Developmental Biology of Wood Formation – Finding Regulatory Factors Through Functional Genomics

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Abstract

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The wood-forming vascular cambium is responsible for the production of a large part of the biomass on this planet. Yet, there is only limited knowledge on how cell proliferation and differentiation in the cambial meristem are regulated. In this thesis the wood-forming tissues of aspen were used as a model system to identify and characterize molecular factors related to cambial meristem activity.

An important regulator of cambial meristem activity is the plant hormone auxin. As polar transport is crucial for the delivery of auxin to the cambial zone, we identified homologues of known regulators of polar auxin transport and described their regulation by environmental and developmental factors. Translating changes in auxin concentration into changes in gene expression involves members of the *Aux/IAA* gene family. Aspen homologues of *Aux/IAA* genes were cloned and found to be expressed in a highly tissue-specific fashion, which is further influenced by developmental events and changes in the environment.

A major response of trees to environmental changes is the suspension of meristematic growth during winter dormancy. A comparison of gene expression in active and dormant cambia revealed dramatic changes in the transcriptome including the expression of many cold and stress related genes during winter.

During the process of wood formation, cells originating in the vascular cambium go through an elaborate process of cell division, cell expansion, secondary wall formation and programmed cell death. Large-scale analysis of gene expression was used to create transcriptional maps of the differentiation process. This extensive dataset allowed us to confirm the proposed functions of various genes involved in wood formation, assign other known genes to specific stages along the developmental gradient and identify a large number of novel potential regulators of wood formation. The data further suggest that the cambial meristem shares regulatory mechanisms with other meristems in addition to its own, specific factors.

Key words: Populus tremula x tremuloides, development, microarrays, stem cells, AUX1, PIN1

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The most exciting phrase to hear in science,
the one that heralds the most discoveries,
is not "Eureka!", but "That's funny..."

Isaac Asimov (1920-1992)

Contents

Appendix

List of Papers

The present thesis is based upon the following papers, which will be referred to by their Roman numerals:

I. Schrader, J., Baba, K., May, S.T., Palme, K., Bennett, M., Bhalerao, R.P. & Sandberg, G. (2003) Polar auxin transport in the wood-forming tissues of hybrid aspen is under simultaneous control of developmental and environmental signals. *Proceedings of the National Academy of Sciences of the United States of America* 100(17):10096-101

II. Moyle, R., Schrader, J., Stenberg, A., Olsson, O., Saxena, S., Sandberg, G. & Bhalerao, R.P. (2002) Environmental and auxin regulation of wood formation involves members of the Aux/IAA gene family in hybrid aspen. *Plant Journal* 31(6):675-85.

III. Hertzberg, M., Aspeborg, H., Schrader, J., Andersson, A., Erlandsson, R., Blomqvist, K., Bhalerao, R., Uhlen, M., Teeri, T.T., Lundeberg, J., Sundberg, B., Nilsson, P. & Sandberg, G. (2001) A transcriptional roadmap to wood formation. *Proceedings of the National Academy of Sciences of the United States of America* 98(25):14732-7.

IV. Schrader, J., Hertzberg, M. Berglund, A., Nilsson, P. & Sandberg, G. (2003) A high-resolution transcript profile across the cambial meristem identifies potential regulators of vascular cell differentiation. *Manuscript*

V. Schrader, J., Moyle, R., Bhalerao R., Hertzberg, M., Lundeberg, J., Nilsson, P. & Bhalerao, R.P. (2003) Cambial meristem dormancy in trees involves extensive remodelling of the transcriptome. *Manuscript*

Introduction

Plant growth displays an astounding degree of plasticity; plants can adjust their development in response to a multitude of environmental factors like light, temperature, wind or nutrients. They are also able to compensate for damages caused by animals, fungi or bacteria. At the same time there is strong genetic control of plant development; a birch might be growing in a crack on a rock and not become larger than half a meter or it might grow on fertile ground and become 30 m tall with a massive crown and a thick stem. But both trees will always have the same characteristic leaf shape, colour of the bark and branching pattern that distinguish them from other species.

On a cellular level, plant growth requires the sequential execution of genetically encoded programs of cell division and differentiation but also the continuous adjustment of these programs according to the input from the plant's environment. Elucidating the molecular and physiological details of this process is a formidable task, to which this thesis aims to provide a small contribution.

Plant growth and development involves the continuous generation of new organs and tissues. This is achieved by populations of undifferentiated, proliferating cells which are located in specialized structures known as meristems. Meristems are the ultimate source of all plant tissues and they play an essential role in determining the architecture of the mature plant body. And because plant growth depends on meristems, they are the structures we have to study if we want to understand how plants achieve their developmental flexibility.

The vascular cambium is a lateral meristem found in species undergoing secondary growth. Located between phloem and xylem in tree stems it forms a continuous ring, which produces the characteristic regular files of secondary phloem and xylem cells. In its role as the wood-forming meristem of trees, the cambium is responsible for producing most of the plant biomass on this planet. Understanding cambial biology has consequently a considerable economical value. But above all the cambium presents a unique model system to study aspects of meristem identity and cell differentiation. The highly regular organization of the cambial region makes it possible to sample cells at different stages along a developmental gradient at high purity. These samples can then be analysed for metabolites, hormones or gene activity thus providing information on how gene expression translates into physiological changes, which finally lead to cell differentiation.

The activity of the cambium and other meristems is affected by a number of regulatory factors. External influences like light, temperature or nutrient availability can modulate developmental programs, as do internal cues like the ratio of crown size to root area and stem thickness or the transition to reproductive development. Environmental effects can cause profound changes in meristems as for example during winter dormancy when the normal proliferation is suspended completely and the meristems change their physiology to cope with freezing temperatures. Our picture on how all these regulatory signals reach the cambium and how their various effects are processed into differential gene activity is not very complete. One important player are plant hormones like auxin, cytokinin, gibberellin, or ethylene. Auxin has a particularly strong connection to meristem biology based on its effects on cell proliferation, differentiation and pattern formation. Auxin supply and signal

transduction are therefore important aspects which need to be considered when one tries to understand the biology of the cambial meristem.

Objectives

The importance of the plant hormone auxin for wood formation is well recognized and numerous studies have investigated the effects of changes in auxin supply on cambial activity. There are, however, a number of questions that need to be addressed at the molecular level:

Auxin is distributed in a steep concentration gradient across the wood-forming tissues where it influences the activity of the cambial meristem (Little & Savidge, 1987; Uggla *et al.*, 1996). Since the auxin gradient coincides with a developmental gradient of cambial derivatives it has been suggested that auxin might act as a morphogen in these tissues (Uggla *et al.*, 1998). The mechanisms responsible for the establishment and maintenance of this gradient are not well understood but are likely to involve active transport of auxin. Auxin transport is also a central element of the canalization hypothesis, which tries to explain vascular pattern formation (Sachs, 1991). Paper I tries to shed some light on the role of auxin transport in wood formation by analysing the expression of a family of auxin transport carriers in hybrid aspen.

Auxin can have different effects on plant growth depending on tissue and developmental stage. This implies that at some stage the auxin signal transduction pathway has to interact with information from tissue context, developmental status and environmental factors. Good candidates for a role in this integration mechanism are genes of the *Aux/IAA* family; I have therefore studied their expression patterns in the aspen stem as well as their response to environmental signals in Paper II.

As a cell progresses from the cambium to become a mature xylem or phloem cell it goes through a number of distinct developmental stages. Each stage involves the activation of specific genetic programmes like the cell cycle machinery during division or lignin biosynthesis during secondary wall maturation. Since the vascular cambium presents a unique system where it is easy to distinguish and sample cells at different developmental stages, these developmental stage-specific samples can be combined with functional genomic tools to map the genes involved in individual stages of cell differentiation. As a first step towards such a map we have used cDNA microarrays to identify genes involved in the differentiation of xylem cells and the specification of cambial meristem identity (Papers III and IV).

The activity of meristems is regulated in response to environmental signals. One such signal is perceived at the end of a growing season, when the decreasing day length and temperatures announce the arrival of winter. Trees respond to this environmental signal by suspending the activity of apical and cambial meristems. This transition into dormancy is a complex process that involves massive changes in the tree's physiology. Molecular data on this process is scarce, so in Paper V we used functional genomic tools to provide a first description of how the transcriptome of the cambial zone is affected by dormancy.

The remainder of the thesis is organized as follows:
I will begin by providing background information on processes that play a central role in this thesis like auxin transport and response, wood formation from the cambial

meristem as well as dormancy. Next is a section highlighting some of the aspects concerning methodology that have been of importance during the preparation of the thesis. The results part summarizes the data presented in Papers I-V and adds some additional information and interpretation. Finally, some concluding remarks and future prospects can be found in the last section.

Background

Auxin and plant development

Plant growth and development is profoundly affected by a class of growth regulators, or hormones, known as auxins. Indole-3-acetic acid or IAA is considered to be the major biologically active auxin although a number of related compounds with auxin activity like indole-3-butyric acid or 4-chloroindole-3-acetic acid have also been identified in various plant species (Davies, 1995). Unless otherwise stated, the terms auxin and IAA will be used synonymously.

Auxin is required for a multitude of processes during plant growth like cell expansion (Cleland, 1995; Fu & Harberd, 2003), cell division (Casimiro *et al.*, 2001) and pattern formation (Sabatini *et al.*, 1999; Sachs, 1991). The same simple molecule can have a variety of effects on plant cells depending on the exact tissue, organ and developmental stage-specific context. In case of tropic responses, for example, the role of auxin is both that of a messenger and an effector. The asymmetric distribution of auxin following gravistimulation in maize coleoptiles relays the message from the gravisensing cells in the endodermis to the cells in the elongation zone which are then directly stimulated by auxin to undergo cell elongation (Friml *et al.*, 2002b; Fukaki *et al.*, 1998; Philippar *et al.*, 1999). During embryogenesis auxin has a role in morphogenesis and pattern formation as indicated by several Arabidopsis mutants with altered embryo development that affect genes involved in auxin response (Hamann *et al.*, 1999; Hardtke & Berleth, 1998) or the generation of auxin gradients (Friml *et al.*, 2002a).

The effect of a hormone like auxin on a given cell is determined by the concentration of the hormone in that cell, the sensitivity of the cell to the hormone and the response potential of that cell which is determined by the tissue and developmental context. Like with other plant hormones, the concentration of auxin depends on the rates of synthesis, catabolism and conjugation. Traditionally, it was believed that developing apical tissues, young leaves and buds are the major sites of auxin biosynthesis (Davies, 1995). Recent evidence suggests, however, that all parts of young plants have the capacity to synthesize IAA (Ljung *et al.*, 2001a) but that nevertheless there is strong developmental control of both synthesis and catabolism (Ljung *et al.*, 2001b).

Polar Auxin Transport

In contrast to other plant hormones, auxin concentrations are also strongly affected by transport of the hormone (Lomax *et al.*, 1995; Rubery & Sheldrake, 1974). There are two major pathways for auxin transport. Polar auxin transport (PAT) is unidirectional, carrier-mediated, consumes energy and is specific for biologically active auxins (Lomax *et al.*, 1995). Additionally, auxin is also found in the phloem stream (Baker, 2000) where it can move relatively fast (5-20 cm/h) compared to the 5-20 mm/h found for polar transport (Friml & Palme, 2002). There are indications that both pathways may be linked since labelled IAA applied to leaves was initially loaded into the phloem but later detected in the PAT system. Thus phloem transport would be responsible for rapid, long-distance movement while PAT mediates the local redistribution of the hormone (Cambridge & Morris, 1996; Marchant *et al.*, 2002).

The mechanism for PAT can be explained by the chemiosmotic model, which states that IAA can enter the cell through the plasma membrane either by diffusion or with the help of dedicated influx carriers (Fig. 1). At the low apoplastic pH (≈pH 5.5) the weak acid IAA (pK_a 4.7) will exist mainly in its protonated form making it more lipophilic, thus facilitating diffusion across the membrane. Once inside the symplast, the higher pH (≈pH 7.0) will favour the ionic form, essentially trapping the molecule inside the cell. IAA can only exit the cell through specialized efflux carriers located in the plasma membrane. By controlling the location of these membrane carriers, the cell can regulate the direction of the auxin flux.

The existence of a dedicated influx carrier was originally inferred from physiological studies that showed auxin uptake to be saturable and dependent on the proton motive force (Lomax *et al.*, 1985; Rubery & Sheldrake, 1974). The efflux carrier on the other hand can be specifically inhibited by a number of drugs, with 1-N-naphtylphtalamic acid (NPA) and triiodobenzoic acid (TIBA) being the most widely used compounds. Since auxin efflux activity and NPA binding can be separated, it has been suggested, that the auxin efflux is mediated by separate carrier and NPA-binding proteins, linked by a third, labile component (Morris *et al.*, 1991).

Candidate molecules for the influx and efflux carrier

The details of the molecular machinery involved in PAT are still not completely resolved but Arabidopsis molecular genetics and mutants impaired in PAT have provided a number of candidate genes. The *aux1* mutant shows an agravitropic root phenotype, which can be rescued by growing seedlings on medium containing the membrane permeable auxin analog 1-naphtaleneacetic acid (NAA) (Marchant *et al.*, 1999; Yamamoto & Yamamoto, 1998). The phenotype of *aux1* can be mimicked by the application of specific inhibitors of auxin influx (Parry *et al.*, 2001). When the *AtAUX1* gene was cloned, its sequence showed high similarity to a family of aminoacid permeases (Bennett *et al.*, 1996). The structural similarity of auxin and the amino acid tryptophane together with the phenotype of the *aux1* mutant led to the conclusion that the AtAUX1 protein probably is the auxin uptake carrier, although direct evidence for this function is still lacking. The Arabidopsis genome contains three additional genes with a high degree of homology to *AtAUX1*, named *AtLAX1-AtLAX3*. No clear data on their biological function is available yet but

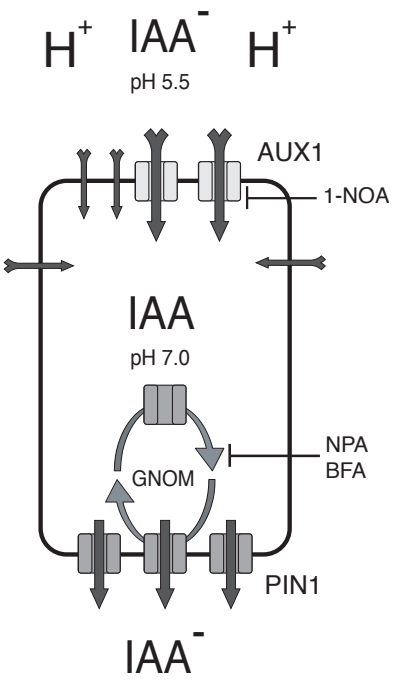

Fig. 1. Schematic representation of an auxin transporting plant cell. Auxin flux is indicated by black arrows. Light and dark grey boxes represent the auxin influx and efflux carriers, respectively. The efflux carrier cycles rapidly between the plasma membrane and an endosomal compartment, This cycling involves the GNOM protein and can be inhibited by drugs like NPA or BFA.

initial experiments suggest that they, too, are involved in auxin responses (Parry *et al.*, 2001).

Similar to the *AUX1* gene, Arabidopsis mutants also provided the most promising candidates for the efflux carrier. The *AtPIN1* gene was cloned from the corresponding *pin1* mutant which has a drastic phenotype where the inflorescence is reduced to a pin-like structure (Gälweiler *et al.*, 1998). Shortly thereafter several laboratories found a homologous gene named *AtPIN2* (or *EIR1/AGR1*) from a mutant deficient in root gravitropism (R. J. Chen *et al.*, 1998; Luschnig *et al.*, 1998; Müller *et al.*, 1998; Utsuno *et al.*, 1998). Like for AtAUX1, there is no direct biochemical proof that the AtPIN proteins are efflux carriers. An extensive body of evidence, however, strongly supports a role for PIN proteins as either permeases or closely associated regulatory components of the PAT machinery: i) The topology of PIN proteins is similar to transporters of the major facilitator class (R. J. Chen *et al.*, 1998; Luschnig *et al.*, 1998; Müller *et al.*, 1998; Palme & Gälweiler, 1999; Utsuno *et al.*, 1998). ii) Yeast cells overexpressing *AtPIN2* show increased resistance to fluoroindole, a toxic component with structural similarity to IAA (Luschnig *et al.*, 1998). These cells also retain less labelled IAA than control yeast (R. J. Chen *et al.*, 1998). iii) Plants mutant in PIN proteins show reduced PAT (Okada *et al.*, 1991; Rashotte *et al.*, 2000). iv) The phenotypes of several *pin* mutants can be phenocopied by auxin efflux inhibitors (Friml *et al.*, 2002b; Gälweiler *et al.*, 1998; Maher & Martindale, 1980). Even the *AtPIN* genes form a small family in Arabidopsis with a total of eight members.

A particularly nice confirmation of previous hypotheses on PAT with molecular methods was achieved by studying the subcellular localization of AtPIN and AtAUX1 proteins. According to the classical chemiosmotic model, the influx and

efflux carriers should be located at the apical and basal ends of the transporting cells, respectively. And this was indeed the case for both AtPIN1 in the inflorescence (Gälweiler *et al.*, 1998) and AtPIN2 and AtAUX1 in the root epidermis where influx and efflux carriers localized at opposite ends of the cells, in an orientation predicted by the known direction of auxin flux (Müller *et al.*, 1998; Swarup *et al.*, 2001).

The most basic version of the chemiosmotic model for PAT does not require the existence of a dedicated influx carrier, since protonated IAA can enter the cell directly through the membrane. Available data from knockouts in the putative influx carrier *AtAUX1* in Arabidopsis as well as the use of the influx-specific inhibitor 1-naphthoxyacetic acid (1-NOA) indicate however, that loss of influx carrier activity has an impact on root gravitropism (Bennett *et al.*, 1996) and organ initiation at the shoot apical meristem (Stieger *et al.*, 2002), thus demonstrating the biological relevance of a dedicated influx protein.

Recently, the classical chemiosmotic model for PAT has been challenged based on new data about the mechanism for PIN protein localization. AtPIN1 appears to be cycling rapidly between an endosomal compartment and the plasma membrane (Steinmann *et al.*, 1999). This cycling can be interrupted by mutations in the *AtGNOM* gene or by using the chemical Brefeldin A (BFA) which leads to internalization of PIN protein from the plasma membrane to endosomal compartments (Steinmann *et al.*, 1999). Surprisingly, the effect of PAT inhibitors like NPA on PIN1 was similar to that of BFA in that the cycling of the protein was blocked. It was also possible to phenocopy the effects of NPA by using BFA (Geldner *et al.*, 2001). This indicates that the rapid cycling is essential for correct functioning of the PAT machinery. The cycling could simply be a means to rapidly relocate the carrier protein to different parts of the membrane but it is also possible that PAT involves a mechanism where IAA is bound to membrane vesicles inside the cell and released by polar exocytosis (Friml & Palme, 2002). There are indeed a number of observations which suggest that PAT might more closely resemble a vesicle mediated secretion process similar to that of animal neurotransmitters rather than the classical plasma membrane permease model (Baluska *et al.*, 2003). Secretion-based PAT could also explain the failure to confirm the molecular function of AtAUX1 and AtPIN in biochemical assays testing for membrane permease function.

Auxin signal transduction

The auxin receptor

In order to exert its role as a plant growth regulator, the presence of auxin in a cell has to be sensed and this information has to be translated into changes in gene expression and protein activity. At the top of such a signal transduction cascade there has to be a receptor molecule. In the case of auxin response, only one good candidate exists to date, the auxin binding protein ABP1. Originally identified over thirty years ago in corn coleoptiles by affinity chromatography (Hertel *et al.*, 1972) there is now an extensive collection of data showing that ABP1 binds auxin at the extracellular face of the plasma membrane and that the protein can mediate auxin related effects such as the activation of proton pumps and membrane channels (Reviewed in Napier *et al.*, 2002). A knockout in the single copy Arabidopsis *AtABP1* gene leads to an embryo lethal phenotype in homozygous plants, supporting an important role for ABP1 in

plant development (J. G. Chen *et al.*, 2001b). On the other hand the existing data suggests that ABP1 clearly plays a role in auxin-induced cell elongation but that cell division and the activation of auxin inducible genes are probably mediated by a different receptor (J. G. Chen *et al.*, 2001a). There is a possibility that components of the PAT pathway also act as auxin receptors although no experimental evidence in this direction is available to date (Friml & Palme, 2002).

Early response genes

One very important aspect of auxin response is the modulation of gene expression. Several screens for auxin responsive genes have been performed, resulting in numerous transcripts which are up or down regulated upon addition of auxin (Hagen, 1995; Sitbon & Perrot-Rechenmann, 1997). The most extensively studied subgroup are the so-called early response genes whose induction occurs within minutes of exposure to elevated IAA levels and does not require protein synthesis. These early genes represent three unrelated classes of proteins, the *SAUR*, *GH3* and *Aux/IAA* families. Although all three families were identified in the mid 1980s (Hagen *et al.*, 1984; McClure & Guilfoyle, 1987; Theologis *et al.*, 1985) information on the function and activites of *SAUR* and *GH3* genes is still very limited while research on *Aux/IAA* genes has progressed more rapidly. The *SAUR* genes form families in many plant species with more than 70 genes found in the Arabidopsis genome. They encode small proteins of 9-10 kDa and the corresponding transcripts are rapidly, and in many cases specifically, induced by IAA. There are also several *SAUR* genes that can be induced by cycloheximide or cytokinins. The role of these genes in plant development remains elusive, however (Hagen & Guilfoyle, 2002). The *GH3* family has 19 members in Arabidopsis and homologues are found in other plants as well as in blue-green algae and even vertebrates, but not in yeast or Drosophila. Most *GH3* genes tested so far were auxin inducible. The predicted proteins with masses of 65-70 kDa do not offer any clues for the function of *GH3* in plants but the identification of the Arabidopsis mutants *far-red-insensitive 219* (*fin219*) and *dwarf in light 1* (*dfl1*) affecting two GH3 genes provide a first hint on a connection between light and auxin signalling mediated by *GH3* genes (reviewed in Hagen & Guilfoyle, 2002).

Aux/IAA genes

Like the *SAUR* and *GH3* genes, even the first members of the *Aux/IAA* family were identified in auxin treatment experiments. Their response to IAA is specific, very rapid, in the order of minutes, and their induction does not require protein synthesis. Indeed, inhibiting protein synthesis with cycloheximide (CHX) causes an increase in *Aux/IAA* gene expression, which led to the conclusion that their activity is controlled by a labile repressor, which disappears rapidly when its synthesis is blocked by CHX (Abel *et al.*, 1995; Abel & Theologis, 1996). *Aux/IAA* genes are present as multigene families in a number of plant species but not in other organisms; the Arabidopsis genome harbours 29 members (Liscum & Reed, 2002). The predicted proteins range in size from 20 – 35 kDa, are short-lived and nuclear localized (Abel *et al.*, 1994; Abel & Theologis, 1995). The analysis of multiple Aux/IAA proteins identified four conserved motifs referred to as domains I, II, III and IV (Fig. 2; Abel *et al.*, 1995;

Ainley *et al.*, 1988). While first identified based on sequence conservation only, it has later been possible to assign different functions to the individual domains. Domains I, III and IV are involved in protein-protein interactions, in case of domain I this has been shown for homodimerization while domains III and IV seem to be involved in both homo- and heterodimers (Abel & Theologis, 1995). Domain IV also contains a functional nuclear localization signal (Abel & Theologis, 1995). Domain II on the other hand is involved in the controlled degradation of Aux/IAA proteins. A fusion of domain II to the luciferase protein leads to rapid degradation of the reporter while mutations in the highly conserved qvvgWPPirs motif of domain II can increase the half life of Aux/IAA proteins (Colon-Carmona *et al.*, 2000; Ouellet *et al.*, 2001; Ramos *et al.*, 2001; Worley *et al.*, 2000).

Several Arabidopsis mutants with a stabilizing mutation in domain II of an *Aux/IAA* gene show auxin related phenotypes, indicating that the degradation of Aux/IAA proteins is essential for normal auxin signalling (Worley *et al.*, 2000). The link between protein degradation and auxin signalling was further strengthened when it was shown that auxin could affect the degradation rate of Aux/IAA proteins (Gray *et al.*, 2001; Zenser *et al.*, 2001) and that domain II can interact with the ubiquitin ligase SCF^{TIR1} in an auxin dependent manner (Gray *et al.*, 2001).

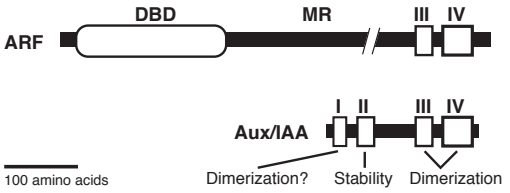

Fig. 2. Domain structure of ARF and Aux/IAA proteins. The conserved domains I-IV are represented by white boxes and their proposed functions are indicated. DBD, DNA binding domain; MR middle region of ARF proteins, responsible for transcriptional activation or repression.

Auxin response factors

The promoters of many auxin inducible genes contain so-called auxin response elements (AuxREs) which contain the motif TGTCTC. When present in direct or palindromic repeats or together with other constitutive elements, the TGTCTC motif can confer auxin responsiveness to a minimal promoter (Ulmasov *et al.*, 1997a, 1999). Using palindromic repeats of TGTCTC as bait in a yeast one-hybrid system, Ulmasov *et al.* (1997) were able to isolate the auxin response factor AtARF1 from Arabidopsis and show that it binds AuxREs specifically. Analysis of the complete Arabidopsis genome later identified 22 ARF transcription factors (Hagen & Guilfoyle, 2002). *AtARF1-AtARF10* are expressed in most major plant tissues and their expression is not influenced by auxin (Hagen & Guilfoyle, 2002). Analysis of ARF protein sequences identified a plant-unique DNA binding domain at the N-terminus as well as potential transcriptional activation or repression sites in the middle region (Fig. 2; Guilfoyle, 1998; Ulmasov *et al.*, 1997a). Surprisingly, the ARF proteins also contain two domains with high similarity to domains III and IV of the *Aux/IAA* genes. This suggested that ARF proteins might form heterodimers with Aux/IAAs and other ARF proteins; a hypothesis that was later confirmed experimentally (Kim *et al.*, 1997; Ouellet *et al.*, 2001; Ulmasov *et al.*, 1999).

The current model for auxin regulated gene expression

Findings that overexpression of some *Aux/IAA* genes could repress the transcription from an AuxRE (Ulmasov *et al.*, 1997b) together with the ability of Aux/IAA and ARF proteins to form dimers and the auxin dependent degradation of Aux/IAA proteins can be combined into a model for auxin regulated gene expression (Fig. 3; Hagen & Guilfoyle, 2002). At low auxin concentrations, Aux/IAA proteins dimerize with ARF transcriptional activators. This prevents transcription of early genes either by preventing binding of the ARF to the target AuxRE or - if the Aux/IAA-ARF dimer is bound to the AuxRE, by active repression from the Aux/IAA protein. Increasing auxin levels leads to the degradation of Aux/IAA genes and possibly also to their dissociation from ARFs. Now early gene transcription will be increased due to the lack of an Aux/IAA repressor and/or the ability of ARF activators to bind as homodimers. Since many *Aux/IAA* genes are early genes themselves, increased expression of these genes might, after some delay, feed back on their own transcription and down-regulate expression again.

This basic model does not cover all the complexities of Aux/IAA and ARF mediated signalling. A complete model will have to accommodate the fact that some ARF proteins act as repressors, not activators. It will have to be clarified which of the many potential homo- and heterodimers of Aux/IAA and ARF proteins actually occur *in vivo*. Neither is it clear how the increased interaction of Aux/IAAs and SCF^{TIR1} is achieved and whether auxin controls dissociation of Aux/IAAs from ARFs prior to degradation. Recent data suggest the involvement of peptidyl-prolyl isomerase in auxin-mediated binding of Aux/IAAs to SCF^{TIR1} (Dharmasiri *et al.*, 2003).

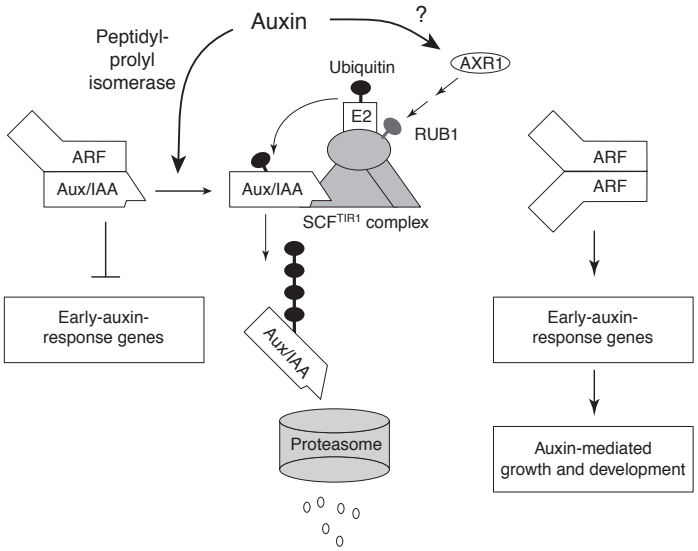

Fig. 3. Model for auxin signal transduction. The expression of early response genes is blocked by the association of Aux/IAA repressor proteins with ARF transcription factors. Auxin promotes the ubiquitination of Aux/IAA proteins via the SCF^{TIR1} complex, leading to their rapid degradation. In the absence of Aux/IAA repressors, the early genes will get activated by ARF transcription factors. The exact point of auxin input into this pathway is currently unknown but there are indications that auxin affects the binding of Aux/IAA proteins to the SCF ubiquitination complex. (Adapted from Gray & Estelle, 2000)

From molecular mechanisms to developmental function

In the preceding paragraphs I have described findings that led to a better understanding of the molecular mechanisms of both PAT and auxin signalling. But while understanding molecular mechanisms is indeed a rewarding research objective in itself, it is equally interesting to know the role of PAT and auxin signal transduction genes in the context of plant growth and development. Most of the breakthrough information concerning the effect of auxin on plant growth and development during the recent years has been provided by the analysis of Arabidopsis mutants. In the following paragraphs I will try to highlight some of the details and concepts, which have emerged from this analysis.

Auxin and embryo development

There is an emerging picture of how auxin and PAT contribute to pattern formation during embryogenesis and post-embryonic development. Mutations in the *AtPIN4* efflux carrier gene show defects in the patterning of the root meristem with aberrant cell divisions and an enlarged quiescent centre. These changes can already be detected during embryo development where the asymmetric division of the hypophysis happens prematurely. The alterations in development are paralleled by changes in auxin distribution as judged by an auxin responsive reporter gene. A well-defined auxin maximum below the quiescent centre becomes enlarged and diffuse in *Atpin4* mutant roots (Friml *et al.*, 2002a). These phenotypes can be explained by a failure to establish a correct auxin gradient due to the lack of AtPIN4-mediated efflux carrier activity in the *Atpin4* mutant.

Mutations in the *MONOPTEROS* (*MP*) and *BODENLOS* (*BDL*) genes affect the initiation of the root meristem (Berleth & Jürgens, 1993; Hamann *et al.*, 1999). In either single mutant as well as in the double mutant the hypophysis fails to undergo an asymmetric division necessary for the creation of the quiescent centre. Both genes were cloned and found to encode AtARF5 and the Aux/IAA protein AtIAA12, respectively (Hamann *et al.*, 2002; Hardtke & Berleth, 1998). Both proteins are expressed in the same cells and were able to interact in a yeast two-hybrid assay and are thus the first candidates for a functional pair of ARF and Aux/IAA proteins (Hamann *et al.*, 2002). The conclusion drawn from these mutants is that an Aux/IAA- and ARF-mediated auxin signal is required for the correct patterning of the embryo root.

Taken together these data suggest that auxin can provide positional information during root development through the PIN-mediated formation of gradients and a signalling pathway involving *Aux/IAA* and *ARF* genes. Many more examples are available, where mutations in individual members of auxin transport or ARF genes lead to distinct developmental defects:

AtPIN2 and AtAUX1 play a role in gravitropic response and lateral root formation (Marchant *et al.*, 1999; Müller *et al.*, 1998). Shoot gravitropism involves the *AtPIN3* gene (Friml *et al.*, 2002b). The *ettin* (*AtARF3*) mutation affects floral patterning (Sessions *et al.*, 1997) while *nph4* (*AtARF7*) has defects in translating auxin gradients into differential growth (Harper *et al.*, 2000).

In case of the *Aux/IAA* genes the situation is much less clear. Most of the mutations identified in *Aux/IAA* genes were gain-of-function stabilizing mutations in domain II. The corresponding plants show pleiotropic and often overlapping phenotypes

making it difficult to assign specific functions to individual genes. Loss-of-function alleles on the other hand seem to have only very subtle effects on the plants (Liscum & Reed, 2002).

Thus, while some of the pathways start to emerge with some clarity, many of the PAT or auxin response genes are still awaiting the assignment of a specific function. Furthermore, the functions of individual genes might vary depending on tissue and developmental context. Some gene family members might be functionally redundant while others might interact in subtle ways, which will further complicate their functional characterization.

Wood formation

Wood is a material with a vast array of applications, and its use as a construction material and for paper production is of significant economical importance. Different tree species produce wood of very different quality with a wide variation in parameters like density, colour, tensile strength, elasticity, fibre length and diameter or durability. Despite this wide spectrum of properties, the basic makeup of wood is very uniform and the observed variation is the result of subtle differences in cell division, cell expansion or cell wall formation as wood is made by the vascular cambium.

Origin and structure of the vascular cambium

The first anatomically observable stage of vascular differentiation in shoots of growing plants is the formation of provascular strands at the shoot apex connecting emerging leaf primordia with the existing vasculature. In the subapical elongation zone the innermost cells of the procambium will differentiate into protoxylem elements. Further down along the stem metaxylem will form in more centrifugally located cells. In parallel proto- and metaphloem develops from the centrifugal parts of the procambium (Esau, 1960; Steves & Sussex, 1989). In species undergoing secondary growth, a layer of undifferentiated procambium remains between the metaxylem and the metaphloem. These cells constitute the fascicular cambium, which will produce secondary xylem and phloem through periclinal (tangential) divisions. Later, parenchyma cells located between the vascular strands will de-differentiate and form the interfascicular cambium connecting the fasciular cambia of individual vascular bundles. At this stage there is a complete cylinder of cambial cells extending along the entire length of the elongated root and shoot - the vascular cambium (Esau, 1960; Larson, 1994; Steves & Sussex, 1989).

The cells in the vascular cambium increase the diameter of the shoot by periclinal divisions, producing the regular files of xylem and phloem cells characteristic for secondary growth (Fig. 4). Since the increase in stem diameter is paralleled by an increase in girth this is accounted for by occasionally adding new files through anticlinal (radial) divisions in the cambium.

The maturation of a xylem cell originating in the cambium can be subdivided into a number of distinct developmental zones based on anatomical and cytological cues (Fig. 4). Following cell division, cells enter the expansion zone and enlarge both radially and longitudinally to their final sizes. Next, the secondary cell wall is formed which is subsequently lignified. The final stage involves programmed cell

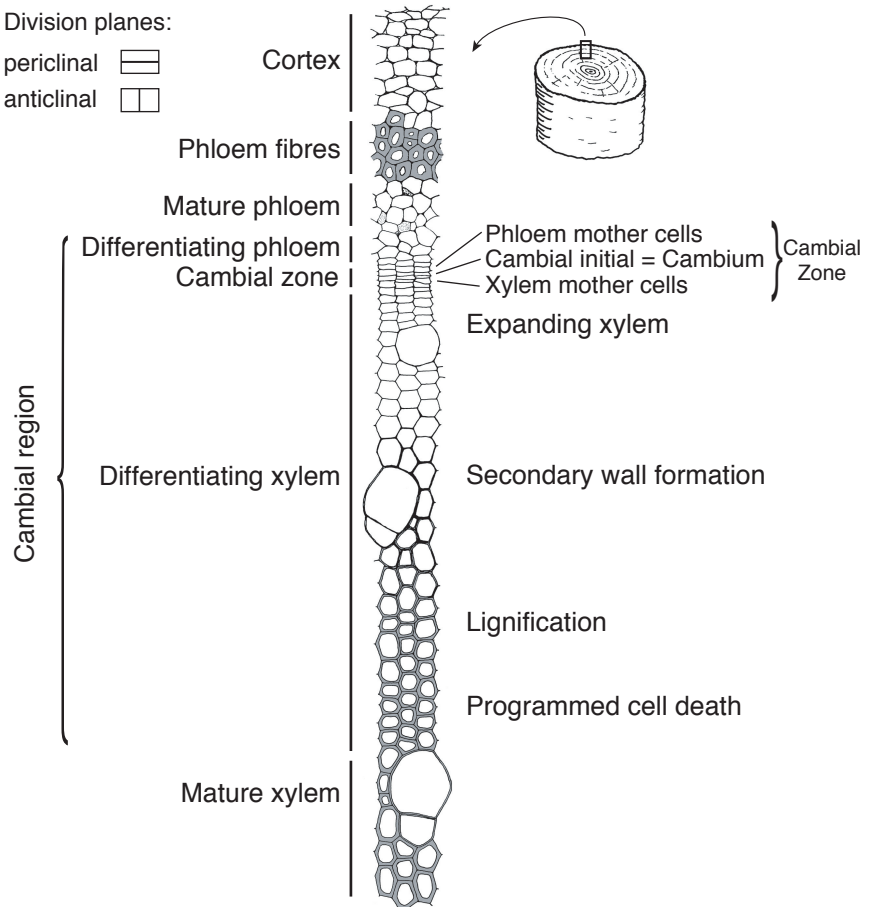

Fig. 4. Terminology of wood-forming tissues.

death of vessels, tracheids and fibres. In angiosperms, fibres and vessels mature at different rates leading to an overlap of the different zones. The axial elements of fibres, tracheids and vessels are responsible for mechanical support and water conductance. Wood also contains a system of ray cells, which play a role in radial conductance and storage of nutrients.

Cambial initials and stem cells

The exact cellular organization of the vascular cambium has been subject of a long-standing debate, which is still not completely resolved to date (reviewed in Larson, 1994). The main controversy is concerned with the existence and position of a cambial initial, which serves as the ultimate source of all the cells in a radial file. The uniseriate theory originally proposed by Sanio (1873) describes the existence of a single cambial initial per radial file (Schmid, 1976). This initial would divide continuously to produce xylem and phloem mother cells, which in turn divide only once or twice before proceeding to maturation. On the other side Raatz (1892) and others believed in a multiseriate structure of the cambium where all cells have an equal capacity to divide (Catesson, 1980). Not surprisingly, there are corresponding differences in the terminology used to identify the different layers of the cambium.

In this thesis I will adopt the definition of Wilson (reproduced in Larson, 1994 p. 68) where the term cambium denotes the layer of initial cells while the initials together with the dividing xylem and phloem mother cells are referred to as cambial zone (Fig. 4).

One of the reasons for the controversy concerning the cambial initial lies in the difficulty of unequivocally identifying this cell as there is no clear difference in anatomy or ultrastructure between the proposed initial and the adjacent mother cells (Larson, 1994 p. 64). There is general agreement that more than one cell in the cambium is capable of division and that initial cells in adjacent files need not be tangentially aligned. It is further generally accepted that only the initial is capable of undergoing anticlinal divisions to initiate new cell files. But the matter of debate is whether the cambial initial is biological reality or just a theoretical concept. The question is whether there exists one single dedicated initial cell per radial file, which is distinct from all other cells in that file or whether any member of a subset of cells in every file can perform the functions we ascribe to the initial. This argument is not purely academic either, as the two different models imply different mechanisms for the regulation of cambial initial identity, as will be detailed below.

In the literature describing the architecture of apical meristems, the term 'stem cell' is frequently used to describe the undifferentiated initial cells that are the ultimate source of all tissues originating from the meristem (Gross-Hardt & Laux, 2003; Nakajima & Benfey, 2002; Traas & Vernoux, 2002). The stem cell concept was originally developed for animals where only stem cells have the capacity to produce all the differentiated cells of a certain tissue or organ while mature cells normally lose this ability. In plants, on the other hand, most living cells are able to regenerate an entire plant, if placed in an appropriate environment (Steves & Sussex, 1989). Such an environment exists in the centres of the shoot and root apcial meristems where groups of initials – the plant stem cells – are able to generate all the cells of the mature plant body. It follows from this that being a stem cell in plants is a reversible state, which is imposed upon a cell by its environment.

Using the above definitions, even the cambial zone should contain stem cells and the question of a uni- or multiseriate cambium translates into the question whether there is a single stem cell – the cambial initial – or a pool of stem cells in the cambial zone. Similar questions have been asked about the identity of initials or stem cells in the shoot and root apical meristems. The powerful tool of Arabidopsis developmental genetics has provided a lot of insight into the regulation of apical meristems (Doerner, 2003; Fletcher, 2002; Nakajima & Benfey, 2002); so the paradigms established there might provide the basis for elucidating the regulation and number of cambial stem cells.

In the shoot apical meristem (SAM) the stem cells form a group of relatively slowly dividing cells located in the central zone (Fig. 5). Their identity is regulated on a population level so that cells inside the stem cell zone remain undifferentiated while cells that get pushed out into the adjacent peripheral zone will proliferate and undergo differentiation. The stem cell population is characterized by the expression of the *CLAVATA3* (*CLV3*) gene (Fletcher *et al.*, 1999). The CLV3 protein can move to a group of cells termed the organizing centre, which is located at the bottom of the central zone. There it is bound by the CLAVATA1 (CLV1) receptor kinase, which in its ligand-bound state becomes a negative regulator of the homeobox gene *WUSCHEL* (*WUS*) (Brand *et al.*, 2000; Laux *et al.*, 1996; Rojo *et al.*, 2002). WUS

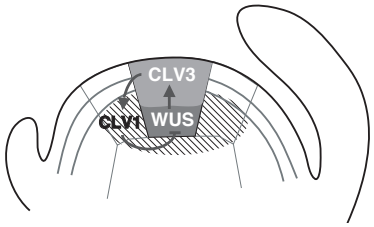

Fig. 5. Model for stem cell maintenance in the shoot apex of Arabidopsis. Shading indicates stem cells (light gray) organizing centre (dark gray) and domain of CLV1 expression (hatched). See text for further details.

in turn is required for maintenance of the *CLV3*-expressing stem cell pool, forming a feed back loop (Schoof *et al.*, 2000). This loop ensures a stable size of the stem cell pool: If the stem cell population becomes too large, it will produce more CLV3, which will down regulate WUS. Lower levels of WUS will then result in a smaller stem cell pool (for reviews see Gross-Hardt & Laux, 2003; Nakajima & Benfey, 2002).

In the root apex the situation is somewhat different. At the heart of the root meristem the quiescent centre forms a group of undifferentiated, slowly dividing cells. Surrounding the quiescent centre is a layer of initials, which will give rise to the different tissue layers of the root (Dolan *et al.*, 1993). The cell divisions in these initials or stem cells are always asymmetric; one daughter will undergo further divisions and differentiate into a functional root cell while the other, which is in contact with the quiescent centre, retains its identity as a stem cell. Studies using laser ablation have demonstrated that the quiescent centre is the source of a signal that prevents the differentiation of surrounding stem cells (Sabatini *et al.*, 2003; van den Berg *et al.*, 1997).

If we apply these models to the situation in the vascular cambium, then the uniseriate theory would resemble the situation in the root, where stem cell identity is regulated at the level of an individual cell (Fig. 14A,C), while the multiseriate model is more in line with what we find in the SAM where stem cell fate is defined on the level of a cell population which undergoes asymmetric divisions (Fig. 14B,D). In order to resolve the question about the specification of cambial initials, we will need to employ modern molecular and genetic tools. First steps towards this goal are discussed in Paper IV and the Results section.

Auxin and wood formation

Auxin seems to be involved whenever plant cells divide and differentiate, therefore it is not surprising that even the activity of the vascular cambium is dependent on auxin. If auxin supplies are manipulated by decapitation, debudding or the application of auxin or PAT inhibitors there is a subsequent effect on wood production (Little & Pharis, 1995; Little & Savidge, 1987; Savidge, 2001; Sundberg *et al.*, 1994). A detailed quantification of auxin concentrations in the cambial region revealed a steep concentration gradient of the hormone in both gymnosperm as well as angiosperm species (Tuominen *et al.*, 1997; Uggla *et al.*, 1996). This led to the suggestion that the role of auxin in wood formation could be that of a morphogen (Uggla *et al.*, 1998). In the centre of the cambial zone, where IAA concentration is at a maximum, auxin would promote cell division. As cells get displaced towards the middle of the stem, they experience subsequently lower auxin concentrations, which would then lead to cell expansion and further differentiation. Such a model is supported by studies with transgenic aspen overexpressing IAA biosynthetic

genes. These plants show a wider auxin gradient with a lower peak concentration. Correspondingly the rate of cambial cell division is lower but fibre diameter is increased. The interpretation of these data was that the lower peak concentration was responsible for the decreased rate of cell division while the wider auxin gradient meant that cells experience expansion-promoting auxin concentrations for a longer time thus resulting in larger cells (Tuominen *et al.*, 1997). On the other hand auxin is not the sole, and maybe not even the most important determinant of cambial organisation. No specific auxin concentration could be associated with a specific developmental stage (Tuominen *et al.*, 1997), nor did the overproduction of IAA in the cambium alter the basic order of developmental zones in the cambial region. This implies that there have to be additional mechanisms like cell-cell communication via moving molecules (Lenhard & Laux, 2003; Nakajima *et al.*, 2001), which are involved in patterning the wood-forming tissues.

Another example of a process where auxin could act as a patterning agent is the establishment of the primary vascular network connecting all parts of the plant. The vascular network in leaves is a particularly beautiful example of such pattern formation (Nelson & Dengler, 1997). Over the years, two basic theories have emerged that try to explain the mechanism underlying the observed patterns. According to the diffusion-reaction prepattern hypothesis (Koch & Meinhardt, 1994; Meinhardt, 2003) small initial variations of an activator molecule can be amplified through a positive feedback mechanism coupled to long-range inhibition by a fast-diffusing antagonist. The activator would induce both its own synthesis and the differentiation of vascular tissue while the antagonist ensures a certain minimum distance between sites of activator production. In computer simulations such a system can recreate a variety of patterns found in animals and plants including leaf vasculature and phyllotaxis (Meinhardt, 2003). In the case of vascular development the activator could be auxin while there is no candidate for a specific inhibitory substance as yet (but see also Parker *et al.*, 2003).

The signal flow canalization hypothesis, on the other hand, predicts that small initial variations in the auxin transport capacity of cells would be amplified by a positive feedback of auxin on PAT (Sachs, 1991). A transporting cell would induce transport in an adjacent cell along the same transport vector and remove auxin from other surrounding cells, thereby creating channels for auxin flux. The elevated auxin level in the channels would then induce vascular differentiation.

While both theories are able to explain certain aspects of vascular differentiation, they fail to account for all the observed features of vascular anatomy (for a detailed discussion see Nelson & Dengler, 1997). The reality might very well be a combination of both models. It is clear from the available data, however, that auxin and PAT play an important role in establishing and patterning the vascular system (Mattsson *et al.*, 1999; Ye, 2002).

Molecular regulators of vascular differentiation

The process of cell differentiation as we observe it in the cambial region is the result of the sequential execution of genetic programs that are necessary for steps like cell division, cell expansion or secondary wall formation. Understanding how the timely execution of these programs is achieved, is one of the central questions of current plant research. On the anatomical level, our picture of the events involved

in xylem and phloem differentiation is fairly comprehensive (Esau, 1960; Larson, 1994). From a molecular point of view, however, we have barely begun to scratch the surface. There is considerable knowledge on downstream processes like cell expansion (Cosgrove *et al.*, 2002; Ketelaar & Emons, 2001; Rose & Bennett, 1999), cell wall synthesis (Mellerowicz *et al.*, 2001; Reiter, 2002) or lignification (Boerjan *et al.*, 2003). Many of the key players in the cell cycle machinery have been identified and their connection to upstream regulators is starting to emerge (Menges *et al.*, 2002; Stals & Inze, 2001). But only very few genes are known that are involved in specifying vascular patterns, procambial or cambial identity, or the differentiation into either phloem or xylem.

A number of screens in Arabidopsis have been aimed at the identification of genes involved in vascular development (Candela *et al.*, 1999; Carland *et al.*, 1999; Carland & McHale, 1996; Deyholos *et al.*, 2000; Hanzawa *et al.*, 1997; Koizumi *et al.*, 2000; Parker *et al.*, 2003; Zhong *et al.*, 1999; Zhong & Ye, 1999). Several of the mutants isolated in these screens have islands of vascular tissue that are not connected to existing vasculature thereby challenging the signal flow canalization hypothesis which predicts the formation of continuous strands (Sachs, 1991). Instead they are more compatible with the diffusion reaction model. The *vascular network* (*van*) mutants for example, show a fragmented differentiation of provascular cells in leaves leading to a similarly fragmented development of minor veins (Koizumi *et al.*, 2000). Mutations in *continuous vascular ring 1* (*COV1*) lead to the formation of ectopic vasculature in the interfascicular regions of Arabidopsis inflorescences. The function of the *COV1* gene is unknown but it might be associated with the negative regulator of vascular differentiation predicted by the diffusion reaction model which inhibits the formation of new vascular strands close to existing ones (Parker *et al.*, 2003).

Alignment of vascular strands is also affected in the *cotyledon vascular patterning 1* (*cvp1*) mutants while overall growth is normal. Since the *CVP1* gene encodes a sterol methyltransferase, vascular differentiation might require the production of sterol-based signals. Alternatively the product of CVP1 is needed for membrane integrity resulting in improper positioning of membrane components involved in polarizing and aligning vascular cells (Carland *et al.*, 1999). A connection between vascular differentiation and sterols is also provided by the HD-ZIPIII family of homeodomain leucine zipper proteins with a lipid/sterol-binding domain. Mutations in two members of this family *phavoluta* (*ATHB9*) and *phabulosa* (*ATHB14*) affect the sterol binding domain and lead to defects in leaf polarity. *ATHB-8*, the first discovered member of this family, is one of the earliest known markers of provascular cell fate (Baima *et al.*, 1995; Baima *et al.*, 2001). *ATHB-8* expression can be identified in precursor cells of the vascular system before the appearance of any clear anatomical difference. Transgenic plants overexpressing *ATHB-8* show precocious xylem differentiation (Baima *et al.*, 2001). Plants mutant in *IFL1*, another HD-ZIPIII gene, lack the interfascicular fibres normally found in the inflorescence stem (Zhong & Ye, 1999). This lack of fibres might be a secondary effect of altered apical meristem activity though, as the *revoluta* allele of *IFL* leads to an abnormal ratio of apical versus non-apical meristem proliferation (Ratcliffe *et al.*, 2000; Talbert *et al.*, 1995). Analysis of HD-ZIP III genes in aspen and *Zinnia* further suggests an involvement of this gene family in vascular development (Hertzberg & Olsson, 1998; Ohashi-Ito *et al.*, 2002).

The role for cytokinins in vascular differentiation has recently been supported by the finding that the lack of phloem formation in roots of the Arabidopsis *woodenleg* mutants is due to a knockout of the cytokinin receptor *CRE1* (Inoue *et al.*, 2001; Mähönen *et al.*, 2000).

Apart from genes defined by mutations, there is considerable worldwide effort to identify genes involved in wood formation by means of functional genomics. The basis for this strategy are several large-scale EST sequencing projects in trees like aspen (R. R. Bhalerao, 2003; Kohler *et al.*, 2003; Sterky *et al.*, 1998), pine (Kohler *et al.*, 2003; Sterky *et al.*, 1998), spruce and birch (R. P. Bhalerao *et al.*, 2003). In case of aspen the genomic sequence is being read and should be available in early 2004 (JGI Populus sequencing project). But even annual species can contribute to wood genomics as indicated by specific EST projects in Arabidopsis (Zhao *et al.*, 2000) and *Zinnia elegans* (Demura *et al.*, 2002).

The *Zinnia* system for in vitro transdifferentiaton of leaf mesophyll cells to tracheary elements is probably the most promising non-tree model for xylem development (Fukuda, 1997; Milioni *et al.*, 2001). Based on 8000 *Zinnia* EST clones Demura and coworkers created a comprehensive map of the genes involved in various stages of tracheary element formation (Demura *et al.*, 2002). They expanded the previously available set of markers for the different stages of the transdifferentiation process and data from the *Zinnia TED3* gene suggests that these markers might also be useful in other organisms (Demura & Fukuda, 1994; Demura *et al.*, 2002). At present, the work in *Zinnia* and the experiments presented in this thesis (Papers III and IV) are the only examples of large scale functional genomics applied to problems of wood-formation but the EST collections mentioned above will surely lead to a number of exciting discoveries in the near future.

Environmental control of wood formation

The ability to temporarily suspend growth of meristems has been an essential step in the evolution of land plants. By putting their meristems into dormancy plants are able to survive harsh environmental conditions like draught, heat or cold which would otherwise destroy those delicate structures. The phenomenon of dormancy can be observed in a number of systems like seeds, terminal and axillary buds or lateral meristems. In this section I will focus on aspects of dormancy as they relate to the wood-forming vascular cambium.

Winter dormancy in trees can be divided into distinct stages (Lang, 1987). During ecodormancy growth is inhibited by unfavourable exogenous conditions. In temperate-zone species the most important signal triggering ecodormancy are short days (Howe *et al.*, 1995). At this stage growth inhibition is reversible if the plant is returned to a favourable environment (Vegis, 1964). The following stage of endodormancy is controlled by intrinsic factors and thus growth will not occur even under favourable conditions (Samish, 1954). Endodormancy can be broken by a chilling signal upon which the plant will return to be ecodormant. The final release of dormancy is then triggered by favourable growing conditions.

The dormant state has been the subject of numerous physiological, anatomical and ecological studies (for revies see e.g. Dennis, 1994; Lang, 1994; Samish, 1954; Vegis, 1964). Despite this effort details of the mechanism responsible for

establishment and maintenance of dormancy have remained elusive (Rohde *et al.*, 2000). Phytochrome-mediated sensing of the short-day signal and the involvement of the plant hormones abscisic acid and gibberellin are among the few generally recognized factors (Davies & Jones, 1991; Olsen *et al.*, 1995). Dormant plants also exhibit changes in their auxin physiology. Attempts to correlate changes in auxin levels with seasonal activity of the cambium gave conflicting results in different species (Little & Wareing, 1981; Savidge, 1991). A detailed analysis in Pinus, however, indicates that while the total amount of auxin in the stem does decrease, the concentration in the meristematic tissues is unaffected (Uggla *et al.*, 1996). Rather than changing auxin levels, dormancy appears to affect tissue sensitivity to the hormone. In endodormant stems, IAA is no longer able to promote the activity of the vascular cambium (Little & Bonga, 1974). But even PAT is affected in dormant stems, transport capacity becomes minimal and the polarity of the transport stream is lost (Lachaud & Bonnemain, 1984; Odani, 1975). But whether loss of auxin transport capacity and auxin responsiveness is the cause or an effect of cambial growth cessation has not been determined.

Transition to dormancy involves a number of physiological changes, some of which are associated with dormancy per se (Dennis, 1994), while others are related to the acquisition of cold hardiness (Rowland & Arora, 1997; Thomashow, 1999), stress response (Kozlowski & Pallardy, 2002) or the accumulation of storage compounds but a clear distinction between those processes is not always possible (Rohde *et al.*, 2000). The amount of molecular biological data on cambial dormancy in trees is limited, somewhat more is known about events taking place in buds (Rohde *et al.*, 2000). One example is the short-day responsive induction of a vegetative storage protein in aspen, which is believed to store nitrogen recovered from senescing leaves (Clausen & Apel, 1991; Coleman *et al.*, 1991; Zhu & Coleman, 2001). Cold tolerance, which develops in parallel with winter dormancy, involves resistance to dehydration (Close, 1997), and correspondingly members of the water stress induced family of dehydrins are also induced by short day treatment (Karlson *et al.*, 2003). In many trees a short day signal is the most important trigger for growth cessation and a likely sensor involved in this response is phytochrome A, based on studies using wildtype and transgenic aspen (Howe *et al.*, 1996; Olsen *et al.*, 1997). In the case of bud dormancy, the *PtABI3* was found to be essential for correct bud formation following the short day signal possibly in an interaction with abscisic acid (Rohde *et al.*, 2002).

The comparatively small amount of molecular biological work on dormancy in trees is likely connected to the lack of resources like gene sequences or mutants. Even cloning and sequencing tree orthologues of genes which have been well characterized in other species involves a considerable amount of work which might often preclude researchers from performing otherwise simple experiments.

Methodological considerations

The choice of a model system

The study of biological questions, especially those of fundamental importance, relies heavily on the use of model systems. By focusing research on a few selected species, progress can be made faster because experimental results can easily be transferred between labs and double work is avoided. The choice of a particular model organism is usually dictated by a number of factors; of particular importance is the availability of tools and resources such as genomic- and EST sequences, mutant collections, seed stocks, microarrays etc. for a given model organism. The overwhelming success of the small cruciferan Arabidopsis as the prime model for plant biology in the recent years can be attributed to the wide availability of genomic resources and an extensive collection of mutants.

There are also disadvantages with Arabidopsis, however. The small size of Arabidopsis plants – one of the main advantages when growing large numbers of individuals – makes it difficult to obtain samples with tissue specific resolution. There are further a number of biological processes, which cannot be studied in Arabidopsis simply because they do not normally occur during the life of an annual plant. Wood formation is such a process, as is the seasonal suspension of growth known as dormancy. Finally, the information obtained from the study of model systems must be tested on other species as well, since this is the only way to create theories which are valid for a large number of species and do not just describe the peculiarity of our model organism.

The main model used in this thesis is the aspen *Populus*, represented either by greenhouse-grown clones of *Populus tremula x tremuloides* or wild individuals of *Populus tremula*. Choosing a tree as a model system provides opportunities to study the process of wood formation, which is a rewarding research objecitve *per se* (Taylor, 2002). But on a more fundamental level, the layered, highly organized structure of the vascular cambium makes it an excellent model to study general principles of cellular differentiation. The technique of tangential cryosectioning allows the sampling of tissue at near-cellular resolution and the subsequent quantification of hormones, metabolites and gene transcripts. The process of dormancy, which trees like aspen undergo each winter, provides an additional angle for the study of cell division and differentiation, since here an environmental signal triggers a major change in the entire tree's physiology.

Microarray technology

A major part of the data presented in this thesis has been obtained using microarray technology (for a general introduction to microarray technology se the 'Chipping forecast' supplement to Nature Gentics, Phimister, 1999). Briefly, DNA probes for a large number of genes are immobilised on a solid surface, typically a glass microscope slide. RNA from the biological samples is reverse transcribed, labelled with fluorescent dyes and hybridized to the DNA on the array. The fluorescent signal from the probes is detected using a laser scanner, quantified with image analysis software and after appropriate normalization serves as a measure of gene expression

levels. Originally developed in 1995 (Schena *et al.*, 1995), microarrays have rapidly become a very important tool for modern molecular biology; a literature search using "microarray" as a key word produces about 3.000 hits for the year 2002 alone. The increased popularity of microarrays has been paralleled by significant improvements in the technologies involved in array and probe creation as well as experimental design and data analysis. Especially on the analysis side method development continues at a high rate, so that some of the techniques employed in this thesis, will already have been replaced by superior methods by the time of publication. In the following section I will try to highlight some aspects of the technological side of microarray analysis which have been of particular importance during the preparation of this thesis.

Details of the aspen microarrays

All experiments in this thesis have been performed on spotted cDNA microarrays using targets labelled with two colours (cyanine dyes Cy3 and Cy5) (Schena *et al.*, 1995), as opposed to the other major array platform, the Affymetrix system, which uses *in situ* synthesised oligonucleotides and hybridization with only one target at a time (Lipshutz *et al.*, 1999). The first generation array used in Paper III contained 2995 EST clones obtained from cambial zone and leaf cDNA libraries (Sterky *et al.*, 1998). Papers IV and V are based on the POP1 arrays with 13824 spots representing 13526 genes from six different libraries (R. R. Bhalerao, 2003). A number of control spots are present on each array, these include human and archaebacterial cDNA clones as well as the synthetic controls of the Lucidea Scorecard Kit (Amersham Biosciences, Uppsala, Sweden). The identity of the spotted clones has been verified by resequencing from the 5' end, and in the case of the POP1 array even the 3' end. Annotation of the clones is a work in progress, since the available sequence information continues to increase. Most of the data in this thesis (except that in Paper III) is based on contigs built from 120 000 EST sequences of PopulusDB (R. R. Bhalerao, 2003) which were BLAST searched against the Arabidopsis genome, GenBank and SwissProt. All clones that are discussed in any detail in the Papers have been manually BLAST searched to verify their annotation. For reasons of simplicity I will often refer to the clones spotted on the arrays as genes, fully aware of the fact, that they do not always represent single genes or that they might even be synthetic control spots.

Experimental design

The design strategy for a microarray experiment needs to take into account the biological question at hand as well as the possibilities and limitations of the array technology and available analysis tools. With two colour arrays, each slide is hybridized with two targets labelled with different coloured dyes and the resulting ratio of dye signals is the main quantity of interest.

The experiments reported in Papers III and IV measured gene expression across a series of tangential sections through the wood-forming tissues. In this case individual samples were hybridized against a common reference consisting of pooled aliquots from all samples. This design was chosen because i) There is no natural reference point in a spatial series as opposed to time 0 in a time series. ii) Pooling all

samples insures that even genes expressed in only one tissue will be represented in the reference. iii) Other designs like loop structures (Kerr & Churchill, 2001) offer theoretical advantages concerning the efficiency of the experiments since comparisons are made directly between all samples and not via a reference. These advantages diminish, however, as the number of samples increases, moreover, all samples need to be hybridized successfully and extension of the experiments at a later stage is difficult. Finally, loop designs require advanced analysis tools which have only become available recently, and their theoretical advantages might not hold true in practical experiments (Dobbin & Simon, 2002; Kerr *et al.*, 2000; Wolfinger *et al.*, 2001).

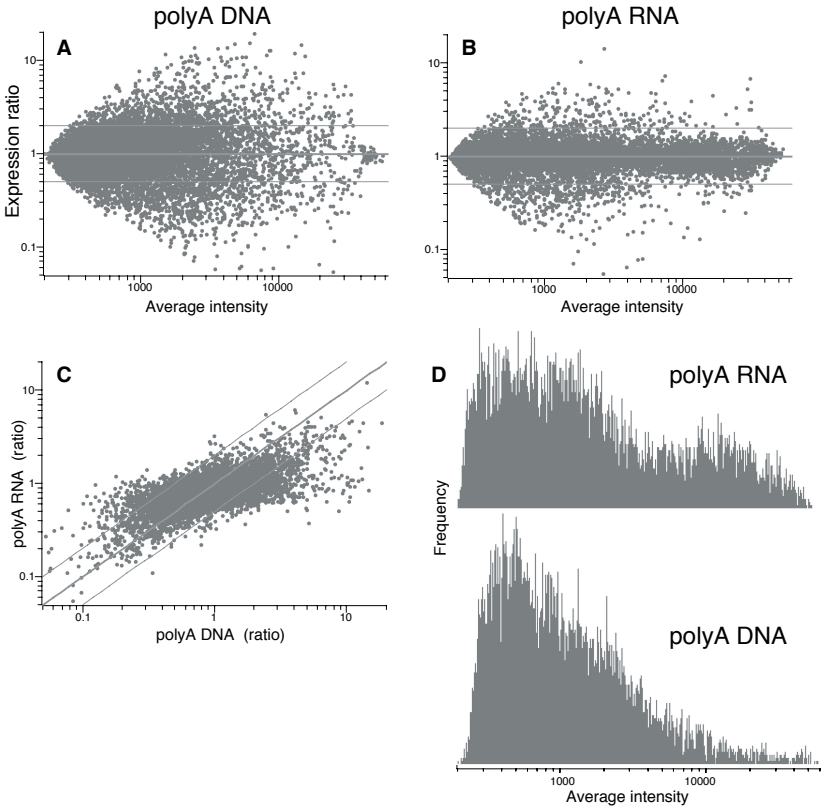

Fig. 6. Effects of polyA DNA or RNA as a blocker of unspecific binding to polyA tails in microarray hybridisations. The data shown are results from two hybridisations of stem versus leaf RNA. A, average signal intensity and expression ratio when using polyA DNA as a blocker. B, as in A but using polyA RNA, note the decreased number of differentially expressed genes. C, expression ratios from A and B plotted against each other. D, histograms showing intensity distribution of all genes in both hybridisations. Note increased number of high-intensity genes in the polyA RNA hybridisation.

Challenges in target generation and hybridization

The generation of target for microarray hybridization usually requires about 20 µg of total RNA, which is equivalent to about 50 mg of tissue. Since several studies in this thesis were performed on cryosections weighing in the order of 1 mg, a PCR-based cDNA amplification protocol developed by Hertzberg *et al.* (2001) was used to generate the necessary amounts of labelled target.

Common hybridization protocols for microarrays specify the use of polyA nucleotides as a competitor to reduce unspecific binding of the polyT tails of target cDNA to the spots on the array. Initially polyA DNA was used in our experiments but we decided to investigate, whether the less expensive polyA RNA could be used instead. When target prepared from the same two samples was hybridized using either polyA RNA or DNA as a blocker, the samples containing polyA RNA showed considerably less differential expression than those blocked with polyA DNA (Fig. 6 compare A with B). Plotting ratios from both hybridizations against each other revealed that the basic trend in expression change was the same for both blockers; polyA RNA only severely compressed the observed ratios (Fig. 6 C). At the same time the intensity distribution was changed towards more genes with higher intensity in the polyA RNA samples (Fig. 6 D). Taken together these data suggest unspecific binding of target cDNA to the array. We could not identify the reason for the poor performance of polyA RNA but it is possible that the RNA is degraded by contaminating RNAses and is thus not able to block unspecific binding. Interestingly, when PCR amplified target was used the choice of blocker had no effect on the number of differentially expressed genes (data not shown). A possible explanation for this phenomenon lies in the double-stranded nature of the amplified target, which consequently contains equal amounts of polyA and polyT which are able to mutually block each other's unspecific binding to probe DNA.

Data normalization

Data normalization is a crucial step in microarray analysis and depending on the nature of the experiment, this step can be straightforward or require careful consideration (Quackenbush, 2002). There are three main types of normalization strategy, which all have their specific advantages and disadvantages; the basic concept is the same however: we assume, that a subset of the spots on the array have a known true expression ratio, usually equal to one. Normalization is then achieved by dividing the observed ratio of all spots by the observed ratio of the subset.

i) *Internal standard*. For this technique the array has to contain spots with heterologous DNA which has no close homologues in the species studied. Equal amounts of mRNA or cDNA corresponding to these spots are added to both samples. Following hybridization, it is assumed that the true signal ratio from these spots is equal to one, and all observed ratios on the array are adjusted accordingly. This technique works equally well with arrays containing many clones or a few, specially selected genes. Moreover, accuracy is not affected by the number of genes that show differential expression in the experiment. The major drawback is that the accuracy of the system is dependent on careful quantification of the starting material for labelling and accurate pipetting of the internal standard. Furthermore the control spots should be present in multiple copies on the array in order to allow reliable quantification of their signal ratios.

ii) *Housekeeping genes*. The basic assumption here is that a set of housekeeping genes will not change in expression between the samples of the experiment. Unfortunately, there are no true housekeeping genes that are equally expressed under all experimental conditions. One would therefore have to verify the constitutive expression of the housekeeping genes for each experiment individually. With the increasing number of microarray experiments performed, however, one can use meta-analysis to identify genes which are stably expressed in a large number of different experiments (S. H. Wu *et al.*, 2001).

iii) *Median ratio*. This is probably the most widely used normalization method. It assumes that the majority of genes represented on the array do not change their expression in the experiment; or, if a larger number of genes do change, that this change is symmetrical, i.e. there is an equal number of up- and down regulated genes. The median of all observed expression ratios then becomes the normalization factor. If the number of differentially expressed genes is not small, this can severely affect the normalization result so alternative methods might be more appropriate (Paper V; van de Peppel *et al.*, 2003).

In addition to the above mentioned procedures, which are based on correcting the ratios for each array separately, the relative changes of gene expression in an entire array experiment can be estimated using analysis of variance (ANOVA). This approach at the same time provides a framework for identifying differentially expressed genes (Kerr *et al.*, 2000; Wolfinger *et al.*, 2001).

The normalization process must also try to remove any systematic bias in the data. In two colour array experiments one often observes a correlation between the average intensity of both channels and the observed expression ratio (Yang *et al.*, 2002). There can further be systematic differences in the observed ratio in different areas of the array. Such bias can be removed under the assumption that the number of differentially expressed genes is small and that their change is symmetrical. In the case of intensity-dependent bias, a scatter plot of average intensity versus observed ratio is produced for all genes. A curve is then fitted through these spots, typically using the LOWESS procedure, and the normalization factor for a gene with a certain average intensity can be determined using this curve (see Yang *et al.*, 2002 for a detailed description of intensity dependent normalization). Combined space- and intensity-dependent normalization is then an extension of the same basic concept into three-dimensional space (position on the array and intensity; H. Wu *et al.*, 2002).

The three array experiments reported in this thesis used different normalization methods, which partly reflects differences in experimental design but is also a result of technology development so that experiments in Paper IV could benefit from methods which were not yet available when Paper III was published.

In Paper III we used an internal standard consisting of human cDNA clones for normalization. This choice was primarily motivated by the fact that the experimental samples spanned a wide developmental gradient, ranging from meristematic cells to tissues undergoing programmed cell death. Combined with the fairly small number of genes on the first array, we expected the number of differentially expressed genes

to be too large for reliable normalization by the median method. The larger number of clones on the POP1 array and the narrower developmental gradient examined in Paper IV, allowed us to use space- and intensity-dependent normalization based on the majority of genes.

The comparison of active and dormant cambial meristems in Paper V posed a particular challenge for normalization. The number of differentially expressed genes in this experiment is very large, and using either an internal standard or the majority of genes produces vastly different sets of up or down regulated genes. The biological implications of this are discussed in more detail in Paper V and the Results section.

Results

The principal subjects of this thesis are factors involved in the regulation of meristem activity and cell differentiation in the vascular cambium. The first half of the results deals with the influence of a single factor, auxin, and focuses on genes needed for transport and signal transduction of the hormone. In the second half I will describe a functional genomics approach which identifies a number of factors involved in various stages of wood development as well as the regulation of cambial activity during dormancy.

Molecular components of the auxin transport machinery

The study of polar auxin transport and its influence on plant development has for a long time been restricted to physiological experiments (Davies, 1995). It was the cloning of auxin transport genes from the Arabidopsis *pin1* and *aux1* mutants at the end of the last century that enabled the study of PAT on a molecular level (Friml & Palme, 2002). In order to allow similar studies in our aspen model system, the decision was taken to clone and analyze homologues of *AtPIN1* and *AtAUX1* from hybrid aspen *Populus tremula x tremuloides* (Paper I).

A note on terminology: Currently there is no biochemical data supporting the notion that AtAUX1 or AtPIN1 are actual auxin transporters (see 'Candidate molecules for the influx and efflux carrier' in the introduction). It is clear from the available evidence, however, that they are at least very closely associated with this function and for the sake of simplicity I will therefore use the term auxin carrier for *AtAUX1* and *AtPIN1* like genes in this thesis, fully aware that when the details of their biochemical function are unravelled in the future, this might necessitate the use of a different term.

The LAX family of AUX1 – like auxin influx carriers.

In Arabidopsis the *LAX* (**l**ike ***AUX1***) gene family has four members, *AtAUX1* and *AtLAX1-AtLAX3 (Parry et al., 2001)*. In aspen on the other hand, there is evidence for the existence of more than four *PttLAX* genes (Table I). Based on the Arabidopsis sequences these aspen influx carrier homologues were originally identified either in a library of ESTs from the wood-forming tissues (PopulusDB; Sterky *et al.*, 1998),

through the use of PCR with degenerate primers matching highly conserved regions of the LAX protein or in a screen of a phage cDNA library. Since neither the original *PttLAX1* ESTs nor the PCR fragments were ful-length, a cDNA phage library was screened with probes derived from those fragments. Full-length clones for *PttLAX1* and *PttLAX3* were identified in this way while the missing 5' and 3' ends of *PttLAX2* were obtained by RACE-PCR. Subsequently, as the EST sequencing progressed, clones corresponding to new *PttLAX* genes appeared in PopulusDB but they were not included in the detailed analysis and consequently lack full-length sequences (Table I).

Table I. Summary of available sequence for PttLAX genes. Accession: GenBank Accession number for full-length cDNA sequences. Isolated: Method by which the first representative for that gene was isolated, library refers to a phage cDNA library. Other PttLAX refers to EST sequences in PopulusDB which do not match any of the other clones. These could correspond to one or several more PttLAX genes

Gene	Accession	ESTs in PopulusDB	Isolated
PttLAX1	AF115543	A068P18, C052P65, A040P24, UB22CPA06, X060C04, A040P42, C058P54	EST
PttLAX2	AF190880	UB52DPD07, UB17CPE04, UB59CPE10, UB20CPB01, UB46DPG08, UB46DPD10	PCR
PttLAX3	AF263100	-	PCR
PttLAX4	-	P062F10	Library
Other *PttLAX*		UB22CPD06, UB55BPB10, UA47BPB08, X047E07	

While there are at least one or two more *LAX* genes in aspen than in Arabidopsis, some of the predicted PttLAX proteins are extremely similar, over 92% in the case of PttLAX1 and PttLAX4 for example, and might represent recent duplications. The soon-to-be-completed aspen genome will provide a more complete picture of the exact number of *PttLAX* genes and their phylogenetic relationship. This will also make it easier to identify potential functional orthologues.

In general, the predicted PttLAX proteins are very similar to each other and to LAX proteins from Arabidopsis and other species (Fig. 7; Paper I, Fig. 5). Amino acid identity ranges from 72% to 92% within aspen and 72% to 85% between aspen and

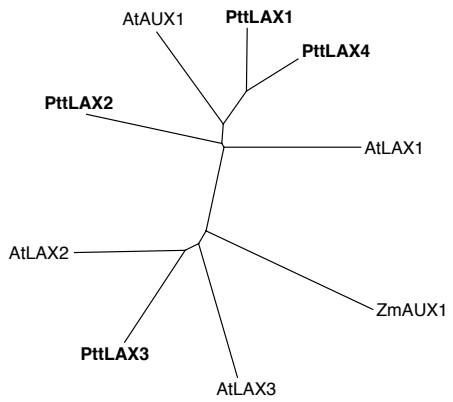

Fig. 7. Unrooted phylogenetic tree of LAX proteins from aspen, Arabidopsis and maize. The tree was constructed using the neighbour-joining algorithm of the phylip software. Aspen proteins are shown in bold.

Arabidopsis. Like for the Arabidopsis AtLAX proteins, diversity is highest at the N- and C-termini. The high degree of sequence similarity raises the question whether the proteins perform the same biochemical function and thus could be substituted for each other. This possibility was tested by trying to complement the agravitropic and auxin resistant root phenotype of the Arabidopsis *aux1* mutant with *PttLAX* transgenes. The Arabidopsis *aux1-100* null allele was transformed with coding sequences for PttLAX1 - PttLAX3 under the control of the *AtAUX1* promoter. In the case of *PttLAX1*, which is the closest homologue of *AtAUX1*, several independent lines of T2 plants showed a partial restoration of root gravitropism while they still remained resistant to 2,4-D (Fig. 8; data not shown). All lines expressing the cDNA for *PttLAX2* or *PttLAX3* on the other hand, did not differ markedly from the *aux1-100* phenotype (data not shown). Based on sequence, *PttLAX1* and *PttLAX4* are the closest homologues of *AtAUX1*. The partial complementation of *aux1* by *PttLAX1* suggests that despite its similarity, *PttLAX1* might not be a full functional orthologue of *AtAUX1*. Negative results in complementation assays are generally hard to interpret, however, because there can be multiple reasons for non-complementation, including insufficient expression of the transgene. The data nevertheless suggest that individual *LAX* genes have distinct biological functions despite the high degree of homology between them. Similar data were also obtained in Arabidopsis where none of the *AtLAX* genes was able to fully complement the *aux1* phenotype (M. Bennett, personal communication). Data from knockouts in *AtLAX* genes suggest a role in auxin response for all *LAX* genes (Parry *et al.*, 2001), therefore all LAX proteins might be auxin transporters but individual members might interact with different cellular partners leading – for example – to different intracellular distributions of different LAX proteins.

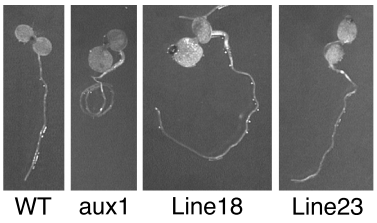

Fig. 8. Complementation of *Ataux1* with *PttLAX1*. Ten-day-old T2 seedlings grown on MS medium. Numbers 18 and 23 indicate two transgenic lines expressing *PttLAX1* from the *AtAUX1* promoter in an *aux1* background.

The PIN family of auxin efflux carriers

Hybrid aspen contains at least four *PIN* genes, all of which were identified as EST clones in PopulusDB. Some attempts to identify further members of the family were made using degenerate PCR or low-stringency screens of phage libraries but they did not result in any further sequences. Again, given that there are eight *PIN* genes in Arabidopsis (Friml & Palme, 2002), further *PttPIN* sequences might be found in the completed aspen genome. Full-length clones for *PttPIN* genes were obtained by screening cDNA libraries or by RACE-PCR. Table II summarizes the available information on different *PttPIN* clones in aspen.

The AtPIN proteins are characterized by two conserved predicted transmembrane domains and an intracellular loop. This structure is equally present in the PttPIN proteins (Paper I, Fig. 6). The intracellular loop is the most variable part of the protein, both within and between species, but it contains nevertheless smaller domains which are conserved between Arabidopsis and aspen.

Table II. Summary of available sequence for PttPIN genes. Accession: GenBank Accession number for full-length cDNA sequences. Isolated: Method by which the first representative for that gene was isolated, library refers to a phage cDNA library. Other PttPIN refers to EST sequences in PopulusDB which do not match any of the other clones. These could correspond to one or several more PttPIN genes

Gene	Accession	ESTs in PopulusDB	Isolated
PttPIN1	AF190881	A071P53, G063P23	EST
PttPIN2	AF515435	F051P48, T016A02	EST
PttPIN3	AF515434	Y001B03	EST
PttPIN4	-	A083P26, B007P21	EST
Other *PttPIN*		P042F09, UL70B04, T083H08, T056E12, UL66F09	

Six PAT genes with available full-length sequences, *PttLAX1*-*PttLAX3* and *PttPIN1*-*PttPIN3*, were chosen for further detailed studies

Antisense studies of aspen PAT genes

The most valuable information concerning the function of *LAX* and *PIN* genes in Arabidopsis has come from studies of knock-outs. An attempt was therefore made, to study the function of *PttLAX1* and *PttPIN1* in aspen using a genetic approach.

We generated several lines of transgenic aspen expressing *PttLAX1* or *PttPIN1* in antisense orientation under the control of the 35S promoter. None of the lines (10 PttLAX1, 7 PttPIN1) showed any obvious phenotype, however. Growth rate, morphology and wood anatomy did not differ from wildtype aspen (data not shown). The presence of the transgene was confirmed by RT-PCR but when the expression of the targeted genes were quantified using northern hybridization, it appeared that none of the lines had less than about 30% of wildtype levels of *PttLAX1* or *PttPIN1* mRNA (data not shown). The residual gene activity was apparently sufficient to allow wild type growth behaviour.

It is possible that the tissue culture method of aspen transformation has selected against any transformants with a stronger phenotype. Since auxin transport plays a central role in the establishment and maintenance of active meristems, cell lines with reduced PttPIN1 or PttLAX1 levels might have difficulties regenerating shoots or roots. Using an inducible expression system might be advisable for future experiments with any genes whose up or down regulation can interfere with regeneration in tissue culture.

Early auxin response genes in *Populus*

Members of the Aux/IAA family of early auxin response proteins have emerged as key regulators of auxin-modulated growth and development in plants. The size of the gene family (29 Aux/IAA proteins in Arabidopsis) and the phenotypes of plants mutant in *Aux/IAA* genes indicate that these proteins are involved in a diverse range of auxin mediated processes (Liscum & Reed, 2002; Reed, 2001). One of these processes is likely to be cambial activity; therefore, as a first step towards understanding auxin regulation of wood formation, a decision was made to identify members of the *Aux/IAA* family from hybrid aspen.

Table III. Aux/IAA genes in hybrid aspen. Where available, the GenBank accession numbers of full-length cDNA clones are indicated. For each gene one reference EST is given, which was used for all hybridisation experiments. Except for PttIAA4 all genes are represented by one or more contigs in PopulusDB

Gene	Accession No.	EST Clone	Contig in PopulusDB
PttIAA1	AF373100	A016P04	POPLAR.185.C2, POPLAR.185.C3
PttIAA2	AJ306825	A069P49	POPLAR.3603.C1, POPLAR.3603.C2
PttIAA3	AJ306826	A048P31	POPLAR.242.C1, POPLAR.242.C2
PttIAA4	AJ306827	A083P72	
PttIAA5	AJ306828	C054P56	POPLAR.2689.C1
PttIAA6	AJ306829	C056P70	POPLAR.9805.C1, POPLAR.9805.C2
PttIAA7	AJ306830	C024P79	POPLAR.1595.C1
PttIAA8	AJ306831	G086P74	POPLAR.918.C1
PttIAA9		F030P20	POPLAR.2279.C1
PttIAA10		UB11CPC07	POPLAR.5256.C1, POPLAR.5256.C2
PttIAA11		UB60BPD09	POPLAR.4866.C1, POPLAR.4866.C3
PttIAA12		I021P58	POPLAR.5256.C3
PttIAA13		A062P49	POPLAR.918.C2
PttIAA14		UM64TE04	POPLAR.10828.C1
PttIAA15		V047F08	POPLAR.10127.C1
PttIAA16		F021P42	POPLAR.1802.C1

The aspen EST sequencing project proved invaluable in the identification of aspen *Aux/IAA* genes. At least 16 different *Aux/IAA* homologues are found among the 120 000 ESTs of PopulusDB (Table III). High-quality, full-length sequences were obtained for *PttIAA1* – *PttIAA8* (Paper II, Fig. 1) and their relationship to Arabidopsis proteins can be seen in Fig. 9.

Detailed comparison of the predicted protein sequences revealed some interesting differences in the structure of domain II, which is necessary for auxin-dependent degradation of Aux/IAA proteins (Ramos *et al.*, 2001; Worley *et al.*, 2000; Zenser *et al.*, 2001). PttIAA6 contains two tandem copies of domain II, albeit with exchanges in two conserved aminoacids in the second copy (Paper II, Fig. 1). This second copy could allow faster degradation of PttIAA6 or the interaction with a different SCF ubiquitination complex (Gray *et al.*, 2001). It is equally possible, however, that this duplication has no specific function but arose in a recombination event and has subsequently been rendered non-functional due to the two mutations. This model would be supported by the fact that none of the Arabidopsis Aux/IAA proteins has such a duplication which indicates that duplications in domain II are not a common feature of Aux/IAA proteins.

In PttIAA3 and PttIAA4 as well as in the closest Arabidopsis homologues AtIAA30 and AtIAA31 the otherwise well-conserved glycine of the qvvgWPPirs motif in domain II is replaced by aspartic acid. A similar replacement of this glycine with glutamic acid in AtIAA3 leads to the *shy2* mutant phenotype (Tian & Reed, 1999), furthermore a replacement with alanine or glutamic acid increased the stability of domain II-luciferase fusions in tobacco protoplasts (Ramos *et al.*, 2001) suggesting that PttIAA3, PttIAA4, AtIAA30 and AtIAA31 might be more stable than other Aux/IAA proteins. These proteins further share common motifs in

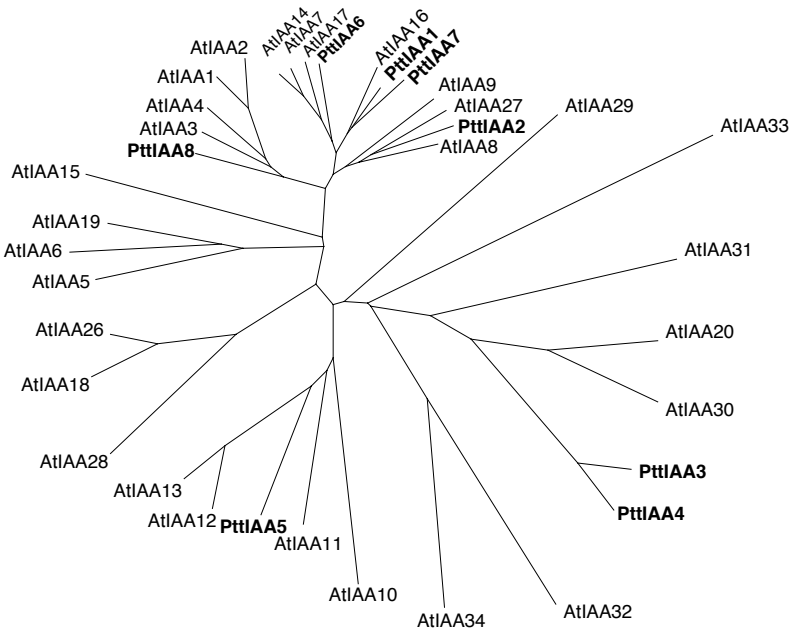

Fig. 9. Unrooted phylogenetic tree of Aux/IAA proteins of aspen and Arabidopsis. The tree was constructed using the neighbour-joining algorithm of the phylip software. Aspen proteins are shown in bold.

domains III and IV, which differ from the remaining Aux/IAA proteins suggesting different specificity in their interaction with ARFs and other Aux/IAAs. PttIAA3 and PttIAA4 therefore represent a subclass of the Aux/IAA family, which might be less responsive to the degradation promoting effect of auxin and might bind a different set of interactors.

Auxin and vascular pattern formation

Feed back regulation of auxin transport

During plant development a number of patterning processes take place that shape the final organism. One of these processes is the establishment of the intricate network of vascular tissue connecting all parts of the plant. The signal flow canalization hypothesis explains the formation of vascular networks based on the channelling of auxin flux into narrow files of cells.

A central prediction of the canalization hypothesis is that there should be a positive feedback of auxin on PAT. Since auxin influx and efflux carriers are likely effectors of PAT regulation, studying the corresponding genes might shed some light on their role in pattern formation. We tested this by measuring the expression of aspen PAT gene expression in stems that had been decapitated and immediately capped with lanolin containing 5 mg/g IAA. No clear effect on the expression of PAT or Aux/IAA genes could be detected in these samples (data not shown). We then hypothesised that the IAA concentrations in the cambium might be sufficient for maximum expression of auxin inducible genes and any externally applied auxin could not lead to further induction. Removing IAA by first incubating stem tissue

in MS medium led to a reduction in auxin content (Paper I) which could then be increased again by adding 10 µM IAA into the medium. Now all PAT genes with the exception of *PttLAX2* showed clear responsiveness to IAA levels (Paper I, Fig. 3). These data indicate positive feed back of auxin on the expression of PAT genes and are thus in line with the predictions of the canalisation hypothesis. There is bound to be additional control on the post-transcriptional level, however, because it is not only the amount of transporter mRNA but also the correct localisation of the corresponding protein in the plasma membrane which has to be regulated according to the amount of auxin and the direction of auxin flux. The most likely candidates for auxin transporters involved in early vascular differentiation and the setting-up of vascular patterns are *PttLAX3* and *PttPIN2*. Both genes are expressed in young internodes and especially in young, developing leaves during the process of vascular differentiation (Paper I, Figs. 1B and 2C).

While young and developing tissues have traditionally been seen as the major sources of IAA in plants (Davies, 1995), recent findings suggest, that all tissues, including mature leaves, can play a role in supplying the plant with auxin (Horvath *et al.*, 2002; Ljung *et al.*, 2001a). Mature leaves, for example, are able to export externally applied IAA into the phloem stream (Cambridge & Morris, 1996). Our data shows expression of *PttPIN3* in mature leaves suggesting a need for PAT in these tissues and a possible role for PttPIN3 in supplying IAA to the phloem stream.

Auxin and cambial development

It has been well established over the years that auxin is necessary for active cambial growth and that it can induce the formation of new vascular strands (Jost, 1942; Little & Bonga, 1974; Little & Savidge, 1987; Sachs, 1981; Savidge, 1996; Uggla *et al.*, 1996). The discovery of a concentration gradient across the wood-forming tissues raised the possibility that auxin not only promotes cambial cell division but that it could also guide the development of cambial derivatives by acting as a morphogen (Uggla *et al.*, 1998; Uggla *et al.*, 1996). A role for auxin as a morphogen would imply a mechanism that controls the extent and shape of the auxin concentration gradient. That such a mechanism indeed exists, is suggested by attempts to modify the gradient by overexpressing auxin biosynthetic genes from a constitutive promoter, which only led to relatively moderate changes in the width of the gradient (Tuominen *et al.*, 1995). Since the capacity of the wood-forming tissues to metabolize IAA is relatively low (Sundberg, 1987), PAT represents the most likely mechanism for setting up and maintaining the auxin gradient. In Paper I we were able to identify *PttLAX3, PttPIN1* and *PttPIN2* as candidates for genes involved in controlling the auxin gradient in the cambium.

The morphogen concept for auxin implies a mechanism whereby cells along the gradient can sense the prevailing auxin concentration and translate this information into the activation of specific genetic programs. At the moment there seems to be no clear data showing that certain auxin concentrations activate distinct sets of genes. It is therefore likely, that auxin influences the differentiation of cambial derivatives in concert with other patterning factors like regulatory proteins or other metabolites.

In Paper II we studied members of the *Aux/IAA* gene family as likely candidates for genes involved in regulating cambial differentiation in response to auxin. We

could show that these early response genes are auxin inducible (Paper II, Fig. 2), but that their expression in the cambial region does not necessarily follow the auxin gradient (Paper II, Fig. 3). There is, in fact, strong developmental-stage specific control of *PttIAA* gene expression as indicated by very steep expression gradients with opposite direction for *PttIAA6* and *PttIAA3* in the vicinity of the cambial initials (Paper IV, Fig. 6). We obtained detailed expression profiles across the wood-forming tissues for 16 *PttIAA* genes (Fig. 10 and Paper II, Fig. 3) but there were never more than two genes showing the same pattern. This large variety of distinct expression profiles supports the idea that *PttIAA* genes could be responsible for providing tissue specificity to the auxin response in aspen stems. At the same time this implies that the tissue information is provided independently of auxin concentration, supporting the idea that there have to be other important factors besides auxin that specify the pattern of developmental zones in the cambial region.

Tissue context and auxin levels are not the only factors affecting the expression of *PttIAA* genes, however. When aspen trees were bent in order to induce tension wood, the expression of *PttIAA7* increased 5 days after the treatment while levels of *PttIAA1* and *PttIAA2* decreased. Since all three genes largely overlap in their expression domains, this must represent a response specific to individual genes.
When the expression of several PttIAA genes was analysed in an auxin depletion - induction time course, *PttIAA3* showed an unexpected increase during the depletion phase which was not observed for the other genes tested (Figs. 11 A and B). A possible explanation is wound responsiveness of *PttIAA3* expression and indeed, in an experiment where aspen stems were wounded with razor blades, *PttIAA3* was induced four hours after the treatment (Fig. 11C).

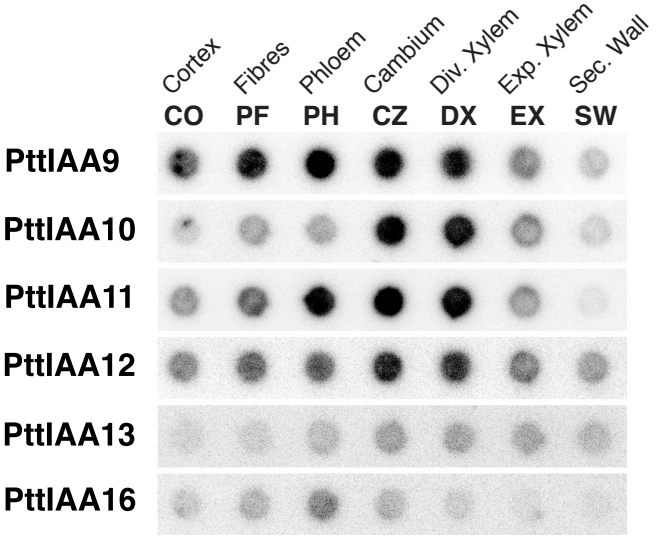

Fig. 10. Expression of *PttIAA* genes in the wood-forming tissues of aspen. PCR amplified cDNA from tangential sections through an aspen stem were blotted onto nylon membranes and hybridized with radiolabelled probes. The samples are CO, cortex; PF phloem fibres; PH, mature phloem; CZ, cambial zone; DX, dividing xylem mother cells; EX, expanding xylem; SW, secondary wall formation. See Paper II for method details.

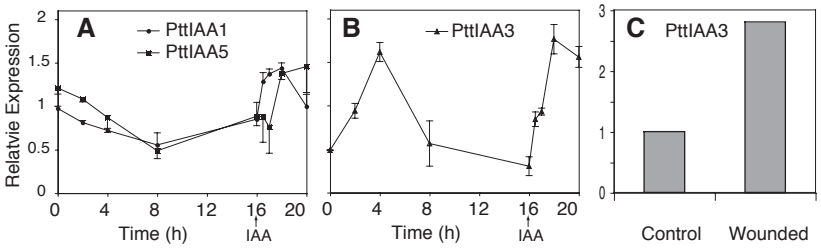

Fig. 11. Wound responsiveness of *PttIAA3*. A and B, tissue culture-grown stems were decapitated, defoliated and depleted of internal auxin for 16 h in MS medium. IAA was added to 10 µM and incubation continued for 4 h. Expression of *PttIAA* genes was followed using RT-PCR (see Paper I for method details). C, greenhouse-grown aspen stems were wounded by vertical cuts with a razor blade and sampled 4 h after the treatment. Gene expression was measured as above.

The responsiveness of *PttIAA* genes to auxin further appears to be under developmental control, since auxin induced their expression in young, but not in mature leaves (Paper II, Fig. 2). It remains to be determined, however, whether this is a feature specific to *Aux/IAA* genes in aspen or whether it is a sign of a general loss of auxin sensitivity in mature leaf tissue.

If we try to define a role for *Aux/IAA* genes based on the available data, it could be that of integrator molecules, which combine information from tissue and developmental context with auxin levels and specific environmental or stress signals and translate this information into the regulation of downstream genes. The ability of phytochrome to phosphorylate Aux/IAA proteins (Colon-Carmona *et al.*, 2000) would provide another example of environmental (light) input into Aux/IAA mediated gene regulation.

A model where *Aux/IAA* genes act as signal integrators must take into account the fact that Aux/IAA proteins act as transcriptional repressors on auxin inducible genes including themselves (Tiwari *et al.*, 2001; Ulmasov *et al.*, 1997b) and that thus a high level of *Aux/IAA* gene expression could actually be the result of low levels of the corresponding protein. One has to further consider that Aux/IAA proteins are believed to act through dimerisation with ARF transcription factors which can act as activators or repressors and which appear to be constitutively expressed in many but not all cases. (Guilfoyle & Hagen, 2001 and data not shown). As an example, if the expansion zone-specific PttIAA8 interacts with a constitutive ARF activator this would result in expansion zone-specific down regulation of downstream genes; but this block could be relieved by IAA. Alternatively, interaction with an ARF repressor would allow expansion zone-specific expression of downstream genes but only at low IAA levels. These examples show that we need to learn more about the true in vivo interactions between Aux/IAA and ARF proteins and identify their target genes before we can begin to truly understand this regulatory network.

Finding novel regulators of wood development

A developmental process like the transformation of a xylem mother cell into a mature vessel or fibre involves a multitude of different genetic programs that have to be co-ordinated in both space and time. One of the goals of developmental biology is to identify the component genes of these programs and map their regulatory interactions. We have used microarray technology to both assign genes with known functions to specific stages of wood formation and identify new, potential regulators or effectors of this developmental process (Papers III and IV). Starting point for these experiments were thin tangential cryosections through the wood-forming tissues. Amplified cDNA from these sections was hybridized to cDNA microarrays resulting in transcriptional maps of the of the cambial region. Two such maps are presented in this thesis, the first, published in Paper III encompasses the entire cambial region from phloem mother cells to the zone of programmed cell death. Paper IV then describes a more detailed map of meristematic cells in the cambial zone.

The process of xylem formation can be subdivided into a number of distinct steps like cell division, cell expansion, secondary wall formation, lignification and programmed cell death (Fig. 4). Cluster analysis of gene expression profiles identified groups of genes that are expressed during one or several of these developmental steps (Paper III, Fig. 1; Paper IV, Fig. 3). Figure 1 in Paper IV shows an overview of the sections used for the different samples. For numerous genes, the observed expression profile across the wood-forming tissues matched the pattern predicted from the known function of these genes. For example, genes known to be involved in cell division and cell cycle regulation, like Cyclin A1 (*CYCA1*), Cyclin dependent kinase B (*CDKB*) or the histone H4 were all expressed in the zone of cell proliferation (Paper III, Fig. 3A; Paper IV, Fig. 5). Similarly, genes coding for expansin or xyloglucan endotransglycosylase (*XET*), which play a role in cell expansion, were expressed in the proliferation and expansion zones, as predicted from their proposed function. Finally, genes coding for enzymes involved in lignin biosynthesis were co-ordinately up regulated in the zones of secondary wall formation and lignification (Paper III, Fig. 4B).

In many cases we identified several genes encoding highly similar proteins, which nevertheless had clearly distinct expression profiles. Based on our microarray data we can therefore distinguish isoforms of enzymes and regulatory proteins from each other and provide new information about their potential roles in wood formation. Examples include 4-coumarate:CoA ligase and cinnamyl alcohol dehydrogenase where the expression pattern of one isoform suggests an involvement in lignification while the other genes appeared to have less tissue-specific functions as judged from their even expression profiles (Paper III, Fig. 4B). Similar results were obtained for tubulins (Paper III, Fig. 3) or for members of the cell cycle machinery like the cyclins (Paper IV, Fig. 5).

As mentioned before, forward genetic screens are not a viable option in *Populus* for identifying genes involved in a certain process. Reverse genetics therefore becomes an important strategy but the cost and labour associated with the generation of transgenic aspen allows for only a small number of genes that can be tested this way; it is thus essential that the selection of target genes is based on as much relevant

data as possible. The PopulusDB EST collection contains thousands of clones with unknown function. Others can only be assigned a generic function like ‘protein kinase’ or ‘transcription factor’. For these genes, the expression profiles across the wood-forming tissues can provide valuable clues to their role in plant development. The power of microarray analysis for finding candidate regulatory genes is illustrated in Paper IV where we assembled a set of 159 genes with a peak of expression in the cambial zone (Paper IV, Fig. 4). 46 of them had higher expression in the cambium than in either the root or shoot apical meristem. The latter set contains 33 clones with an unknown function but their specific expression profile strongly suggests a role for these clones in the organisation of the cambial meristem. This moderate number of genes can now be tested using RNA interference without having to employ large, industrial scale growth facilities but with nevertheless a good chance of identifying among them genes with important regulatory functions.

Cross comparison of microarray experiments

The value of microarray experiments lies not only in the immediate conclusions that can be drawn from the expression profiles. It is above all the assembly of databases with the results of multiple transcript profiling experiments, which in the future will allow us to obtain a wealth of information on a given gene using data mining techniques without having to enter the laboratory. While this is already becoming a reality for organisms of major interest like Arabidopsis, Yeast or Human (Gollub *et al.*, 2003; Rhee *et al.*, 2003), in the case of trees, we have just begun this work. In the following I will give some examples on how the combination of results from different array experiments can be used to verify previous data and to provide considerable new information on the genes involved in a biological process.

Global versus local maps

The cutting series experiment in Paper III produced a map of the wood-forming region from the cambium to the zone of mature xylem, while Paper IV contributed a fine mapping of the cambial zone, which extended further to the phloem side. It is of obvious interest to combine information from both experiments to obtain a full picture of a gene’s expression profile in the wood-forming tissues. An example of such an analysis for genes associated with the cell cycle can be seen in Fig. 5 of Paper IV. In general genes with a similar expression in the cambial zone also showed a similar profile across the whole wood-forming region so that an increase of expression towards the xylem side in the cambial zone series corresponds to high expression in sample A of the global series. But while some genes like *Cyclin B2* are down regulated upon leaving the proliferation zone (samples A and B), others like *Cyclin A2* remain highly expressed even during secondary wall formation. Consequently post-transcriptional regulation might be more important for *Cyclin A2* or the gene plays a role even in non-dividing cells as has been suggested for its Arabidopsis homolog (Burssens *et al.*, 2000).

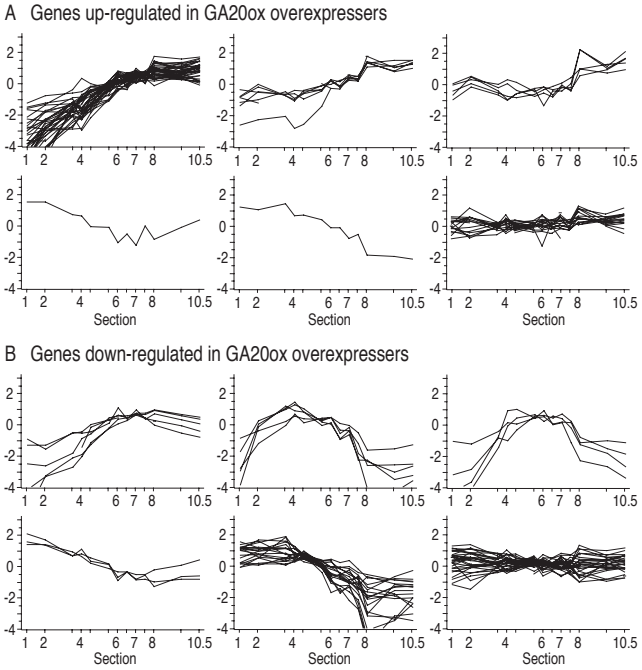

Fig. 12. Cambial zone expression profiles of genes differentially expressed in transgenic aspen with elevated GA levels (Israelsson et al., 2003). The expression profiles are taken from the dataset of Paper IV. The horizontal axis shows individual sections of the cambial zone. Please refer to Paper IV, Fig. 1 for details on the sections. The vertical axis shows relative expression in $\log_2$ scale.

Hormone induction versus tissue distribution

Transgenic trees overexpressing the gibberellin (GA) biosynthetic enzyme GA 20-oxidase have elevated levels of GAs and show increased secondary growth (Eriksson *et al.*, 2000). In a recent publication, Israelsson and co-workers studied how the elevated GA levels affect the transcriptome of such trees and found 92 genes to be induced in the transgenic trees relative to wildtype controls (Israelsson *et al.*, 2003). They then obtained the tissue-specific expression profiles of the same genes from the stem cutting series of Paper III. Interestingly, a majority of these genes had highest expression in samples A-C where cell proliferation and expansion take place. From this the authors concluded that meristem and expansion zone cells are most strongly affected by the elevated GA levels.

Very similar behaviour is observed when the induced genes are mapped onto the cambial zone cutting series of Paper IV (Fig. 12). The majority of genes up regulated in GA 20-oxidase overexpressors show an increase towards the zone of cell proliferation and expansion. The opposite is true for the cambial expression profiles of the genes down regulated by elevated GA. Here we find many genes with specific expression in the cambial zone or an increase towards the phloem side (Fig. 12B). This would suggest that some processes associated with undifferentiated meristematic cells and phloem are down regulated in the transgenic trees. There is, however, an alternative explanation for the observed down regulation. The samples from wildtype and GA 20-overexpressors were obtained by peeling off the bark

and scraping the exposed developing xylem. The peeling process mainly breaks the thin-walled cells in the zone of rapid radial expansion, consequently these samples approximately correspond to sections X9 and onward of the series in Paper IV. If the elevated GA levels cause changes in the cell wall properties of the transgenic trees, as suggested by the increased expression of cell wall loosening enzymes, the peeling process might break the tissue at a different position relative to the cambial zone. The apparent down-regulation of cambium and phloem-specific genes could therefore at least partly be a result of differences in the cellular composition of wildtype and transgenic samples. While such a tissue effect does not invalidate the major conclusions drawn by Israelsson *et al.*, as they mainly focussed on up regulated genes, it clearly illustrates the point that specificity of tissue sampling is a crucial factor in any large-scale expression analysis. Furthermore, examining the tissue distribution of genes changing in response to drug treatments or transgene expression can reveal important systematic effects, which might otherwise be overlooked.

Tissue effects in active dormant comparisons

In Paper V we observed a correlation between the exprresion profile of a gene in the active cambium and its active/dormant cambium expression ratio. Clusters of genes with high expression on the xylem side of the cambium had a larger number of genes up regulated in active cambium while phloem associated clusters showed more genes up regulated in dormant cambium (Fig. 13A; Paper V, Fig. 4). It was concluded that the observed changes between active and dormant cambium could partially result from changes in tissue composition. More specifically, it is possible that the dormant cambium sample was shifted to the phloem side relative to the active cambium sample. This hypothesis was tested *in silico* by creating 'virtual' active and dormant samples from the data in Paper IV. First, cluster analysis was used to determine that sample A4 at the centre of the cambial zone was most similar to the active sample, which also was in line with anatomical data on the position of the sections. The 'virtual' active sample then consisted of averaged expression values of sections A3 to A5 while the corresponding 'virtual' dormant sample was shifted towards the phloem side encompassing sections A1 to A3 (Fig. 13C). We hypothesized, that if the changes observed in Paper IV are mainly due to a shift in tissue composition, there should be a high correlation between the ratios from the real active dormant experiment and the ratios of 'virtual active' versus 'virtual dormant'.

The graph resulting from this experiment is presented in Fig. 13B. There is a certain amount of correlation between genes that are up regulated in active cambium (red dots) and those with higher activity on the xylem side of the cambium. There are, however, also numerous genes that show opposite behaviour and for genes up regulated in dormant cambium (green dots) there is no clear correlation with tissue specificity. Based on this *in silico* experiment we can conclude that while changes in tissue composition might be responsible for some of the changes in gene expression between active and dormant cambium, there are many genes where such an explanation is not sufficient and which thus show genuine dormancy-specific regulation.

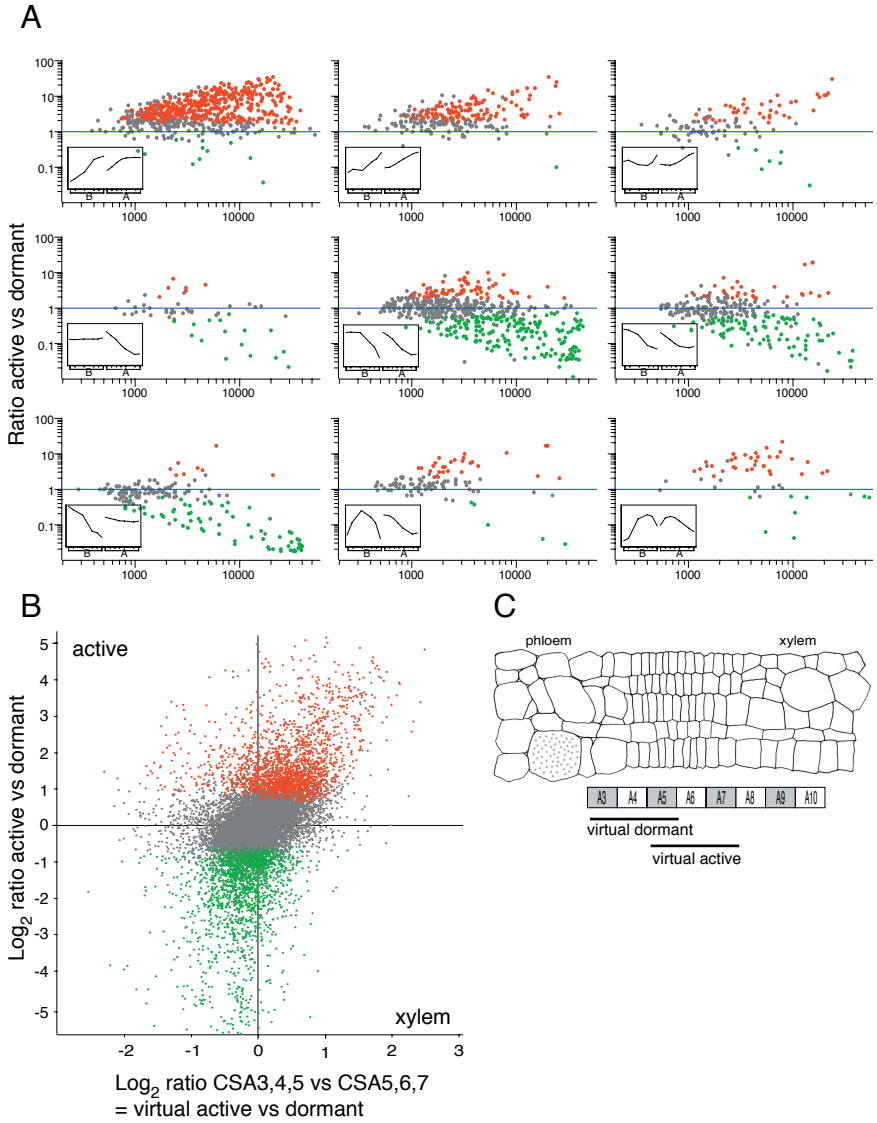

Fig. 13. Comparison of active/dormant and cambial zone expression data. A, expression data from the comparison of active and dormant cambium in Paper V subdivided into cambial zone expression clusters obtained from Paper IV. Graphs show average signal intensity versus active-dormant expression ratio. Red and green spots correspond to genes significantly up regulated in active and dormant cambium, respectively. The small insets show the average expression profiles across the cambial zone for genes in that cluster, similar to those shown in Paper IV, Fig. 2. B, a 'virtual' active dormant experiment was created by dividing average expression values of sections A3 to A5 by the average of A5 to A7 in an attempt to simulate a shift of tissue composition between active and dormant samples. The graph shows ratios from the true active dormant comparison plotted against the 'virtual' data. C, schematic of the cambial zone, showing the location of the 'virtual' samples.

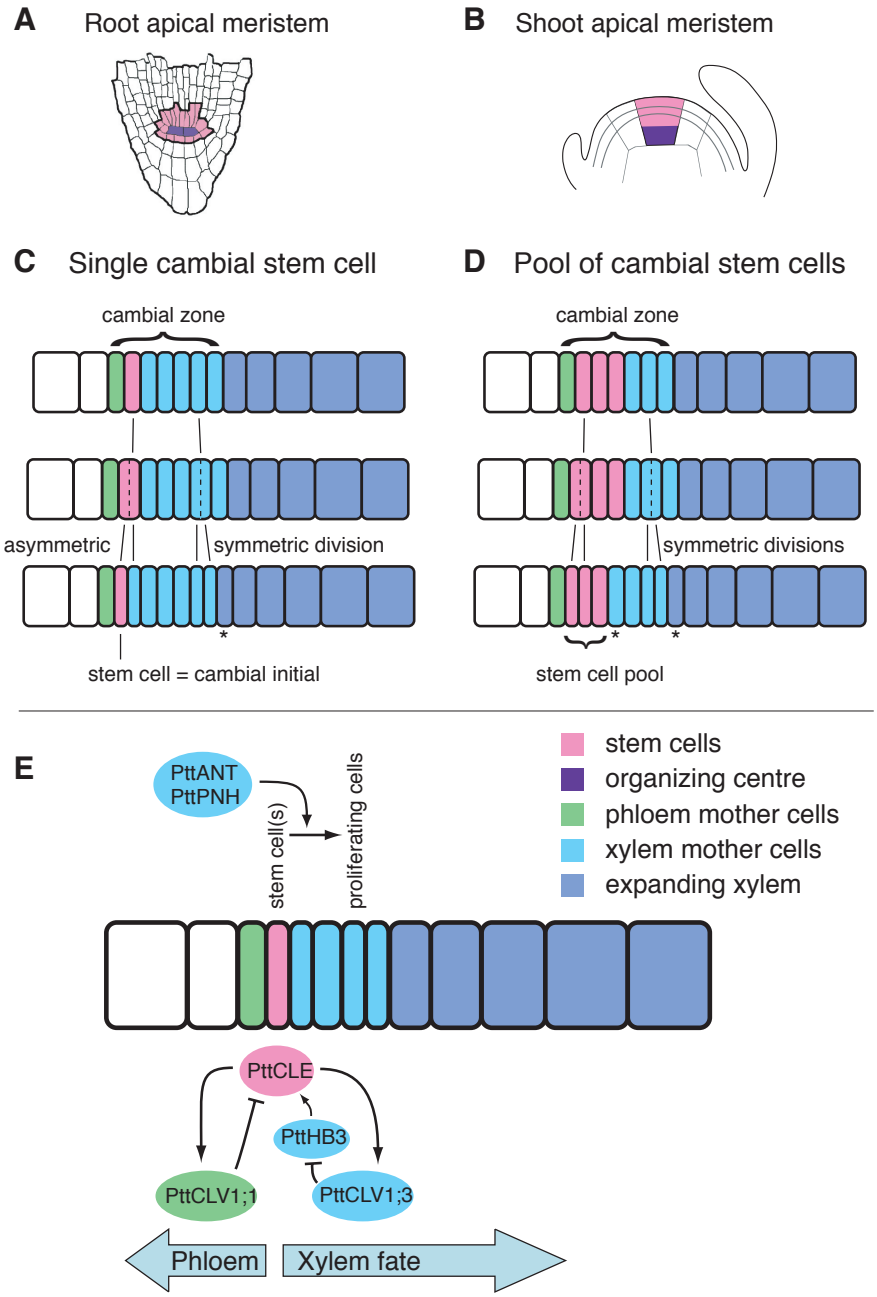

Fig. 14. Models for the specification of cambial stem cells. The cambial zone might contain a single layer of stem cells (A,C) whose identity is regulated on a single cell basis. Such a model is analogous to the situation in the root meristem where the quiescent centre acts as an organizing centre keeping the immediately surrounding cells in a stem cell state. In this case divisions of the stem cells will always be asymmetric since only one daughter retains the stem cell identity. Xylem and phloem mother cells are more numerous and should thus be specified on a population basis.

In an alternative model (B,D) both stem cells and mother cells form small populations. Cells change identity if they get pushed out of a zone as a result of cell divisions. This model is similar to the situation in the shoot meristem where a population of stem cells is maintained by signals emanating from the underlying organizing centre. Asterisks mark cells that have changed their identity as a result of being pushed out of the stem cell or mother cell zone.

E, hypothetical model for cambial meristem regulation. The cambial region is divided into cells with phloem fate (green) and xylem fate (blue). Separating both is the layer of cambial initials or stem cells (pink), which could express a member of the CLE family of CLV3 like ligands. This ligand would be bound by CLV1-like receptors expressed on either side of the stem cell layer. Binding of CLE to the receptors would then restrict expansion of the stem cell pool in that direction in a process involving WUS-like genes. Daughter cells of the cambial initials on the xylem side will enter the zone of xylem proliferation (light blue). This entry could be regulated by aspen homologues of ANT and PNH.

Using microarrays to study stem cells

In the background section I discussed the controversy concerning the number of stem cells in the cambial zone and suggested how information from Arabidopsis genetics could provide useful paradigms for resolving this problem. A major goal of the work in Paper IV was to identify potential markers for stem cell identity in the cambium and to investigate, to what extent there are similarities in the regulation of apical and cambial meristem identity.

On the anatomical and ultrastructural level the cells of the cambial zone are very homogenous (Larson, 1994). The existence and location of a cambial stem cell has therefore been inferred from division patterns of its derivatives. In Paper IV we were able to identify clusters of genes that showed differential expression within the cambial zone (Paper IV, Fig. 4). This is therefore the first molecular evidence that differences indeed exist between the individual cells of the cambial zone. While the resolution of the experiment was not sufficient to verify the existence of a single layer of stem cells, we were able to narrow down the location of this layer to the cells around section X5. The data strongly argues against a cambial zone made up of equivalent cells but rather suggests a subdivision into proliferating mother cells and a less active stem cell layer. Given this subdivision, there still remain two main models for the organization of the cambial meristem. i) A uniseriate model with only a single cambial stem cell (Fig. 14A,C). This cell will always undergo asymmetric divisions producing a stem cell and a xylem or phloem mother cell. Asymmetric in this context means that the daughter cells differ with respect to their developmental fate and the genes they express, but not necessarily regarding their morphology or ultrastructure. In analogy to the root meristem the identity of the stem cell would be regulated on a single cell level, possibly by direct cell-to-cell communication (Sabatini *et al.*, 2003; van den Berg *et al.*, 1997). ii) A modified multiseriate model where we can distinguish between a stem cell zone and a zone of proliferating mother cells (Fig. 14B,D). The stem cell layer would encompass one or several cells – depending on growth rate – whose identity is regulated on a population level similar to the cells in the central zone of the SAM. In this model any of the cells in the cambial zone has the capacity to act as an initial and this fate decision would be based on positional cues from the surrounding cells (Gross-Hardt & Laux, 2003).

Ontogenetically, the cambium is a derivative of the SAM. One could argue, therefore, that the cambium might also have inherited some of its regulatory mechanisms from the shoot apex. This idea is supported by the tissue specific

expression profiles of homologues of known regulators of SAM development like *CLAVATA1*, *PINHEAD* or *AINTEGUMENTA* in the cambial zone (Paper IV, Fig. Q6). On the other hand, two important regulators of SAM identity, *CLAVATA3* and *WUSCHEL* appear not to be expressed in the cambial zone. There are further major differences in the structure of both meristems. The SAM produces a number of different tissues, and has consequently a rather complex, three-dimensional architecture with several distinct layers and zones. Cells that leave the stem cell pool of the central zone do not immediately begin to differentiate; they can, for instance, be incorporated into leaf primordia where they will give rise to the various tissues of a mature leaf. The cambium, on the other hand, shows a clear bilateral symmetry, where the derivatives of the stem cell immediately face a basic decision, whether they become phloem or xylem cells. Since the same stem cell layer is able to produce both phloem and xylem in opposite directions, there has to be a mechanism, which is able to control proliferation on both sides of the cambium independently. So even if the cambium and apical meristems share a number of regulatory mechanisms, it is clear that the unique biology of the cambial zone necessitates the existence of a dedicated system for its regulation, which cannot be understood by looking at apical meristems alone. A speculative model on how cell identity and proliferation might be regulated in the cambial meristem is presented in Fig. 14E. It is based on the known functions of apical meristem regulators and the expression of their aspen homologues in the cambial zone. While such a model obviously lacks the functional evidence to support it, it can nevertheless serve as a starting point for the design of functional studies aimed at testing the role of its different components.

Environmental regulation of wood formation

Dormancy, auxin transport and response

When trees enter dormancy, the cambium looses its ability to respond to exogenously applied IAA (Little & Bonga, 1974) but the underlying mechanism has not been determined. In Paper II, Fig. 5 we describe the down regulation of a number of *PttIAA* genes in dormant stem tissues. This opens up the possibility that the lack of responsiveness is at least partly caused by alterations in auxin signal transduction. It would be interesting to see whether the expression of these genes can still be induced in dormant tissues and if not, whether there is any mechanistic connection to the loss of inducibility in mature leaves (Paper II, Fig. 2). Since dormant stems are also impaired in PAT (Lachaud & Bonnemain, 1984; Odani, 1975) lack of response could also result from auxin not reaching the cambium.

The molecular basis for the reduced PAT capacity in dormant stems was investigated in Paper I. Transcriptional control of the PAT machinery appears to offer at least a partial explanation as the transcription of all *PttPIN* and *PttLAX* genes was reduced in dormant twigs (Paper I, Fig. 4). On the other hand, our data point to an important post-transcriptional component in the regulation. In early spring the expression of aspen PAT genes increased dramatically without having a strong effect on PAT capacity. A possible mechanism is the vesicle transport-dependent localization of PIN proteins in the plasma membrane, which might be inactive in dormant twigs before bud break (Steinmann *et al.*, 1999).

Functional genomics of cambial dormancy

The transition of meristems from activity into dormancy provides an interesting model for studying factors involved in specifying meristem identity and regulating cell proliferation. While stem cell identity should be preserved even in dormant meristems, factors related to proliferation can be expected to disappear, thereby allowing a functional separation of the two systems. While the cambial meristem can be easily sampled and dormancy can be induced by exposure to short days, expression studies are dependent on the availability of DNA sequences of the genes that are expressed in active and dormant cambia. Providing the necessary sequence information and clones for microarray and reverse genetic studies of active and dormant cambia was one of the major goals of the work presented in Paper V.

The most striking finding in this Paper was the magnitude of transcriptional change between active and dormant cambium. Nearly 3000 genes, about one fifth of all the genes on the POP1 array, differed in their expression (Paper V, Fig. 2). Together with the decrease in complexity of the dormant cambium, where two genes produce 10% of all transcripts, it becomes apparent that the transition from active growth to dormancy represents a major change in the physiology of cambial cells.

Such a system provides a number of challenges for functional analysis, however. Particularly the interpretation of a change in gene expression is no longer clear-cut, as it has to be seen in the context of other, interacting genes. If all members of a regulatory network, activators and repressors alike, are down regulated to similar extent, what conclusions can we draw about their downstream targets?

Despite these difficulties we were able to identify a number of processes that are clearly up or down regulated in dormant cambia. The accumulation of storage proteins cannot be overlooked, and our data agree well with the existing literature (Coleman *et al.*, 1991; Zhu & Coleman, 2001). It can also be concluded that a cambium at the early stages of dormancy devotes a majority of its transcriptional activity to processes related to cold acclimation and general stress response. Changes were also observed in a number of metabolic genes, including an increased proportion of secondary metabolism-related transcripts in dormant cambium. The expression levels of some metabolic genes like amylase did not correlate well with measurements of enzyme activity. While such a lack of correlation might be due to differences in ecotype and timing of the entry into dormancy it could also be due to post-transcriptional regulation mechanisms which we might be able to test in a near future using proteomic tools.

Interesting results are obtained from the dormancy expression of potential meristem regulators in Paper IV. We speculated that the *PttANT* gene might be involved in regulating meristem proliferation, based on the activity of its Arabidopsis homologue. This gene is strongly down regulated in dormant cambium (about seven fold), supporting a role specific for actively growing tissues. Similar conclusions can be drawn from the expression changes of *PttCLV1-3* and *PttHB3*, which are equally down regulated.

Conclusions and Future Prospects

Summary

In this thesis I have tried to shed some light on the factors regulating growth and development in the wood-forming tissues of aspen. For one of these factors, auxin, I have provided new information on the molecular mechanisms underlying auxin transport and signal transduction. PAT in the wood-forming tissues involves several distinct auxin transport genes that are under tissue- and developmental stage-specific control. Auxin transport is regulated on both transcriptional and post-transcriptional levels in response to environmental cues. There is further a positive feed back of auxin on the expression of PAT genes as predicted by the canalization hypothesis. Auxin signal transduction in the cambial region is likely to involve members of the *PttIAA* family of auxin responsive genes, which could play a role as integrators of tissue specific and environmental signals. Our data further suggests a developmental switch in auxin sensitivity as tissues mature. Finally we provide indications for a role of auxin in late stages of xylem and phloem fibre maturation.

The amount of molecular biological information on the wood-forming tissues is limited, especially when compared to other meristems. Our transcriptional maps of the cambial region therefore provide a valuable basis for future studies. They provide candidate genes for novel regulators of vascular development and molecular markers for the individual stages of wood formation. The data further suggest a certain overlap between the regulatory mechanisms of apical meristems and the vascular cambium while at the same time identifying unique properties of the wood-forming tissues.

Cambial dormancy is an example of a massive switch in the physiological status of a tissue. The creation of dormancy-specific EST libraries together with gene expression data provides the first description of the transcriptome of a dormant meristem. We could identify which processes are most affected by the transition into dormancy and have provided a basis for future investigations on individual processes and regulatory networks.

From description to function

A large part of the data in this thesis is of descriptive nature. This is partly a result of the limitations of aspen as a model system, but also due to the fact that molecular data on wood formation is scarce so that one cannot use previous work from other authors as a starting point. Providing basic descriptions of a system, however, provides the necessary framework for functional studies in the future. In the following I will present some examples of hypotheses and questions that have emerged from the data in this thesis and try to outline approaches for functional investigations.

In Paper I we described how the transcription of PAT genes is activated in spring before a major increase in auxin transport capacity. A hypothesis was presented whereby auxin emerging from newly formed leaves would activate the transport machinery post-transcriptionally. The low transport capacity could be due to a block in mRNA translation, improper localisation of the transporter proteins or

a negative regulation of transport protein activity. As a first step we would have to measure the levels and subcellular localisation of PttPIN and PttLAX proteins during spring activation and determine whether these parameters can be changed by auxin application. At least in pine the auxin concentrations in the cambial zone do not change between activity and dormancy (Uggla *et al.*, 1996). The activating factor might thus be auxin flux resulting from the presence of both sinks and sources for auxin rather than steady-state auxin levels. This possibility could be tested by exposing stem sections to auxin without creating a gradient and recording changes in transport capacity. In a general dormancy context it would also be interesting to know, whether the activation of transcription in early spring only concerns auxin transport genes or whether it reflects a general increase in gene transcription.

The exact nature of cambial meristem organization remains unsolved. We were able to identify a subdivision of the cambial zone and suggest the involvement of a number of known gene families in the regulation of cambial cell identity and differentiation. A lot of work remains to be done, however.

Firstly, we need to determine the precise location and extent of the cambial stem cell layer. One approach would be clonal analysis (Subtelny & Sussex, 1978), essentially creating mutant sectors within the cambial zone that express a marker gene like GUS or green fluorescent protein (GFP). By following the spread of marked sectors in a large number of cell files, one should be able to infer the position of the cambial initial as only a mutated initial will spread the marker to both phloem and xylem side. Similar techniques have been instructive in determining the structure of both shoot and root apical meristems (Steves & Sussex, 1989). The experiments in Paper IV identified a number of genes with highly specific expression in the stem cell region, providing us a set of potential stem cell markers. The expression of a stem cell marker should be restricted to one or a few cells and the vast majority of anticlinal divisions should occur in cells expressing the marker. In order to achieve the necessary single cell resolution, techniques like in situ hybridizations, immunolocalization or promoter-reporter constructs will have to be employed. Genes involved in specifying cambial stem cell identity might also be discovered by creating transcript maps across dormant cambial meristems. During dormancy cell division ceases so most factors associated with cell proliferation will be absent. The stem cells, however, need to retain their identity throughout the dormant period. Genes showing specific expression in the cambial zone of dormant stems would therefore be likely candidates for factors specifying stem cell identity. The model presented in Fig. 14E makes a number of predictions on the cambial functions of known regulatory genes. These will remain speculative, of course, until they have been tested functionally. If the *PttCLV1* genes flanking the stem cell region have a function in restricting the size of the stem cell pool, then loss of *PttCLV1* function should lead to an increased number of cambial stem cells with a concomitant increase in cambial cell proliferation. Similarly increased and decreased activity of *PttANT* should lead to more or less cambial proliferation, respectively (Mizukami & Fischer, 2000), while loss of *PttPNH* function should interfere with the maintenance of the cambial stem cells, analogous to its function in the SAM (Moussian *et al.*, 1998).

Most of the experiments suggested above require changing the expression levels and expression patterns of genes using overexpression or RNA interference. This approach poses a number of challenges, however. In case the gene in question plays a role in SAM activity, global change of its expression will affect the SAM as well as the cambium, making it difficult to distinguish between primary and secondary effects. Especially the necessary regeneration of transgenic trees in tissue culture will select against any transgene that interferes with shoot initiation and elongation. Inducible systems might be a viable alternative here. In cases where there is a clear sequence orthologue in Arabidopsis it might be more convenient to do initial characterisations in that system since the Arabidopsis hypocotyl shows most of the central features of secondary growth (Chaffey *et al.*, 2002).

The comparison of gene expression between active and dormant cambium only provided a snapshot of the dormancy process. For a deeper understanding it will be necessary to generate time courses of gene expression activity in order to try and separate the different subprocesses involved in establishment and maintenance of dormancy. Comparison of results from in vitro induction experiments with data from trees grown in a natural setting could be used to estimate the influence of environmental factors on the expression of different genes. The microarray experiments also raised issues concerning the influence of changes in tissue composition on gene expression results, which could be resolved by creating tangential cutting series at different timepoints during dormancy induction.

In the end: Pros and cons of Populus

The work in this thesis has shown both the possibilities and the limitations of aspen as a model system. If one wants to answer fundamental questions on plant development and cell biology like how polarity is established and maintained, how a hormone activates gene expression or what the factors are that determine leaf shape, then aspen is clearly not the model of choice. Here Arabidopsis offers an unrivalled selection of mutants, markers and methods that greatly facilitate experimental work. But Arabidopsis is not a tree. It does not form a massive stem, it does not make heartwood, and it does not go into winter dormancy. Arabidopsis will never be used as a construction material or for papermaking. Tree-specific questions will have to be answered using trees, but the most effective approach will be the one that combines the strength of multiple model systems, where each part of the question is approached with the tool best suited for the job at hand.

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