# Molecular Analysis of Factors Regulating Wood Formation and Seasonal Growth Cycles in Hybrid Aspen

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Doctoral Thesis
Swedish University of Agricultural Sciences
Umeå 2011

Acta Universitatis Agriculturae Sueciae
2011:24

Cover: All seasons aspen tree
(Artist: Gun Lövdahl)

ISSN 1652-6880
ISBN 978-91-576-7559-0

Print: Arkitektkopia, Umeå 2011

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Of all of the organisms on the planet, trees are perhaps the ultimate survivors. Their unique growth characteristics allow them to become both the largest and the oldest organisms on Earth. Their size can be attributed to their secondary growth (wood formation), which results in the formation of their massive trunks, while their ability to survive and continue growing for several hundred years depends on their ability to modulate their growth according to the surrounding environment.

This thesis explores wood formation and seasonal growth cycles on a molecular level. These tree-specific processes were studied separately, but several common factors that influence both of them were identified. One of these is the control of the core cell cycle machinery. The cell cycle machinery is subject to significant transcriptional regulation during the seasonal growth cycle; it was found that this transcriptional regulation primarily affects plant cyclins. A transcription factor of the AP2 family, AINTEGUMENTALIKE1 (AIL1), was shown to play a significant role in short-day- induced growth cessation, possibly by regulating the expression of the D-type cyclins, which are core regulators in the plant cell cycle. AIL1 is also involved in cambial growth; its misexpression causes severe developmental effects in the stem tissues.

This thesis also provides new insights into the influence of the plant hormone auxin on tree growth and development. The known loss of auxin responsiveness during winter was shown to be a gradual process controlled by the intricate auxin signaling network. The same network was also studied during normal growth and shown to have a major influence on cambial activity and maintenance as well as wood development.

*Keywords:* meristem, growth cessation, dormancy, cell cycle, wood, auxin, AIL1, ANT, short day, hybrid aspen

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*I almost wish I hadn't gone down that rabbit-hole- and yet- and yet- it's rather curious, you know, this sort of life!*

Alice in Wonderland

# 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. Karlberg A*, Englund M*, Petterle A, Molnar G, Sjödin A, Bako L and Bhalerao RP. (2010) Analysis of global changes in gene expression during activity-dormancy cycle in hybrid aspen apex. *Plant Biotechnology* 27(1), 1-16

II. Karlberg A, Bako L and Bhalerao RP. AINTEGUMENTA-LIKE1 is the target of short day signal in the regulation of growth cessation response in hybrid aspen. *(Manuscript)*

III. Baba K*, Karlberg A*, Schmidt J, Schrader J, Hvidsten TR, Bako L and Bhalerao RP. (2011) Activity-dormancy transition in the cambial meristem involves stage-specific modulation of auxin response in hybrid aspen. *Proceedings of the National Academy of Sciences of the United States of America* 108 (8), 3418-3423

IV. Nilsson J*, Karlberg A*, Antti H, Lopez-Vernaza M, Mellerowicz E, Perrot-Rechenmann C, Sandberg G and Bhalerao RP. (2008) Dissecting the molecular basis of the regulation of wood formation by auxin in hybrid aspen. *Plant Cell* 20, 843-855

* To be considered joint first authors

# Abbreviations

| | |
|---|---|
| ABA | Abscissic acid |
| AIL1 | AINTEGUMENTALIKE1 |
| ANT | AINTEGUMENTA |
| ARGOS | AUXIN-REGULATED GENE INVOLVED IN ORGAN SIZE |
| AS1 | ASYMMETRIC LEAVES1 |
| At | *Arabidopsis thaliana* |
| ARF | Auxin Response Factor |
| AUX/IAA | Auxin/indole-3-acetic acid |
| AuxRE | Auxin-responsive element |
| CCA1 | CIRCADIAN CLOCK ASSOCIATED1 |
| CDK | Cyclin-dependent kinase |
| cDNA | complementary DNA |
| CHE | CCA1 HIKING EXPEDITION |
| CLV3 | CLAVATA3 |
| CO | CONSTANS |
| CZ | Central zone |
| CYC | CYCLIN |
| DNA | Deoxyribonucleic Acid |
| EMSA | Electrophoretic mobility shift assay |
| EST | Expressed sequence tag |
| FT | FLOWERING LOCUS T |
| FR | Far red (light) |
| G (as in G1) | Gap (cell cycle) |
| GA | Gibberellic acid |
| GI | GIGANTEA |
| GUS | β-glucuronidase |
| IAA | Indole-3-acetic acid |
| LHY | LATE ELONGATED HYPOCOTYL |
| LP | Leaf primordia |

| | |
|---|---|
| miRNA | microRNA |
| mRNA | messenger RNA |
| PAT | Polar auxin transport |
| PCR | Polymerase Chain Reaction |
| PH | Phloem |
| PHY | Phytochrome |
| PM | Pith meristem |
| PZ | Peripheral zone |
| QTL | Quantitative trait loci |
| RAM | Root apical meristem |
| R | Red (light) |
| RBR | Retinoblastoma-related |
| REV1 | REVEILLE1 |
| RNA | Ribonucleic acid |
| S (as in S-phase) | Synthesis (cell cycle) |
| SAM | Shoot apical meristem |
| SCF | SKP-Cullin-F-box |
| SD | Short days |
| siRNA | short interfering RNA |
| STM | SHOOTMERISTEMLESS |
| tasiRNA | trans-acting short interfering RNA |
| TIR1 | TRANSPORT INHIBITOR RESPONSE1 |
| TOC1 | TIMING OF CAB EXPRESSION1 |
| VC | Vascular cambium |
| WOX | WUSCHEL-RELATED HOMEOBOX |
| WUS | WUSCHEL |
| XY | Xylem |

# 1 Introduction

All living organisms interact with the surrounding environment, and evolve so as to better adapt to environmental changes. When it comes to adaptation, plants in general and especially perennials such as forest trees are the true "ultimate survivors" - they cannot evade changes by moving as do animals, nor can they avoid seasonal changes by setting seed as do annual plants. Instead, they must simply remain in place and adapt to any changes that confront them.

## 1.1 Why study trees?

One could answer this question simply by asking "why not?" While humans tend to consider themselves the crown of evolution trees stand taller and grow older than any other organisms on Earth. Since the dawn of humanity we have relied on trees to provide raw materials for construction and energy generation, amongst other things. But what makes trees so remarkable? Firstly, their growth habits allow them to form a massive trunk providing support even in the most challenging conditions. The trunk allows them to rise higher towards the sunlight then lesser plants. Secondly, they can grow for thousands of years in the same spot as a result of exquisite adaptation to the environment. This thesis set out to explore processes that make trees the fantastic organisms they are, namely wood formation and seasonal growth cycles. The processes involved in wood formation provide the tree with its massive trunk while the seasonal growth patterns is part of the adaptation allowing the trees to survive the harsh conditions during winter.

So, instead of asking why one would chose to study trees the question should rather be why the most studied plant today is *Arabidopsis,* a small annual weed. The answer to that question is partly related to space requirements and time but can mostly be found in the easy way to study

genetics. *Arabidopsis* is small, self-fertilizing and has a very short rotation time while trees take up a lot of space, crossings are unfeasible due to long generation times and they grow slowly. The first plant genome to be sequenced was that of *Arabidopsis*; its sequence was published in 2000 (Kaul *et al*. 2000). Since then, substantial collections of databases and online resources have been made available to the *Arabidopsis* research community, and have proven to be extremely useful. It was not until 2006 that the first tree genome was sequenced (Tuskan *et al.* 2006); the genetic resources and genome annotations available for trees are still comparatively underdeveloped and lack much of the quality and user-friendliness an *Arabidopsis* researcher might be used to. However, *Arabidopsis* is not a suitable model for all plant traits. The work described in this thesis focused on short-day induced growth cessation and dormancy in trees, and also touched on the processes involved in wood formation; consequently, *Arabidopsis* would have been a poor choice of model organism. Some efforts have been made to use secondary growth in *Arabidopsis* as a model for wood formation (Nieminen *et al.* 2004) but integration of the results obtained using this model system are not always easily transferrable to an actual tree (Edvardsson. 2010). As more tree genomes are sequenced it will be of immense interest to see if the results from *Populus* will be proven valid. The draft sequence of the *Eucalyptus grandis* genome is now available (www.phytozome.net/eucalyptus) and tree researchers around the world are eagerly awaiting the first conifer genome. They should not have to wait much longer; the effort to sequence Norway spruce (*Picea abies)* is well under way.

## 1.2 Plant meristems

All plant growth and development originates from meristematic cells (i.e. stem cells). During normal development, these cells reside in meristematic regions throughout the plant body. The major meristems are the shoot apical meristem (SAM), the root apical meristem (RAM) and the vascular cambium (VC) (Fig 1). In this thesis, only the shoot apical meristem and the cambium will be further discussed. Of these meristems, it is the SAM that has been most extensively studied; one could easily fill a sizable book with a summary of what is currently known about the regulation and maintenance of the SAM. Consequently, the following sections of this thesis contains only a very brief overview of the regulation of the SAM, emphasizing the similarities between the regulation and maintenance of the SAM and those of the vascular cambium.

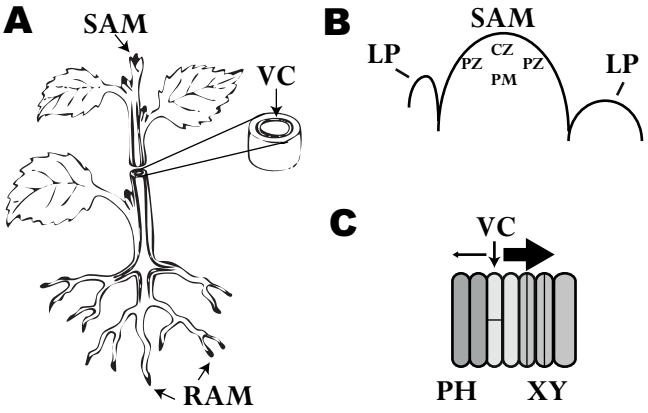


**Figure 1. Plant meristems**
A) Main plant meristems; shoot apical meristem (SAM), root apical meristem (RAM) and vascular cambium (VC). B) SAM. The meristematic cells reside in the central zone (CZ). When cells are pushed out towards the peripheral zone (PZ) or the pith meristem (PM) differentiation starts. (LP: leaf primordia). C) The vascular cambium produces xylem (XY) and phloem (PH). Cell division activity is highest in the xylem mother cells while the anticlinal divisions only occur in the meristematic cells (cambial initials).

### 1.2.1 The shoot apical meristem

The shoot apical meristem, like all plant meristems, is highly organized. It contains a small pool of stem cells that divide to form new leaf and stem tissue as well as new meristem cells. The meristematic competence is under strict positional control- cells that are pushed out of the meristematic region begin to undergo differentiation, and while they may continue to divide, their progeny will not be new stem cells. The workings of the system responsible for the maintenance of the stem cell pool have been thoroughly reviewed by Barton (2010); this thesis necessarily contains only a brief summary of these findings. The management and maintenance of the meristems is governed by a complex system of transcription factors and ligand-receptor interactions. The meristem itself is quite small and is located in the central zone (CZ) of the apex. Its position is clearly important, since cells pushed out of the meristematic zone differentiate and obtain relevant cell identity. One gene required for SAM identity and function is *SHOOTMERISTEMLESS* (*STM*). *Arabidopsis* mutants lacking

STM activity have no meristem and exhibit no apical growth (Leyser and Day 2003). STM functions by repressing a gene called *ASSYMMETRIC LEAVES 1* (*AS1*), which in turn represses the meristem identity genes *KNAT1* and *KNAT2* (Leyser and Day 2003). KNAT1 and KNAT2 can themselves give rise to a functional meristem in *stm* plants provided that *AS1* is knocked out as well. Another gene that is essential for meristem maintenance is *WUSCHEL* (*WUS*). *WUS* expression is restricted to a small number of cells constituting the meristem organizing center just below the actual meristem. It promotes transcription of *CLAVATA3* (*CLV3*) in the meristem (Leyser and Day 2003). CLV3 is not a transcription factor, but its initial product undergoes post-translational modification to form a small signaling peptide (Kondo *et al.* 2006, Ohyama *et al.* 2009) that interacts with the receptor kinase CLAVATA1 to restrict *WUS* expression (Leyser and Day 2003). A signaling system resembling the WUS/CLV loop has been proposed to be operational in the RAM as well (Sarkar *et al.* 2007, Stahl *et al.* 2009).

### 1.2.2 The vascular cambium-an expanding meristem

The vascular cambium is a lateral meristem (in contrast to the apical meristems of the shoots and roots) and is the source of secondary growth (wood formation). Despite a general interest in wood formation through the history of plant science the molecular mechanisms involved in the regulation of cambial activity are poorly understood. Although it is generally assumed that there are stem cells in the cambium (called "initials") and that these are defined positionally, no general consensus has been reached on how to identify the true stem cells in the cambium (Larson 1994). One of the most convincing attempts towards this goal was made by Schrader et al in 2004. On the basis of the generally accepted definition of a stem cell (i.e. a cell that can divide to make new stem cells), these workers hypothesized that the position of cambial initials could be determined by pinpointing the positions of anticlinal divisions. Most cell divisions in the cambium are periclinal and produce xylem or phloem. Anticlinal divisions give rise to new cell files, i.e. new cambium (Larson 1994, Catesson 1994). Indeed, anticlinal divisions occur only in a narrow window close to the phloem side of the cambium (Fig 1, Schrader *et al.* 2004*a*). It thus seems likely that the stem cells' positions are tightly controlled, as is the case in the apical meristem. Schrader and co-workers also used high resolution transcriptional profiling covering the cambial zone to identify potential regulators of stem cell activity and maintenance. Their results indicated that the cambium exhibits feedback mechanisms similar to those operating in

the SAM (Schrader *et al.* 2004*a*). These findings provide a strong basis for research on the functional properties of candidate genes and indeed, it seems that the proteins operating in the cambium are identical or at least very similar to those operating in the SAM. For instance, a STM homolog is expressed in the cambium of poplar (Schrader *et al.* 2004*a*), and overexpression of At*STM* or *ARBORKNOX1* (a *Populus* STM homolog) gives similar overexpression phenotypes in poplar (*Populus tremula x Populus alba*) (Groover *et al.* 2006). The closest homolog of AtWUS in *Populus* is not expressed in the cambium (Schrader *et al* 2004*a*), but WUS is a member of the broader WOX family (WUSCHEL-RELATED HOMEOBOX), several members of which are involved in meristem maintenance. WOX-genes are known to be active in the RAM (Sarkar *et al.* 2007, Stahl *et al.* 2009), and the WUS-related *WOX4* gene is a component of a system that has been implicated in the maintenance of the vascular cambium in *Arabidopsis* and which closely resembles that involved in SAM maintenance (Hirakawa *et al.* 2010). A probable *WOX4* ortholog, *HB3*, is highly expressed in the cambium of hybrid aspen and has been proposed to participate in a regulatory loop in the same way as does WUS in the SAM (Nilsson 2010). One important difference between the VC and the SAM is that the positional information has to be derived from both phloem and xylem. In the SAM cells only have one way to go when leaving the meristem (downwards) while cells leaving the cambium can move out of the meristematic zone in two different directions (Fig 1). In a model proposed by Nilsson a signaling peptide is produced at the phloem side and diffuses through the cambium to interact with a receptor-like kinase expressed in the xylem. This interaction would positively regulate *HB3* expression in the cambium (Nilsson 2010).

## 1.3 Seasonal growth cycles in perennials

All plants living in temperate climates need a strategy to survive recurring periods of non-permissive growth conditions (i.e. winter). Annual plants spend the last of their energy to set and disperse seeds, thereby promoting their survival and distribution. Trees, which do not flower until they reach maturity after several years' growth, need an alternative and less drastic approach to ensure their survival. To protect the meristems, trees halt their growth and form buds that protect the apical meristem inside. The timing of bud set in autumn and bud flush in spring is not only critical for survival; it also determines how well a tree can compete in terms of optimizing its

growth potential as premature growth cessation or delayed bud flush shortens the active growth period (Wareing 1956).

### 1.3.1 Short-day induced growth cessation

As winter approaches, day length gets shorter and the temperature drops. Both of these events can potentially serve as signals that can be detected by trees and trigger preparations for unfavorable conditions. Many trees, including *Populus,* seem to rely more or less exclusively on the shortening of length of the day as a reliable signal that it is time to stop growth and prepare for winter (Wareing 1956, Nitsch 1957). These preparations involve several distinct events that occur simultaneously, (growth cessation, cold hardiness acquisition and the establishment of dormancy) but can nevertheless be regarded as separate processes (Rohde and Bhalerao 2007). Other species may use temperature as the key signal of oncoming winter (e.g. apple and pear) (Heide and Prestrud 2005), or a combination of the two.

In *Populus,* the length of the day is the key signal that triggers the sequence of events that ultimately lead to a dormant, hardy plant that can withstand a long, cold winter. The critical daylength is cline-dependent, i.e. more northern ecotypes typically have a longer critical day length compared to southern ecotypes (Howe *et al.* 1995, Luquez *et al.* 2007). When the daylength drops below the critical value, elongation growth stops, expression of cold-hardiness related genes is initiated, and eventually bud scales develop and form a protective shell around the apical meristem (Rohde *et al.* 2002, Resman *et al.* 2010). The cessation of elongation growth and bud formation are easily visible by the naked eye, whereas the processes involved in the acquisition of cold hardiness and the establishment of dormancy occur at the cellular level.

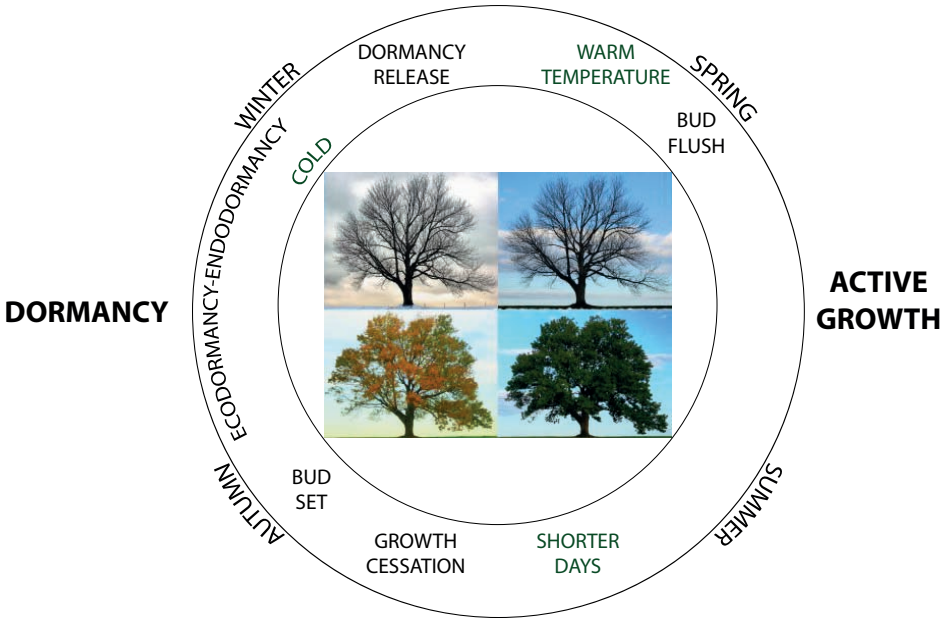


**Figure 2. Seasonal growth cycling in *Populus***
When autumn approaches and day length falls below the critical value growth cessation is induced. Growth cessation is followed by budset, and the tree is ecodormant at this stage. If short day conditions continue, the dormant state is established and the tree becomes endodormant. To release the tree from the endodormant state a prolonged period of chilling is required. When the chilling requirement is fulfilled the tree once again becomes ecodormant and can resume growth under favorable conditions.

### 1.3.2 Dormancy

The term 'dormancy' is generally used to describe a state of the entire plant structure, but in reality it is only the meristematic tissues that become dormant. Dormancy is typically discussed using the terms originally defined by Lang, although it should be noted that the terminology used in this field is not always entirely consistent. According to Lang, dormancy can be defined as a temporary suspension of growth in any plant structure containing a meristem. Three dormancy states are defined: Endodormancy, ecodormancy and paradormancy (Lang *et al.* 1987). Paradormancy refers to dormancy whose continuation is dependent on growth-inhibiting signals from tissues outside of the meristem (e.g. apical dominance). Endodormancy (which is also known as rest or "true" dormancy) is a state in which the plant meristems will not resume growth, even if the conditions

are favorable. Ecodormancy (also called quiescence) is somewhat harder to define. This is a state where growth has completely ceased, but the plant will immediately resume growth under permissive environmental conditions (the plant is effectively "on standby"). Lang states that ecodormancy is a response to "unexpected" stressful environmental conditions, and does not include photoperiodic responses in this definition. However, in more recent articles, the term "ecodormancy" has been used to denote the state that follows the photoperiodic short-day response and growth cessation and precedes the establishment of endodormancy (Fig 2).

Endodormant trees requires a period of chilling temperatures to revert to an ecodormant state and thereby regain the ability to resume growth (Wareing 1956). Dormancy is a highly adaptive trait and is crucial for survival in a climate with harsh winter conditions. Failure to establish dormancy properly makes a plant very vulnerable to for early frost nights during an otherwise mild autumn or cold spells in spring.

### 1.3.3 Bud dormancy vs cambial dormancy

A majority of the research into growth cessation and dormancy has focused on bud dormancy, probably because the bud is the most unambiguous visual indicator of the cessation of growth. It is also easy to follow the resumption of growth in the form of the bud flush. Cambial dormancy on the other hand is not visible from the outside, although histological studies have established that there are obvious differences between active and dormant cambium (Sitbon *et al.* 1993, Catesson 1994,). There are some specific advantages associated with studying cambial dormancy rather than bud dormancy. First, bud dormancy-related processes in the meristem are easily occluded by parallel events connected to bud formation rather than cessation of growth and dormancy establishment. Second, the sampling resolution of bud tissues is very low compared to the relatively pure meristematic tissue obtained using cryosectioning of tree stems (Uggla *et al.* 1996).

Several attempts have been made to elucidate changes in gene expression during short-day induced growth cessation and dormancy establishment, most of which have focused on bud dormancy (Paper I, Ruttink *et al.* 2007, Rohde *et al.* 2007, Park *et al.* 2008). Of these studies, only that described in Paper I utilized a full genome array (for more technical information see chapter 3). While the use of a full genome array should in principle provide the greatest possible amount of information in such a study, the focus of the work described in Paper I was events occurring after cessation of growth (i.e. bud formation and dormancy

establishment). Consequently, the first data point in this study was recorded after five weeks of short-day conditions, by which point growth had already ceased. Ruttink et al used the smaller POP2 array and focused on growth cessation and the early events in the induction of dormancy; the last data point in their study was recorded after six weeks of short-day treatment. These two studies can thus be considered to complement one-another. However, they have one common technical weakness: in both cases, the array used for hybridization was not tailored specifically to the *Populus* species used in the study. Microarrays have also been used to study cambial dormancy (Schrader *et al.* 2004*b*, Druart *et al.* 2007, Resman *et al.* 2010).

It is tempting to compare bud dormancy and cambial dormancy on the basis of the expression data obtained from the studies described above. However, such comparisons should be made with care; it is important to account for differences between the studies in terms of the experimental conditions used, species studied, sampling resolution, and various other factors. Ruttink et al. used data from Schrader et al. to compare expression in growth to dormancy transitions and found striking similarities. They hypothesize that genes that are expressed in the apex but not the cambium during the transition from activity to dormancy might play specific roles in the morphological changes that occur during apical bud development (Ruttink *et al.* 2007). A finding that is common to all of the studies discussed above is that the transition to dormancy is associated with major changes in the transcriptional regulation of the core machinery of the plant cell cycle and genes related to meristematic activity. These changes occur during the early phases of short-day treatment or in early autumn (Ruttink *et al.* 2007, Druart *et al.* 2007), so it is likely that they are related to growth cessation processes rather than to the establishment of dormancy. However, transcriptional analysis is blind to the effects of post-transcriptional regulation, and it is vital to consider the potential influence of this complicating factor when discussing the results of these studies. This is apparent in the case of cell cycle regulation during short-day treatment. On the basis of transcriptional data alone, one might conclude that the expression and activity of all cyclin-dependent kinases (CDK:s) except for CDKB are unchanged or fluctuate very little during growth cessation and dormancy (Paper I, Ruttink *et al.* 2007, Schrader *et al.* 2004*b*, Druart *et al.* 2007). However, analyses of actual protein levels revealed a more complex picture; the activity of CDKA was found to be subject to strong post-transcriptional control under these conditions (Espinosa-Ruiz *et al.* 2004). This type of regulation has yet to be demonstrated for other CDK:s, but it

seems likely that post-transcriptional control plays an important role in activity-dormancy cycling (Paper I).

Physiologically, the shoot apical meristem and the cambium are very different, both before and after growth cessation and dormancy. In the SAM, bud scales protect the meristematic cells and cell-cell communication via plasmodesmata is interrupted by the formation of sphincter complexes (Rinne and van der Schoot 1998). In the cambium, the initials themselves develop thick protective cell walls (Catesson 1994, Farrar and Evert 1997, Druart *et al.* 2007) and even though symplasmic connections change with the seasons, no changes in the morphology of the plasmodesmata have been observed – particularly, there is no evidence for the formation of sphincter complexes (Fuchs *et al.* 2010, Sokolowska and Zagórska-Marek 2007). Thus, there are both striking similarities and significant differences between bud and cambial dormancy.

## 1.4 Light signaling and daylength perception

Short-day-induced growth cessation and the subsequent establishment of dormancy are dependent on the tree properly sensing and responding to the onset of short-days (SD). The SD response not only requires the perception of light but also an internal time keeping mechanism to determine the length of the day. In this section, I briefly describe the means by which plants can distinguish short days from long days and some similarities and differences between the induction of flowering and short-day-induced growth cessation.

### 1.4.1 Photoreceptors

All plants, from the simplest unicellular cyanobacteria to the magnificent redwoods, utilize a number of different photoreceptors to differentiate not only light from dark, but also different light qualities. Higher plants have more of them but the basic concept remains the same throughout the plant kingdom. The impact of different light qualities on growth cessation and bud set has been investigated in detail in Norway spruce (*Picea abies*) (Mølmann *et al.* 2006). Blue light, detected by cryptochromes, can delay growth cessation and bud formation but not completely prevent it. Red (R) and far-red (FR) light is recognized by phytochromes and has been implicated in several photoperiodic and environmental responses (e.g. shade avoidance) (Morelli and Ruberti 2002). The ability of phytochromes to control short-day induced growth cessation has been demonstrated in *Populus,* both by night-break experiments demonstrating R/FR reversibility

(Howe *et al.* 1996) and by the inability of hybrid aspen overexpressing oat phytochrome A (*PHYA*) to stop growth when subjected to short-day treatment (Olsen *et al.* 1997, Kozarewa *et al.* 2010). Downregulation of endogenous *PHYA* levels in hybrid aspen also modifies photoperiodic responses (Kozarewa *et al.* 2010). Additional evidence of phytochrome involvement in short-day induced growth cessation comes from QTL analysis, which established that the *Populus PHYB2* maps to a locus affecting bud set and bud flush (Frewen *et al.* 2000). Interestingly, there is a correlation between the natural sequence variation in *PHYB2* cDNA and latitude (Ingvarsson *et al.* 2006).

### 1.4.2 Circadian rhythm

A plant must be able to keep track of time in order to trigger the onset of growth cessation and dormancy once the daylength has fallen below the critical value. This is necessary because the short-day response is dependent on a light-sensitive phase occurring at a set time. A functional circadian clock is thus crucial for the coordination of endogenous processes with daily changes and also sets the stage for seasonal variation by enabling external coincidence. The plant circadian clock depends on interlocking feedback loops that generate rhythmic gene expression patterns; genes exhibiting these expression patterns are said to be circadic. Within one organism (animal or plant), several clocks may be active in various cell types, each one of which controls a specific group of processes (Harmer 2009, Imaizumi 2010). However, the central circadian system remains the same. In plants, the clock is based on three transcriptional feedback loops (reviewed in Harmer 2009). The first of these loops to be identified contains three key components. *TIMING OF CAB EXPRESSION1* (*TOC1*) is a circadic gene whose expression peaks in the evening and whose function is to induce expression of the dawn-genes *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*). This induction is believed to be relayed through an as-yet unidentified unknown factor because TOC1 itself possess no DNA binding activity. Moreover, an additional component, CCA1 HIKING EXPEDITION (CHE), which represses *CCA1* expression during the day has recently been identified (Pruneda-Paz *et al*. 2009). CCA1 and LHY also repress their own transcription and that of *TOC1*. In addition to this central oscillator, there are two additional feedback loops. One is evening-phased, and is expected to contain one as-yet unidentified positive regulator of *TOC1* (designated Y). The expression of this unknown regulator is expected to be negatively regulated by TOC1, CCA1 and LHY. The activity of Y can in part be

explained by the activity of GIGANTEA (GI), which is a negative regulator of TOC1 (Locke *et al.* 2006). However, one or more as-yet unidentified elements have to be involved. The existence of this loop is suggested by mathematical modeling, which indicates it to be necessary to explain the experimental data on circadian regulation. Similar arguments for the existence of one or two morning loops have also been proposed on the basis of collaborative studies between mathematicians and biologists (Zeilinger *et al.* 2006, Harmer 2009).

Many genes are subject to circadian control. In *Arabidopsis*, at least 6% of the genes are rhythmically expressed but it is plausible that the true number is significantly higher than this (Harmer *et al.* 2000, Schaffer *et al*. 2001). The need for plants to keep accurate track of time is highlighted by the fact that *Arabidopsis* clock mutants with shorter or longer internal rhythms exhibited decreased biomass production compared to the wild type over a 24 hour photoperiod, whereas the opposite was true when the photoperiod was modified to match the mutant clocks (Dodd *et al.* 2005). Environmental changes can have profound impacts on clock function: the cold response of the chestnut involves a complete loss of the core oscillators' diurnal expression pattern (Ramos *et al.* 2007, Ibanez *et al* 2008). Modulation of expression of the core clock regulators in hybrid aspen leads to changes in the critical daylength of the growth cessation response (Ibanez *et al.* 2010). The circadian clock genes are also part of the pathway that leads to short-day-induced growth cessation. GI acts upstream of the genes *FLOWERING LOCUS T* (*FT*) and *CONSTANS* (*CO*) in both flowering promotion and in the short-day growth cessation response (Mizoguchi *et al.* 2005, Bohlenius 2007). There are still many aspects of circadian regulation that remains unclear, but it is obvious that the circadian clock influences environmental responses, even when those responses are not directly coupled to light or day length.

### 1.4.3 The external coincidence model

The perception of different light signals depends on photoreceptors, but if the day length is to be measured, it is important to know at what time of day a light given signal is perceived. The circadian clock can function as a "gating" system, ensuring that events requiring a certain gene or protein can only occur at specific times. One example of this is the day length sensing mechanism, which is also known as "external coincidence". The most famous process controlled by external coincidence is the induction of flowering, in which the labile CONSTANS (CO) protein plays a critical role. CO is a strong promoter of flowering in *Arabidopsis* and promotes

flowering by induction of *FLOWERING LOCUS T* (*FT*). It is regulated by the circadian clock at the transcriptional level and its expression peaks towards the end of the day on long days. On short-days, *CO* expression peaks after nightfall. The observation that expression of *FT* closely mirrors that of *CO* when *CO* expression peaks before nightfall provided important insights into how changes in day length control flowering (Suarez-Lopez *et al.* 2001). A hypothesis called "the external coincidence model" was proposed, which states that CO protein is somehow inhibited by darkness and thus is unable to activate FT during short-days (Yanovsky and Kay 2002). It is now known from *Arabidopsis* that CO protein is stable only during daylight hours, especially under conditions where light is detected by PHYA (Valverde *et al.* 2004). In darkness, the protein is destroyed by light-regulated proteasomal degradation and thus cannot induce expression of downstream targets. The working of this system depends on a complex interplay of different factors that control CO expression and protein levels (reviewed in Imaizumi 2010). The term "external coincidence" refers to the concept that FT is only activated when the endogenously-controlled peak of *CO* expression coincides with an external cue (light). The active FT protein is then transported from leaf to apex (Corbesier 2007), where it triggers developmental processes such as the switch from vegetative to reproductive growth.

### 1.4.4 The CO/FT regulon- the day length controlled switch

It is generally accepted that the external coincidence model, in which the CO/FT regulon plays the central role, provides a good description of the mechanism by which flowering is controlled in *Arabidopsis*, and probably in other long day flowering plants as well. CONSTANS has also been suggested to be a general regulator of other photoperiodic responses, such as tuberization in potatoes (Martinez-Garcia *et al.* 2002). Bohlenius et al. showed that a *Populus* ortholog of FT1 is a strong promoter of flowering in hybrid aspen, and that trees overexpressing *FT1* flower very early, even when still in tissue culture (Bohlenius *et al.* 2006). Similar results have been shown for FT2 (Hsu *et al.* 2006). However, since trees flower only after several years' growth, the processes that control their flowering cannot be as simple as a day length-dependent on/off switch. Both FT1 and FT2 seem to accumulate with age, and one attractive hypothesis is that FT needs to reach a threshold level to be able to induce flowering under inductive conditions (Bohlenius *et al.* 2006, Hsu *et al.* 2006). Bohlenius and co-workers also showed that short-day induced growth cessation is impaired in *FT1*-overexpressing trees and accelerated in trees with reduced

*FT1* or *CO* expression. Additionally, they found that the clinal variation in critical day length is dependent on differences in the endogenous rhythm of *CO* expression. Overall, their data strongly support the hypothesis that the short-day-induced growth cessation is gated by the CO/FT regulon. In contrast to flowering, for which several downstream targets of CO/FT have been identified (Turck *et al.* 2008), almost nothing is known about the early events in short-day induced growth cessation. It has been demonstrated that *FT* expression levels drop within days of the shortening of the light period (Bohlenius *et al.* 2006, Resman *et al.* 2010), but there is very little information on the other events that occur within that time frame. Several microarray studies examining short-day-induced growth cessation and the establishment of dormancy have been performed but none have focused on these very early short-day responses. It is tempting to assume that some of the known targets of FT in the flowering pathway would be involved, but as many of these are transcription factors controlling a developmental switch from a vegetative meristem to an inflorescence meristem it is more likely that growth cessation processes will be controlled by a different, possibly tree-specific, set of genes.

## 1.5 The plant cell cycle – the key to growth control

Plant growth can be divided into two types of processes: expansion of cells and cell division. These processes are tightly linked as organs grow - if there are too many cells, they will expand less and if there are too few, they will be larger. Plant growth control is therefore closely linked to cell cycle control, but there are mechanisms to compensate for the effects of aberrant cell cycle behavior should they occur. Differentiation and cell cycle are also interconnected – for example, the differentiation of cells undergoing aberrant division might be delayed. Differentiated or differentiating cells can also send specific growth-controlling signals to adjacent cells. In the context of annual growth cycles, expansion growth ceases before cell cycle arrest, and cell cycle arrest precedes dormancy. The aim of the work described in this thesis was to identify regulators of cell division activity, both in the context of active growth and wood formation and in the context of short-day induced growth cessation.

### 1.5.1 Core cell cycle regulators

The core cell cycle machinery of plants, yeast, and metazoans is very similar, at least on the surface. However, it has been shown that although plants have many proteins that are homologous to those of yeasts and

metazoans, the plant homologues does not necessarily share the same function and may not have the same importance as their counterparts from other organisms (Harashima and Schnittger, 2010). There are also plant specific groups of cell cycle regulators (e.g. B-type cyclin dependent kinases) as well as genes present in animals that have no counterpart in plants. The cyclin dependent kinases (CDK:s) and the cyclins that control their activity are crucial components of the cell cycle machinery. Different CDK-Cyclin complexes control the progression of different phases of the cell cycle. The G1-S transition is the critical point at which the cell "decides" whether or not it will undergo division. If it enters S-phase, the process of cell division begins, and new DNA is synthesized; strict control of this checkpoint is therefore needed for normal growth. The G1-S transition is controlled by CDKA, which is activated by D-type cyclins. The active CDKA phosphorylates retinoblastoma-related protein (RBR), thereby relieving the inhibition of the E2F transcription factor. E2F is a strong promoter of cell cycle progression, and its release leads to the expression of genes required for entry into S-phase, triggering the process of cell division (reviewed in Harashima and Schnittger, 2010) (Fig 3). These processes seem to function in much the same way in both animals and plants. One intriguing difference is that there is one additional level of CDK control in animals, in which CDK1 (a homolog of CDKA) is inhibited by phosphorylation and needs to be dephosphorylated to become functional. The proteins related to this phosphorylation and dephosphorylation are present in *Arabidopsis* but are not necessary for progression of the cell cycle (Dissmeyer *et al* 2009).

External control of the cell cycle is largely exerted *via* different plant hormones. The growth-promoting hormone cytokinin activates cell division by exerting control over the activity of D-type cyclins (Riou-Khamlichi *et al.* 1999). It has also been suggested that auxin control the activity of D-type cyclins *via* the auxin-inducible protein ARGOS and its downstream target AINTEGUMENTA (Mizukami and Fischer 2000, Dewitte *et al.* 2003, Hu *et al.* 2003). Because the levels of hormones are dependent on environmental factors, hormonal control of the cell cycle *via* regulation of cyclin activity is one important way by which plants can adapt to the challenges of a sessile lifestyle. Hormones and their influence on the regulation of growth are discussed in more detail in section 1.6. Many processes that affect cell division ultimately exert control over S-phase entry. It is therefore not surprising that when studying processes such as short-day induced growth cessation or control of meristems, one often finds that while the proteins being studied may not actually be the products of

core cell cycle genes, they nevertheless have a relatively direct impact on cell cycle progression.

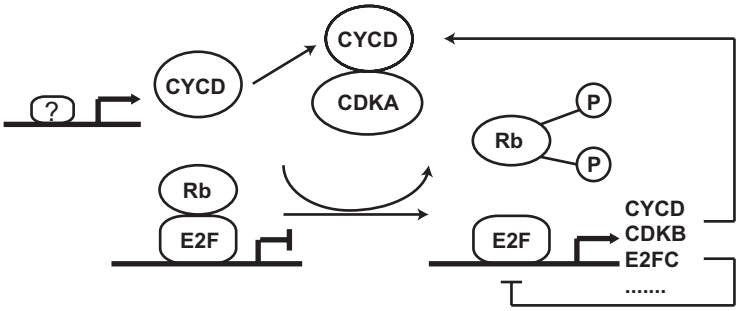


**Figure 3. G1-S transition**
For the plant to enter S-phase the activity of CDKA is required. CDKA is activated by D-type cyclins. The CDK-Cyclin complex releases inhibition of cell cycle progression genes by phosphorylating Rb.
(Modified from Dewitte and Murray 2003)

Aintegumenta (ANT) is a transcription factor belonging to a very large plant-specific family of transcription factors, the AP2/EREBP family. Transcription factors of this family have been connected to a plethora of developmental processes and responses to a variety of external signals, hormones, and stresses (Riechmann and Meyerowitz 1998). The ANT subfamily of AP2 transcription factors is relatively small; in *Arabidopsis*, it has nine members (Shigyo *et al.* 2006). Interestingly, all of these are expressed in actively dividing and /or meristematic tissues (Nole-Wilson *et al.* 2005). Some genes in this subfamily have been further characterized and found to be involved in meristem activity (Galinha *et al.* 2007) and/or meristem identity (Boutilier *et al.* 2002). ANT itself has been shown to increase cell proliferation in *Arabidopsis*, leading to larger organs due to prolonged cell division activity (Mizukami and Fischer 2000). This increase in cell division activity has been proposed to be a consequence of transcriptional activation of CYCD3:1 (Mizukami and Fischer 2000, Dewitte *et al.* 2003), but to date no evidence supporting a physical interaction between ANT and any promoter sequence has been published. *In vitro*, ANT has been shown to bind DNA (Nole-Wilson and Krizek 2000) and function as a transcriptional activator (Vergani *et al.* 1997, Krizek and Sulli 2006). Little is known about the functions of the ANT-

group genes in *Populus*, but microarray studies of the cambial zone in hybrid aspen stems have shown that they have two ANT homologs whose expression peaks at the expected position of the meristem and extends out into the zone of actively dividing xylem mother cells (Schrader *et al.* 2004*a*). In microarray experiments focusing on growth cessation and the transition to dormancy, AINTEGUMENTA-like genes were found to be significantly downregulated in both the apex and the cambium (Ruttink *et al.* 2007, Schrader *et al.* 2004*b*, Druart *et al.* 2007). The research described in Paper II and some of the hitherto unpublished work described in this thesis focused on a *Populus* ANT homolog, AINTEGUMENTALIKE1 (AIL1), and its functions in tree-specific processes such as wood formation and short-day induced growth cessation.

## 1.6 Plant hormones- key players in the regulation of growth

Any thesis covering developmental processes in plants would be incomplete without a discussion of the roles of plant hormones. In fact, any major effort to study any plant developmental processes will eventually have to take hormone actions into account. This section provides a brief description of what is known about the involvement of the plant hormone auxin (IAA) in short-day induced growth cessation and dormancy. In addition, the consequences of auxin signaling and hormone action on the maintenance and activity of the cambium are discussed. The research described in this thesis led to new insights into the role of auxin involvement in the regulation of the cambium meristem and wood formation as well as in the cessation of short-day induced growth. Other hormones besides auxin are of equal importance in many of these processes but will not be extensively covered in this thesis.

### 1.6.1 Auxin signaling

One of the primary questions in auxin biology is how it is that one single molecule can be involved in so many different processes and perform so many diverse functions. Part of the answer lies in the complexity of auxin signaling. Auxin responses are relayed through a large family of transcription factors called Aux/IAA (auxin/indole-3-acetic acid) proteins. These transcription factors repress auxin responses by binding to another group of transcription factors, the Auxin Response Factors (ARF:s) (Tiwari *et al.* 2003, Tiwari *et al.* 2004). ARF:s play critical roles in auxin regulated processes: they control the expression of auxin regulated genes, including the Aux/IAA:s, and are therefore part of a self-regulating negative feedback

loop (Fig 4) . There are two major types of ARF:s - activating and repressing (Guilfoyle and Hagen 2007). ARF transcription factors bind to auxin response elements (AuxRE:s) in promoters of auxin responsive genes and activate or repress their transcription (Liu *et al.* 1994, Ulmasov *et al.* 1997, Ulmasov *et al.* 1999). The numerous combinations of Aux/IAA:s and ARF:s allow for a high degree of complexity and fine-grained control in the auxin signaling system. Variation in the number and sequence of auxin response elements further increases this complexity (Chapman and Estelle 2009). Additionally, these proteins have different spatial distribution in the plant as well as different degrees of auxin responsiveness. Many ARF:s contain micro-RNA (miRNA) or trans-acting small interfering RNA (tasiRNA) target sites that give rise to additional levels of control by rendering them more or less stable (Mallory *et al.* 2005, Fahlgren *et al.* 2006). The stability of AUX/IAA repressors is also variable (Dreher *et al.* 2006). All of these levels of control make this seemingly simple transcriptional control system extremely versatile.

Aux/IAA:s function as both stabilizers and direct targets of the $SCF^{TIR1}$ complex (Fig 4). When auxin is present, it binds to the F-box protein TRANSPORT INHIBITOR RESPONSE1 (TIR1) (or other homologous proteins) and the Aux/IAA (Kepinski and Leyser 2004, Kepinski and Leyser 2005, Tan *et al.* 2007). This interaction leads to ubiquitination and subsequent degradation of the Aux/IAA (Gray *et al.* 2001). Aux/IAA:s contain a so called “degron” sequence that is necessary for this process (Ramos *et al.* 2001). Mutations in the degron sequence stabilize the repressor and lead to aberrant auxin signaling and auxin insensitivity, making them useful tools for the study of auxin-controlled processes. Importantly, this approach works as well in trees as it does in *Arabidopsis*; it was exploited in the study described in paper IV, which focused on auxin-regulated control of gene expression in hybrid aspen stem. The control of auxin biosynthesis and auxin transport is not discussed in detail in this thesis, but these processes are of course also important for complete understanding of auxin biology.

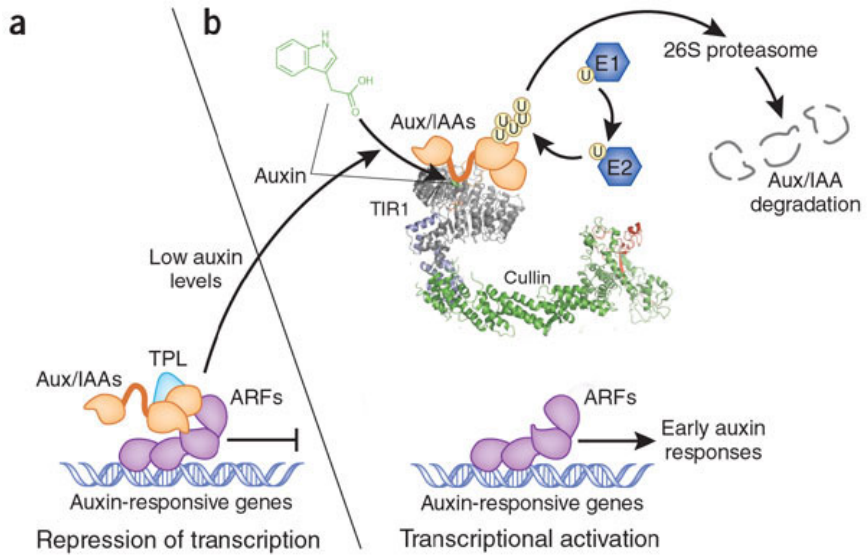


**Figure 4. Auxin signaling**
*Left panel:* In the absence of auxin Aux/IAA repressors bind to ARF:s thereby inhibiting transcription of auxin responsive genes
*Right panel:* When auxin is present the auxin repressor TIR1 that is part of the $SCF^{TIR1}$ complex binds to auxin, which allows the complex to stably bind to the Aux/IAA repressor targeting it for degradation. This relieves the inhibition of ARF:s and allows expression of early auxin response genes.
(Figure modified from Santner *et al.* 2009)

### 1.6.2 Auxin and the environment

As stated above, plants obtain information about their environment in part by means of a plethora of photoreceptors. If the plant is to respond appropriately to changes in its environment, these signals must be translated into developmental responses; responses of this kind are of the utmost importance in the maintenance of a sessile lifestyle. As auxin is one of the key regulators of plant growth and development, it was perhaps inevitable that auxin responses would be found to be involved in light signaling, directly or indirectly (reviewed in Halliday *et al.* 2009, Covington and Harmer 2007). Most of our current understanding of the connection between light and auxin from classical de-etiolation or shade avoidance studies using *Arabidopis* (Morelli and Ruberti 2002, Symons and Reid 2003). For the most part, such studies have focused on monitoring auxin levels and/or biosynthesis. Interestingly, microarray experiments

have shown that there is a higher degree of overlap between genes regulated by light and genes regulated by auxin than is the case for other hormones (Tepperman *et al.* 2006) and a surprisingly high proportion of auxin regulated genes show a circadic expression pattern (Covington and Harmer 2007). In conjunction with the complex network of transcription factors described above, the fact that plants can integrate external signals into the auxin signaling network gives rise to immense scope for fine-grained control over developmental processes. Remarkably, it has recently been shown that there is a connection between the circadian rhythm and auxin levels (Rawat et al. 2009). This is one of the first unambiguous pieces of evidence connecting light regulated processes and auxin-mediated control at the molecular level. The transcription factor identified in this study, REVEILLE1 (REV1), is closely related to the clock components CCA1 and LHY, which are conserved throughout the plant kingdom. It therefore seems likely that REV1 may also be highly conserved; it would be highly desirable to conduct studies in species other than *Arabidopsis* to see whether this is indeed the case and to see how far the findings observed in *Arabidopsis* can be generalized across other plant species.

Light, unsurprisingly, is important in the regulation of short-day induced growth cessation. Auxin is a growth-promoting hormone that stimulates cambial cell division. Variation in auxin levels is often attributed to variation in auxin transport (in tissues where auxin synthesis is relatively low), and auxin levels and transport decreases in tree stems during short-day treatment (Lachaud and Bonnemain 1984, Sundberg *et al.* 1987). It is therefore tempting to suggest that the cessation of growth and subsequent dormancy are triggered by a decrease in auxin levels. However, several additional factors need to be considered. First, auxin levels in the meristematic region remain unchanged during the dormant period (Uggla *et al.* 1996, Eklund *et al.* 1998). Secondly, the maximum auxin levels in in the cambial zone are not found at the expected position of the cambial initials; rather, they occur in young dividing xylem (Savidge *et al.* 1982, Uggla *et al.* 1996, Paper IV). Additionally, the observed decline in auxin levels show poor temporal correlation to key growth transitions (Sundberg *et al.* 1987, Sundberg *et al.* 1991). It is now established that at the cambium, i.e. the tissue that eventually becomes dormant, the seasonal variation in auxin levels is quite modest (Uggla *et al.* 1996). Moreover, the application of auxin to balsam fir (*Abies balsamea*) dormant cambium had no effect, suggesting that even if auxin levels were high during winter, growth would not be affected (Little and Bonga 1974). It therefore appears that the impact of auxins on growth cessation and dormancy may be mediated by changes

in the cells' auxin responsiveness rather than through changes in auxin levels themselves. The study described in paper III of this thesis provides evidence for a connection between the short-day response and a continuous change in auxin responsiveness at the transcriptional level. That is to say, it provides evidence for a connection between seasonal changes and auxin responses.

Other hormones are involved in the seasonal cycle between growth and dormancy. Abscissic acid (ABA) is a well-studied plant hormone known to be induced by various stresses (e.g. drought stress) and is involved in seed dormancy (Sondheimer *et al.* 1968, Wareing and Saunders 1971, Karssen *et al.* 1983). Not surprisingly, ABA was early suggested to function as an inhibitory factor during bud dormancy, but proof of this regulation has been elusive (Wareing and Saunders 1971). Careful measurements of ABA levels during seasonal changes or induced short day response did not clarify the issue (Lenton *et al.* 1972, Druart *et al.* 2007). A recent study using transgenic ABA-insensitive hybrid aspen revealed that ABA has no discernable effect on growth cessation, but maintaining bud dormancy requires ABA response (Resman 2010). Gibberellins (GA) are often described as antagonistic to ABA. This seems to hold true when it comes to seasonal dormancy, as GA levels drop as the length of day falls below the critical value (Olsen *et al.* 1995) and overexpression of a key enzyme in GA biosynthesis (GA20 oxidase) leads to delayed growth cessation and impaired dormancy establishment. Further evidence for GA involvement in short day induced growth cessation is the fact that low levels of GA can lead to growth cessation but not subsequent dormancy in trees overexpressing PHYA (Mølmann *et al.* 2005). Exogenous GA treatment can be used as a means to break bud dormancy in some species and induces bud flush and reactivation in ecodormant trees (Lavender and Silim 1987).

### 1.6.3 Auxin and wood formation- old news?

Our current understanding of the molecular aspects of auxin biology was largely obtained by studying *Arabidopsis*. However, auxin was a popular subject of study long before *Arabidopsis* became the model plant of choice. Many classic auxin experiments have been carried out in a diversity of species, including trees, and many early publications focused on the connection between plant hormones and cambial activity (wood formation) (e.g. Hejnowicz and Tomaszewski 1969, Digby and Wareing 1966). Historically, most studies were done using conifers because of their economic importance. More recently, *Populus* has emerged as the predominant model species because its genome has been sequenced. When

studying auxin and wood formation in *Populus*, it is important to verify the relevance of results previously obtained in distantly related species such as conifers or closer relatives with a very divergent growth pattern such as *Arabidopsis.* In the case of auxin biology, it seems valid to assume that the behavior of gymnosperms and angiosperms is similar. The auxin gradient over the cambial zone is very similar in both types of plant (Uggla *et al.* 1996, Tuominen *et al.* 1997, Uggla *et al.* 1998), and both exhibit a similar reduction in auxin sensitivity during short-day induced cambial dormancy (Little and Bonga 1974, Paper III).

Wood formation is a consequence of the cambial activity that gives rise to phloem and xylem (fig 1). The bulk of the *Populus* stem is made up the xylem fibers and, to a lesser extent, xylem vessels. IAA influences all aspects of cambial cell division activity and xylem formation. Cambial activity is dependent on IAA transported from source tissues such as young leaves; it ceases in decapitated defoliated trees, but resumes if external auxin is applied (Little and Savidge 1987, Savidge 1988). Cambial activity is thus also inhibited in trees in which polar auxin transport has been disrupted. Similar results can be seen when performing the corresponding experiments in *Arabidopsis* floral stem indicating conserved cambial regulation by auxin in herbaceous and woody species (Little *et al.* 2002). Cambial activity can be restored by the application of auxin alone, but normal wood formation requires a "natural" mix of the various hormones - auxin, cytokinins, and gibberellins (Hejnowicz and Tomaszewski 1969). The presence of an auxin concentration gradient (Uggla *et al.* 1996, Tuominen *et al.* 1997) in both pine and hybrid aspen has led to the suggestion that auxin might act as a morphogen, providing positional information to cambial cells and their derivatives (Uggla *et al.* 1998). The studies described in paper IV of this thesis focused on the relationship between IAA and wood formation at the molecular level. The roles of cytokinins and gibberellins have been investigated by other workers; in conjunction with their studies, the results described in this thesis confirm that a complex interplay between plant hormones is required for optimal growth (Hejnowicz and Tomaszewski 1969, Nieminen *et al.* 2008, Björklund *et al.* 2007).

# 2 Objectives

This thesis is an effort to increase understanding about plant growth in general and tree growth specifically. The order of papers included is scientific rather than chronological and should help to answer the following questions:

*How are core cell cycle regulators controlled during perception of short days and cessation of growth?*
(Paper I and II)

*How does the responsiveness to plant hormone IAA change during short day induced growth cessation and dormancy?*
(Paper III)

*How is cambial growth and maintenance affected by modulation of IAA response?*
(Paper IV)

*Can we find common regulatory factors controlling cessation of growth and maintenance of the vascular cambium?*

# 3 Methodological overview

This section provides a brief overview of the various experimental methods used in the studies described in this thesis and discusses the types of questions they can be used to answer. It does not provide instructions on how to perform the experiments in question – details of that kind can be found in the experimental sections of the included papers.

## 3.1 *Populus*- the tree of choice for molecular biologists

Working with trees has become increasingly straightforward since the publication of the first complete tree genome (Tuskan *et al*. 2006). The species that was sequenced was *Populus trichocarpa* (black cottonwood), which has a high degree of sequence similarity with other *Populus* species such as the European aspen (*Populus tremula*) and the hybrid aspen (*Populus tremula x tremuloides*); as such, all of these species are good candidates if one is looking for a tree to study. In fact, the largest EST collection for any *Populus* species is PopulusDB, which is a database that consists largely of hybrid aspen sequences (Sterky *et al.* 2004). Hybrid aspen is also more easily transformed and propagated than *P. trichocarpa*, which makes it very attractive for molecular biology studies. These traits make it quite easy to generate transgenic lines using *Agrobacterium* mediated transformation, a technique used to great effect in *Arabidopsis*. In *Arabidopsis*, the single most successful strategy for elucidating gene function has been the use of mutants. This approach is not feasible in *Populus* since the long generation time prohibits the use of crosses to make homozygous lines. Instead, functional analysis is normally performed by overexpressing the target gene, either under the control of the 35S promoter (which essentially results in the transgene being expressed everywhere in the organism) (Nilsson *et al.* 1992) or by means of targeted expression

using tissue-specific promoters (Mauriat and Moritz 2009). However, there are certain drawbacks associated with the use of ectopic expression in functional studies, and so alternative systems are needed. Inducible overexpression would overcome some of the problems with constitutive overexpression, but it is difficult to identify systems that provide satisfactory levels of induction without causing extensive secondary effects. For the targeted downregulation of specific genes, RNA interference (RNAi) and the use of artificial microRNA have proven to be useful methods, and can at least partly compensate for the lack of *Populus* knockout mutants (Chuang and Meyerowitz 2000, Li *et al.* 2008, Ossowski *et al.* 2008). Another alternative to loss of function alleles in *Populus* is to induce the expression of modified versions of the target proteins; this strategy was successfully used in the study described in paper IV of this thesis.

## 3.2 Gene expression analysis

The results presented in this thesis were largely obtained through gene expression analysis. Paper I presents data from a full genome microarray, and papers III and IV both include the use of cDNA arrays. In addition to these large scale expression studies, paper II presents results obtained using the real time polymerase chain reaction (real time-PCR) while paper III contains results obtained using the more "old-school" reverse transcriptase polymerase chain reaction (RT-PCR). All of these methods share the same major weakness: they provide information on the expression of the gene, but not on the abundance of the actual protein or on the extent to which the protein is subject to regulatory modification. Despite this weakness, transcription analysis is a powerful tool. The following sections provide a brief overview of the methods used in this work.

### 3.2.1 Microarray

*Populus cDNA arrays*

Even before the publication of the poplar genome sequence, poplar cDNA arrays had been manufactured using sequence information from PopulusDB – the world's largest poplar EST collection. A poplar transcriptomics platform was jointly established by the Umeå Plant Science Centre and The Royal Institute of Technology, and became a world-leading institution in the field of *Populus* genomics. The first publications emanating from this facility used the so-called "wood-chip", a spotted cDNA array of 2995 probes (Hertzberg *et al.* 2001, Israelsson *et al* 2003). The EST:s used in this

first array were obtained from a xylem cDNA library. Later arrays used EST:s derived from various tissues and more probes. The first chip containing EST:s from various tissues was POP1, which contained a double spotted unigene set of 13526 clones (used in paper IV). The latest chip, POP2, is a 25k array (used in paper III). To make it easier for scientists interested in using the POP arrays a local database, UPSC-BASE, was set up. This database can be used for all steps in an experiment, from experimental design to pathway analysis, and has a user-friendly interface (Sjödin *et al.* 2006).

The basis of cDNA microarray based expression analysis is quite straightforward. Two samples of single stranded cDNA from RNA samples that are to be compared are coupled to two different fluorescent dyes, mixed in equal amounts, and hybridized to the array. After hybridization, the slide is scanned and spot intensity is quantified. The color intensity of each spot depends on the difference in expression between the samples (Schena 1996). Spotted cDNA arrays are considered to be old-fashioned compared to full genome arrays, but the data generated using the POP1 and POP2 arrays have in many ways revolutionized our understanding of how trees grow. Both of these arrays are represented in the papers included in this thesis.

*Full genome oligoarrays*

Nowadays, all of the major manufacturers of high-density full genome oligoarrays produce *Populus* arrays, including Affymetrix, Agilent, and Nimblegen. A detailed comparison of the different manufacturers' platforms can be found elsewhere (Tsai *et al.* 2009); a whole genome array made by Nimblegen was used in the study described in paper I. This array contains 65,911 unique probes including predicted gene models, mitochondrial- and chloroplast gene models, and aspen EST sequences. EST sequences aside, the array is based entirely on sequences from *Populus trichocarpa*; despite the high sequence similarity between *Populus* species, this point should still be borne in mind when using this array with other *Populus* species such as hybrid aspen. Specifically, if this array is used with other *Populus* species, there is a slight risk of increased cross-hybridization, and sequence diversity might cause the signal intensity to fall below the threshold value even when the relevant gene is in fact being expressed.

*Microarrays- pros and cons*

Arrays are used to monitor gene expression on a large scale. This is very useful when trying to elucidate transcriptional networks or patterns of gene expression in a time series or after particular treatments. It is less useful if you already have some clue as to what specific genes you are interested in studying. Microarrays generate large amounts of data that require careful analysis, and will always include some false positives and false negatives. The best way to avoid pitfalls in the analysis is to adopt an unbiased approach. This might seem easy but there are many examples where extensive biological reasoning has been based on the expression of a single gene from a microarray experiment. Conclusions drawn from expression patterns of single or very few genes should always be corroborated by single gene expression analysis (e.g. real-time PCR). Cross-hybridization, especially within gene families, is also an issue.

Microarrays are powerful tools for global expression analysis and can be used to identify transcriptional networks and global patterns of expression. They are also very useful for analyzing transcriptional regulation of metabolic pathways. This thesis describes the use of microarrays both as tools for simultaneously studying the expression patterns of many genes and as a means of identifying candidate genes for further functional analysis.

### 3.2.2 Reverse Transcriptase Polymerase Chain Reaction

The Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) has long been the method of choice for studying the level of expression of specific genes of interest. Put simply, it is a method for measuring the amount of a particular mRNA in a sample. In order to compare expression levels between samples, it is also important to measure the levels of one or more reference genes in each sample. The reference genes are often so-called "housekeeping" genes whose expression supposedly varies very little between samples. The method gives a relative expression value based on the difference between the level of expression of the reference gene and that of the gene of interest. This is the basis of both "old-school" RT-PCR, in which the quantities of the amplified products are measured on a gel, and the more modern real-time PCR technique, which uses non-specific fluorescent dyes that make it possible to measure the quantity of double stranded DNA after each PCR cycle. The drawback of both old and new PCR techniques is that they can only be used for the analysis of one gene at a time, although if multiplexing techniques are used, it is possible to

simultaneous analyze a small number of genes. On the other hand, because the primers used are specific, it is a very exact and reliable way to measure gene expression.

## 3.3 DNA-protein interaction

Gene expression is dependent on many external and internal factors. Control of gene expression is dependent on transcription factors that can either repress or activate specific genes. One way to identify specific target genes controlled by a certain transcription factor is to use an inducible overexpression system and monitor gene expression (e.g. using a microarray or some other means of analyzing expression) to find co-regulated genes that are induced or repressed. This method can facilitate identification of targets, but cannot be used to determine whether the targets are primary (i.e. their expression is directly promoted by the binding of the transcription factor to their promoter) or secondary (genes that function downstream of the transcription factor, but do not interact directly with it). Misexpressed genes in transgenic plants are often hypothesized to be targets of the gene of interest. None of these methods can reliably identify transcription factor- promoter interactions. In the study described in paper II, a method for identifying the specific binding of a transcription factor to a promoter *in vitro* was used to show a direct link between AINTEGUMENTALIKE1 and a CYCD3.2 promoter. This method, the electrophoretic mobility shift assay (EMSA), is based on the fact that the mobility of protein-bound DNA in a polyacrylamide gel differs from that of DNA that is not so bound (Hendrickson and Schleif 1984). While the method might sound somewhat simplistic, there are pitfalls to consider when using it. First, it is necessary to decide whether to use a purified protein or total cell extracts enriched in your protein of choice. The use of a pure protein would minimize the risk of non-specific binding, but since many transcription factors require co-factors to bind to DNA, this might eliminate the protein's DNA-binding ability. The drawback of using cell extracts is that they will contain many DNA-binding proteins besides the transcription factor of interest, any one of which might bind to your DNA-sequence and cause a gel shift. To avoid this, only small DNA-fragments can be used, and since regulatory sequences can reside far from the start codon, this can make it difficult to identify the right sequence.

## 3.4 Monitoring growth and cell cycle activity

The work described in this thesis deals mainly with plant growth. As such, it was necessary to employ reliable methods to study and compare growth, which is not always as straightforward as it might seem. Short day induced growth cessation is mainly defined by the cessation of elongation growth (measured using a simple measuring stick approximately once per week). However, it can also be defined by the cessation of cambial cell division (Espinosa-Ruiz *et al.* 2004, Resman *et al.* 2010). In some extreme cases, such as those involving AIL1 overexpressors, none of these methods are viable because the transgenic trees exhibit almost no apical growth and cell division in the stem is not exclusively limited to the cambium (Fig 5 and Paper II). In such cases, the absence of bud formation was used as a marker of continued growth and aberrant growth cessation. In the study described in paper IV, cell division counting was used to monitor two related but distinct phenomena. Total cambial cell division activity was used as a measure of wood formation, since most cambial cell divisions produce xylem. Additionally, anticlinal divisions were counted to determine the position of stem cells in the cambial region (Schrader *et al.* 2004*a*). This method can be used to locate the position of the cambial initials and to thereby identify potential problems in meristem maintenance.

# 4 Results and discussion

This thesis addresses some fundamental questions about hormonal and environmental control of plant growth and development in perennial trees. The papers included in the thesis focus on seemingly separate processes, but it was found that the transcriptional pathways that are downregulated during short-day-induced growth cessation are related to the complex processes involved in the maintenance of actively growing meristems. Moreover, it was found that auxin signaling processes that control wood development in actively growing trees is modified in response to the onset of shorter days.

## 4.1 Cell cycle control- to grow or not to grow? (Paper I and II)

Growth in plants can be attributed to two key processes, cell division and cell expansion. Both of these processes can be used as markers to investigate the timing of short-day-induced growth cessation, by monitoring changes in elongation growth or cell division activity (Espinosa-Ruiz *et al.* 2004, Resman *et al*. 2010). There is an obvious relationship between growth cessation and cell cycle arrest, and the connection between short day signal and the termination of cell cycle activity was investigated in the studies described in papers I and II. Paper I presents the results of a study in which a full genome array was used to examine the expression patterns of all known core cell cycle regulators during key phases of seasonal growth cycling. Additionally, the involvement of histone modification and the plant hormones GA and ABA during activity- dormancy cycles was analyzed at the transcriptional level. These latter subjects, while interesting, fall outside of the scope of this thesis and will not be further discussed. Paper II presents a more detailed analysis of the connection between environmental signals and cell cycle

control; the role of a growth promoting transcription factor, AINTEGUMENTALIKE1, that acts upstream of the cell cycle machinery is elucidated.

### 4.1.1 Transcriptional control of core cell cycle genes (Paper I)

The regulation of several core cell cycle regulators during seasonal growth cycles has been investigated previously, in both the apex and the cambium (Ruttink *et al.* 2007, Schrader *et al.* 2004*b*, Druart *et al.* 2007). However, the study described in Paper I was, to the best of the author's knowledge, the first effort to examine the expression of all known core cell cycle regulators simultaneously using a full genome array. The results of this study, in conjunction with those previously reported, clearly demonstrate that transcriptional regulation plays an important role in cell cycle control during activity-dormancy cycles. However, the levels of a subset of core cell cycle regulators do not change significantly in response to changes in day length. It has previously been shown that post-transcriptional regulation is an important factor in cell cycle control during cambial dormancy (Espinosa Ruiz *et al.* 2004); the results obtained in this study are consistent with that finding.

The large scale microarray study described in Paper I was conducted to investigate transcriptional changes during the following key steps in the activity-dormancy cycle in the apex of hybrid aspen: growth cessation, dormancy establishment, dormancy release, and reactivation/bud burst. Growth cessation occurs during the first 0-5 weeks of short days. Dormancy is then established (i.e. the apex undergoes the transition from eco- to endo-dormancy) after 5-11 weeks of short days. Dormancy release occurs after approximately 4 weeks' exposure to cold conditions, and reactivation/bud burst occurs after trees have been subjected to conditions permissive of growth. It should be noted that during the reactivation phase, samples were acquired only after visual inspection of the trees indicated bud burst to have begun. The core cell cycle regulators examined in this study exhibited a surprisingly limited range of transcriptional profiles during the transitions between active growth and dormancy (Fig 1 in Paper I). Analysis of these regulators on the basis of their predicted functions revealed some interesting patterns. With the exception of the plant-specific CDKB:s, the cyclin dependent kinases (CDK:s) were not subject to transcriptional regulation at all (Fig 1 in Paper I). B-type CDK:s were downregulated during short-day and cold treatment and upregulated during reactivation/bud burst. Interestingly, the downregulation of *CDKB* continued well beyond the point at which growth ceased. This seemingly

excessive downregulation has no obvious function, but could reflect a defensive mechanism preventing premature reactivation due to CDK activity. The levels of expression of the other CDK:s remained unchanged during the course of the experiment. We know from earlier studies that CDKA protein in the cambium disappears while its mRNA is still detectable (Espinosa-Ruiz *et al.* 2004), indicating that the intracellular levels of the CDKA proteins are subject to post-transcriptional control. Protein levels of CDK:s in the apex have not been studied, but since the expression patterns of the CDK:s are very similar in both tissues, it seems highly probable that the CDK:s in the apex would be subject to similar temporal and functional regulation as they are in the cambium. This hypothesis should be investigated further, preferably by direct measurement of the levels of the relevant proteins in the apex.

As the name "cyclin dependent kinase" implies, cell division also requires the presence of cyclins. The levels of the cyclins mirrored those of *CDKB*, which is transcriptionally controlled; it appears that like CDKB (and unlike the other CDK:s), the activity of the cyclins is also subject to transcriptional control (Fig 1 in paper I). Like CDKB, none of the cyclins were upregulated during the release of dormancy (i.e. in response to cold treatment), so the release of dormancy is not accomplished by modifying the expression of these core cell cycle genes. It seems likely that the increased downregulation of cell cycle related transcripts during cold treatment is an efficient strategy to prevent growth until the onset of permissive temperatures. The conclusion of this large scale study is quite simple: cyclins are primarily regulated transcriptionally, whereas most CDK:s are not. However, biology is seldom simple and so there are exceptions to this general conclusion, e.g. CDKB. Of the cyclins, only some D-type cyclins deviated from the general trend; while most of the D-type cyclins' expression patterns closely resembled those of the other members of the family, some were clearly subject to a very different regulatory regime (Fig 1 in Paper I). At this point, not enough is known about the functions of different D-type cyclins to permit the drawing of any strong conclusions about the significance of these results, but it seems that the role of D-type cyclins in cell cycle regulation may be more complex than that of simple on/off switches for CDK:s. D-type cyclins are targets of external factors that affect cell cycle activity; it may be the case that different signals target different cyclins.

The levels of known negative regulators of the cell cycle machinery either remained unchanged during the activity-dormancy cycle or exhibited the same expression pattern as did *CDKB* and most of the cyclins (Fig 1 in

paper I). This downregulation might seem contradictory but actually reflects the function of these negative regulators. The core cell cycle machinery is complex and contains a number of checkpoints at which the progression of cell division can be arrested. These negative regulators function as an additional level of CDK control (Menges *et al.* 2005, Wang *et al.* 2008) but can also function as "gate-keepers" at cell cycle checkpoints (e.g. WEE1) (Michael and Newport 1998, De Schutter *et al.* 2007). In either case, there is no need for these negative regulators to supervise cell cycle progression after growth cessation and during dormancy because cell cycle activity is completely abolished under these conditions.

### 4.1.2 AINTEGUMENTALIKE1- a growth-promoting transcription factor in *Populus*

*Searching for candidate genes involved in growth control*

The existing body of knowledge concerning the behavior of core cell cycle components during short-day treatment suggests that both transcriptional and post-transcriptional control are essential in the control of growth. It is fairly easy to identify genes that may be involved in the control of growth in *Populus* by using transcriptional data from microarrays covering different tissues and timelines in conjunction with the existing body of data concerning factors that contribute to cell proliferation in the model plant *Arabidopsis*. However, it is more difficult to determine which of these candidates will be most worthwhile to pursue. In these studies, the selected candidates were transcription factors that were expected to be positive regulators of growth on the basis of their transcriptional profiles across the cambial region and the nature of their regulation during growth cessation. In *Populus* the technique that affords the greatest resolution of different cell types, from actively dividing undifferentiated cells to mature tissues, is cryosectioning of stem samples (Uggla *et al.* 1996, Schrader *et al.* 2004*a,* Schrader *et al.* 2004*b*). Microarray experiments covering the wood-forming zone of hybrid aspen (Hertzberg *et al.* 2001, Schrader *et al.* 2004*a*) can be used to identify genes specifically expressed in the meristematic and proliferating cells. One transcription factor that stood out as being highly expressed in actively dividing cambium close to the proposed position of the cambial initials was a homolog of the *Arabidopsis* gene *AINTEGUMENTA* (Schrader *et al.* 2004*a*). As described in the introduction, this transcription factor is a known positive regulator of cell proliferation, and its overexpression in *Arabidopsis* results in an increase in

the number of cells in the plant and in the size of its organs (Mizukami and Fischer 2000). Furthermore, a homolog of this gene has been shown to be downregulated in both the cambium and apex of *Populus* during short-day-induced growth cessation (Ruttink *et al.* 2007, Schrader *et al.* 2004*b*). This downregulation of *Populus AINTEGUMENTA* homologs was confirmed by Real-Time PCR (Fig 1 and S2 in Paper II). One of these homologs, *AINTEGUMENTALIKE1* (*AIL1*), was targeted for further study. As would be expected of a gene that controls cell growth and proliferation, *AIL1* is expressed in actively growing or meristematic tissues (Fig 3 in Paper II). GUS experiments established that it is strongly expressed in the apex and cambium, but also in the dormant axillary buds (Fig 5, Fig 3 in Paper II). Its expression is thus detectable in dormant meristematic tissues as well as actively dividing cells.

*Overexpression of AINTEGUMENTALIKE1 in hybrid aspen reveals multiple functions of AIL1 in growth control*

Paper II presents an analysis of the role of AINTEGUMENTALIKE1 (AIL1), a homolog of AtANT, along with more limited analyses of the roles of other closely related transcription factors from the same family. A phylogenetic analysis revealed that *Populus* has two pairs of close homologs to AtANT (Fig S1 in Paper II). The four homologs are very similar, especially within each pair. The high degree of similarity between these four genes means that loss-of-function techniques are unlikely to be useful when studying their function because of the risk of functional redundancy: if one is disabled, it is likely that one or more of the others will be able to fulfill its function. Indeed, the introduction of RNAi constructs targeting *AIL1* had little effect on the overall morphology of experimental trees. On the other hand, overexpression of *AIL1* in hybrid aspen caused drastic growth effects that superficially had very little in common with the overall size increase observed in *Arabidopsis* mutants that overexpress *ANT*. *AIL1*-overexpressing (*AIL1*oe) hybrid aspen showed severely stunted growth, twisted stems and a bushy phenotype caused by a loss of apical dominance (or the loss of axillary bud dormancy) (Fig S3 in Paper II) . This mutant also exhibited severe growth-related effects on cambial activity (Fig 5). Specifically, parts of the cambium collapsed into the xylem as a result of runaway cell division, causing severe disruption of xylem formation. In addition to this invasion of the xylem, there were islands of non-differentiated dividing cells scattered throughout the xylem (Fig 5). As *AIL1* is normally expressed in dividing cells the activity of the endogenous AIL1 promoter was examined in *AIL1*oe lines- The introduction of a

transcriptional fusion of GUS with the endogenous AIL1 promoter into *AIL1*oe had significant effects on the activity of the endogenous AIL1 promoter, which was active in all areas with actively dividing cells (Fig 5).

This result indicates that AIL1 positively regulates its own activity, although the detailed molecular mechanism by which this regulation is effected remains unclear. Clearly it is very important that *AIL1* expression

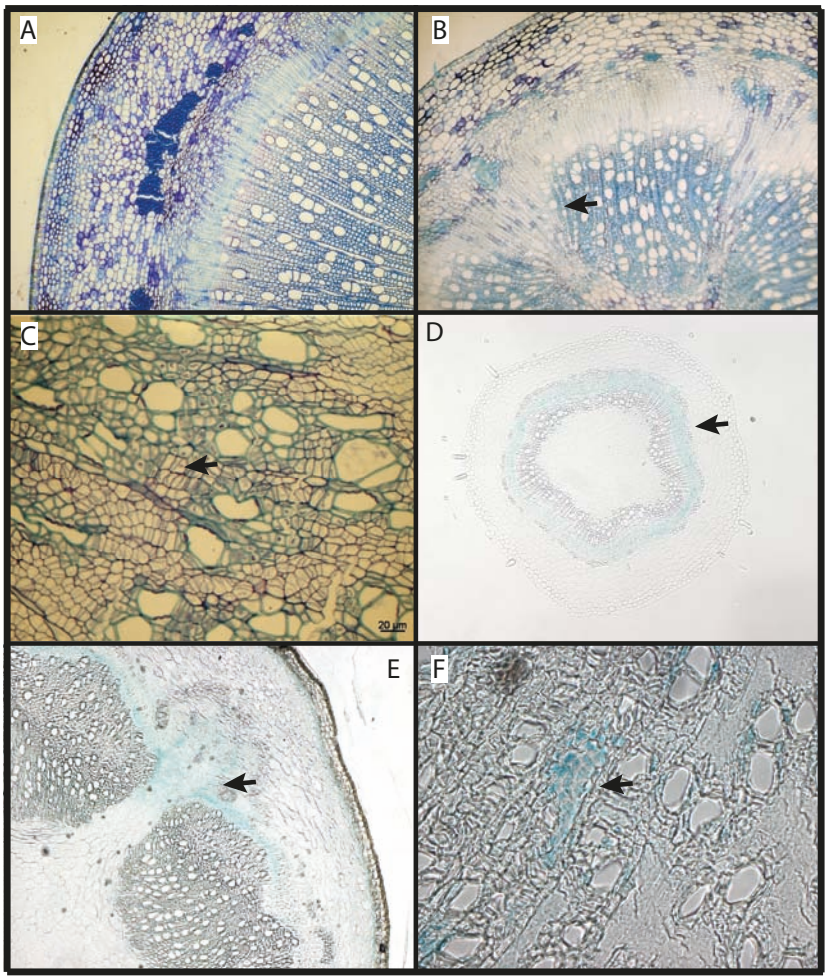


**Figure 5. Overexpression of *AIL1* have severe developmental effects in hybrid aspen stem**
A) Cross section of wild type hybrid aspen stem (internode 20). B) *AIL1*oe internode 20. Arrow indicate runaway cell division activity. C) Area with dividing cells in the xylem. *AIL1*oe internode 20. D) pAIL:GUS in wt internode 10. Arrow indicate *AIL1* expression in the cambium. E and F) pAIL1:GUS in *AIL1oe.*

is spatially restricted in order to maintain normal growth and development; elucidating the mechanism by which it is so controlled is an enticing topic for future study.

Another aspect of growth control studied using *AIL1*oe was the short day response. The *AIL1*oe transgenic lines exhibit an aberrant growth cessation response in response to short-day treatment, and bud formation is severely disrupted in both *AIL1*oe and in trees that overexpress another ANT homolog, *AIL3* (Fig 4 in Paper II). Both of these genes are normally downregulated during short day growth cessation and *AIL1* exhibit a temporal expression profile that mimics those of the core cell cycle genes *CYCD3:2* and *CYCD6:1* (Fig 1 in Paper II). These results further strengthens the hypothesis that there is an important connection between the AIL genes and cell cycle control that has a profound influence on growth and development in *Populus*.

*AINTEGUMENTALIKE1 functions downstream of the CO/FT switch and regulate cell cycle activity through D-type cyclins*

One important question is: Where does AIL1 fit into the complex pathway leading from shorter day length to cell cycle arrest? Downregulation of *AIL1* in wild type trees during short day treatment coincides with cessation of growth and downregulation of core cell cycle regulators (Fig 1 in Paper II). To further investigate the connection between shorter days and *AIL1* expression, the relationship between day length sensing and *AIL1* expression was analyzed in mutant trees with a defective short day response, namely 35S:*PHYA,* 35S:*FT* and *FT*RNAi (Olsen *et al.* 1997, Bohlenius *et al.* 2006). Because PHYA functions upstream of FT and the rapid drop in *FT* expression is the first known indication that the daylength has fallen below the critical value (Bohlenius *et al.* 2006, Resman *et al.* 2010), it was decided that *FT* levels in *AIL1*oe should be investigated. A rapid drop in FT levels was observed shortly after the commencement of the short day treatment (Fig 5 in Paper II), indicating that *AIL1*oe trees retain the ability to respond to the day length signal. The conclusion that AIL1 functions downstream of day length sensing is further supported by the finding that *AIL1* expression is unaffected in 35S:*PHYA* and 35S:*FT* during SD treatment and that *AIL1* levels fall prematurely in *FT*RNAi trees that exhibit premature growth cessation and bud set when subjected to SD (Fig 2 in Paper II). In *Arabidopsis*, AINTEGUMENTA has been suggested to function closely upstream of D-type cyclins but any interaction between AtANT and cyclin promoters has not been shown (Mizukami and Fischer 2000, Dewitte *et al.* 2003). In paper II, electrophoretic mobility shift assays

were used to probe the relationship between the AIL1 transcription factor and cyclin D 3:2 promoter sequences. This D-type cyclin study is down-regulated during short-day induced growth cessation in wild type trees but not in the *AIL1* overexpressing lines (Fig 6 in Paper II). The assays show that the AIL1 transcription factor can bind to CYCD3:2 promoter fragments (Fig 7 in Paper II). The assay does not reveal if this binding requires other transcriptional regulators or cofactors. The result suggests that AIL1 influences cell division by controlling transcription of core cell cycle components.

The question of how the downregulation of *AIL1* downstream of the CO/FT regulon is mediated remains open. It is unlikely that AIL1 is a direct target of FT because there is a clear temporal delay between the downregulation of the one and that of the other. *FT* levels drop in a matter of days after the commencement of short-day treatment, whereas *AIL1* downregulation coincides with the cessation of growth, some weeks later. Some of the known targets of FT are meristem identity genes that promote the transition from vegetative to reproductive growth in the flowering pathway (Turck *et al.* 2008) and it cannot be excluded that the same genes have a function during short-day-induced growth cessation. However, the current evidence suggests that there is at least one regulatory step between loss of FT and downregulation of AIL1. The regulation of cell division via the FT regulon is likely due to functional divergence in perennials, which supports the hypothesis that the CO/FT regulon acts as a general day length sensing switch that acts on different subsets of regulators depending on the plant's developmental and environmental status. This conclusion is strengthened by the fact that the CO/FT regulon controls other day-length-controlled processes in addition to flowering and seasonal growth cessation i.e. potato tuberization (Martinez-Garcia *et al.* 2002). It should be noted that there is a fundamental difference between the above-mentioned processes and SD induced growth cessation; while the first depends on induction of FT the latter is a result of FT downregulation.

Studies in *Arabidopsis* indicate that there is a correlation between ANT expression and auxin levels. It has been suggested that the enhanced organ growth is an auxin response that is relayed through ARGOS (Hu *et al.* 2003). The story in *Populus* seems to be more complicated and it is likely that the results from *Arabidopsis* do not provide a complete picture. There is no indication that AIL1 is induced by auxin in hybrid aspen, and its expression is not induced by the overexpression of homologs of the proposed upstream regulator ARGOS.

## 4.2 Auxin responsiveness and cambial growth activity (Paper III and IV)

Auxin is generally described as a growth promoting plant hormone and it is well known that applied and endogenous auxin stimulates cambial cell division. It is also known that the cambium loses the ability to respond to this growth stimulus during the process of dormancy establishment (Little and Bonga 1974) (Fig S1 in Paper III). In the work described in this thesis, considerable effort was made to elucidate the role of auxin both during active growth and during the process of short-day-induced growth cessation and dormancy establishment. The most important conclusion is that even though auxin levels and transport are important, modulation of auxin signaling and response is the key to understanding how auxin influences cambial growth.

### 4.2.1 Microarray strategies for studying auxin responsiveness and auxin regulated genes in hybrid aspen stem

Two distinct microarray strategies were used to elucidate how auxin affects cambial growth and how auxin responsiveness is regulated during short days. In paper III the auxin-responsive transcriptome was investigated at different stages of growth cessation and dormancy establishment. Using this novel approach (Fig S3 in Paper III) it was established that subsets of auxin responsive genes cease to respond to auxin at specific stages in the activity-dormancy cycle. Interestingly, this loss of responsiveness is strongly correlated with the number of AuxRE:s in the promoter of the gene (Table S2 in Paper III).

Auxin levels in actively growing tree stems seem to follow the same gradient in both hardwood trees and conifers (Uggla *et al.* 1996, Tuominen *et al.* 1997, Uggla *et al.* 1998). This prompted the suggestion that auxin could act as a morphogen in the wood forming zone (Uggla *et al.* 1998). If this is the case, one might expect the expression of auxin responsive genes to follow this gradient in an auxin concentration dependent manner. Paper IV describes an investigation of this hypothesis in an effort to identify the auxin responsive transcriptome in hybrid aspen stem. What sets this study apart from previous experiments is that it specifically targets stem tissue and that the definition of responsiveness requires differential response during both depletion and induction. In other words, auxin responsive genes should react to both reductions and increases in the concentration of auxin. The auxin responsive transcriptome identified was further studied to determine whether the expression of auxin responsive genes in the wood forming zone mirrors the auxin concentration gradient. Interestingly, the

expression patterns of auxin responsive genes in the wood-forming zone of hybrid aspen stem do not match the endogenous auxin gradient (Fig 2 in Paper IV). This is not all that surprising since auxin responsive genes probably respond to dynamic changes in auxin levels rather than to high or low steady states in a rigid gradient.

### 4.2.2 Auxin plays a crucial role in maintaining the vascular cambium and the position of the cambial initials

The study described in paper III established that some genes retain their auxin responsiveness during both ecodormancy and endodormancy. The reason for this is not clear, but since auxin plays an important role in defining and controlling the activity of the meristems, it seems likely that these genes are involved in maintaining the cambial initials in the meristem. The idea that auxin is involved not only in cell proliferation but also in defining the identity of the meristem is supported by the results of the study described in paper IV, in which it was found that the distribution of the anticlinal cell divisions in the cambium was abnormal in trees with abnormal auxin responses. This suggests that the cambial initials occupy abnormal positions in these transgenic trees (Fig 7 in Paper IV). It is also known that auxin levels in the cambial region fluctuate very little during seasonal changes (Uggla *et al.* 1996). Taken together, these results suggest that there may be some form of active auxin signaling that maintains the identity of the meristems during periods of dormancy. Interestingly, the polar auxin transport (PAT) machinery remains functional during winter, but transcriptional control of PAT components is lost during ecodormancy (Fig S2 in Paper III). The functional importance of this loss of control is unclear. One explanation could be that since IAA synthesis and transport from source tissues ceases during the autumn and winter, the need to modulate its transport in response to increases or decreases in auxin levels is temporarily abolished. It may be the case that a functional transport system is required to maintain auxin levels in cambium even though auxin transport activity is expected to be low during winter (Schrader *et al.* 2002).

### 4.2.3 Auxin-regulated control of wood formation

Previous attempts at elucidating the effects on wood development by perturbing auxin levels as opposed to auxin responses have been met with limited success (Tuominen *et al.* 1995) suggesting that an alternative approach to study auxin influence on wood formations is needed. Some of the components of various auxin signaling pathways are known to have distinct expression patterns across the wood forming region. One of these is

the AUX/IAA transcriptional repressor IAA3 (Moyle *et al.* 2002). To explore auxin mediated regulation of wood development, a mutated form of the AUX/IAA transcriptional repressor IAA3 (called IAA3m) was overexpressed in hybrid aspen (paper IV). The mutated protein contains a disrupted degron and is thus resistant to proteasomal degradation; a similar strategy has been used to study auxin-regulated development in *Arabidopsis* (Tian *et al.* 2002). Hybrid aspen overexpressing *IAA3m* showed severe overall morphological effects due to its auxin insensitivity, but the studies described in paper IV focused on the developmental effects in the cambium and its derivatives (xylem and phloem). The mutants' diametric growth was reduced, which is a sign of reduced cambial activity; this reduced activity was confirmed by an analysis of cell division activity (Fig 6 in Paper IV). On closer inspection, most of the observed effects on stem development were attributable to abnormal development of the xylem tissue, even though the 35S promoter (which is also active in phloem tissue) was used. More detailed studies revealed that the transgenic trees' xylem fibers and vessels were unusually small (Fig 8 in paper IV); in combination with the reduced rate of cell division, this gave them a low ratio of xylem to phloem (Fig 5 in paper IV). The phloem appeared to be normal in the transgenic lines, showing that disruption of a specific AUX/IAA repressor can have tissue-specific effects even when using a general overexpression system. Clearly, auxin has a profound influence on wood formation, not only in regulating the amount of wood formed, but also in controlling developmental processes that give rise to specialized cell types such as vessels and fibers (Fig 8 in paper IV).

#### 4.2.4 The auxin response is naturally modulated by stabilization of AUX/IAA during seasonal growth cycles

The connection between IAA3 and cambial cell division activity is of course also interesting in the context of seasonal growth cycles. Indeed, it was found that IAA3 is one of the auxin responsive genes whose responsiveness is lost during short day treatment, in this case at the endodormancy stage where cambial activity cannot be stimulated by external auxin (Fig S1 in Paper III). In paper III, the functional background to this loss of auxin responsiveness was probed in more detail. It was suggested that the loss of auxin responsiveness might be due to changes in the proteasome pathway involving the auxin receptor, TIR1. However, this hypothesis can partly be rejected because key components of the pathway continue to be expressed after the onset of short-day treatment, although *TIR1* expression is reduced by nearly 50% (Fig 5 in Paper III). This

reduction cannot completely explain the loss of auxin response, which is not surprising given that a subset of the genes retain their auxin responsiveness. It is possible that the auxin-responsive pathways might exhibit some functional redundancy and that the role of TIR1 could be fulfilled by one or other of its close homologs in hybrid aspen. Alternatively, the different F-box proteins that could function as auxin receptors might target the products of different AUX/IAA genes for destruction. In the case of IAA3, ubiquitination of the repressor is somehow inhibited in endodormant trees (Fig 4 in Paper III). This could in turn explain the loss of responsiveness in a whole set of genes repressed by IAA3. It should be noted that the hypothesis regarding TIR1 is based purely on expression data. If short day treatment led to post-transcriptional regulation of the auxin receptor, this could of course influence auxin sensitivity. On the other hand, the strong correlation between AuxRE abundance in a promoter and the loss of auxin responsiveness would support a strong role for transcriptional control during the seasonal growth cycle.

# 5 Summary and future perspectives

The work presented in this thesis was conducted as part of an effort to understand the processes that make a tree a tree and not just a mere plant. So, what makes trees unique? The most impressive features of trees are their size and lifespan. I think most of us remember the trees we climbed as children, and chances are that our great-grandchildren will be able to climb the same trees long after we are gone. To reach such impressive sizes and ages, trees must be able to grow indefinitely and adapt to an ever-changing environment. This work is concerned with processes that contribute to both of these abilities. Specifically, it focuses on wood formation, which enables the tree to develop its impressive trunk, and seasonal dormancy, which allows the tree to survive in the same spot year after year.

To summarize my work and findings, I would like to explore how well I have managed to answer the objectives outlined in section 2:

- *How are core cell cycle regulators controlled during perception of short days and cessation of growth?*

Undoubtedly, there is still a lot to learn regarding the role of core cell cycle regulators and the seasonal growth cycle. In the work described in this thesis, I have shown that both transcriptional and post-transcriptional regulation are important during activity-dormancy cycling. This hypothesis may have been explored before, but not on the global scale discussed in paper I. Additionally, we have identified the transcription factor AINTEGUMENTALIKE1 as a crucial element in short-day induced growth cessation. On the basis of the results reported in paper II, we conclude that AINTEGUMENTALIKE1 works upstream of the core cell cycle machinery and relays environmental signals by controlling the transcription of D-type cyclins thereby providing a crucial link between day-length sensing and growth arrest.

*-How does the responsiveness to plant hormone IAA change during short day induced growth cessation and dormancy?*

The plant growth regulator auxin is known to play an extremely significant role in controlling the seasonal growth cycle. As stated in the introduction, auxin is implicated in every aspect of plant growth and development. The ability of trees to modulate auxin responsiveness over the course of the year has been extensively documented, but it has proved harder to determine the mechanisms behind this phenomenon. In paper III, we show that the loss of auxin response is gradual, that specific subsets of auxin responsive genes lose their responsiveness at distinct stages during activity-dormancy transitions, and that one subset of auxin-responsive genes is always responsive. Thus, the loss of auxin responsiveness is a specific process rather than a general one. The loss of auxin response is in part achieved by modulation of the proteasomal degradation of AUX/IAA transcriptional repressors; the precise mechanism by which this modulation is effected remains to be determined. Another key feature of this loss of response is a significant correlation between the number of AuxREs in a promoter and at what stage response is lost. Overall, I conclude that the loss of auxin response is achieved by transcriptional control, since it is accompanied by the stabilization of transcriptional repressors and is affected by the number of regulatory sequences in the promoters of specific auxin responsive genes.

*-How is cambial growth and maintenance affected by modulation of IAA response?*

Paper III dealt with activity-dormancy transitions in the cambium and the natural modulation of the IAA response during seasonal growth cycles. The results presented in that paper, together with other findings previously reported in the literature, suggested that auxin might be important in maintaining the stem cell pool in the vascular cambium during both active growth and during dormancy. Paper IV describes a study of the molecular aspects of auxin control over wood formation, using transgenic trees with an artificially modulated auxin response. The results obtained suggest that in addition to its influence on meristem activity, auxin may be important in imposing positional restrictions on the stem cell pool. It was also shown that perturbing the auxin response has severe effects on cell division and developmental processes during wood formation.

*-Can we find common regulatory factors controlling cessation of growth and maintenance of the vascular cambium?*

This is the key question, and answering it is perhaps the ultimate goal of this thesis. Can we draw connections between the seemingly diverse processes of seasonal growth cycles and wood formation on a molecular level? I am confident that in this work I have shown that the processes that make a tree a tree are closely connected and share several features. The AINTEGUMENTALIKE1 transcription factor is part of a signaling pathway that relays environmental signals to the core cell cycle machinery, but is also involved in regulation of cambial growth. Additionally, modulations of auxin signaling like those that occur naturally in response to seasonal changes were shown to have severe effects when artificially imposed on actively growing trees. Despite the vast amount of work that has been conducted on auxin signaling and wood formation, it is clear that much remains to be learned concerning the details of auxin signaling and its role in wood formation and seasonal growth cycles.

The future holds many new and enticing possibilities for tree research. The sequencing of more tree genomes will make it possible to further investigate the properties that set trees apart from other plants. In particular, these resources can be used to explain why poplar and spruce have many traits in common that are seemingly absent in *Arabidopsis*, even though poplar is more closely related to *Arabidopsis* than to the phylogenetically distant conifers.

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