabidopsis* and the complexity and number of genes involved is steadily increasing (Imaizumi, 2009). Classically, the circadian clock is divided into three components: the *input pathway*, senses and transmits light signals that synchronize the *central oscillator*, which functions as a pendulum that maintains rhythmicity through multiple *output pathways* that connect the oscillator to various developmental responses (Mas, 2005). This is a simplification, but it provides a model describing how light signals are connected to biological responses.

The present theory is that the circadian clock in *Arabidopsis* is composed of interlocking loops (Harmer, 2009). The core genes in these feedback loops are consisting of (*inter alia*): CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1)/LATE ELONGATED HYPOCOTYL (LHY), PSUEDO RESPONSE REGULATORS (PRRs), TIMING OF GROWTH CESSATION (TOC1) and ZEITLUPE (ZTL) amongst others (Locke *et al.*, 2006; Zeilinger *et al.*, 2006). The MYB transcription factors (CCA1)/(LHY) comprise together with (PRR7)/PRR9 one loop. In another loop the expression of CCA1 and LHY peak in the morning and act together to repress the transcription of the PRR protein TOC1 (Penfield, 2008). By evening the expression of CCA1 and LHY has declined and TOC1 expression increases. The third loop consists of evening-peaked TOC1 and

a hypothetical clock component Y. The CCA1/LHY–PRR7/9 loop and the TOC1–Y loop are connected by a negative feedback loop, which is formed by CCA1/LHY and TOC1 (Mas & Yanovsky, 2009; Alabadi *et al.*, 2001).

1.2.2 Regulation of developmental responses by day length signaling and circadian clock

Control of developmental responses by change in day length is expected to involve the integration of day length signaling and circadian clock. There have been many different suggestions of how the light and circadian signalling is integrated to control developmental responses in plants (Yanovsky & Kay, 2003), but the "external coincidence model" is the predominant model today (Saunders 2005). This model was originally proposed by Bünning (1936) based on observations in soybeans. Bünning stated that a circadian clock drives a rhythm in a light-sensitive process, and that photoperiodic responses are promoted (LDPs) or inhibited (SDPs), when the illuminated part of the day overlaps with the most sensitive phase of this endogenous rhythm (Fig.3) (Yanovsky & Kay, 2003). Later Bünnings hypothesis was refined with the addition of light as a synchronizing factor, also called entraining (Imaizumi & Kay, 2006). The transition from active growth to flowering in *Arabidopsis* is an example of such external coincidence.

1.2.3 Photoreceptors and regulation of plant development

Studies in Arabidopsis have shown how photoperiod sensing is integrated with the functioning of circadian clock to the regulation of flowering time. Analysis of this process shows that photoreceptors phytochromes and cryptochromes act in close relation with the circadian clock (Eriksson & Millar, 2003). With respect to regulation of the timing of growth cessation in *Populus* trees the role of Phytochromes is better studied whereas that of cryptochromes remains to be established. For example, it has been found that trees overexpressing the oat PHYA (Phytochrome A) photoreceptor in hybrid poplars do not cease growth or set bud in response to short photoperiods (Welling *et al.*, 2002; Olsen *et al.*, 1997). These PHYA overexpressing plants are also unable to induce cold hardiness properly supporting a role for SD-signaling in cold hardiness. PHYB might also play

an important role in controlling bud set in Poplars, since PHYB2 coincides with a QTL affecting bud set and it has been demonstrated that PHYB2 has a clinal variation in SNPs (Ingvarsson *et al.*, 2006; Frewen *et al.*, 2000b). In agreement with the interaction between day length sensing via photoreceptors and circadian clock, in hybrid aspen, reduction of the expression of PHYA leads to changes in phasing of clock associated genes and alteration of growth cessation responses (Kozarewa *et al.*, 2010).

Phytochromes play a central role in day length signaling as indicated above and are made of a holoprotein consisting of two dimerized apoproteins, each binding a chromophore for light capture (Rockwell *et al.*, 2006). The phytochromes are synthesized in their inactive Pr form but when it is exposed to light in the red spectrum they undergo a conformational change to the active Pfr form (Rockwell *et al.*, 2006). Conversely, when exposed to light in the far red region they revert to their inactive Pr form. The PHY A and PHY B then interact with a family of basic helix-loop-helix (bHLH) transcription factors called phytochrome-interacting factors (PIFs). Different PIFs have different biological functions, cotyledon expansion, seed germination, chlorophyll biosynthesis is some examples (Shen *et al.*, 2005; Oh *et al.*, 2004). In recent years studies mainly in Arabidopsis have shed light on the role of PHYTOCHROMES and their interactions with PIFs in regulation of downstream responses mentioned above and this appears to involve hormonal signaling e. g. in hypocotyl elongation (de Lucas *et al.*, 2008; Feng *et al.*, 2008). In contrast less is known about the PHYTOCHROME-PIF interaction in short day mediated growth cessation and other responses in trees.

1.2.4 The CO/FT regulon

In addition to phytochromes two regulators namely CONSTANS and FLOWERING LOCUS T have a central role in day length of circadian regulation of plant development (Searle *et al.*, 2006; Michaels *et al.*, 2005; Michaels & Amasino, 1999). Below I discuss mainly their role in flowering time regulation and control of growth cessation in trees. The CO gene encodes a B-box-type transcription factor that induces expression of the floral activator FT in a light dependent manner (Fig. 2) (Yanovsky & Kay, 2002). It has been shown that CO expression peaks in the late afternoon regulated by the circadian clock via GIGANTEA (GI) (Suarez-Lopez, 2001), and that CO and FT are expressed in tissue-specific patterns; CO being expressed in leaves and the phloem in stems, while FT is only

expressed in leaves (Imaizumi & Kay, 2006). Under long day conditions the CO protein activates the production of FT, and then the FT protein is translocated from the leaf to the shoot apical meristem where it interacts with FLOWERING FOCUS D (FD), which in its turn induces flowering (Fig. 3) (Corbesier et al., 2007).

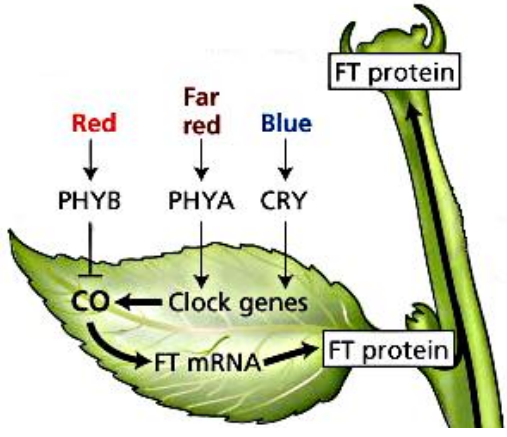

Figure 2. Schematic figure describing perception and signal transduction of the FT/CO regulon in flowering time regulation. In *Arabidopsis* light is perceived by phyA and cry2 Modified figure from Plant Physiology, fourth edition.

However, when *Arabidopsis* is grown under short photoperiods CO expression peaks during the night and no FT expression is induced because the CO protein is unstable in darkness, hence there is no flower development (Valverde *et al.*, 2004; Suarez-Lopez *et al.*, 2001). Parallels to *Arabidopsis* flowering have been observed in SD-induced growth cessation in trees. Notably, Böhlenius showed that expression of PtCO2 and PtFT1 in poplar followed the same pattern as in *Arabidopsis*, suggesting that a regulon analogous to the CO/FT regulon is involved (Bohlenius *et al.*, 2006).

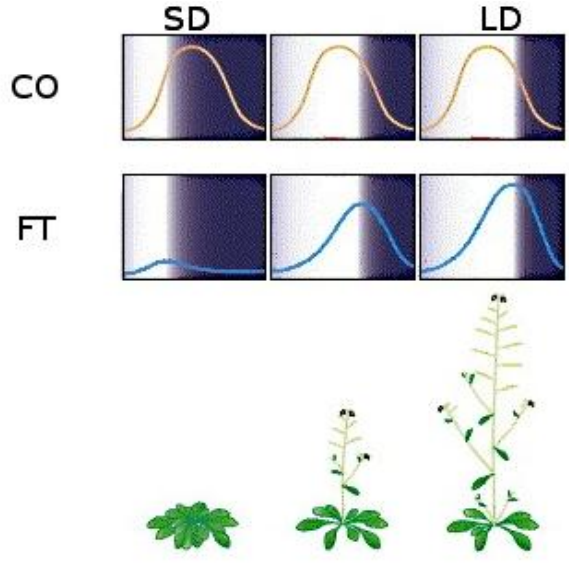

Figure 3. Regulation of flowering by CO/FT regulon. The yellow curved line describes expression of CO mRNA. CO is a clock regulated protein and a known regulator of FT expression. If CO protein levels coincidence its peak of expression during day light, it remain stable and is able to induce expression of FT which expression induce flowering in *Arabidopsis*. If CO expression peaks at night the protein is degraded and FT is not expressed. Modified image from: Imaizuma and Kay 2006.

The role of this regulon was further elucidated when it was discovered that poplars with reduced expression of PtFT1 initiated growth cessation and set bud earlier than wild type poplars. In contrast, poplars overexpressing PtFT1 failed to set bud and enter dormancy in short photoperiodic conditions (Bohlenius *et al.*, 2006). The regulatory pathway downstream of FT controlling growth cessation is to date not known.

1.3 Seed dormancy

The kind of dormancy that has been most thoroughly studied at a molecular level is seed dormancy, thus analysis of bud and cambial dormancy can benefit from findings regarding dormancy in seeds. Therefore, I will briefly describe key features of seed dormancy here.

Seed dormancy is regarded as a state in which an intact, viable seed is unable to germinate under favorable conditions (Bewley, 1997). There are strong selective pressures for such a state, since it is highly important for seeds to germinate at a time when conditions are not only favorable for germination *per se*, but there will also be a prolonged period after germination in which conditions are appropriate for growth and reproduction. Thus, dormancy is an adaptive trait that increases a plant's chances of survival. There are two different kinds of seed dormancy: coat-enhanced and embryo dormancy (Bewley, 1997). Coat-enhanced dormancy is, as the term indicates, enhanced by a physical barrier that prevents environmental signals penetrating into the embryo and initiating germination; this is sometimes not regarded as "true" dormancy. In embryo dormancy, the embryonic tissue *per se* is dormant even when environmental signals are growth-promoting. In this case the molecular signaling inside the seed inhibits successful germination. In this section I will briefly discuss embryonic seed dormancy.

It has been shown that several hormones, especially ABA and GA, influence seed dormancy and germination in numerous ways. Overexpression of genes involved in ABA biosynthesis can increase seed ABA contents and enhance seed dormancy and/or delay germination (Nambara & Marion-Poll, 2003; Thompson, 2000; Frey *et al.*, 1999). In contrast to excess ABA, ABA deficiency is associated with absence of primary dormancy of the mature seed (Kucera *et al.*, 2005). Several abscisic acid insensitive (ABI) mutants have been reported to have impairments of seed dormancy that are identical to those of ABA-deficient mutants (Kucera *et al.*, 2005).

Another factor that plays a key role in seed dormancy is the plant hormone Gibberellic acid. It is thought that GA:s play a role in another temporal phase of seed dormancy. GA levels increase when the seed is released from dormancy and starts germinating. Thus, while ABA plays a role in maintaining seed dormancy, GA appears to participate in breaking seed dormancy and initiating germination. However, GA alone cannot stimulate release from dormancy in fully dormant seeds (Derkx & Karssen, 1993). Seed dormancy and germination seem to be net results of many interacting factors, of which GA and ABA are two of the most important (Debeaujon *et al.*, 2000).

1.3.1 Similarities between bud and seed dormancy?

It seems likely that seed dormancy shares regulatory pathways with bud dormancy, since several traits are common to both states. The requirement for a long period of low temperatures to break them is the most obvious

similarity. In addition, both seeds and dormant buds are dehydrated when entering dormancy and subsequently rehydrated when dormancy is broken. Given the physiological similarities, one might hypothesize that common regulatory pathways maintain dormancy in seeds and plants. Physiological findings are also supported by transcription data showing similarities between dormant bud and seed transcriptomes (Ruttink *et al.*, 2007).Thus, knowledge about seed dormancy, which has been more extensively examined at a molecular level than bud dormancy, could provide new insights about dormancy in trees (Penfield & King, 2009).

1.4 Physiological responses accompanying growth cessation and dormancy

Parallel to induction of growth cessation, several other biological processes occur after exposure to SD. These include: induction of cold hardiness, bud set, and a shift in metabolism as the synthesis of components that play important roles in cold hardiness, such as sugars and cryoprotectants, is started.

Bud set always precedes dormancy, and both processes are induced by shortening of the photoperiod. The tissues inside the bud become dehydrated and harden, which is essential to prevent damage by freezing during periods with sub-zero temperatures (Rohde *et al.*, 2002; Welling *et al.*, 2002). Another parallel process to dormancy induction is autumn senescence, which occurs after bud set and is highly regulated, involving *(inter alia)* nutrient salvage from senescing tissues via the ordered degradation of macromolecules (Larry *et al.*, 1997). The reuse of nutrients, especially nitrogen, is very important since nitrogen is generally the growth-limiting factor in various ecosystems, including boreal forests (Nasholm *et al.*, 1998). Like bud set, the main trigger for the initiation of autumn senescence is shortening of the photoperiod (Keskitalo *et al.*, 2005). Bud set and autumn senescence are also similar in that the timing of their initiation shows latitudinal adaptation, although the variation in the timing of senescence is greater than that of bud set (Ingvarsson *et al.*, 2006). Furthermore, although shortening of the photoperiod is the main trigger for both bud set and senescence in some trees there may be up to three months differences in the timing of their initiation (Fracheboud *et al.*, 2009). This suggests that two different critical photoperiods control the induction of senescence and bud set.

1.4.1 Temperature interactions with photoperiod

In contrast to photoperiodic light signaling, the molecular pathway underlying perception of temperatures is poorly understood (Franklin, 2009).

There are several tree species such as apple (*Malus domestica)* and pear (*Pyrus communis)* that, in contrast to most boreal zone woody plants, do not respond to SD-treatment (Nitsch, 1957). Instead, these trees cease growth and enter dormancy in response to low temperatures (Heide, 2008). In some species of *Prunus* it has recently been shown that interactions between photoperiod and temperatures influence the timing of growth cessation and dormancy (Heide, 2008). The occurrence of photoperiod-temperature interactions is also well known from studies of flowering, in which the photoperiodic requirements are dependent on temperature (Wilkins, 2005). In addition, Molmann *et al.* (2005) found that treating PHYA-overexpressing hybrid poplars growing in SD and low night temperature conditions with paclobutrazol (a GA inhibitor) resulted in bud set and growth cessation.

1.5 Cold hardiness

Although the studies this thesis is based upon primarily focused on growth cessation and dormancy, some background information should be provided about cold hardiness, and the reasons why cold temperatures are potentially damaging to plants (since there would otherwise be no need for dormancy).

1.5.1 Why do freezing temperatures pose threats to plants?

Freezing temperatures pose major threats to plants in the temperate zones of the world and at high latitudes because ice starts to form inside their tissues when the temperature sinks below 0°C. Ice mainly forms in the intercellular spaces between the cells rather than in the cells (Thomashow, 1998), since the higher osmotic pressure inside the cells hinders ice formation. When ice forms in the intercellular parts of plant tissues a second related problem arises for the plants, since the imbalance in water potential between intra- and inter-membrane spaces forces water out of the cell, thereby causing dehydration (Webb & Steponkus, 1992). Cold temperatures also affect all kinds of enzymatic reactions. This poses severe threats to the photosynthetic machinery, since low temperatures slow the enzymatic reactions involved in photosynthetic electron transport and other processes, while having no effect on the photochemical reactions that occur in the photosystems

(Benedict *et al.*, 2005; Asada, 1996). This leads to increased risks of the creation of damaging reactive oxygen species. Hence, in trees short days together with low temperatures induce the expression of cold hardiness genes (Welling *et al.*, 2002) and it has been shown that trees overexpressing PHYA are unable to induce cold hardiness genes in response to short days.

1.5.2 How do plants respond to freezing temperatures?

Plants have evolved diverse mechanisms that enable them to withstand low temperatures and prepare for the oncoming winter. Cold tolerant plants have the ability to create antifreeze proteins, which (as the name suggests) have the ability to lower the temperature at which ice crystals are formed. The antifreeze proteins are primarily found in the intercellular tissues, where they bind to the surfaces of newly formed ice-particles, thereby preventing their aggregation. Plants also react to low temperatures by changes in lipid composition, which increase the stability of their membranes at low temperatures. Cold acclimation also involves reductions in the size of vacuoles, and often their splitting into several, smaller vacuoles (microvacoulation) (Kuroda & Sagisaka, 1993).

In low, but non-freezing, temperatures the COR (COLD RESPONSIVE) gene is induced by the C-repeat/drought-responsive element (CRT/DRE) DNA regulatory binding factor (CBF:s/DREB:s) (Jaglo-Ottosen *et al.*, 1998; Thomashow, 1998). CBF:s have been extensively studied and characterized, mainly in *Arabidopsis*. Overexpression of the *Arabidopsis* CBF1 transcription factor induces expression of COR-genes and enhances cold hardiness in *Arabidopsis* (Benedict *et al.*, 2006). CBF:s have also been found to play a role in winter hardiness in birch (Welling & Palva, 2008) and cold hardiness in poplar leaves and stems (Benedict *et al.*, 2006). The most important upstream regulator of CBF:s is ICE1 (INDUCER OF CBF EXPRESSION1), a helix-loop-helix transcription factor that is a constitutively expressed promoter of CBF expression (Chinnusamy *et al.*, 2003).

There is also abundant evidence that plant hormones play important roles in mediating developmental responses to cold signals. Notably, gibberellins have been shown to participate in many temperature-sensitive developmental processes (Penfield, 2008; Stavang *et al.*, 2005). For instance, in *Arabidopsis*, overexpression of the Reduced height3 gene (Rht3) perturbs GA signaling and results in a dwarf phenotype. In cold temperatures (10 ^{0}C) Rht3-mutants grow like wild type plants, but unlike wild type plants when switched to 20 ^{0}C growth conditions their growth rate does not increase (Tonkinson *et al.*, 1997). ABA is another plant hormone that is intimately

connected to cold stress and many genes that are known to be responsive to cold have also been found to be responsive to ABA. However, there are also many ABA-independent pathways that have been found through analysis of ABA-insensitive or ABA-deficient plants (Llorente *et al.*, 2000; Gilmour & Thomashow, 1991). It remains to be seen how hormonal signaling pathways are involved in the regulation of cold hardiness in trees.

1.6 Vernalization

Dormancy and vernalization (acquisition of the competence to flower) share several common features, notably both are promoted by a long period of cold temperatures. Flowering has been intensively studied and the knowledge acquired in flowering investigations may help elucidate the regulation of dormancy release. In *Arabidopsis,* flowering competency is induced by extended cold treatment, in which FLOWERING LOCUS C (FLC) expression is repressed though chromatin remodeling, which in turn releases FT repression and the plant becomes competent to flower (Hepworth *et al.*, 2002; Michaels & Amasino, 1999). FLC-like genes have been shown to be differentially expressed during the completion of the chilling requirement in poplar buds, indicating that they may be involved in bud dormancy regulation (Chen & Coleman, 2006). However, there are also some fundamental differences between vernalization and bud dormancy. Notably, in *Arabidopsis,* the repression of FLC is probably reset during meiosis (Mylne *et al.*, 2006). However, if tree dormancy is regulated by FLC-like genes through chromatin remodeling, as in vernalization, it must be reset in a different way since meiosis does not occur in cycling meristem cells following reactivations (Penfield & King, 2009).

1.7 Hormonal control of activity-dormancy transitions

1.7.1 Abscisic Acid (ABA)

In trees ABA is known to affect several aspects of plant development and responses to environmental stresses such as cold, drought and salinity. However, separating dormancy processes from freezing/dehydration and distinguishing causes from effects is often difficult. For example, it is difficult to tell if an increase in ABA levels in the apical meristem has induced dormancy or is merely a response to freezing and dehydration, two processes in which ABA is known to play an important role. However, as described above, ABA has been shown to play a role in seed dormancy (Kucera *et al.*,

2005; Thompson, 2000; Frey *et al.*, 1999), and increases in cambial ABA levels during the transition to dormancy have been observed in several studies, e.g. in the shoot apices of silver birch (Li *et al.*, 2005) and hybrid poplars (Rohde *et al.*, 2002). Further, Li found that ABA levels remained high during dormancy, indicating a possible role of ABA in its maintenance. Contrary indications, that ABA does not play such a role (or at least that its role is more complex) have also been observed, notably that ABA levels do not decrease during dormancy release in spring time, and in some cases increases in ABA levels have been observed in paper I. Two key regulators of ABA responses include the 2C protein phosphates (PP2Cs) ABI1 and ABI2. Transgenic *Arabidopsis*, carrying mutations in either of these enzymatic genes are insensitive to ABA (Ma *et al.*, 2009). When an interacting protein called RCAR (REGULATORY ELEMENT OF ABA RECEPTOR) binds to either ABI1 or ABI2, the resulting complex binds strongly to ABA, and initiates the ABA signaling cascade by shutting down enzyme activity (Pennisi, 2009). In Paper III we (i.e. my co-authors and I) show that hybrid aspen overexpressing a dominant negative mutant allele $abi_{1\text{-}1}$ cease to grow at the same time as wild type plants, but are unable to maintain dormancy. Thus, ABA has a potential role in bud dormancy, but not in growth cessation.

1.7.2 Gibberellins (GA)

GA is well known to be involved in stem elongation in plants and in response to SD early downregulation of GA:s has been observed in both angiosperms and gymnosperms (Olsen *et al.*, 1995). As previously described, GA is also a known antagonist to ABA in regulating dormancy in seeds (Kucera *et al.*, 2005; Bewley, 1997).

In trees transgenically expressing the oat PHYA, GA levels are not downregulated as in wild type trees after SD treatment, and since GA is a well known growth-regulating hormone this could be why they fail to undergo growth cessation and enter dormancy (Molmann *et al.*, 2005; Olsen *et al.*, 1997). It has also been shown that wild type poplars grown in long days set bud if their gibberellin production is reduced, but they do not enter dormancy (Molmann *et al.*, 2005).

Similarly, cell division and elongation ceases in willow (*Salix pentandra)* in response to short days but can be re-initiated by GA application (Hansen *et al.*, 1999; Olsen *et al.*, 1995) and hybrid aspen plants that overexpress GA20 oxidase have high levels of GA and display a delayed growth cessation in response to SD-treatment.

Furthermore, it has been shown that not only levels of GA, but also sensitivity to GA changes during prolonged SD-treatment (Junttila & Jensen, 1988). DELLA-proteins are known to negatively regulate GA-signaling. Hence, the reduced sensitivity to GA could be explained by the strong upregulation of known DELLA-proteins, such as GA-INSENSITIVE (GAI) and REPRESSOR OF ga1-3 in response to SD-treatment (Paper I). It has also been noted that PIF4 (PHYTOCHROME INTERACTING FACTOR 4) and PIF3-LIKE1, two genes that are known activators of GAI and RGA, are induced in response to SD (Castillon *et al.*, 2007; Ruttink *et al.*, 2007). The cited studies indicate that regulation of GA probably plays an important role in growth cessation.

2 Objectives

The molecular regulation of the transition from active growth to dormancy is not well understood. Therefore, the main aim of the work underlying this thesis was to increase knowledge about this process. In the studies reported in Papers I and II, my co-authors and I investigated growth cessation and dormancy at a global transcriptional level by examining the transcription patterns of both outdoor-grown aspen trees and greenhouse-grown ecotypes of hybrid poplar that differ in the timing of growth cessation.
As reported in Paper III, we also investigated the role of the plant hormone ABA in dormancy using transgenic hybrid aspen plants with reduced ABA sensitivity.

The main specific questions addressed in these studies (using metabolomic analyses, microarray-based transcript profiling, transgenic trees, and both visual and physiological observations) were:

I. How is the transition from active growth to dormancy regulated at transcriptional and metabolic levels in aspen trees? (Paper I);

II. What molecular mechanisms contribute to the differences in timing of growth cessation between different poplar and aspen ecotypes? (Paper II);

III. What roles does ABA play in growth cessation and dormancy in poplars? (Paper III).

3 Methodological overview

3.1 The central dogma of biology

The central dogma of biology is (simplistically) that "DNA makes RNA makes protein". DNA carries the genetic information required to construct cells, and consists of thousands of genes arranged in chromosomes. Each gene serves as a code for a specific messenger (mRNA) "transcript", the non-protein coding parts of the mRNA are removed and the mRNA is transported from the nucleus to the cytosol, where it is "translated" into proteins.

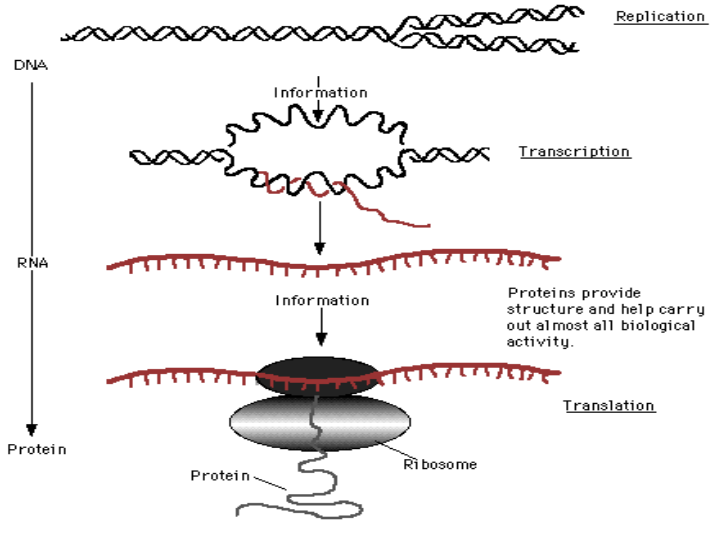

Figure 4. The central dogma in biology. In reality, there are many complicating aspects. Figure modified from access central, National Health Museum, USA.

The basic objective in gene expression analysis is to explain phenotypic or environmental responses by measuring mRNA levels. There are several ways to do this, but two of the most recent and frequently applied are microarray analysis and quantitative Real Time PCR (qRT) analysis. These transcriptional methods are well established and both robust protocols and reaction kits have been developed that allow transcriptional patterns to be conveniently and rapidly explored. However, several factors complicate the interpretation of gene expression as explanations of phenotypes (Anderson & Seilhamer, 1997). Notably, it is well known that post-transcriptional processes may uncouple the correlation between mRNA and protein levels, e.g. RNA turnover and protein turnover (Chang & Gallie, 1997). The discovery of microRNA as a major regulatory element has added further complexity to our understanding of the regulation of mRNA (Zhang et al., 2006).

3.2 Microarrays

Much of the information presented in Papers I-II was obtained using microarrays; therefore I will describe this technique in some detail.
Microarrays allow levels of thousands of mRNA transcripts to be robustly measured, and hence global gene expression profiles in diverse organisms to be monitored. They also provide opportunities to acquire huge amounts of expression data and hence obtain new insights about complex phenomena, e.g. wood formation, pathogen responses and responses to many biotic stresses such as cold, heat and drought in tree species (Street *et al.*, 2006; Schrader *et al.*, 2004a).'

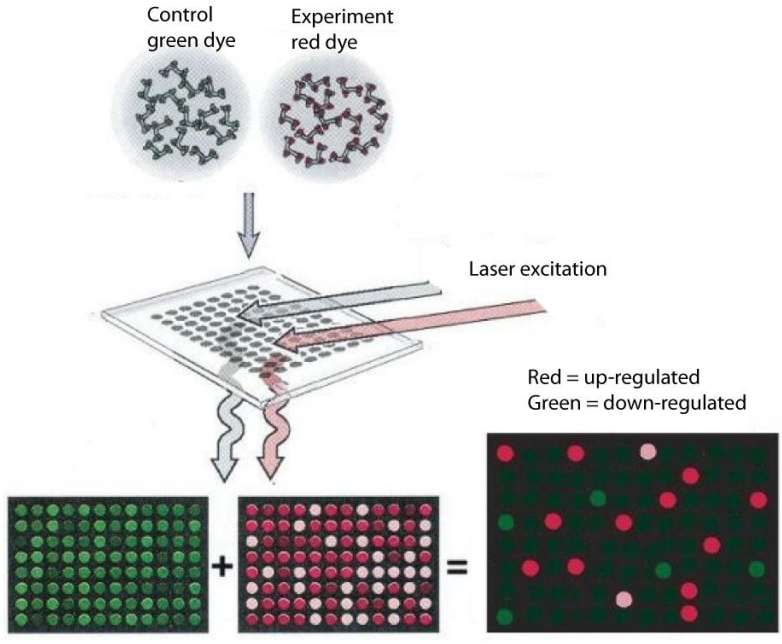

Figure 5. Schematic illustration of a cDNA microarray analysis, figure modified from Gibson and Muse 2002.

The two main types of DNA microarray system that are available are: cDNA microarrays Schena et al. (Schena *et al.*, 1995) and oligo-nucleotide microarrays (Lockhart *et al.*, 1996). The microarray data presented in this thesis are derived from cDNA microarrays, hence I will only describe that technique.

Briefly, cDNA-probes for thousands of genes are immobilized on glass-slides. RNA from the samples of interest is extracted, reversely transcribed and separately fluorescence-labeled using the cyanine dyes Cy3 (green) and Cy5 (red), and then mixed in equal proportions into a single solution which is hybridized with cDNA probes on the microarray slide. The resulting fluorescence is examined using a scanner that measures signals at the wavelength of reflecting laser emissions and quantified by image analysis software. The ratio of the green to red fluorescence is indicative of the relative abundance of the corresponding DNA probes on the microarray slide (Yang & Speed, 2002). The approach allows the levels of thousands of mRNA transcripts to be simultaneously monitored, and hence enables global gene expression patterns to be evaluated in diverse organisms. The ability to investigate the expression of huge numbers of genes

simultaneously has provided new insights about complex phenomena such as wood formation, and responses to pathogens and many biotic stresses, e.g. cold and drought in tree species (Street *et al.*, 2006; Schrader *et al.*, 2004b). It should be noted that the interpretation of the data must be biologically relevant, hence common standards that should be met by all published microarray studies have been developed, called MIAME (Minimum of Information About a Microarray Experiment) (Brazma *et al.*, 2001).

3.2.1 Experimental Design

The success of a microarray experiment is to a high degree determined at the design stage. There are several issues to take into account when designing a scientific experiment, the most important including the cost, time and amount of material required. The limitations in these factors influence the feasibility of some studies, and the optimal approach with available resources. Furthermore, some designs generate much more complex data than others, and in some cases data processing may be an extreme constraint.

3.2.2 Normalization

Several factors may affect the reliability of microarray data. Variations in the efficiency of dye incorporation, dust or irregularities on the microarray slide and differences in the probe spotting are all possible sources of error in the quantification of the data. Normalization provides a way to handle systematic sources of error, under the assumption that few genes are differentially expressed between samples (e.g. samples of trees that have been subjected to different treatments or have different genotypes) and that the changes are roughly symmetrical (e.g. expression levels of similar numbers of genes increase and decrease).
There are a number of possible ways to normalize microarray data. The data presented in Papers I and II were normalized using the print-tip lowess method (Smyth & Speed, 2003) implemented in BASE in the statistical software R (Ihaka & Gentleman, 1996).

3.3 RT-PCR and qRT-PCR

Reverse transcriptase polymerase chain reaction (RT-PCR) analysis is a quantitative, gene-specific method used for quantifying gene expression. The method is very sensitive and enables the detection of mRNA from very weakly expressed genes. In the studies this thesis is based upon, two RT-PCR methods were used: semi-quantitative RT-PCR and Quantitative

RT-PCR. A common step in both methods is the reverse transcription of mRNA into cDNA. This cDNA is then used as a template for gene-specific PCR-amplifications. In semi-quantitative RT-PCR, it is necessary to adjust quantities of samples to account for differences in starting amounts of the cDNAs of reference gene(s) and the gene(s) of interest. It is also important to quantify relative gene expression when the amplification of both target and reference cDNAs is in logarithmic phase. PCR products are generally quantified by agarose gel electrophoresis followed by ethidium bromide staining, optical imaging of the gels and finally processing of the acquired data using appropriate software.

A newer and more powerful method is qRT (quantitative Real-Time)-PCR, which differs from semi-quantitative PCR in that amplification of gene products is measured after each cycle, using an incorporated fluorescence dye (for experiments presented in this thesis, SybrGreen) (Wong & Medrano, 2005). Thus, since gene expression is measured in each cycle, it's easier to avoid saturation of signals and gene product levels are always quantified when their amplification is in the logarithmic phase. Hence, there is no need for competitive primers to decrease the amplification efficiency of reference genes. Furthermore, the quality of the amplified products is determined by analyzing their melting points. Melting curves provide an indication of the purity of the reaction products and reveal primer-dimer artifacts. qRT-PCR was used in studies described in Papers I and II to determine gene expression of FT and ABI1, respectively, and RT-PCR was used to confirm successful transformation of *35S::abi1* transgenic poplars (Paper II).

3.4 Poplar as a model tree

The first plant genome that was completely sequenced was that of thale cress (*Arabidopsis thaliana)*, a weed with little commercial value growing in Europe, Asia and North Africa. Due to its small genome, short rotation time and ease to transform it became the workhorse of the plant molecular science world. However, to address the questions posed in this thesis another model organism is required. Poplar has a clear advantage relative to *Arabidopsis* for this purpose since poplars are perennial, which is an obvious requirement for studies of post-embryonic dormancy. In addition, the poplar genome has been completely sequenced (Tuskan *et al.*, 2006) (the

third plant genome to be elucidated, after *Arabidopsis* and rice), poplars are fast growing, and easy to both transform and propagate in sterile environments.

3.5 Plant material used in the studies

Hybrid poplar (*Populus trichocarpa x Populus deltoides)* clones that differ in timing of growth cessation were kindly provided by professors Glenn Howe and Tony Chen of Oregon State University (Howe *et al.*, 2003), and used in the study described in Paper II. In the study reported in Paper I, we used outdoor-grown aspens (*Populus tremula*), and in the study reported in Paper III we used *in vitro*-grown sterile hybrid aspens (*Populus tremula x tremuloides*), some of which were transformed to express the cDNA from the *Arabidopsis* $abi_{1\text{-}1}$ (abscisic acid insensitive 1) gene.

4 Results and Discussion

4.1 Summary of my t(h)ree papers: Elucidating growth cessation and dormancy.

The establishment of dormancy in trees involves massive physiological and metabolic alterations. When a tree progresses from active growth to dormancy extensive remodeling of the transcriptome occurs, which initiates many pathways needed for survival in harsh winter conditions. In the apical meristem there is a transition from growth to bud formation and the plant tissue dehydrates, while sugars and storage proteins are produced as part of the process of acclimation to freezing temperatures.

In order to provide a molecular framework for the processes involved in growth cessation and dormancy induction, or processes associated with dormancy, we monitored transcriptional changes (using microarray analysis) in the cambial meristem of aspen trees during the transition from active growth to dormancy and subsequent reactivation.

More specifically, in the study reported in Paper I, we examined changes in levels of transcripts involved in cold hardiness, storage protein production, cell cycling and carbon metabolism in cells from the vascular cambium during both the induction and release of dormancy. We also measured levels of metabolites to monitor metabolic changes during the activity-dormancy cycle and investigated the levels of phytohormone ABA, given its proposed role in dormancy and cold hardiness.

The tree samples analyzed in these investigations were collected from plants growing outside that had been affected by different environmental conditions. To extend the analyses, in subsequent studies (Paper II) we explored the causes of variations in timing of growth cessation, especially the role of factors acting downstream of SD signal perception.

In contrast to the previous study, the samples examined in this experiment were from trees grown in a controlled greenhouse environment with a day length of 8 hours. Using this experimental set up we hoped to reduce the complexity of the transcriptome data by eliminating environmental variations that may affect gene expression unpredictably. For this experiment we used two hybrid poplar clones originating from an interspecific cross between *Populus trichocarpa* and *P. deltoides*, which differed in their timing of growth cessation and bud set. The clones were grown with an 8-hour photoperiod, shorter than the critical daylength of both clones, since we wanted to identify processes that differed between them downstream of perception of SD-signals.

It has been previously shown that ABA plays an important role in seed dormancy, displaying an antagonistic effect towards GA (Nambara & Marion-Poll, 2003). Many earlier studies have reported elevated ABA levels during growth cessation and/or dormancy induction, prompting speculation that ABA may play a role in the signal transduction mediating growth cessation (Li *et al.*, 2005; Rohde *et al.*, 2002). Therefore, in further studies (Paper III), we used transgenic hybrid aspens with reduced sensitivity to ABA to explore the putative involvement of ABA in tree dormancy.

In the following three chapters I describe the results obtained in the studies in more detail.

4.2 Regulation of transcriptional and metabolic networks in the cambial meristem during activity-dormancy cycles (Paper I).

Samples of cambial meristem were taken from aspen trees grown outdoors in Umeå (Sweden, 65°N) from 20 April (before bud break) until 13 December, covering the reactivation period as well as the transition to dormancy. Tangential sections through the cambial region of the stem were obtained using a cryomicrotome (Uggla *et al.*, 1996) and analyzed using microarrays. The microarray results show that substantial changes occur in the cambial tissues during distinct phases of the activity-dormancy cycle**,** which corresponds well with earlier observations (Ruttink *et al.*, 2007; Schrader *et al.*, 2004a). The transcriptional modifications that occur after

growth cessation are likely associated with induction of cold hardiness and the establishment of dormancy.
When we examined cell cycle-related genes we observed an increase in their expression in late May and a decrease in expression in mid-July. Interestingly, anatomical analysis revealed an increase in the number of dividing cambial cells between 20th of April and 26th of May, which was not mirrored by an increase in the transcription of cyclin-dependent genes. A possible explanation for this is that the increase in dividing cells is under post transcriptional control and the transcription of key cell-cycle genes is maintained at low levels during winter, but translation is repressed until short day signals are perceived during spring, releasing the repression of translation. Similarly, in seed dormancy it has been shown that translation of stored transcripts plays an important role (Nakabayashi *et al.*, 2005; Rajjou *et al.*, 2004).

In aspens growing in Umeå cold hardiness starts to develop in mid-August, well before cold temperatures are experienced. We found that transcript levels of genes encoding dehydrins, cold-regulated proteins (CORs) and cryoprotectants, such as raffinose, increase in cambial tissues in the autumn, accompanied by increases in phospholipid biosynthesis and lipid desaturation. We also detected very strong induction of cold hardiness genes in spring reactivation, which is surprising since previous studies have found that cold hardiness rapidly decreases during reactivation of growth in the closely related genus *Salix* (Sennerby-*Forsse* & von *Fircks, 1987*). The gene expression data might be indicative of the vulnerability of trees to sudden drops in temperature in the spring, so cold hardiness genes could be induced during this phase during spring when temperature vary substantially. Since we did not measure cold tolerance *per se*, it is possible that the increased expression of these genes may not necessarily lead to increased cold tolerance. However, it is also possible that increased expression of cold hardiness genes might instead serve as a precautionary measure and the mRNA may be translated only if there is a sudden drop in temperature.

GA and ABA are plant hormones that are known to have important regulatory functions in various aspects of the activity-dormancy cycle in trees (Ruttink *et al.*, 2007; Olsen *et al.*, 1995). ABA is known to be heavily involved in drought/water stress, and in seed dormancy (Gusta *et al.*, 1992). We found that ABA levels started to increase in mid-August, later than the initial peaks in expression of short day-regulated genes. Since the ABA levels

increase after growth has stopped we concluded that ABA does not play a role in the induction of growth cessation. Similarly, if ABA is involved in early short day-induced cold hardiness, it is not mediated by increases in ABA levels. When we examined ABA levels during reactivation in the spring, we observed high cambial ABA levels in early spring, at the same time that cold responsive genes were strongly upregulated, suggesting a possible role for ABA in the induction of cold hardiness at this stage.

GA is another plant hormone that has suggested involvement in growth cessation. When trees are exposed to short days their GA levels decrease, and it has been suggested that this leads to growth cessation in the apical meristem (Molmann *et al.*, 2005; Olsen *et al.*, 1995). Moreover, growth cessation is delayed in hybrid aspen plants with increased GA levels (Olsen *et al.*, 1997; Olsen *et al.*, 1995; Junttila & Jensen, 1988). However, we noticed no downregulation of the GA-20 oxidase represented on the microarray in response to the transition from active growth to dormancy. Instead, we noticed the induction of PttRGA, a homologue of *Arabidopsis* RGA that has been shown to act as a negative regulator of GA signaling (Thomas & Sun, 2004). Thus, it is possible that in cambial meristems reduced sensitivity to GA after short day treatment is involved in growth cessation.

In addition to cold hardiness, substantial modulations of metabolism occur during activity-dormancy transition. This includes massive increases in storage protein production and sucrose accumulation. Many of the detected metabolites with differential levels have known associations with specific processes connected to growth cessation and dormancy, e.g. starch catabolism and synthesis of cryoprotectants. Increases in contents of the same groups of metabolites have also been observed in *Arabidopsis* in response to low temperatures (Sauter *et al.*, 1988). However, the induction of cold tolerance in aspen and *Arabidopsis* differs in an important respect. In aspen cold acclimation is to a high degree triggered by shortening of the daylength, and not solely by low temperatures. Our studies and previous findings (Li *et al.*, 2009) also show that temperature plays a role in cambial reactivation, but not cambial growth cessation.

The breakdown of starch coincides with the development of cold hardiness, suggesting that the synthesis of cryoprotectants requires substantial amounts of energy. It has also been observed that decreased beta-amylase expression affects the ability of *Arabidopsis* plants to degrade starch, and thus inhibits their ability to develop freezing tolerance (Kaplan & Guy, 2005). Starch breakdown was previously proposed to be induced by low temperatures

(Sauter *et al.*, 1998). However, we found that the induction of key enzymes involved in starch breakdown, such as glucan-water dikinase (SEX1), beta-amylase and starch phosphorylase, began before the temperature dropped, suggesting that a signal other than low temperatures triggers the induction, namely short days.

Our data show that massive changes occur in the cambium transcriptome during the transition from active growth to dormancy, suggesting a possible role for chromatin remodeling. Of particular interest in this process is the induction during dormancy initiation of a poplar homolog of the *Fertilisation Independent Endosperm* (*PttFIE*) gene (Ohad *et al.*, 1999). It has been shown that suppression of FIE expression leads to aberrant activation of the regulator of meristem activity SHOOTMERISTEMLESS (Katz *et al.*, 2004). Further, FIE appears to participate in dormancy regulation as its downregulation leads to lack of dormancy in hybrid aspen plants (Englund et *al.*, unpublished). Therefore, it will be interesting to investigate its role in growth cessation and dormancy.

4.3 Components acting downstream of SD perception regulate differential cessation of cambial activity in early and late clones of hybrid poplar (Paper II).

Substantial variation can be found in the timing of growth cessation and the regulation of short day responses accompanying growth cessation in trees originating from different latitudes. These variations are controlled genetically since several QTLs controlling the traits have been found in clones originating from crosses between northern and southern genotypes of the same species, e.g. in hybrid poplars (Frewen *et al.*, 2000a; Howe *et al.*, 1995). Recent evidence has suggested that the differences in the timing of short day-regulated growth cessation are most likely due to differences in sensing short days, e.g. differences in critical daylengths. To explore the molecular basis of differential regulation of growth cessation and related responses we examined aspen clones and two hybrid clones originating from an interspecific cross between *P. trichocarpa* and *P. deltoides*: one in which growth ceases early and another in which it ceases late, after 14 and 30 short days, respectively (Frewen *et al.*, 2000a). By growing the two clones in a controlled environment, with an 8-h photoperiod (well below the critical daylength for both clones) in the greenhouse, we were able to investigate the effects of environmental factors e.g. day length sensitivity, and whether

factors other than daylength sensing contribute to variation in responses, e.g. growth cessation and cold hardiness.

Transcriptomal analysis showed that global changes in gene expression varied significantly between the early and late clones, in both timing and magnitude; the early clone showed stronger and faster responses to short day-signals than the late clone. This finding suggests that the clones exhibit quantitative differences in response to SDs, rather than simply initiating an identical SD program after sensing their respective critical daylength.
We also investigated changes in the expression of genes involved in processes whose regulation is closely associated with growth cessation and the induction of dormancy. These included auxin-related responses, since auxin is a plant hormone that may play a role in the regulation of cambial growth cessation and dormancy (Nilsson *et al.*, 2008; Schrader *et al.*, 2004a; Little, 1974). Furthermore, it has been shown that auxin-regulated genes are downregulated in response to short days (Schrader *et al.*, 2004a). Interestingly, we observed changes in the expression of auxin-responsive genes, which were stronger and earlier in the early clone, suggesting that auxin responsiveness may be among the targets for SD-induced growth cessation pathways, and that a divergence in auxin regulation could potentially contribute to differences in timing of cambial growth cessation.
Our data also revealed differences in the expression patterns of cold-regulated (COR) genes between the two genotypes. The up- or down-regulation of COR genes occurs about two weeks later (and more weakly) in the late clone than in the early clone. We also found that the expression profiles of the CBF/DREB transcription factors were very similar to those of the COR genes, suggesting that differential expression of these transcription factors could contribute to the difference in responses to short days between the clones.
Differences in timing of growth cessation could be due to differences in the perception of short days or responses to short day signals, or a combination of both of these factors. It is believed that short days are sensed by photoreceptors in the leaves and that short day signals lead to rapid down-regulation of the poplar homologs of the flowering time gene FT (ptFT1 and PtFT2) (Bohlenius *et al.*, 2006). FT1 and FT2 expression was strongly downregulated in both clones, but no difference between the clones was found in this respect. These findings suggest that the two clones do not differ in their SD perception under the growth conditions used and the difference in their dormancy timing is presumably due to factors acting downstream of FT. To further strengthen this hypothesis we measured the

downregulation of PtFT2 in *P. tremula* genotypes collected from along a latitudinal cline in Sweden (55.9 to 66^{0}N). These trees, which differ in their timing of growth cessation, showed no differences in downregulation of FT-expression when shifted to an 8-hour photoperiod regime. This further strengthens our hypothesis that the difference in timing of growth cessation in these trees when grown in an 8 hours photoperiod regime is not due to differences in their perception of short days, but rather in factors acting downstream of FT.

4.4 Hybrid aspen with reduced sensitivity to ABA have impaired bud dormancy (Paper III).

4.4.1 Plants overexpressing the *abi1-1* genes display reduced sensitivity to ABA.

How bud set and dormancy is regulated is currently unclear, but ABA is a strong candidate for involvement. There are two main strands of circumstantial evidence that ABA plays a role in dormancy. Firstly, ABA levels rise during dormancy, and secondly ABA is known to be a regulator of seed dormancy and there are similarities between seeds and buds with respect to gene regulation (Penfield & King, 2009; Ruttink *et al.*, 2007). We assessed the possibility that ABA plays a role in dormancy regulation by transgenically expressing cDNA corresponding to the $abi_{1\text{-}1}$ mutant allele coding for an ABI protein phosphatase in hybrid aspen.
This provided an opportunity to elucidate ABA's role in short day response mechanisms. It has previously been shown that the dominant negative mutation *abi1-1* gives rise to ABA insensitivity in *Arabidopsis* (Leung *et al.*, 1994; Meyer *et al.*, 1994), and we found that its transgenic expression reduces sensitivity to ABA in poplar plants, a result also confirmed by Arend et al., (2009). We then proceeded to investigate whether growth cessation and dormancy is affected in these plants.

4.4.2 ABA is not involved in short day-induced growth cessation

It has been previously shown that ABA levels peak after growth cessation (Paper I), strongly indicating that ABA is not responsible for growth cessation. However, this does not preclude a role for ABA in growth cessation since it is possible that short days increase ABA responsiveness and thus contribute to growth cessation. If so, decreased sensitivity to ABA should affect the timing of growth cessation and endodormancy induction. However, when we shifted the light regime from 16 to 8 h light, the wild type and *abi1-1* expressors stopped elongation growth at approximately the

same time, after 15-18 days in short day conditions. This finding strongly suggests that ABA does not play a role in SD-induced growth cessation.

4.4.3 ABA plays a role in short day-induced bud dormancy.

ABA is known to be a key factor in the maintenance of dormancy in seeds, and given the similarities between dormancy in buds and seeds, e.g. dehydration, we investigated ABA's role in bud dormancy by exposing wild type and *abi*$_{1\text{-}1}$ expressors to 10 weeks of SD treatment, which induces bud dormancy in wild type plants. At this stage bud burst and reactivation cannot occur in wild type plants unless they are exposed to chilling temperatures before exposure to long days. Accordingly, when we exposed wild type and *abi1-1* expressing plants to long days after 10 weeks of short days, the wild type remained dormant, but bud flush and reactivation of growth occurred in the *abi1-1* mutants (Figure 6).

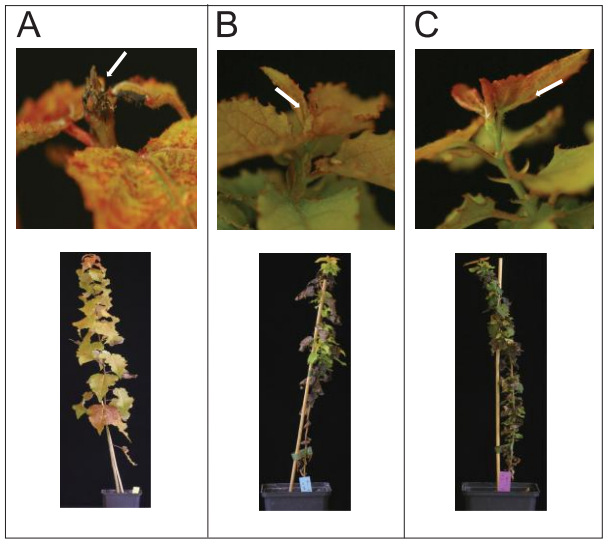

Figure 6. Photographs of buds and plants of two aba1-1 lines and wild type. The wild type plants (A) maintain bud dormancy when moved to LD conditions while in the *abi1-1* plants (B, C) buds burst and growth is reinitiated.

Our results show that ABA regulates bud dormancy and in the future it will be possible to identify the mechanisms whereby ABA regulates bud dormancy in trees.

5 Conclusions and future perspectives

The work presented in this thesis has contributed new knowledge at a molecular level on the mechanisms whereby trees initiate and maintain dormancy. The results show that the transition from active growth to dormancy is accompanied by massive changes in the transcriptome. We have also examined processes closely connected to dormancy, such as cold hardiness, starch metabolism and phytohormones. Many of these processes are regulated at least partly by shortening of the photoperiod. Although it has previously been postulated that variations in the timing of growth cessation are due to differences in critical daylength, we have shown that signaling components downstream of the CO/FT regulon also play an important role. Our microarray data show that the clones differ not only in the timing of their short day responses, but also in the strength of the up- or down-regulation. Finally, using poplar plants with reduced sensitivity to the phytohormone ABA, we have shown that ABA does not play a role in short day-induced growth cessation but is involved in the maintenance of bud dormancy.

Until now, most research on dormancy induction has been focused on the effects on photoperiod. However, we still do not know which factors contribute to differences in the timing of growth cessation downstream of the CO/FT regulon and more extensive studies are needed to identify these components. A logical follow up to my work would be to functionally characterize candidate genes e. g. those of hormone signaling that might act downstream of the day length signaling in growth cessation response. Secondly, a number of different sub-processes, e.g. cold hardiness and dehydration are induced simultaneously with apical growth cessation. It would be of great interest to try to separate these processes from each other. Although they are partly overlapping, experiments have suggested that the different sub-processes may be induced by partly different environmental

signals. While all my work has been performed on angiosperm (*Populus),* many gymnosperms, which are more important from a commercial point of view, do not need chilling requirement to break dormancy. Comparative studies between poplar and gymnosperms (e. g. spruce whose genome sequence would be available in not too distant future) would be very interesting. For the ABA-insensitive plants we are planning a microarray experiment that could identify processes other than dormancy that are affected by ABA-insensitivity as well as identify the genes mediating ABA control of dormancy in trees. With the help of improved microarray methods and by integrating more genotypes and biological replicates into microarray studies one might be able to increase the resolution of the transcriptional analysis and find downstream components involved in the timing of growth cessation and dormancy. I am convinced that the integration of expression data, genome sequence information and protein studies will provide much new knowledge about dormancy and dormancy related processes.

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