Wood Formation and Transcript Analysis with Focus on Tension Wood and Ethylene Biology

Sara Andersson Gunnerås

Faculty of Forest Science

Department of Forest Genetics and Plant Physiology

Umeå

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Abstract

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New molecular tools were used to study wood formation in hybrid aspen (*Populus tremula* L. x *tremuloides* Michx.), with a focus on tension wood (TW) formation. TW is a gravitational response, and forms on the upper side of the stem to maintain an optimal position. TW formation is associated with an increased growth rate, modified fiber cell walls with low lignin, and high cellulose content.

The plant hormone ethylene is induced during TW formation, but mechanisms regulating its level in wood forming tissues, and its exact function in wood development, are not well known. Several ethylene related transcripts were found in expression sequence tags (EST) libraries from wood forming tissues. A 1-aminocyclopropane-1-carboxylate oxidase (ACO) was cloned, observed to be expressed in developing xylem, and highly up-regulated exclusively in (upper side) TW forming tissues. Simultaneously, the precursor of ethylene, ACC, accumulated in opposite (lower) wood. This finding demonstrated that ACO expression, rather than ACC availability, regulated ethylene production in this system. Further, an ACC deaminase (ACD) that metabolizes ACC was identified from EST libraries. Previously thought to be specific to microorganisms, this is the first report of an ACD endogenous to plants. The poplar ACD was mainly expressed in the cambial zone and phloem, complementing the expression of ACO in the xylem, which suggests that ethylene synthesis within the stem is tightly controlled.

A poplar microarray was used to study gene regulation in TW. The analysis was focused on genes related to the flux of carbohydrates to cell wall components, plant hormones and a group of highly up-regulated fasciclin-like arabinogalactan proteins. The study demonstrates several genes important for the flux of carbohydrates from lignin and hemicelluloses towards cellulose production.

Microarray analysis was also used to study the effect of gene expression in transgenic trees down-regulated in lignin biosynthesis genes, either the enzyme caffeic acid O-methyl transferase or cinnamyl alcohol dehydrogenase (CAD). Interestingly, several of the genes uniquely affected by CAD down-regulation where related the plant clock function. This finding appears to be caused by a secondary event, due to the red coloration of the xylem observed in the transgenic lines.

Keywords: populus, wood formation, tension wood, ethylene, ACC, lignin, CAZY, fasciclin-like arabinogalactan proteins, microarray

Author's address: Sara Andersson Gunnerås, Umeå Plant Science Centre, Department of Forest Genetics and Plant Physiology, SLU, S-901 83, UMEÅ, Sweden

“Fääääääääääääääääääääääärdig!”

ur Beppes godnattstund

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Appendix

List of papers

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

I. Andersson Gunnerås, S.,* Hellgren J.M.,* Björklund, S., Regan, S., Moritz, T. and Sundberg, B. Asymmetric expression of a poplar ACC oxidase controls ethylene production during gravitational induction of tension wood. *Plant Journal* 2003 May; 34 (3): 339-49.

II. McDonnell, L., Andersson Gunnerås, S., Sundberg, B., Glick, B., Willmore, W. and Regan, S. Identification of a functional plant-encoded ACC deaminase - A mechanism to regulate ethylene levels. *Manuscript*.

III. Andersson Gunnerås, S., Mellerowicz, E.J., Ohmiya, Y., Nilsson, P., Henrissat, B., Love, J., Moritz, T. and Sundberg, B. Making cellulose enriched gelatinous fibers in poplar: global analysis of transcripts and metabolites identifies biochemical and developmental regulators in secondary wall biosynthesis. *Manuscript*.

IV. Holst Christensen, J., Storme, V., Andersson Gunnerås, S., Sundberg, B. and Boerjan, W. Dissection of the transcriptional/biochemical response to down-regulation of the lignin biosynthesis gene cinnamyl alcohol dehydrogenase in poplar. *Manuscript*

*To be considered joint first authors

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Introduction

Trees provide us with wood, a natural and renewable resource used for a diversity of products; e.g. building materials for house construction, furniture, energy, pulp and papermaking and even for clothes. With a growing human population the demand for wood and woody products is constantly increasing, and large areas of natural forests have therefore been exploited. Much recent research on forest trees is focused on improving not only wood biomass production, but also the properties of wood and wood fibers and new techniques in biology have provided us with research tools to accelerate tree breeding (reviewed by Campbell et al., 2003). To a large extent, future forestry is likely to rely on plantations with highly productive trees grown for specific purposes. Such plantations would allow for larger areas to be kept as natural forests.

Wood anatomy and its physical development are well described (e.g Savidge, 2000; Mellerowicz et al., 2001; Ye, 2002), but still much research remains in order to elucidate the molecular regulation of wood formation. One of the first initiatives to study molecular regulation of wood formation in a global perspective was done by Sterky and co-workers (1998), who produced over 5,000 expressed sequence tags (ESTs) from a complementary DNA (cDNA) library made from wood forming tissues (cambial region) of hybrid aspen (*Populus tremula* L. x *Populus tremuloides* Michx.). Nearly half of the sequences either showed no similarity to existing sequences available in public databases or represented genes of unknown function. Hertzberg and co-workers (2001) used a unigene set from this EST library to produce a microarray with nearly 3,000 ESTs. By combining micro-dissection with polymerase chain reaction (PCR) amplification, gene expression across the wood forming tissues was visualized. This study revealed, for example, genes specifically expressed during secondary wall formation and lignification, two important steps in the wood forming machinery. Schrader and co-workers (2004) elaborated on this approach further using a 13 K poplar array (Andersson et al., 2004), demonstrating gene expression across the cambial meristem regulated according to a developmental gradient. To date, more than 100.000 EST have been sequenced from *P. tremula x P. tremuloides* and *P. trichocarpa* T.&G. (Sterky et al., 2004), and recently the poplar genome (*P. trichocarpa*) was fully sequenced by the Joint Genome Institute, US Department of Energy (http://www.jgi.doe.gov/). These efforts have established poplar as an important model system for molecular tree biology and biotechnology (Bradshaw et al., 2000; Brunner et al., 2004). The softwood species Loblolly pine (*Pinus taeda* L.) has been exploited by a similar global effort (http://pine.ccgb.umn.edu/), including EST sequencing of wood related libraries and production of microarrays (Allona et al., 1998; Whetten et al., 2001). There are also other wood related EST databases made from several other tree species like cider tree (*Eucalyptus gunnii* Hook) (Paux et al., 2004) and maritime pine (*Pinus pinaster* Ait.) (http://www.pierroton.inra.fr/Lignome/Posters/lignomeforet.jpg).

Although EST sequencing and array experiments result in enormous amounts of data, transcript abundance does not give the complete story of molecular events in the experimental tissue(s). Ideally, transcript data should be combined with protein and metabolite information, because regulation also occurs post-transcriptionally. Global studies of proteins and metabolites in wood forming tissues are still in

its infancy and only few examples in the literature are available. For example, Mijnsbrugge and co-workers (2000) compared protein patterns between bark and wood tissues from aspen, and Morris and co-workers (2003) performed metabolic profiling on differentiating xylem tissue of loblolly pine. Much research in this field is in progress and these techniques are likely to be used on a more regular basis in the near future.

The information on gene expression in public databases is a valuable source that can be used for comprehensive functional genomics approaches to identify key components in the process of wood formation. In this thesis, I have explored information emerging from the Swedish poplar-sequencing project (http://www.populus.db.umu.se/). In the first library sequenced from wood forming tissues (Sterky et al., 1998) several ethylene related genes were identified. In particular the roles of an ACC oxidase and an ACC deaminase were investigated. I have also used microarray technology to study gene expression during tension wood formation and in transgenic poplars with modified lignin.

Background

Wood formation

The lateral meristem of a tree, the vascular cambium, is responsible for the production of wood. The outer side of the vascular cambium (bark side) forms phloem, which is responsible for the transport of photosynthates from source to sink tissues. Towards the inner side the cambium forms xylem (wood). The wood provides support to the stem and is responsible for upward transport of water and nutrients from the root system. Wood formation is a highly organized process (Fig. 1) where the xylem mother cells first divide, then differentiate through the phases of radial expansion and elongation, secondary wall deposition, lignification and eventually the cell's protoplast is hydrolyzed and broken down. Recent understanding of wood development has been reviewed by e.g. Mellerowicz and co-workers (2001) and Plomion and co-workers (2001) and based on their reviews I will briefly describe this process.

In angiosperm trees like poplar, wood is composed of vessel elements, fibers and parenchyma cells. These cell types have different function and originate from two types of initials in the cambial meristem: 1) the fusiform initials giving rise to the axial wood elements, such as vessel elements, fibers and axial parenchyma cells, and 2) the ray initials giving rise to horizontally oriented ray cells. Fiber cells are the most abundant cell type and serve mainly to provide mechanical support. Vessel elements mature faster then fibers and are responsible for water transport. The secondary wall of vessel elements is thinner and the cell diameter wider than that of fibers. Horizontally aligned ray cells also form a secondary wall, but stay alive for several years. Their function is to store nutrients like starch and for its transport to and from the phloem. The proportion of the different cell types varies between tree species. For example, Panshin and De Zeeuw (1980) reported poplar wood to be composed of 53-55 % fibers, 33 % vessels and 12-15 % ray cells.

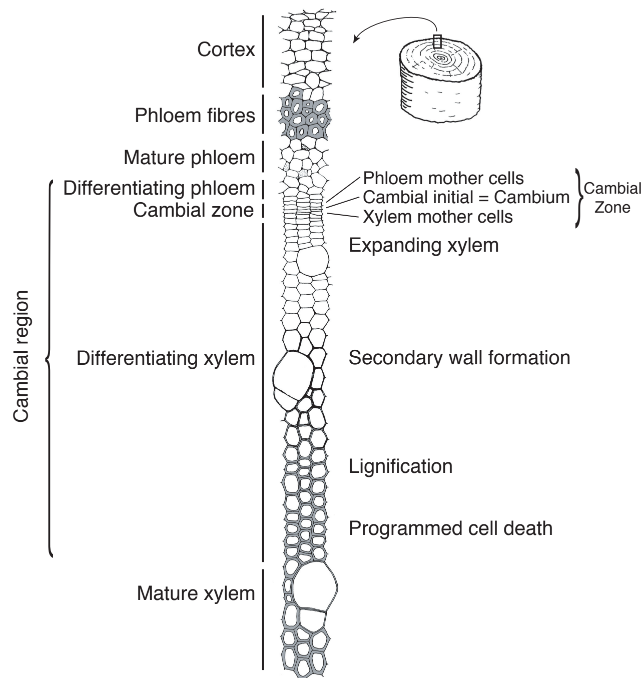

Figure 1. Schematic overview and terminology of the cambial region in *Populus* (from J.Schrader, thesis 2003)

Variations in wood

The structure and composition of wood is highly variable within a stem. This variation is due to both intrinsic and environmental factors. Examples of intrinsic wood variation are the formation of juvenile/mature wood and heartwood formation. Juvenile wood is normally formed during the first 10-15 years of cambial activity from inception and is in general characterized by shorter fibers with greater micro fibril (MF) angles as compared to mature wood (reviewed by Parresol and Cao, 1998). Heartwood is formed in the central core of the stem after a period of time that varies depending on species. Heartwood accumulates extractives, does not conduct water and contains only dead cells (reviewed by Panshin and De Zeeuw, 1980).

Examples of environmentally induced wood variations are earlywood/latewood and reaction wood. Earlywood/latewood are typical characteristics in trees growing in temperate regions and results in annual rings. Earlywood is associated with fast early seasonal growth and has xylem cells with large lumen and relatively thin secondary walls. Latewood cells form later in the growing season and have cells with narrow lumina and thicker cell walls. Annual ring formation is more or less species dependent. Poplar is a diffuse-porous tree, with an even distribution of vessels and less pronounced seasonal variation within an annual ring. However, increase in xylem cell length and altered lignin composition has been reported in latewood (Liese and Ammer 1958; Takabe et al., 1992), beside the characteristic cell wall morphology. Formation of reaction wood enables stems and branches undergoing secondary development to grow in more favorable directions. This

response results in a drastic developmental switch, with changes in several aspects of the wood produced e.g. altered growth rate and cell wall composition.

Clearly, there are large variations of wood properties within a tree stem that will affect its use as a raw material. Because wood is mainly composed of cell walls, their biosynthesis is an important aspect in understanding the molecular control underlying wood properties and wood variations.

The cell wall

Plant cells deposit a primary cell wall. Major components of the primary wall are pectins, cellulose and hemicelluloses. Xylem cells eventually develop a secondary cell wall, which is deposited between the plasma membrane and the primary cell wall after cell expansion is complete. Major components of the secondary cell wall are cellulose, hemicelluloses and lignin. There are major differences in the composition and properties of the primary and secondary cell walls. Pectins and xyloglucan are only deposited into the primary wall although a recent study reported that the majority of xyloglucan is deposited when the secondary wall is already partially formed (Borquin et al, 2002). The organization of cellulose also differs between the primary and secondary walls. Several long (1-4) β-D-glucan chains of paracrystalline cellulose are linked together by many hydrogen bonds to form MFs that in the primary wall are randomly oriented tending to the longitudinal axis whereas MFs in the secondary wall lie parallel to each other in a helicoidal pattern (Fig. 2A). The MF angle in the cell wall contributes significantly to its properties. In the secondary wall the MF angle differs across the wall and results in the formation of two to three distinct layers termed, S1, S2 and S3 (Timell, 1986). In the S1 the MFs are usually almost transverse to the cell axis, whereas the thickest layer S2, has more longitudinally arranged MFs. In the S3 layer, the MFs have again a more transverse angle (Fig. 2A).

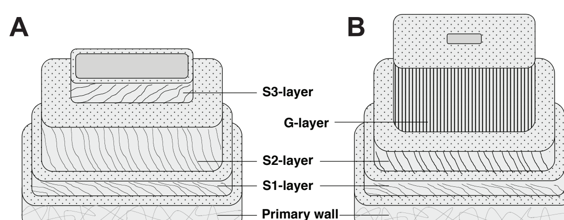

Figure 2. Three-dimensional structure of a *Populus* wood fiber (inspired from Plomion et al 2001). The cell wall is divided into different layers, each layer having its own particular arrangement of cellulose MFs, which determine the mechanical and physical properties of the wood in that cell. These MFs may be aligned irregularly (as in the primary cell wall), or in a particular angle to the cell axis (as in layer S1, S2, and S3). A, Normal wood fiber. B, G-fiber: part of S2 and S3 is replaced by the thick gelatinous G-layer.

The cell wall also contains structural proteins such as glycine-rich proteins, proline rich proteins, arabinogalactan proteins, and hydroxyproline-rich glycoproteins (or extensins) that are mainly cross-linked with wall carbohydrates (Brett and Waldron, 1996). The group of arabinogalactan proteins (AGPs) may also have a function in growth and development. This idea is supported by the finding that some AGPs are anchored to the plasmalemma by a glycosylphosphatidylinositol (GPI)-anchors

(Youl et al., 1998; Sveteck et al., 1999). GPI-anchored proteins in animals have been found to be involved in cell-to-cell signaling (Selleck, 2000). A recent paper from Motose and co-workers (2004) identified a proteoglucan with AGP domains and a GPI-anchor that functioned as a xylogen in *Zinnia* cell cultures, and was demonstrated to be required for normal vascularisation in *Arabidopsis*. This AGP-like protein was claimed to function as a signal for vessel development.

Synthesis of polysaccharides like cellulose, hemicelluloses, pectins and glucosylated cell wall proteins is mediated by a large group of carbohydrate active enzymes (CAZYs). Coutinho and Henrissat (1999) initiated the Carbohydrate-Active Enzymes server (http://afmb.cnrs-mrs.fr/CAZY/), compiling families of structurally related enzymes with catalytic and carbohydrate-binding modules, or functional domains that degrade, modify, or create glycosidic bonds. CAZYs are collected from different phyla including several eukaryotes, *Arabidopsis* being the only plant species included at present. Thanks to the information obtained from genome sequencing together with bioinformatics resources, several hundred CAZYs have now been identified. Much work is now focused on understanding the function of these enzymes. At the moment only a few have been related to plant cell wall polysaccharide synthesis or modification (reviewed by Mellerowicz et al., 2001; Plomion et al., 2001; Scheible and Pauly, 2004; Aspeborg et al., 2005).

Lignin is a heterogeneous phenolic polymer that gives rigidity and impermeability to xylem cells. Lignin also functions as protection against attack from herbivores. The lignification process starts in the middle lamella after the deposition of S1, and continues through the primary wall and further through the secondary wall (Terashima et al., 1993). Lignin is a polymer of cinnamyl alcohols (monolignols), mainly *p*-coumaryl, coniferyl and sinapyl. Hardwood species mainly incorporate coniferyl- and sinapyl alcohols into their lignin complex. Recent research has shed much light on the lignin biosynthetic pathway and most aspects of the pathway leading to the formation of monolignols are now known, (reviewed by Boerjan et al., 2003; Rogers and Campbell, 2004).

Plant growth regulators and wood formation

Ideas concerning the physiological role of plant hormones in wood formation come from application studies that have shown hormones to affect most processes during xylogenesis (reviewed by Aloni et al., 2000; Mellerowicz et al., 2001). Today, analytical techniques have been developed that can measure the endogenous levels of a hormone with high sensitivity and accuracy adding further understanding to their function in wood formation; see for instance Moritz and Sundberg, (1996), Uggla and co-workers (1996), Tuominen and co-workers (1997), Eriksson and co-workers (2000) and Klintborg and co-workers (2002).

Ethylene is important in many aspects of plant development and has for a long time been suggested to have a role in xylogenesis and wood formation (reviewed by Bleecker and Kende, 2000; Roberts and Miller, 1983). However, ethylene's homeostasis and the mechanism of action in wood forming tissues still remain unclear. In this thesis, ethylene biology in wood forming tissues has been studied using modern analytical and molecular tools in combination with global transcript analysis.

Ethylene biosynthesis, regulation and signal transduction pathways

Ethylene is a small gaseous compound and its biosynthesis, perception and signal transduction pathway have been described in several reviews (recently by Guo and Ecker, 2004). It is produced from L-methionine, which is converted to S-adenosylmethionine (SAM), a reaction that is catalyzed by S-adenosyl-1-Methionine (SAM) synthase. SAM synthase is one of the most abundant transcripts and proteins in developing xylem in both poplar (Sterky et al., 1998; Mijnsbrugge et al., 2000) and pine (Guo and Ecker, 2004; Costa et al., 1999). The first enzyme entirely committed to ethylene biosynthesis is 1-aminocyclopropane-1-carboxylic acid (ACC) synthase converting SAM to ACC. Ethylene is then produced by the action of ACC oxidase (Fig., 3). Both ACC synthase (ACS) and ACC oxidase (ACO) belong to multigene families that are regulated in response to several developmental and environmental factors (Yang and Hoffman, 1984; Boller et al., 1979; Yu et al., 1979). ACO has been suggested to be located in both the cell wall (Rombaldi et al., 1994) and in the cytosol (Chung et al., 2002).

ACC can be conjugated to malonyl-ACC (Hoffman et al., 1982) and to glutamyl-ACC (Martin et al., 1995) (Fig. 3). ACC conjugation is thought to be an irreversible reaction and functions to decrease the ACC pool (Abeles et al., 1992). In soil bacteria, an ACC deaminase has been described that converts ACC to α-ketobutyrate and ammonia. The bacteria interact with plant roots thereby affecting the ethylene status of the root, hence also root growth and development (Hall et al., 1996). ACC deaminase has not been described in plants, but the bacterial gene has been used in biotechnological applications to slow down ripening of tomatoes and decrease stress induced ethylene synthesis (Klee et al., 1991; Robison et al., 2001). However, our research shows that ACC deaminase is a plant enzyme (paper II).

The ethylene signal transduction pathway has been studied in e.g. *Arabidopsis* where five ethylene receptors (ETR1, ETR2, ERS1, ERS2 and ethylene insensitive 4 (EIN4)) have been identified (Hua and Meyerowitz, 1998). Chen and co-workers (2002) found that ETR1 is predominantly located in the endoplasmatic reticulum membrane. Binding of ethylene to the receptor leads to a conformational change in the serine/threonine protein kinase CTR1, identified by Kieber and co-workers (1993) and recently found to be a part of the ethylene-receptor signaling complex (Gao et al., 2003). CTR1 function as a negative regulator possibly inactivating a MAPK cascade (Quaked et al., 2003). Downstream of CTR1, EIN2 functions as a trans-membrane positive regulator (Alonso et al., 1999). Further downstream of EIN2, the transcription factor EIN3 is another positive regulator (Chao et al., 1997). EIN3 activates ethylene responsive element binding proteins (EREBPs), which accordingly result in developmental and growth responses (recently reviewed by Wang et al., 2002).

Ethylene and wood formation

About 3 % of the EST library made from the cambial region of poplar (Sterky et al., 1998) contained hormone related transcripts, and among them several ethylene biosynthetic and signaling related sequences. This indicates a functional role of ethylene in wood forming tissues. Also ACC and ACC conjugates have been observed in the cambial region of several softwood species (Savidge et al., 1983; Eklund, 1991; Klintborg et al., 2002). Ethylene related studies during wood

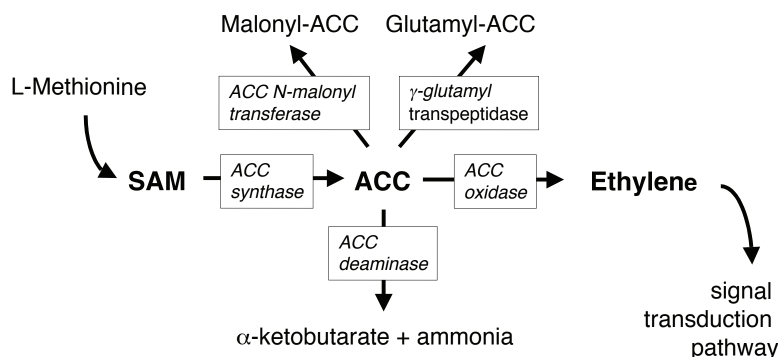

Figure 3. Ethylene biosynthetic pathway. SAM=S-adenosyl-1-Metionine, ACC=1-aminocyclo-propane-1-carboxylic acid.

formation of hardwood species are few. Yamamoto and Kozlowski, (1987) and Yamamoto (1987c) applied ethrel (an ethylene releasing compound) to Norway maple (*Acer platanoides* L.) and American elm (*Ulmus Americana* L.) seedlings, which resulted in increased growth of both bark and xylem. Moreover, ethylene treatments resulted in wider rays with larger cells and a decreased proportion of vessels. In a recent study Junghans and co-workers (2004) applied ethrel to poplar (*P. alba* L. *x P. canescens*) stems and, similar to previous studies, an increased growth of both bark and xylem was observed. They also measured the length of xylem fibers and vessel elements and found that these were much shorter after treatment with ethrel.

Ethylene has also been suggested to affect cell wall composition by altering deposition of carbohydrates. Ingemarsson and co-workers (1991b) found an increased proportion of cellulose with increasing concentration of applied ethrel on Norway spruce (*Picea abies* L.). They also observed a decrease in non-cellulosic polysaccharides whereas the lignin content was not changed. Sitbon et al., (1999) found that tobacco lines overproducing IAA had higher ethylene production, increased peroxidase activity and more lignin compared to wild type plants. The idea that ethylene is inducing peroxidase and enzymes involved in monolignol synthesis, phenylalanine ammonia lyase (PAL) and 4-coumarate:coenzyme (4CL), has existed for a long time (for review see Roberts and Miller, 1983), but conclusive evidence for this hypothesis is still lacking.

Reaction wood

Reaction wood (RW) is a modified type of wood and is a unique feature of woody perennial species. It is formed in leaning trees, or in response to prolonged wind/snow load (Timell, 1969). It can also easily be induced for experimental purposes by bending and fixing the tree with a string as seen in figure 4A. RW is formed to maintain a branch in 'proper' orientation or a stem upright. In conifers, RW is formed on the lower side of a leaning stem and is called compression wood (CW), whereas in angiosperm trees, RW is formed on the upper side of the leaning

stem and is called tension wood (TW). RW is often associated with an increase in diameter growth in order to facilitate stem or branch reorientation while growth is often inhibited on the opposite side of stem (Timell, 1986). The wood formed on the opposite side of stem is referred to as opposite wood (OW).

Originally RW was thought to form in response to mechanically induced growth stresses, because leaning trees are subjected to tension on the upper side and compression stresses on the lower side. This idea was challenged after the experiment by Jaccard in 1938, who showed that CW and TW was not always induced on the compression or tension side, respectively. He demonstrated that RW formed in branches bent to form loops, depended on position in the stem, not necessarily at the site of tension or compression stress (reviewed by Scurfield, 1973). The conclusion from these experiments is that stems and branches react to their position in the gravitational field rather than to the mechanical stress *per se*. This conclusion has been confirmed in many other experiments, employing for example a clinostat (Timell, 1986). However gravity alone cannot explain all aspects of RW induction. For example, if the apical leader is removed, RW will form inside branches to enable them to bend upward and replace the lost leader (Timell, 1986). In addition, Kwon and co-workers (2001) showed that Douglas fir (*Pseudotsuga menziesii* Mirb.) and Loblolly pine (*Pinus taeda* L.) plants grown under micro gravity conditions form compression wood in response to mechanical bending.

Tension wood

Compared to normal wood, TW contains fewer vessels of a smaller diameter, more fibers and fewer rays (reviewed by Hughes, 1965b). The cellulose content is higher, and lignin and hemicelluloses contents are lower (Timell, 1969). TW formation is often associated with increased growth resulting in stems that are eccentric when observed in cross-section. A characteristic feature of TW is a thick gelatinous cell wall layer (G-layer) in the fibers (G-fibers) (Fig. 2B and 4B and C). The G-layer is composed of highly crystalline cellulose, which is loosely attached to the S-layer. Therefore cut surfaces of TW have a "woolly" appearance as the G-layer is partially pulled out. The MFs of the G-layer are arranged nearly parallel to the fiber axis (Fig. 2B). How the G-fiber helps with the restoration of the stem or branch angel is not known. It has been suggested that different shrinkage properties of the G- and S-layers create the physical forces that make the branch/stem to reorient (Norberg and Meier, 1966), but the exact mechanisms behind these forces are still to be demonstrated. Jourez and co-workers (2001) did an extensive investigation on TW properties in poplar (*P. euamericana* Dode). They found that TW fibers were longer than fibers from the OW, although no comparison with normal wood was done.

In wood species that normally form three cell wall layers in fibers like poplar, the S3-layer and partly the S2-layer are replaced by the G-layer in G-fibers (Norberg and Meier, 1966). Mia (1968) studied deposition of the G-layer in poplar and found that cell death is delayed in G-fibers. Mia (1968) also studied the chemical composition of the S1- and S2-layers, which appeared to be similar to that in normal wood. In other studies, however, it has been reported that lignin content is higher in the S1- and S2-layers of TW fibers (Bentum et al., 1969). In general, a lower lignin content of TW compared to normal wood has been reported for many species (Timell, 1969).

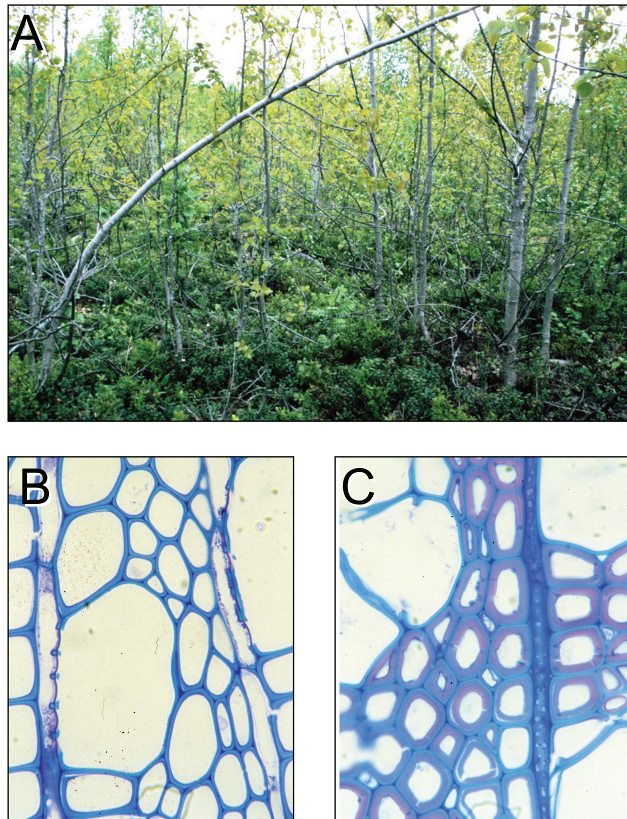

Figure 4. Tension wood in *Populus tremula*. A, Tension wood can eacily be induced by bending and fixing the stem with a string. B and C are cross-section of xylem from B= normal wood and C=tension wood with typical G-fibres and G-layer.

Whether the G-layer contains lignin is still a matter of debate. Norberg and Meier (1966) could not detect lignin when analyzing isolated G-layers from *P. tremula* by quantitative paper chromatography. Mia (1968) used electron microscopy and stained with heavy metallic salts and observed densely stained amphorous substances in the G-layer that could be lignin. Bentum and co-workers (1969) questioned the generality of lower lignin content of TW because they detected an increased level of lignin in the compound middle lamella, S1- and S2-layer in G-fibers compared to normal fibers. Blanchette and co-workers (1994) did not find a change except in the G-layer, were they found no lignin. Recently, Joseleau co-workers (2004) claimed the presence of syringyl units in the G-layer of Populus deltoides (Bartr. ex Marshall) based on immunogold labeling with antibodies.

Plant growth regulators and tension wood formation

Application studies have indicated a role for both auxin and gibberellin (GA) in TW formation. Necesany (1958) applied auxin on the upper surface of leaning

cottonwood (*Populus monilifera*) trees, which prevented TW to develop. Cronshaw and Morey (1965) ringed vertical stems of red maple (*Acer rubrum* L.) with TIBA, an auxin transport inhibitor, and found TW below the application point. These observations resulted in the idea that TW is formed under conditions of auxin deficiency. Recently Hellgren et al., (2004) mapped IAA (natural auxin) distribution across the cambial region tissues of aspen (*P. tremula*) stems forming TW using GC-MS in combination with cryosectioning. IAA levels were lower on the opposite side, compared to normal wood, which contradict the theory that low auxin levels induce TW.

GA involvement in TW formation was suggested from the experiments by Baba and co-workers (1995) who applied GA3 (active form of GA) to branches of the weeping cultivar of Japanese cherry (*Prunus spachiana*). This resulted in the induction of TW on the upper side of branches followed by their upward growth, thus the weeping phenotype was lost. Other studies have shown no affect when applying GA. For example, Cronshaw and Morey (1968), applied various growth substances, including GA, to horizontally placed red maple. Unlike auxin, GA application did not affect TW formation.

Ethylene and tension wood formation

An elevated level of ethylene has been observed on the upper side of stems/branches forming TW in e.g. tuart (*Eucalyptus gomphocephala* DC) and Japanese horse chestnut (*Aesculus turbinata* Bl.) (Nelson and Hillis, 1978; Du and Yamamoto, 2003). Yamamoto and Kozlowski (1987) applied ethrel to vertical stems, which did not induce TW. Furthermore they applied ethrel on the upper half of leaning stems, which was found to inhibit G-fiber formation, but an increased growth on the side of the application point was observed. They concluded that ethylene is not directly involved in G-fiber formation. This was further supported by recent research in our laboratory by Love, Björklund, Tuominen and Sundberg (unpublished). They found that while G-fibers were formed in ethylene insensitive silver birches (*Betula pendula* Roth) that were leaned to induce TW, the eccentric growth was not observed in these trees suggesting that ethylene may be important in the growth aspect of the TW response.

Objectives and summary of the papers

My first aim was to increase the understanding of ethylene biosynthesis during wood formation. The second aim was to increase the general understanding of the molecular regulation of tension wood using microarray analysis. Microarray technology was also used for a study of transgenic poplars with modified lignin.

My results are presented in the following papers:

Paper I describes the identification and characterization of an ACC oxidase (*PttACO1*) from the wood-forming tissues of hybrid aspen. *PttACO1* was found to be primarily expressed in xylem cells depositing secondary cell walls. Despite this localized expression of the ACC oxidase, ACC levels were found to be fairly uniform across the cambial region. Leaning of stems strongly induced *PttACO1* expression and ACC oxidase activity in developing TW, whereas no change was recorded in OW. In parallel, ACC and its hydrolysable conjugates were slightly increased in TW and greatly accumulated on the opposite side of the stem. This suggests that the ACC content increases in association with tension wood induction, and is rapidly converted to ethylene on the TW side by the highly up-regulated *PttACO1*. These observations led to the conclusion that ACC oxidase activity, rather than ACC availability, limits ethylene production during tension wood formation in poplar trees.

Paper II identifies for the first time a plant ACC deaminase (ACD). The ACD from *Arabidopsis* was expressed in *Escherichia coli* to confirm that it encodes an active enzyme. The plant ACD was identified by its expression in the cambium/phloem tissues of hybrid aspen, which correlates with the localization of its activity. This suggests that ACD together with *PttACO1* (presented in paper I) may regulate and direct ethylene biosynthesis in developing wood tissues. Furthermore, enzyme activity of ACD was shown during germination, seed development and in vascular tissues of *Arabidopsis*. It was also expressed during tomato fruit ripening where the expression correlates with the ethylene burst. In summary, ACD may be an additional regulatory step for ethylene production in plants by regulating ACC availability for ethylene biosynthesis.

Paper III is a microarray study on developing tension wood in poplar. Developing xylem tissues from normal wood were compared to developing tension wood tissues. The study was complemented with metabolite profiling of soluble compounds from the same tissues. Most striking was a group of highly up-regulated arabinogalactan proteins. We demonstrate that these up-regulated genes lack true orthologues in *Arabidopsis*. Because of the modified chemistry in G-fibers, i.e. decreased lignin, and hemicelluloses and increased cellulose, we made a special effort to identify differentially regulated genes in these pathways in more detail. We also identified several ethylene and auxin related transcripts that were differentially regulated in TW forming tissues.

Paper IV is a microarray study to identify transcripts affected by the down-regulation of caffeic acid O-methyltransferase (COMT) and cinnamyl alcohol dehydrogenase

(CAD), two enzymes involved in monolignol biosynthesis. These transgenic lines have previously been described by Baucher and co-workers (1996) and Van Doorsselaere and co-workers (1995), respectively. In this study, we particularly focused on transcripts that were uniquely affected in the CAD lines. The CAD antisense line has earlier been demonstrated to incorporate more non-conjugated phenoxy groups into the lignin polymer, and exhibit a red coloration of the xylem. These alterations do not affect growth and development but result in tissue and fiber properties with improved pulping characteristics. Interestingly, we found that many genes related to circadian rhythm were affected in the CAD lines. Possibly this was due to altered light perception in the xylem of the CAD lines due its red coloration.

Methodological considerations

Poplar – a model system for wood formation

Poplar has recently has been established as a model system for woody plants (Bradshaw et al., 2000; Brunner et al., 2004). Poplar is well suited for this purpose as it has a small genome (5x the size of *Arabidopsis*), fast growth, is easy to propagate and it is easily transformed. Much work in this thesis was focused on TW formation, which is specific for hardwood species, therefore it was suitable to use poplar for these studies. In my research I have taken advantage of recently developed tools that established poplar as a tree model species; about 120,000 ESTs from 19 different cDNA libraries sequenced within the Swedish poplar consortium (Sterky et al. 2004; www.populus.db.umu.se/). From this collection of ESTs, microarrays have been produced (Hertzberg et al, 2001; Andersson et al., 2004). In addition, the full genome of *P. tricocarpa* was recently sequenced, by the Joint Genome Institute US Department of Energy (http://www.jgi.doe.gov/).

Plant materials and sampling techniques

Both potted clonal greenhouse grown hybrid aspen (*Populus tremula* x *P. tremuloides* or *P. tremula* x *P. alba*) and field-grown aspen trees (*P. tremula*) were used. Greenhouse grown trees offer the advantage of all year round access to experimental material grown under controlled conditions. However, for studies on tension wood, these young trees have the disadvantage that tension wood is also easily formed in upright control trees. For this reason, experiments were also performed with older field grown trees where spontaneous TW in control trees was rarely observed. The drawback with field experiments is the increased genetic and environmental variation and the limited time window available for experiments.

For the greenhouse experiments TW was induced in approx. 1.5-2m high plants by tilting the pot and supporting the stem at an angle of about 30° from vertical axis. The experiments with field-grown aspen were done on about 15 year old trees selected from a natural stand near Umeå, Sweden (paper I, II and III). TW was induced by bending the stem to an angle of approximately 45°, and fixing it in this position using a string (Fig. 4A). This was done during the most active period of cambial growth. TW with typical characteristics was induced in both systems.

Bending experiments, however, will unavoidably induce mechanical stress, which should be taken into consideration when interpreting, for example, global gene expression studies. The bending does induce TW along more or less the whole stem on the upper side, so to avoid the most mechanically affected areas sampling was done below the point of the stem where visible bending were observed.

To collect the cambial region tissues the bark was peeled and the exposed surfaces were scraped with a scalpel. Anatomical studies showed that the fraction scraped from the wood side contained mostly xylem elements in the stage of secondary wall formation. The fraction scraped from the bark side contained mostly xylem elements in the expansion stage of development and cambial zone cells. In paper II the bark side was first scraped lightly to obtain a sample enriched in cambial zone cells followed by a second scraping yielding developing and mature phloem. Anatomical sections were prepared to confirm tissue identities in the samples.

Microarray analysis

Two of the papers in this thesis include microarray experiments (III and IV). Microarray analysis is a technique that allows global expression analysis of genes, first described by Schena and co-workers (1995), and later reviewed several times (e.g. Phimister, 1999). Briefly, poplar microarray slides are produced by immobilizing cDNA sequences on a glass microscope slide. RNA samples from two biological samples are reverse transcribed, labeled with respective fluorescent dyes and both allowed to hybridize with the DNA on the array. The relative fluorescent signal is detected using a laser scanner, followed by image analysis software, which quantifies signal intensity in channels corresponding to the respective dyes. Signal intensity from the respective channels is normalized to remove systematic variation giving global transcript profile information.

The microarray chips used in this work were the POP1 version with about 13.000 sequences, described in detail by (Andersson et al., 2004). The EST sequences originate from seven cDNA libraries (cambial zone, active cambium, dormant cambium, tension wood, mature leaves, senescing leaves and flower bud). Annotations of all sequences are mainly based on the best *Arabidopsis* hit from the genome model (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html). Clones discussed in any detail in the papers have been manually searched by BLAST algorithm to verify their annotation.

The development of the microarray technique has facilitated great progress in transcript analysis. The technique is fairly rapid and generates copious amounts of data. However, it is important to carefully evaluate the data obtained. For example, in this thesis two developmental event, TW formation and normal wood formation are compared (paper III). The many hundreds of genes affected involved not only the shift in cell wall metabolism, but also modulation of the proportion of cell types (e.g. vessel/fiber ratio). Therefore, although this information identifies many key genes involved in TW formation, it is an intricate task to evaluate the data in order for it to be informative. When comparing tissues from mutant plants, such as the CAD and COMT plants with WT (paper IV), one can expect less downstream genes to be differentially expressed. Even though the emerging picture will be easier to evaluate, genes related to secondary events can also be affected as indicated in paper IV.

Experimental set up

In paper III, the aim was to identify genes differentially expressed between normal- and tension wood at the stage of secondary wall formation. Therefore, only the xylem tissues were used. To account for biological variation the experiment was repeated during two different years and three trees were pooled at each time point.

In paper IV, trees with modified lignin was studied using the greenhouse grown hybrid aspen (*P. tremula* x *P. alba*). Trees with down-regulated CAD and COMT were compared with wild type (WT) trees. Trees from each line were divided into two groups of 5 individuals and each pool of trees was compared with one pool of WT trees.

Data analysis

To identify differentially expressed genes two interconnected ANalysis Of VAriance (ANOVA) models were used according to Wolfinger et al., (2001). Briefly, the first ANOVA is done to remove systematic errors. The residuals from this model representing normalized values are fitted into the second model that considers gene-specific responses to the treatment. P-values are calculated allowing inference to be made about differential expression in the treated/transgene sample compared to control/WT. Both models used PROC MIXED in SAS (SAS Institute Inc., SAS/STAT Software version 8, SAS Institute, Cary, NC, 1999).

Result and Discussion

Characterization of two ethylene metabolism genes, *PttACO1* and *PttACD*, identified from a cambial region EST library made from hybrid aspen (paper I, II)

Ethylene related ESTs were present in a cDNA library made from cambial region tissues

The EST library made from cambial region tissues (AB library) of hybrid aspen by Sterky and co-workers (1998) contained several ethylene related cDNAs. Representatives of the ethylene biosynthesis genes, ACC synthase (ACS) and ACC oxidase (ACO) were identified. Surprisingly we also identified a eukaryotic ACD, an enzyme previously described only in bacteria. The ethylene response pathway was also represented by several clones, including orthologues of two receptors (ETR2 and ERS1), a constitutive triple response 1 (CTR1) - like cDNA and a putative ethylene response element binding protein 1 (EREBP1). The evidence of genes involved in ethylene metabolitism and perception supports the idea that ethylene is synthesized in the cambial region tissues and has a function in the process of wood formation. ACO activity and an ACO protein in stem tissues have previously been reported in Scots pine (*Pinus sylvestris* L.) by Klintborg and co-workers (2002) and maritime pine (*Pinus pinaster*) by Plomion and co-workers (2000). However, neither ethylene biosynthesis nor ethylene response genes have been previously characterized in these tissues.

ACS is generally considered the limiting enzyme for ethylene biosynthesis (Yang and Hoffman, 1984). ACS is tightly regulated, at the transcriptional, post-transcriptional and even post-translational levels (Kende, 1993; Olson et al., 1995; Oetiker et al., 1997). This may explain why only one ACS-like EST (B003P14) was found among over 100 000 ESTs that are now available in the *Populus* database (www.populus.db.umu.se/). Expression of this ACS could not be detected in the cambial region by Northern blotting, but reverse transcription-PCR revealed weak expression throughout the cambial region (Björklund, Andersson Gunnerås, Sundberg, unpublished). To investigate if other ACSs were present in the stem, degenerate primers were tested and the obtained fragments were sequenced. No other ACS was found by this approach (data not shown). Further investigations need to be done to characterize ACS expression within these tissues.

PttACO1 is specifically expressed during the stage of secondary wall formation

Four ACO-like ESTs were found in the AB library, and nine more ESTs were sequenced from the tension wood library (G library). These ESTs were found to represent three different ACOs named *PttACO1*, *PttACO2* and *PttACO3* (Table 1).

Table 1. ACC oxidases present in wood forming EST libraries or differentially expressed in the cambial region.

Poplar name	# ESTs in AB library	# ESTs in G library	Total # ESTs representing this gene
PttACO1	3	7	10
PttACO2	-	2	135
PttACO3	1	-	2
PttACO4	-	-	9

No additional ACO-like ESTs were found in other wood related libraries (i.e. wood cell death, active or dormant cambium). Paper I describes the cloning and the characterization of *PttACO1*. *PttACO1* was found highly expressed during the stage of secondary wall formation in developing wood (paper I, Fig. 2). A similar pattern was indicated by microarray data analyzing gene expression across the cambial region tissues (Schrader et al. 2004) (data not shown). These data showed several ACOs with differential expression within cambial region tissues. *PttACO2* and *PttACO4* had a similar expression pattern as *PttACO1*. However, real time RT-PCR with gene-specific primers for all four ACOs in poplar showed that the major ACO within the stem is *PttACO1* (Björklund, Sundberg, unpublished). A weak signal was also detected from *PttACO2* but the remaining two ACOs did not have a signal of significance. From these data it seems likely that at least *PttACO4* is of little importance in wood forming tissues and the signal on the array may be due to cross hybridization. *PttACO3* was not represented on the array and RT-PCR showed no expression. The localization of *ACO* expression in the developing xylem tissues corresponds well with ACO enzyme activity (paper I, Fig. 3A), suggesting that developing xylem is an important site for ethylene production in the stem and that PttACO1 is the isozyme responsible for this.

ACC is evenly distributed within the stem

To investigate the substrate availability for ACO in the cambial region, ACC levels were mapped across these tissues by GC-MS of cryo-sectioned samples. This revealed that ACC was present, and was evenly distributed, across the cambial region tissues (paper I, Fig. 5A). This distribution pattern correlates well with the expression of ACS, but it does not reflect the xylem specific expression of *PttACO1*, and ACO activity.

PttACO1 and ACC accumulates during TW induction

Several investigators have observed increasing ethylene production from TW forming tissues (Nelson and Hillis, 1978; Du and Yamamoto 2003), We found that *PttACO1* and ACO activity was strongly induced in developing xylem during TW formation (paper I, Fig. 3B). A time course study revealed that transcripts started to accumulate after one day with a very strong up-regulation established after three days (paper I, Fig. 6C). In OW however, no clear induction of *PttACO1* was observed and the expression decreased during the days of TW induction (paper I, Fig. 6D). A similar pattern was observed in the phloem/cambium fraction, but the expression was much weaker (data not shown).

Interestingly, ACC levels were also increased after TW induction, but only on the OW side (paper I, Fig. 6B). On the TW side, the level of ACC was slightly lower than that in control trees. In addition, the distribution of ACC across the cambial region was mapped in TW and OW. At the TW side ACC level and distribution was similar to normal wood. Much increased levels were found on the OW side, again the distribution was rather uniform across the cambial region tissues (paper I, Fig. 5B and C). ACC was also found to increase in the xylem sap after TW induction. At present it cannot be concluded whether this ACC originates from the wood forming tissues or is transported from shoots or roots. Transport of ACC in vascular tissues has been reported in other species, for example in flooded tomato (Finlayson et al., 1991).

Auxin levels in TW forming trees

Measurements of IAA in TW-forming tissues revealed a slight decrease compared to upright trees. However, at the OW side, the auxin levels were dramatically decreased (paper I, Fig. 4B). This was confirmed in a more detailed study by Hellgren et al., (2004), who concluded that the distribution pattern of auxin was not altered in relation to TW formation. These findings contradict the previous idea that deficiency of auxin induces TW. We cannot however exclude the possibility that the tissue sensitivity for auxin was altered.

Regulation of ACC availability in the stem by conjugation or deamination

Although ACS is differentially regulated in time and space in order to secure a controlled production of ACC in plant organs and tissues, conjugation of ACC to malonyl-ACC or glutamyl-ACC has been found to act as an additional way to influence ACC availability (Amrhein et al., 1981; Hoffman et al., 1982; Martin et al., 1995). We found that conjugated ACC was much more abundant than ACC in the cambial region tissues during normal growth (paper I, Fig. 7C). When TW

was induced, the accumulation of ACC on the OW side was accompanied by ACC conjugation. No significant change of ACC conjugates was found on the TW side (paper I, Fig. 7B and C; note the different scales). This indicates that conjugated ACC are not a source of ACC for ethylene production in these tissues. This result supports previous findings that conjugation of ACC is an irreversible reaction (Yang and Hoffman, 1984). The ACC conjugation seems to be a way to assimilate superfluous ACC on the OW side.

Some plant growth-promoting bacteria (PGPB) produce an enzyme that deaminates ACC into ammonia and α-ketobutyrate, and thereby lowers the ethylene level in the plant (Penrose and Glick, 1998). In the AB library, four ACC deaminase-like clones were found representing one gene, named *PttACD*. Southern blot analysis confirmed that these sequences were not of bacterial origin (data not shown). This was later confirmed by the poplar genome sequencing data, which also revealed that *PttACD* was the only ACD-like gene within the poplar genome. The putative amino acid sequence of *PttACD* is 435 bp long and the domain search found a pyridoxal-phosphate dependent (PLP) domain, which is characteristic of ACC deminases. Pyridoxal phosphate is a co-factor important for the activity of the enzyme. A search of the public database, NCBI, revealed that there were other ACD-like sequences in several plants species including *Arabidopsis,* tomato, rice and birch, all with a similarity of about 40 % to bacterial ACDs. An amino acid alignment (paper II, Fig. 1) showed conserved regions between bacterial and plant ACDs.

To confirm the enzymatic activity of plant ACD, the *Arabidopsis* orthologue (At1g48420) was expressed in *Escherichia (E.) coli.* Bacteria expressing the *Arabidopsis* ACD gene grew better than wild type bacteria on a medium containing ACC as the sole nitrogen source (paper II, Table 1). The ACD by-product, α-ketobutyrate was also ten times more abundant in the liquid media from the cultures of the bacteria carrying the ACD gene. In addition, the activity of ACD was demonstrated in enzyme preparations from several tissues and organs of *Arabidopsis* where ethylene has a well-documented function (paper II, Fig. 3). These results confirm that *Arabidopsis* carry a functional ACD.

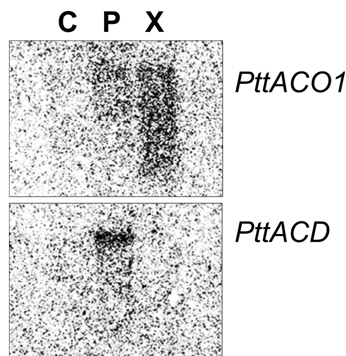

Figure 5. Expression analysis of *PttACO1* and *PttACD* in the cambial region of poplar by Northern blot. C=cortex, P=phloem and cambium, X=developing xylem.

In poplar stem, *PttACD* was mainly expressed in the phloem/cambium fraction (Fig. 5) and a more detailed analysis revealed that the main transcript accumulation occurs in the cambium rather than in the phloem (paper II, Fig. 6). Since the *PttACO1* expression was detected in secondary wall forming xylem tissues, it is clear that *PttACD* and *PttACO1* have different expression patterns in poplar stem (Fig. 5). The activity of the ACD enzyme in the cambial region tissues was indicated by the presence of α-ketobutyrate. The highest activity was found in the phloem/cambium fraction confirming the *PttACD* expression data (paper II, Fig. 6).

Global analysis of developing tension wood (paper III)

To investigate transcriptional changes related to TW formation a microarray experiment was done on developing xylem of tension wood vs. normal wood (paper III). This study focused on changes related to the formation of a modified secondary cell wall layer (G-layer) in tension wood fibers. In particular, genes involved in carbon metabolism and lignin biosynthesis were analyzed. Moreover, hormone related transcripts and transcription factors were identified. To add another dimension to this study a global metabolite analysis was done using the same samples.

Transcript analysis of developing tension wood

To obtain a full spectrum of the TW transcriptome, a cDNA library was made from developing xylem of tension wood from *P. tremula x P. tremuloides* and 5,723 ESTs were sequenced (Sterky et al, 2004). The most abundant transcripts (based on gene models) from the TW library represented sucrose synthase, methionine related enzymes and several fasciclin-like arabinogalactan proteins (FLAs) (paper III, Fig. 1). Dèjardin and co-workers (2004) analyzed 10,062 EST from cDNA libraries made from the cambial zone, differentiating xylem and mature xylem from both developing TW and developing OW of *P. tremula* x *P. alba*). Similar to our results, they found several clusters of arabinogalactan proteins and one cluster of sucrose synthase abundant in TW tissues.

The 13K poplar array (Andersson et al., 2004) was further used to identify differentially expressed genes in developing xylem of TW compared to normal wood. 444 genes were found significantly differentially expressed (paper III, Fig. 3A) at $P \leq 0.001$ and modified by at least ±30%. 75 present of these genes could be assigned a functional class (paper III, Fig. 3B). The group of most up-regulated genes in TW was dominated by fasciclin-like arabinogalactan proteins (FLAs) (paper III, Table 1).

A group of fasciclin domain containing arabinogalactan proteins is strongly up-regulated during tension wood formation

Twenty-one FLAs have been identified in *Arabidopsis*, which were divided into four subgroups by Johnson and co-workers (2003). All FLAs highly up-regulated in TW were similar to FLA12 from *Arabidopsis* (AtFLA12), which belongs to subgroup A together with AtFLA 6, 7, 9 and 11. All these proteins have one fasciclin domain flanked by AGP domains and a glycosylphosphatidylinositol (GPI) anchor.

A search in the poplar genome for orthologues of the subfamily A gave no good hits to FLA6 or 9. FLA 7, 11 and 13 were all represented by two poplar orthologues while as many as 22 FLA12-like genes were found (paper III, Table 3). Finding two poplar genes that match one *Arabidopsis* gene is common, as the poplar genome, to a large extent, has been duplicated (Personal communication, Segerman, B). That, however, cannot explain the presence of the large group of AtFLA12-like poplar genes. Out of these 22 putative genes, 18 were represented by at least one EST and 12 were spotted on the array. A phylogenetic analysis showed that the 10 FLA genes strongly up-regulated in TW were the were most distantly related to AtFLA12 (paper III, Fig. 4A). This suggests that the TW up-regulated FLA12-like genes have

acquired not only the specific expression pattern but also a specific structure. Indeed, all 10 genes that were found up-regulated in TW in our study had an insert in the first AGP domain, while none of the other FLAs had this insert. The presence of this structural motif in TW-up-regulated FLAs was also reported by Lafarguette and co-workers (2004). Interestingly, the expression patters from FLAs, with and without the insert, differed across the cambial region (paper III, Fig. 4B). The FLA11-like genes were also abundant in the wood libraries and these FLAs and the FLA12-like genes with no insert had a similar expression pattern - highly expressed during cell expansion - whereas the genes with an insert peaked later in the secondary wall forming zone. Lafarguette and co-workers (2004) observed a similar expression pattern by semi-quantitative RT-PCR on cambium, differentiating and mature xylem, sampled from both TW and OW. They observed that insert-containing FLAs had a specific expression in mature TW and the others were more evenly expressed in both differentiating and mature TW and OW.

Clearly both FLA11- and 12-like genes seem to have a function during development of xylem cells in poplar. The strong up-regulation of a specific group of FLA12-like genes suggests that these proteins are of importance in TW forming tissues. The $(\beta\text{-D-Glu})_3$ Yariv reagent that detects AGPs strongly reacted with the G-layer in cross-sectioned poplar TW (Nishikubo, Mellerowicz, Sundberg, unpublished). Yariv reagent also stained cell wall proteins of TW and OW tissues separated by rocket electrophoresis with more AGPs found in TW (Lafarguette et al., 2004). The presence of AGP epitopes in the inner G-layer facing the plasma membrane was detected by Lafarguette and co-workers (2004), using JIM14 monoclonal antibodies.

In plants, a GPI anchored protein, COBRA, has been found important for oriented cell expansion (Schindelman et al., 2001). Motose and co-workers (2004) recently identified an AGP-like, GPI-anchored proteoglucan that may function as a signal for vessel development. GPI anchors have been seen to target proteins to the cell surface for extra cellular matrix remodeling and signaling (Borner et al., 2002). Fasciclin domains have been suggested to function as cell-adhesion proteins in *Drosophila* and *Volvox* (Elkins et al., 1990a; Huber and Sumper, 1994). There is only one report of a function of fasciclin domain containing proteins in plants on the sos5 mutant in *Arabidopsis*, with a mutation in the FLA4 gene (Shi et al., 2003). The mutation resulted in thinner cell walls and a cell expansion defect. The difference of the FLA subgroup A between *Arabidopsis* and poplar is interesting. The lack of true *Arabidopsis* orthologues to a big group of poplar *AtFLA12*-like genes is notable and indicates their importance in TW formation since this tissue is not formed in *Arabidopsis* and other herbaceous species.

Carbohydrate Active enZymes (CAZys) and carbohydrate metabolism in TW -a shift in C flow from lignin and hemicellulose to cellulose synthesis

One of the special interests in this study was to identify the presence and regulation of CAZy enzymes in developing TW. CAZys are a highly functionally divergent group of enzymes important for carbohydrate synthesis and modification, and thus synthesis of cell walls (Coutinho and Henrissat, 1999). The enzymes involved in cell wall biosynthesis are still largely unknown and their differential regulation in connection to distinct changes in the cell wall composition between normal wood

and TW would help to pinpoint possible candidates involved in cellulose, pectin, hemicellulose and arabinogalactan biosynthesis. Another question that we were trying to address was to identify key genes in the carbohydrate metabolism that could be responsible for redirecting the C-flow from lignin and hemicelluloses to cellulose biosynthesis.

The CAZY database (http://afmb.cnrs-mrs.fr/CAZY/) was used to identify different gene models in the G-library with CAZy motifs. In total 119 different models were found representing 37 different families. The ESTs representing these gene models where grouped into corresponding CAZy family, to visualize the distribution in different tissues (paper III, Fig. 2). The CAZY families represented by most EST sequences from the G-library could all be related to cellulose biosynthesis (paper III, Fig. 2). The most abundant CAZys in the TW library belonged to the GT4 family, which includes sucrose synthase (SUS) genes, *PttSUS1* and *PttSUS2.* These genes were also significantly up-regulated during TW formation (paper III, Table 1A). Particulate-bound SUS (P-SUS) is important for providing UDP-glucose to the cellulose synthase complex (Delmer and Haigler, 2002). The decrease in sucrose in TW forming tissues (paper III, Table 2) may be indicative of an increased P-SUS activity combined with an increased flow of carbohydrates to cellulose. That more UDP-glucose is synthesized during TW formation is also indicated by the up-regulation of one of the poplar UDP-Glucose pyro-phosphorylase, PttUGP2 (paper III, Table X). Cellulose synthase catalytic proteins (CesA) are members of the abundant family, GT2. Similar to the real time RT-PCR data of Djerby and co-workers (2004) we did not see any major up-regulation of CesA genes in TW, although *PttCesA3-2* and *PttCesA8-3* showed a small increase. Interestingly *PttCesA1* was slightly down-regulated suggesting the possibility of a different composition of rosette complexes in TW forming tissues.

Family GH9 of cellulases was one of the most abundant CAZy families in the TW library (paper III, Fig. 2). The most abundant transcripts in this family corresponded to the poplar *PttCel9A1-1* gene similar to the *Arabidopsis* KORRIGAN (KOR). KOR was identified as a mutant defective in cell elongation and primary wall cellulose content (Nicol et al., 1998). A recent publication on irx2, a weak mutant allele of KOR, by Szyjanowicz and co-workers (2004) showed a deficiency in cellulose of secondary walls, whereas the cellulose content in the primary walls remained unaffected. Even though this cellulase was abundant in the TW library and had a strong signal on the array, it was not differentially expressed in TW (paper III, Table 4). This result indicates that the poplar KOR gene is important in secondary wall formation, which is supported by its up-regulation in the secondary wall forming zone (Aspeborg et al., 2005). Two putative cellulases where slightly up-regulated in TW, however none of these were up-regulated in the secondary wall forming zone. In summary, neither of these two, nor the poplar KOR, seems to have a specific function in G-layer formation.

A strong signal on the array and an up-regulation in SUS, cellulose synthase and KOR-like transcripts is consistent with the high cellulose content characteristic for the G-layer. Earlier studies have shown increased cellulose content in TW and a lower level of hemicelluloses (see e.g. Timell, 1969). This is supported by our transcriptional data where several CAZys down-regulated in TW are putative secondary-wall hemicelluloses biosynthetic enzymes, including members of families GT2, GT8 and GT47. Secondary walls of poplar wood contain glucuronoarabinoxylan

(Mellerowicz et al., 2001). Possible lower glucuronoarabinoxylan biosynthesis in TW was supported by the down-regulation of the genes encoding UDP-glucuronate decarboxylase and UDP-D-xylose 4-epimerase, leading to UDP-D xylose and UDP-L-arabinose, respectively (paper III, Fig. 5).

The other major hemicellulose of secondary cell walls of poplar wood is glucomannan (Mellerowicz et al. 2001). The mannan backbone is formed from GDP-D-mannose. GDP-mannose-pyrophosphorylase has a key function for the formation of GDP-mannose and GDP-L-fucose. It was one of the most down-regulated genes in TW (paper III, Table 1A, Fig. 5). Four isoforms were significantly down-regulated. A GDP-mannose-pyrophosphorylase mutant, *cyt1,* deficient in GDP-mannose-pyrophosphorylase was deficient in N-glycosylation and had a severe defect in cell wall biogenesis (Lukowitz et al., 2001). The recently discovered β-mannan synthase is a member of cellulose synthase-like A family (CslA) (Dhugga et al., 2004). This β-mannan synthase was suggested to synthesize the β-1,4 mannan backbone of galactomannan, which is a hemicellulosic storage polysaccharide in guar seed endosperm walls. A similar gene of poplar, *PttGT2A* was up-regulated during secondary xylem wall formation and down-regulated in TW, making it a likely candidate for the β-1,4-mannan synthase that is important for the biosynthesis of the β-1, 4 mannan backbone of glucomannan.

Several pectin related ESTs, like the pectin methyl esterases, (family CE8) and pectin lyase (family PL1), were found in the EST library, and some members of this family were up-regulated (paper III, Fig. 3, Table 4). This finding was unexpected as pectin is mainly associated with the primary cell wall. However, it is possible that pectin related enzymes reflect a remodeling of primary wall pectin during secondary wall formation. Modulation of the primary wall, even after initiation of secondary wall formation, has previously been shown by Bourquin and co-workers (2002). They found xyloglucan transglycosylase activity at the early stage of secondary wall formation and suggested that the purpose was to create and reinforce connections between the primary and secondary wall layers.

When sucrose is split into UGP-glucose and fructose by the action of SUS, UDP-glucose can be used for cellulose synthesis while fructose can be used for other reactions. Up-regulation of fructokinase indicates that the fructose in TW is converted to D-fructose-6P. This metabolite can provide substrates for the synthesis of mannan and fucose via GDP-mannose pyrophosphorylase or lignin via the pentose phosphate pathway. Both of these pathways were down-regulated in TW. A third possible explanation is that it is channeled back to sucrose via sucrose phosphate synthase (SPS) and sucrose phosphatase (SPP). SPS and SPP were not found differentially regulated, but phosphorylation appears to be the major regulation for the activity of SPS (Huber and Huber 1996), and thus over expression does not necessarily result in higher activity of the enzyme (Huber and Huber 1996). The microarray data gives an indication that SPS may have an important role in supporting the flux of carbohydrate from lignin and hemicelluloses towards cellulose synthesis. This needs somehow to be investigated in more detail e.g. using post transcriptional gene silencing. If down-regulation of SPS/SPP activity could change the carbohydrate flux towards synthesis of hemicelluloses and lignin synthesis is unknown.

Shikimate and lignin biosynthetic pathways are down-regulated in TW

Lignin biosynthesis is linked to primary metabolism (glycolysis and the pentose phosphate pathway) via the shikimate pathway (Borejan et al., 2003). The final product of the shikimate pathway, chorismate, is derived from a series of seven reactions beginning with the reaction between D-erythrose 4-phosphate (E4P) and phosphoenolpyruvate catalyzed by 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAPH) synthase. Chorismate can then be further metabolized into phenylalanine, the first enzyme of the phenylpropanoid pathway. The shikimate pathway is well described in higher plants (Herrmann et al., 1995), but has not been studied in any detail in woody species. We found poplar homologues to all but one enzyme (3-dehydroquinate (DHQ) synthase) on the array, and all were down-regulated in TW (paper III, Fig. 6). Also, the metabolite shikimate was found at reduced levels in developing TW (paper III, Table 2). Interestingly, a plastidic transketolase, the enzyme synthesizing E4P was also down-regulated. This might be a key enzyme controlling the flux of carbohydrates to the shikimate pathway (paper III, Fig.5). The down-regulation of plastidic transketolase in tobacco led to dramatic effects on several aspects of plant growth and development including the down-regulation of phenylpropanoid/lignin metabolism (Henkes et al., 2001).

The phenylpropanoid and monolignol biosynthetic pathways have been under intensive investigation in recent decades (Boerjan et al., 2003). Almost all enzymes from the early phenylpropanoid pathway and monolignol biosynthesis have been studied in poplar, and several isoforms were found down-regulated in TW (Table 4 and III, Fig. 6). Interestingly, PAL1 and CCoAOMT3 had both a strong signal on the array but were not down-regulated along with the other isoforms. This could mean that these isoforms are important during early S2 formation or that they are important for other products from the phenylpropanoide pathway, like flavonoide synthesis. PAL1 and PAL2 did have slightly different expression pattern, PAL2 peaking earlier, over the cambial region (Schrader et al., 2004), which can support this idea.

An interesting finding was that there were two putative poplar homologues for hydroxycinnamoyl transferase (HCT) and one for *p*-coumarate 3-hydroxylase (C3H), which were all weakly down-regulated. These enzymes have been recently identified in *Nicotiana tabacum* (Hoffman et al., 2003) and *Arabidopsis* (Schoch et al., 2001) but they have not been cloned from poplar. The high similarity between the putative homologues, (over 80% identical on amino acid level) supports the idea that these are true homologues. Additional studies must however be done to ensure that these previously not identified poplar isozymes really are involved in lignification, particularly in the xylem. Transgenic trees and substrate specificity studies have been of great help for identified enzymes previously, and could be useful in functional studies of these novel genes.

The last step in monolignol biosynthesis is catalyzed by cinnamyl alcohol dehydrogenase (CAD). On the array, several isoforms of CAD were present, but only one of them, CAD2, identified by Doorsselaere and co-workers (1995b) was significantly down-regulated. Li and co-workers (2001) identified a poplar sinapyl alcohol dehydrogenase (SAD), specific for synthesis of sinapyl-units. This gene was not however found in wood–related EST libraries and thus it was not spotted on the 13K array.

The cytosol-synthesized monolignol units are transported from the cytosol to

the cell wall by an unknown mechanism. There are indications that the units are glycosylated by an UDP-glucose glucosyltransferase (CAZy family GT1), which would then enable the transport through the plasma membrane. In the cell wall, the monolignol units would be then released by a β-glucosidase (CAZy family GH1) and polymerized (Boerjan et al., 2003; Rogers and Campbell, 2004). In *Arabidopsis* a UGT84A2 has been identified that can glucosylate sinapic acid (Lim et al., 2001). We identified two putative poplar GT1 family members that were both down-regulated in TW but none of these were similar enough to be orthologues to *Arabidopsis* UGTs. No putative orthologue to the *Arabidopsis* UGT84A2 was spotted on the array. In lodgepole pine (*Pinus contorta* Dougl. Ex Loud.), an extra-cellular β-glucosidase has been identified but whether this enzyme is related to lignification is not clear (Dharmawardhana et al., 1999). One β-glucosidase was found down-regulated in TW, but whether this gene is somehow involved in the lignification process is unknown.

The polymerization of lignin involves the action of laccases and peroxidases but their roles are not fully understood (reviewed by Borejan et al., 2003). Ranocha and co-workers (1999) identified several laccases in poplar. Poplars down-regulated for these laccases revealed that only one (lac3) showed a xylem phenotype that was different from control trees (Ranocha et al., 2002). It caused deformation of fiber cell walls and their partial separation. The authors speculate that lac3 could be involved in the formation of phenoxy radicals, important for cross-linking wall components. This could explain the loose cell walls in the transgenic trees. Interestingly, lac3 was the only laccase found down-regulated in TW supporting its function in the lignification process.

Peroxidases have also been suggested to be important for the lignification process and Christensen and co-workers (2001) identified several members of syringaldazine-oxidizing peroxidases expressed in differentiating xylem. These peroxidases were not present on the 13K array, which perhaps was not surprising as the corresponding genes were weakly expressed. Among the peroxidases spotted on the array none were found down-regulated but a few were found up-regulated in TW. The expression across the cambial region for the up-regulated peroxidases does not support their role in the dehydrogenative polymerization of the monolignols.

High amounts of nitrogen are used during lignification. It has been found that one cytosolic isoform of glutamine synthase (GS) from Scots pine (*Pinus sylvestris*), PsGS1b, may be important for recovering and re-assimilating of nitrogen in lignifying cells (Singh et al., 1998). Recently, two members of the loblolly pine (*Pinus taeda*) R2R3-MYB family, PtMYB1 and PtMYB4, also expressed in lignifying cells were shown to bind to, and activate transcription of, *PsGS1b* by promoter-driven GUS expression in transient expression assays of protoplasts derived from pine seedling tissues (Gomez-Maldonado et al., 2004). They propose that these genes could possibly be important for the co-regulation of the phenylpropanoid metabolism and nitrogen cycling.

Several isoforms of cytosolic GS were found down-regulated in TW (paper III, Table 1B, supplementary data) unfortunately no orthologues to PtMYB1 and PtMYB4 were found spotted on the array. However, another MYB gene, *PttMYB21a*, identified by Karpinska and co-workers (2004), was found up-regulated in TW. This MYB-gene was previously found induced during TW formation and poplars with a reduced expression of *PttMYB21a* had more lignin indicating that this

gene represses lignin biosynthesis. Another lignin-associated gene that was found to be down-regulated in TW is phenylacumaran benzylic ether reductase (PCBER) identified in poplar by Mijnbrugge and co-workers (2000). LIM transcription factors have also been associated with lignin formation and an antisense repression of a LIM gene, *PLIM1*, in tobacco resulted in a lower lignin content (Kawaoka et al., 2000). However, LIM genes similar to *PLIM1* were found up-regulated in TW.

The present array study showed that not only the monolignol biosynthetic genes were down-regulated, but the shikimate and phenylpropanoid pathway genes as well. Moreover, the entire flux of C from fructose to pentose phosphate and shikimate pathways was down-regulated in TW (paper III, Fig. 5). This result support previous findings of lower levels of lignin in TW compared to normal wood, which have been described for several tree species (Timell, 1969). A recent study by Joseleau and co-workers (2004), who used antibodies specific against syringyl units detected presence of lignin in the G-layer. Apparently, more research is needed to fully understand the nature of the G-fiber anatomy.

What role does ethylene and auxin play in G-fiber formation?

The alteration in wall composition observed during TW formation must be controlled and coordinated by a signaling system. Auxin and ethylene have been proposed as important mediators of TW formation (e.g. Little and Savidge, 1987; Du and Yamamoto, 2003) and indeed we found that the most abundant hormone-related genes that were differentially regulated in TW represented the biosynthesis and/or signaling pathways of these two hormones.

The finding that the major change of auxin levels in stems induced to form TW is actually a decrease on the opposite side (paper I, Fig. 4B and Hellgren et al, 2004) contradicts previous theories that TW is formed in response to auxin deficiency (e.g. Cronshaw and Morey, 1968). However, *PttLAX1* and *PttIAA5* that function as an auxin influx carrier and as an auxin response gene, respectively, were among the most down-regulated hormone-related genes in TW (paper III, Table 6). Schrader and co-workers (2003) and Moyle and co-workers (2002) studied the expression of these genes in the wood forming tissues of poplar. Both *PttLAX1* and *PttIAA5* had a very specific expression in the secondary wall forming zone. Schrader and co-workers (2001) speculated that these two genes might be important for vessel maturation, particularly cell wall perforation or cell death. If it is so, the expression of these genes would be expected to decrease in TW compared to normal wood because the TW is associated with less vessels being produced and fibers that live longer then normal fibers (Mia 1968).

One interesting finding from the EST sequencing of wood related libraries is the identification of transcripts representing more or less the whole ethylene response pathway, which support that these tissues are able to respond to ethylene. EST sequence distribution among different libraries (paper III, Table 6) indicates that there may be a tissue specific regulation of e.g. the receptors, where ETR2 and particularly ERS1 may be particularly important in the xylem. Further studies are needed to conclude that this really is the case as ETR1, ERS2 and EIN 4 is not represented on the array.

To visualize how the expression of ethylene response pathway related genes in the wood tissues are affected by TW induction, a schematic drawing of the response pathway is illustrated in Fig. 6 .The two putative ethylene-receptors, *ETR2* and

ERS1, were found to be up-regulated in TW. *ERS1* has been observed to be induced by ethylene in *Arabidopsis* (Zhong and Burns, 2003) and it may also be the case in poplar during TW formation. A putative *CTR1* was also found up-regulated in TW. This kinase has been found to directly interact with at least one of the ethylene receptors, ETR1 (Gao *et al*., 2003). CTR1 functions as a negative regulator and the presence of ethylene may result in inactivation of a MAPK cascade (Ouaked *et al*., 2003). This event will activate EIN2 a membrane bound protein necessary for downstream ethylene responses (Alonso *et al*., 1999). A putative orthologue for EIN2 was found on the array but the transcript level was not affected by the TW induction. However, the signal was rather strong indicating a role for this protein in developing wood tissues. In the presence of ethylene, EIN2 functions as a positive regulator, activating the transcription factor EIN3, which has been demonstrated to be a principal activator of downstream ethylene effects in *Arabidopsis* (Chao *et al*. 1997). The transcript level for an EIN3-like transcription factor was also affected and induced in TW. In *Arabidopsis*, when ethylene is not present, EIN3 is rapidly degraded by a SCF/proteasome-mediated proteolysis mechanism (Gou and Ecker, 2003; Potuschak et al., 2003). The expression of this transcription factor has not been found induced by ethylene but the presence of ACC or ethylene has been shown to stabilize the protein and delay degradation (Chao et al., 1997; Yanagisawa et al., 2003). Two EIN3-binding F-box proteins, EBF1 and EBF2, have been isolated in *Arabidopsis*, and found to bind to EIN3, tagging it for degradation by poly-ubiquitylation (Gou and Ecker, 2003; Potuschak et al., 2003). We found a putative EBF1 homologue on the array, which was up-regulated in TW. The SCF/proteaosome should lead to less active EIN3 protein and therefore a mitigated ethylene response. Interestingly, Guo and Ecker (2003) found that the expression of both EBF1 and EBF2 were induced by ethylene in *Arabidopsis*. They speculate that EBF1 and EBF2 are involved in a negative-feedback mechanism, which would allow tight

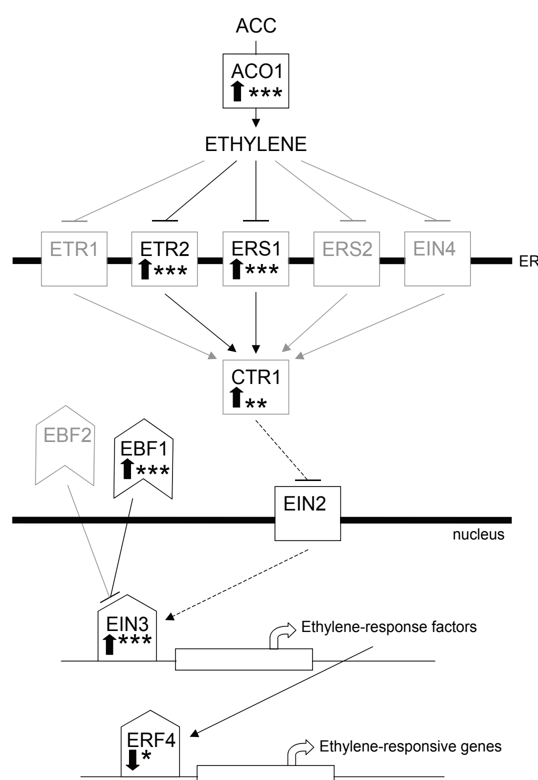

Figure 6. Schematic drawing of the ethylene response pathway. Arrow indicates up or down regulation and asterix indicates the significance level (*= P≤0.05, ** =P≤0.01 and ***=P≤0.001). Grey= no putative orthologue were spotted on the array. Relevant PU IDs are presented in paper III, Table 6.

regulation of EIN3 protein levels.

Induction of TW apparently affects a majority of ethylene–response related genes. The result indicates that the tissues are responding to an increased level of ethylene that is associated with the TW response. This ethylene response also seems to be under tight control by the SCF^{EBF}-dependent proteolysis mechanism. How the transcript regulation is reflecting the actual enzymes/proteins produced and their activity in developing xylem and specifically in TW still remains to be elucidated.

The plot thickens by merging data from different "-omics"

The metabolite analysis identified 26 metabolites that were significantly changed between TW and normal wood according to the PLS-DA. Sixteen of these compounds could be identified (paper III, Table 2). As expected, several sugar related compounds accumulated in different amounts. Sucrose, arabinose and inositol were all reduced in TW, whereas xylose and xylitol were more abundant. The altered sugar composition is understandable as several pathways connected to the carbohydrate pool are modified in TW. The reduced shikimate level, as deduced from metabolomic data, was also consistent with transcriptome changes, corresponding to a down-regulation of the shikimate pathway genes.

Unexpectedly, one of the most down-regulated compounds in TW was gamma-aminobutyric acid (GABA). In animals, GABA is functioning as a signaling molecule (neurotransmitter), and there are reports that GABA may function as a signaling molecule in plants as well (Bouché et al., 2004). GABA appears to be important for the carbon-nitrate balance, for the cytosolic pH and it is accumulating in response to stress (recently reviewed by Bouché and Fromm, 2004). Perhaps it's down-regulation is distantly related to the lignification process as the precursor of GABa is glutamate that has been found important for re-assimilation of nitrogen to phenylalanine (Singh et al., 1998). The lower GABA level could be explained by its rapid turnover, which then would indicate that more GABA is needed during TW formation compared to during normal wood formation. Another possibility is that GABA acts as a signaling molecule and its lower concentration reflects a down-regulation of this signal in TW.Further investigation is mneeded to reveal the unction for GABA in the stem and in particularly during TW formation.

Proteomic studies have also been initiated in TW. Baba and co-workers (2000) did a time course study in *Eucalyptus camaldulensis* (L.) and after 14 days of inclination a different protein pattern was found coinciding with the appearance of G fibers. Five proteins were differentially expressed and all of them were found in the fraction of cell wall and plasma membrane associated proteins. Further, Plomion and co-workers (2003) did a proteomic study on TW in *Eucalyptus gunnii* and found differences in the protein pattern between normal- and TW. To date, no report on identification of the proteins has been reported.

Transcript analysis of poplar down-regulated in cinnamoyl-CoA reductase (CAD) and caffeic acid 3-O-methyltransferase (COMT): two enzymes active in monolignol biosynthesis (IV)

There has recently been much progress in understanding the biosynthetic pathway of monolignols, the precursors of lignin (reviewed by Boerjan et al., 2003). Lignin is a costly compound to remove during chemical pulping. This has lead to many attempts

to reduce its quantity and/or alter its quality by down regulating various key genes in transgenic trees (Boerjan et al., 2003). In this thesis, microarray experiments were performed on wood-forming tissues from poplar trees down-regulated in CAD and COMT. The CAD transgenic lines originally described by Baucher and co-workers (1996) had about 30 % of residual CAD activity compared to wild type trees. The overall growth and development was not affected in these trees but more lignin could be extracted by alkaline treatment compared to WT trees. A slightly lower lignin content (mainly reduction of S-units) were observed in some lines. An obvious phenotype, was a red coloration of the xylem. It was suggested that this coloration was due to the incorporation of coniferyl and syringyl aldehydes into the lignin polymer, as these are substrates for CAD and could presumably accumulate in transgenic trees (Baucher et al., 1996). The COMT trees were originally described by Van Doorsselaere and co-workers (1995) and the COMT activity in these trees was about 5 % of that in the WT. These trees were not affected in lignin amount, but the S/G ratio of lignin monomers was decreased, thus increasing its extractability.

In the antisense CAD and COMT trees, 35 and 37 genes, respectively, were found to be differentially expressed ($P<0.005$) compared to wild type trees. Twenty three genes were similarly affected in both CAD and COMT antisense lines; 12 genes were differentially regulated due to altered CAD expression only; and 14 genes were found to be differentially regulated in the COMT altered lines only. The anti-sensed gene was found down-regulated in respective lines, and was considered a positive control for the experiment.

A suppression of CAD expression results in down-regulation of transcripts involved in flowering and circadian rhythm

Three of the 12 genes exclusively affected by down-regulation of CAD were related to the flowering and circadian rhythm machinery (paper IV, Table 1): a late elongating hypocotyls-like gene (LHY) was up-regulated and a pseudo-response regulator-like (APRR5) and a flavin-binding kelch repeat F-box (FKF1) was down-regulated. LHY is a MYB transcription factor that is normally expressed during the late night and early morning and is part of the central circadian oscillator in plants (Kim et al., 2003). The exact function for FKF1 and APRR5 is not known but they have booth been associated with the SCF complex functioning in circadian specific proteasome-dependent degradation (Yasuhara et al., 2004).

To determine if any of the other genes affected by the down-regulation of CAD were under the control of the circadian clock a search through the TAIR microarray database (http://www.arabidopsis.org) was performed. The best Arabidopsis hits to poplar genes were used to search through experiments related to the circadian clock. Three genes besides CAD were found under the control by the clock (paper IV, Fig. 2): a β−amylase, a cloroplast nucleoid DNA-binding-like protein and a glutathionine S-transferase gene. However, to confirm that the putative poplar orthologues contain circadian regulated motifs, further investigation is necessary. This could be done by studying the diurnal expression of these genes.

The reason for the altered expression of circadian genes in the CAD lines is not obvious but possibly due to a secondary effect where the red colored xylem may be involved. A plausible explanation is that the quality of the light that penetrates into the stem is altered by the red xylem and this affects light-regulated genes. The fact

that these genes are expressed in the stem is of interest, but their function in the stem is at present difficult to predict.

Some of the differentially regulated genes in CAD and COMT down-regulated trees were also differentially regulated in TW forming tissues

Seven of the 49 genes that were differentially regulated in the CAD and/or COMT antisense trees were also found to be affected in developing xylem from tension wood (TW) ($P \leq 0.005$), see Table 2. In tension wood, both the CAD ($P \leq 0.05$) and COMT ($P \leq 0.001$) genes were down-regulated together with most other genes in monolignol biosynthesis (paper III). Among the seven differentially regulated genes, only one, namely PtFLA12P, was regulated in the opposite direction. This gene was strongly up-regulated in TW but down-regulated in the COMT trees. One gene was found up-regulated in all three experiments; a mitogene-activated protein kinase. MAP kinases are important mediators of cellular responses to a wide variety of stimuli (Jonak et al., 2002).

Two genes were down-regulated in both CAD antisense lines and in TW forming tissues. One is of unknown function and the other is a putative β-mannan synthase (Dhugga et al. 2004). Galactoglucomannan and glucomanan are hemicelluloses found in poplar secondary cell walls. Two down-regulated genes were common to both TW and the COMT antisense trees. One of these was a WRKY transcription factor. The *Arabidopsis* homologue (WRKY53) has been studied by Hinderhofer and Zentgraf (2001) and found to be up-regulated in the early stage of leaf senescence. The other gene is a nuclear matrix constituent-like protein. Monoclonal antibodies were raised against its homologue in carrot (Masuda et al., 1997) and the protein was localized to the nucleolus. Why these genes were found coregulated between the antisense lines and tension wood is dificult to speculate in, but it is interesting to se that altered lignification patterns does somehow affect both transcription factors and hemicellulose related transcripts.

Table 2. Genes significantly differentially regulated ($p < 0.005$) in micro array experiments on developing xylem from transgenic lines down regulated for CAD or COMT and tension wood.

PU ID	Regulation in transgene	Description	Regulation in tension wood	AGI ID
Genes differentially regulated in CAD, COMT and TW				
PU04084	up	MAPKKK3	up	At1g53570
Genes differentially regulated in COMT and TW				
PU02638	down	COMT	down	At5g54160
PU09430	down	WRKY family transcription factor	down	At4g23810
PU02973	down	Nuclear matrix constituent protein-like	down	At5g65770
PU03040	down	PtFLA12P	up	At5g60490
Genes differentially regulated in CAD and TW				
PU02990	down	Cellulose syntase-like A9	down	At5g03760
PU12194	down	Expressed gene	down	At3g08780

Comparing array experiment can increase the understanding of gene regulation

When comparing developing TW with normal wood, hundreds of genes were differentially regulated, which can be expected in tissues where developmental patterns are altered. In such experiments it can be difficult determine whether the altered regulation is cased by primary or secondary effects. In mutant plants, where only one gene is affected, a much smaller set of genes are likely to be altered as demonstrated here. The fact that many of the genes differentially regulated in the xylem of these trees were related to the circadian clock indicates that secondary effects (in this case due to the red xylem phenotype) may occur. Both CAD and COMT genes were down-regulated during TW formation, and among the genes co-regulated between the experiments all but one were regulated in the same direction as in TW. Notably is that none of these genes were related to the circadian clock. Whether these co-regulated genes have any significance or not in lignification remains to be demonstrated. However, comparing data from different array experiments is surely a way to facilitate better evaluations of array experiments.

Conclusions and future prospects

PttACO1 was identified as the major ACO in wood forming tissues. It is highly expressed in the zone of secondary wall formation, and one of the most highly up-regulated genes in TW forming tissues. This up-regulation was only observed at the TW side, whereas ACC accumulated on the OW side. Thus it was concluded that ACO activity and not ACC availability is the limiting and controlling factor for ethylene production during TW formation. The origin of ACC produced during the TW response is unknown. It may be synthesized in the stem tissues or transported from other organs and tissues. Identification of ACS genes in poplar and expression analysis of these genes in response to TW would be a way to shed some light on this issue.

Further, an ACD expressed in the wood forming tissues of poplar was identified. An orthologue was also identified in *Arabidopsis* and expression of this ACD in bacteria grown on ACC as the sole nitrogen source showed its functionality. Expression and activity of ACD was also demonstrated in *Arabidopsis* and tomato during seed germination and fruit ripening. Thus we propose that ACD is a part of the system regulating ethylene production in plants and that it has a function in regulating ethylene levels in the poplar stem. At the time for the investigation knock out mutants in *Arabidopsis* was not available, but silencing of the ACD gene would give the conclusive evidence for its function in plants.

To create a platform for understanding genetic and molecular control of TW formation, global transcript analysis of developing TW/G-fiber formation was done together with a metabolite analysis. Genes coding for FLA12-like proteins were among the most up-regulated. This group of 10 genes was all most similar to AtFLA12, which demonstrate that significant gene duplication has occurred in poplar. The AtFLA12-like genes affected by TW are a good example for the importance of a tree model system like poplar, as neither TW nor this particular group of FLAs exists in *Arabidopsis*.

The shift in carbon flows to more cellulose and less lignin and hemicelluloses involved in G-layer formation was reflected in the modification of genes involved in carbon metabolism. The shikimate and lignin biosynthesis pathways were to a large extent down-regulated. Also a transketolase were down-regulated and this gene may be important for the flux from the lignin biosynthesis pathway towards other pathways. We also found some highly expressed isoforms of lignin biosynthesis genes in the early phenylpropanoid pathway that were not down-regulated. This suggests that these isoforms may not be involved in the biosynthesis of lignin, but rather important for synthesis of other substances such as flavanoids

Several hemicellulose related genes were down-regulated. Down regulation of xylan synthesis is indicated by the down-regulated UDP-glucuronate decarboxylase and UDP-D-xylose 4-epimerase that leads to UDP-L-Arabinose and UDP-D-Xylose. Among the most down-regulated hemicelluloses related genes were several GDP-mannose-pyrophosphorylases and a putative β-mannan synthase. As xylan and glucomannan are important hemicelluloses of secondary cell walls of poplar wood this fits well with the lower hemicellulose content previously described. Other down-regulated genes were members of GT8 and GT47 families. Their functions are largely unknown but their up-regulation in secondary wall forming zone in

normal wood and their down-regulation in TW makes these genes interesting for further studies. Interestingly, several pectate lyases were up-regulated, suggesting re-modulation of pectins in G-fibers.

The flow of carbon into cellulose was reflected by up-regulation of genes like SUS1, SUS2, fructokinase and UDP-glucose pyrophosphorylase. However, several of the genes involved in the actual cellulose biosynthesis were just slightly, or not at all, affected in TW. That PtCesA3-2 and PtCesA8-3 were slightly up-regulated and PttCesA1 were slightly down-regulated, suggests that the composition of the rosette may be different in TW forming fibers. A KOR homologue (PttCel9A1-1) and a chitinase (PttCH19), also suggested to be involved in cellulose biosynthesis, were abundant in wood forming tissues, but not affected during TW formation. The data suggest that the limiting factors for cellulose synthesis are the substrate rather than the cellulose biosynthesis machinery. Alternatively, post-transcriptional regulation controls the activity of these enzymes.

Several ethylene and auxin related genes were modified in relation to TW formation. The high up-regulation of ACO1 was followed by an up-regulation of ethylene response pathways related genes, whereas auxIAA and PttLAX1 were found down-regulated. This indicates a role for auxin/ethylene interaction in the TW response.

Taken together, the array data provide a starting point for selecting candidate genes for modifying carbon flow into different wood components. It has to be pointed out, however, that the array analysis is a screening procedure and conclusive expression data are needed for any gene that are taken further into more in depth analysis.

A microarray analysis was also done on poplar trees down-regulated in CAD and COMT with a modified lignin. This experiment demonstrated that the modification of one lignin biosynthesis gene do affect the expression of many other genes, and that phenotypes observed in transgenic plants may be fairly distant from the gene of interest. We found that CAD and COMT trees had many affected genes in common, but it was also concluded that the CAD trees had a whole set of clock related genes altered. This is likely due to the red coloration of the xylem.

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v.g.v.

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