Plant Hormones in Wood Formation

-Novel Insights into the Roles of Ethylene and Gibberellins

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Abstract

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The role of plant hormones in wood development has been studied for decades, and their crosstalk in many biological processes is the subject of increasing focus. In this thesis, modern biological tools have been used to provide novel insights into the roles of gibberellins and ethylene in wood formation in the model tree *Populus tremula x tremuloides*.

A new idea describing the crosstalk between gibberellins (GAs) and indole-3-acetic acid (IAA) is presented. It is demonstrated that GAs stimulate polar IAA transport in secondary stems of *Populus*, a stimuli likely to be caused by the enhanced expression of the auxin efflux carrier *PttPIN1*. Extensive crosstalk between the two hormones is also suggested from global gene transcript analysis of GA and IAA regulated genes in *Populus* stem tissues. Most genes that were induced by GA were similarly induced by IAA, suggesting that both hormones can regulate similar biological processes, independently or in concert.

To conclusively establish the role of endogenous ethylene in wood formation, a reverse genetics approach was used. Ethylene insensitive *Populus* trees were produced by transgenic expression of the mutated ethylene receptor *Atetr1-1*. These trees showed ethylene insensitivity to several wood related responses in pharmacological experiments. More interestingly, these trees were inhibited in their eccentric stem growth pattern in response to leaning. This is one of the characteristic phenotypes in tension wood formation, hypothesized to be ethylene mediated. This is the first conclusive demonstration of the function of endogenously produced ethylene in wood development, and the first documentation of the involvement of ethylene in meristematic activity in plants. We also use a transgenic approach to modify the expression of *PttACCox*, which is coding for the last enzyme in ethylene biosynthesis, and thereby provide solid evidence for its importance in regulating ethylene biosynthesis in wood forming tissue.

Ethylene responses are believed to be mediated via a large number of so-called ethylene response factors (ERFs). Here all ERFs in the *Populus* genome were identified. Putatively important ERFs in stem tissues were found by analysing their expression pattern in response to ethylene. Further, ethylene responsive genes were identified in a global gene analysis.

Keywords: *Populus*, wood formation, auxin, gibberellin, ethylene, tension wood, ACO, ERF.

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Äntligen färdig…

Contents

Appendix

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

List of papers

I. Simon Björklund, Henrik Antti, Ida Uddestrand, Thomas Moritz, Björn Sundberg. Cross-talk between gibberellin and auxin in development of Populus wood: gibberellin stimulates polar auxin transport and has a common transcriptome with auxin. *Plant Journal*, *in press*

II. Sara Andersson-Gunneråsǂ , Jenny Hellgrenǂ, Simon Björklund, Sharon Regan, Thomas Moritz, Björn Sundberg. Asymmetric expression of a poplar ACC oxidase controls ethylene production during gravitational induction of tension wood. ǂAuthors contributed equally to this work. *Plant Journal,* 2003; 34, 339–349.

III. Simon Björklundǂ, Jonathan Loveǂ, Jorma Vahala, Magnus Hertzberg, Jaakko Kangasjärvi and Björn Sundberg. Ethylene is Limited by ACO Activity and is an Endogenous Stimulator of Cell Division in the Vascular Cambium of Populus in Response to Leaning Stress. ǂAuthors contributed equally to this work. *Manuscript*

IV. Simon Björklundǂ, Jorma Vahalaǂ, Jonathan Love, Jaakko Kangasjärvi, and Björn Sundberg. Ethylene response factors and ethylene responsive genes in stem tissues of Populus. ǂAuthors contributed equally to this work. *Manuscript*

Introduction

The demand for wood as a sustainable raw material for many commercial uses is increasing. As such, there is growing interest in the molecular aspects of wood formation. Knowledge of genetic, biochemical and physiological regulation of biomass production and wood traits, together with recently developed tools in forest biotechnology has opened up possibilities to increase the pace of tree breeding. Forestry plantations in the future may well consist of highly productive clones modified for valuable wood characteristics. Such intensively managed plantations could allow for larger areas of forests to be conserved for non-timber values, while still maintaining a high supply of woody raw materials. This is especially important today if we are to mitigate climate change.

In hybrid aspen (*Populus tremula x tremulodies*) the sequencing of Expressed Sequenced Tags (ESTs) from complementary DNA libraries, that are featured on the microarray chip, have enabled the possibility of large scale global analysis of gene expression in wood forming tissues (Andersson-Gunneras *et al.*, 2006; Andersson *et al.*, 2004; Hertzberg *et al.*, 2001a; Schrader *et al.*, 2003; Sterky *et al.*, 2004). These efforts, together with the genome sequencing done by the US department of Energy (http://jgi.doe.org), have established poplar as an important model species for tree-biology and -biotechnology research (Bradshaw *et al.*, 2000; Brunner, Busov & Strauss, 2004). Importantly, in order to achieve an understanding of the molecular aspects of plant growth and development, not only gene expression, but also protein and metabolite profiling are needed.

Plants cannot run. Unlike animals that can move away from unfavourable conditions, plants do not have that physical capacity. Indeed, many tree species must remain at the same location for hundreds of years. Therefore, plants have evolved a capacity to respond in their growth pattern to environment cues in order to survive less propitious biotic and abiotic conditions. This requires a certain plasticity of development. Plant hormones are important signalling molecules in mediating these plastic responses. The definition of a hormone in a zoological context is a substance produced in one tissue of an organism and transported to another tissue, where it elicits a response (Thain & Hickman, 1996). However, plant hormones can also be produced at their site of action. Plant hormones includes the more studied; auxin, gibberellin, ethylene, cytokinin and abscisic acid, and the later discovered; jasmonic acid, brassinosteroids and salicylic acid (Davies, 1995). Hormone interactions, often called crosstalk is often observed in plant species. The crosstalk increases the complexity and levels of control in plant growth and development (Benavente & Alonso, 2006; Vogler & Kuhlemeier, 2003) . In this thesis, I have investigated some aspects of the action of auxin, gibberellin and ethylene in wood formation using the model species hybrid aspen (*Populus tremula x tremuloides*).

Background

Wood formation

The provascular strands in the shoot apex are the first vascular connection between leaf primordia and the existing vascular structure. In these vascular strands, the procambium is forming protophloem and protoxylem, and later metaphloem and metaxylem, which together eventually create vascular bundles. During secondary growth, the layer of undifferentiated procambium (the fascicular cambium) remains inside the vascular bundles. Between the vascular bundles, parenchyma cells will form an interfascicular cambium that connects the fascicular cambium and together creates the vascular cambium which forms a cylinder within the stem (Larson, 1994). Within the cambial meristem, the initial cell divide anticlinally to produce additional initial cells, and periclinally to produce xylem and phloem mother cells, which after further periclinal divisions produces phloem and xylem respectively (reviewed in Larson, 1994). Radial growth is determined by the number of dividing xylem mother cells and the duration of cell their respective cell cycle (Wilson, 1964). The wood forming tissues can be subdivided into specific developmental zones (Schrader, 2003) (Fig. 1). After cell division in the cambium, developing xylem cells enter the expansion zone, where they expand radially and longitudinally to their ultimate sizes. Then they form a secondary cell wall and eventually lignify. In angiosperm trees, fibres and vessels constitute the major building blocks of the xylem. Fibres have mostly a mechanical supporting role, whereas vessels primarily conduct water. A third major cell type in the xylem is the parenchyma cells, which make up the rays, function in radial transport and storage. Poplar wood has been found to be composed of 53-55% fibres, 33% vessel elements and 12-15% ray parenchyma cells. Additionally, another cell type are the contact cells to the vessel elements, which have storage functions (Panshin & Zeeuw, 1980).

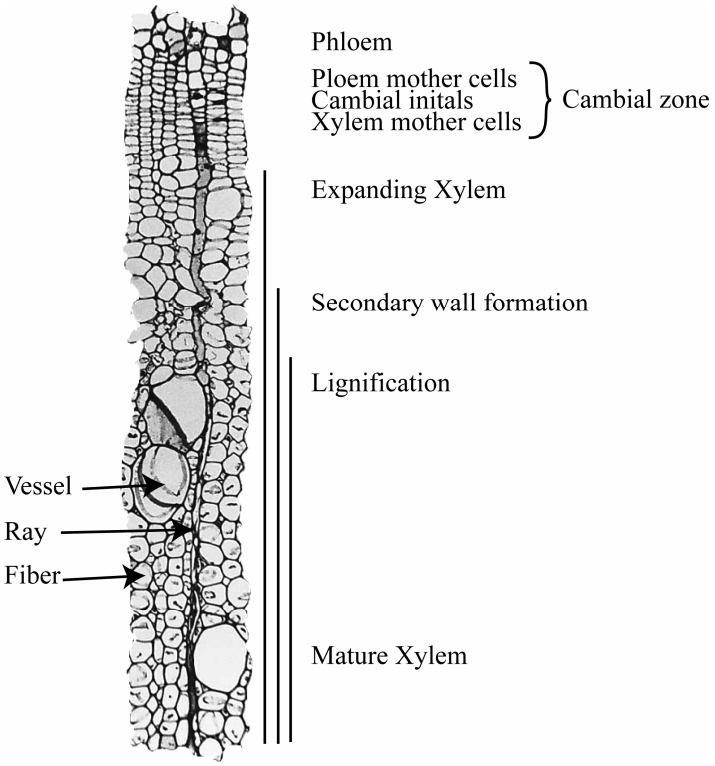

Fig. 1. Schematic overview of the wood developmental gradient in the cambial region in *Populus* located distally to the apical meristem and at the outer part of the stem excluding the bark.

The cell wall in wood forming tissues

The cell wall is an organized structure acting as a surrounding exoskeleton which largely dictates the shape of a plant cell by limiting the rate and direction of cell expansion (Taiz & Zeiger, 1998). Cell wall formation is separated in primary and secondary cell wall deposition. All plant cells have a primary cell wall, which in poplar is composed of mainly pectins, cellulose, matrix glycans and proteins (Mellerowicz et al., 2001). The new primary cell wall is established in the cell plate during cell division and expands to surround the whole cell. Between the divided cells, the middle lamella forms a pectin rich interface between the primary walls.

After expansion, plant cells form a secondary wall between the plasma membrane and primary cell wall. In trees, secondary cell walls create an indispensable highly rigid structure, which further stabilizes the cell shape. Such increased strength stabilizes the plant organs and, and in trees enables growth to significant biomass over centuries. The secondary cell wall deposition in developing poplar wood is first seen in the vessel elements and the contact cells, and then later in fibres (Murakami et al., 1999). In poplar, the secondary cell wall, is composed of mainly cellulose, hemicellulose, lignin and structural proteins (Brett & Waldron, 1996; Mellerowicz*, et al.*, 2001)

Cellulose microfibrils are synthesized at the plasma membrane and arranged in several long (1-4) β-D-glucan chains (Plomion, Leprovost & Stokes, 2001). They are relatively stiff structures that contribute to the cell wall strength. The microfibrils (MFs) are randomly oriented in the primary cell wall, whereas they lie parallel to each other in a helicoidally pattern in the secondary wall (Plomion, Leprovost & Stokes, 2001). In the secondary cell wall, the MF angle differs across the wall and thereby the secondary cell wall can be separated in three distinct layers denoted, S1, S2 and S3. Normally the MFs in S1 and S3 are almost transverse to the cell axis, whereas S2, which is the thickest layer, has more longitudinally arranged MFs (Fig. 2), reviewed in (Mellerowicz*, et al.*, 2001).

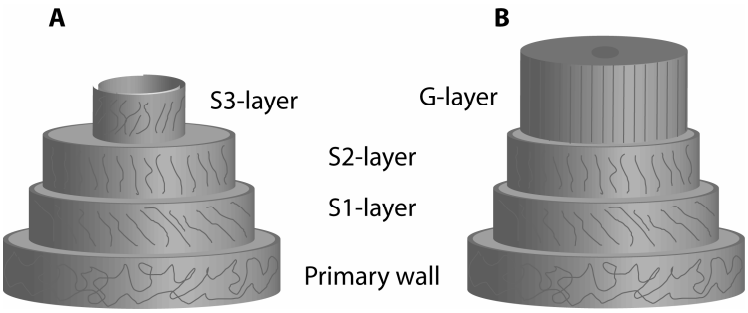

Fig. 2. Three- dimensional structure of a *Populus* wood fibre (modified from Plomion *et al*., 2001). The cell wall is divided into several different layers. Each layer has a characteristic arrangement of cellulose MFs, which determine the mechanical and physical properties of the cell. These MFs can be aligned irregularly (as in the primary wall), or in a particular angle to the cell axis (as in S1-S3 and G-layer). A, Normal wood fibre, B, G-fibre. In the G-fibre, part of S2 and S3-layers are replaced by the thick gelatinous G-layer with its typically vertically oriented MFs.

The matrix glucans in poplar consist of different kinds of hemicelluloses, such as xyloglucan (only in primary wall), xylan, glucamannan and arabinoxylan. These flexible hemicelluloses are attached to the surface of cellulose, binding the cellulose microfibrils together in a cohesive network. The hemicelluloses also act as a slippery coating to prevent direct contact between the microfibrils (Mellerowicz*, et al.*, 2001). In the primary wall, pectins form a gel phase in which the microfibrils and hemicelluloses are embedded. They act as a hydrophilic filler, determining the porosity of the cell wall and also act as a safety mechanism against aggregation and collapse of the cellulose network (Mellerowicz*, et al.*, 2001)

Lignin is a phenolic polymer with hydrophobic properties that improves rigidity and impermeability of xylem cells (Boerjan, Ralph & Baucher, 2003; Mellerowicz*, et al.*, 2001). Like secondary wall formation, lignification is occurring first in vessel elements and their contact cells and thereafter in fibres. When lignification is complete, lignified vessel elements and fibre cells undergoes programmed cell death.

The cell wall also contain structural proteins such as glycine- and proline rich proteins, arabinogalactan proteins and hydroxyproline rich glycoproteins that form cross linkages between the structural carbohydrates. Such linkages may also add mechanical strength to the cell wall (Brett & Waldron, 1996). Moreover, arabinogalactans (AGPs) have been suggested to mediate signal transduction at the primary cell wall-plasma membrane interface (Johnson *et al.*, 2003; Kjellbom *et al.*, 1997; Showalter, 2001).

Plant growth regulators and wood formation

Auxin

In the early 20th century, Went demonstrated the presence of a growth factor from plant extracts that was named auxin (Went FW, 1937). The name auxin originates from the Greek, 'aux-ein' meaning 'to grow'. Auxin is biologically active in sub-microgram amounts and is associated with a great variety of physiological processes including apical dominance, shoot elongation and root initiation. Auxins are also the most recognized stimulator of wood formation, of which indole-3-acetic acid (IAA) is the most abundant endogenous auxin and the most intensively studied (Davies, 1995). Auxin is also required for basic growth processes such as cell division and expansion, and therefore no true auxin deficient mutants have been found (Taiz & Zeiger, 1998).

The major production of auxin is believed to take place in apical shoots and young leaves (Davies, 1995). However, in young *Arabidopsis* plants, also cotyledons and roots were shown to have the ability to produce IAA (Ljung, Bhalerao & Sandberg, 2001). The biosynthetic pathway of IAA is still not completely defined. From studies using feeding of stable isotopes, two alternative pathways have been distinguished; the tryptophan dependent and the tryptophan independent route. The tryptophan dependent pathway is most thoroughly investigated and several of the biosynthetic genes in this pathway are identified (Bonnie *et al.*, 2001; Ljung, Bhalerao & Sandberg, 2001). The discovery of the century in auxin research was the finding of TIR1, the long sought auxin receptor. TIR1 has been shown to be a F-Box subunit of the SCF complex that binds auxin (Dharmasiri, Dharmasiri & Estelle, 2005; Kepinski & Leyser, 2005; Parry & Estelle, 2006). In most studies where physiological responses to exogenous auxin have been investigated, concentration dependent responses have been documented. Moreover, auxin sensitivity has been found to differ between tissues (Davies, 1995; Moyle *et al.*, 2002; Muday & DeLong, 2001).

The transport of auxin can be divided between passive and active transport. Passive transport takes place in the phloem stream at a rate of about 20 cm/h (Baker, 2000). Active transport is an energy consuming and unidirectional transport process of bioactive auxins carried out by special influx and efflux proteins, which are located in the plasma membrane (Geraint *et al.*, 2001; Muday & DeLong, 2001; Paponov *et al.*, 2005; Schrader, *et al.*, 2003). Auxin can enter the cell via diffusion through the plasma membrane in its protonated form (IAAH) or via influx carriers in its anionic form (IAA^-) (Akin et al., 1996). Inside the cell, the anionic form predominates. Exit of IAA from the cell is carried out by efflux

carriers, the PIN proteins (Fig. 3) (Akin*, et al.*, 1996; Petrasek *et al.*, 2006; Vieten *et al.*, 2005). Auxin is stimulating its own transport by feed forward synthesis of efflux carriers (Chawla & DeMason, 2004; Heisler & Jönsson, 2006; Vieten*, et al.*, 2005). During active transport, auxin move at 5-20 mm/h (Friml and Palme 2002).

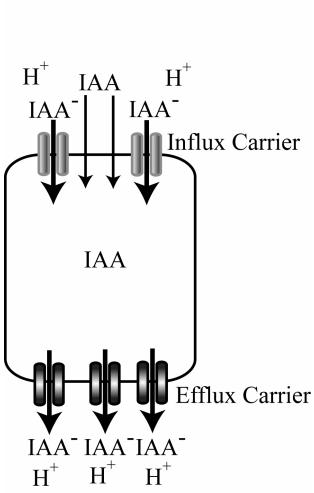

Fig. 3. Schematic representation of an auxin-transporting cell with both passive and active auxin transport visualised. Auxin can diffuse through the plasma membrane or enter the cell via the influx carriers. Auxin is only leaving the cell via the efflux carriers (*e.g.* PIN1). (Modified from Schrader, 2003b)

In wood forming tissues of both angiosperm and gymnosperm trees, IAA is distributed as a step concentration gradient across the developing xylem, peaking in the cambial zone and decreasing towards mature xylem and phloem (Tuominen *et al.*, 1997; Uggla *et al.*, 1996). The auxin gradient has been suggested to provide positional information necessary to establish the developmental pattern of cambial growth (reviewed in (Sundberg, Uggla & Tuominen, 2000). In poplar, Schrader *et. al.,* (2003) showed tissue specific localization of mRNA encoding influx and efflux-like auxin carriers across the cambial region. These results suggest a possible radial route for auxin transport from the cambial zone towards maturing xylem and phloem. The feed forward induction of auxin transporters together with the existence of auxin transporters across the vascular cambium can possibly mediate constant expression of efflux transporters in cells close to the vascular cambium where the auxin concentration is high. The suggested role for PIN proteins are consistent with the well established canalization hypothesis; a hypothesis of active auxin transport which predicts an auxin flow that is under its own feed forward mechanism, inducing both new and continued polarisation. Such cell polarization increases gradually when auxin is channelled and by the increased auxin concentration, vascular patterning is initiated (Berleth & Sachs, 2001; Jonsson *et al.*, 2006; Sachs, 1991; Smith *et al.*, 2006). In *Arabidopsis* roots, the canalization hypothesis was further developed when auxin was shown to act as a polarizing signal in a region of cambial cells during organogenic and regenerative processes. This mechanism was shown to be mediated by re-

localization in the expression of PIN genes and proteins mediated by auxin response factors (*Aux/IAA-ARF)* in a coordinated fashion. Such cell specific polarization of cells can at least in roots establish directed auxin transport to specific cells and thereby determine vascular patterning in a correct orientation relative to already existing body structures (Sauer et al., 2006). To summarize, polar active auxin transport can mediate auxin transport over long distances as well as redistribute auxin locally.

Gibberellin

There are 126 gibberellins (GAs) identified in plants (MacMillan, 2001). The bioactive GA_1, GA_3 and GA_4 have roles in, for example, stem elongation, root growth, flowering and leaf expansion (Davies, 1995; Hooley, 1994; Ross, Murfet & Reid, 1997). The function of active GAs has been investigated in GA biosynthetic mutants of pea and *Arabidopsis* which have a reduced GA level resulting in a dwarfed phenotype (Hooley, 1994; Olszewski, Sun & Gubler, 2002; Silverstone, Ciampaglio & Sun, 1998)

GA Biosynthesis and response pathway

GA biosynthesis starts from isoprenoid (McGarvey & Croteau, 1995). Initially gerabylgeranyl diphosphate (GGPP), is produced via two different pathways, one of which is the mevalonate-dependent pathway taking place in the cytosol and the other (and most important) is the mevalonate-independent pathway that takes place in the plastids (Valerie, 2001), (Lange & Lange, 2006). Briefly, in the plastids *ent*-kaurene is synthesised from GGPP by *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurotene synthase (KS), and further converted to GA_{12} in the ER by cytochrome-P450-dependent mono-oxygenases (Fig. 4). The last stage of the GA biosynthesis can be divided into non-hydroxylation and hydroxylation pathways, which converts GA_{12} to GA_4 and $GA_{1,}$ respectively. This step takes place in the cytosol. In both pathways, the multifunctional enzyme GA 20-oxidase (GA20ox) is responsible for the stepwise oxidation of both GA_{12} and GA_{53} to GA_9 and $GA_{20,}$ respectively. GA 3-oxidase (GA3ox) regulates the last biosynthetic step in production of bioactive GAs. In competition to this production is the conversion to the non-bioactive GA_{51} and GA_{29} by GA 2-oxidase (GA2ox). As a complementary regulatory mechanism, the bioactive GA_4 and GA_1 can be catalyzed by GA2ox to form GA_{34} and GA_8 respectively. The dominant biosynthetic pathway is species dependent. For example in rice and pea GA_1 is the dominating product, whereas in *Arabidopsis* GA_4 is the major end product (José Luis & Joan, 2001; Kamiya & Garcia-Martinez, 1999).

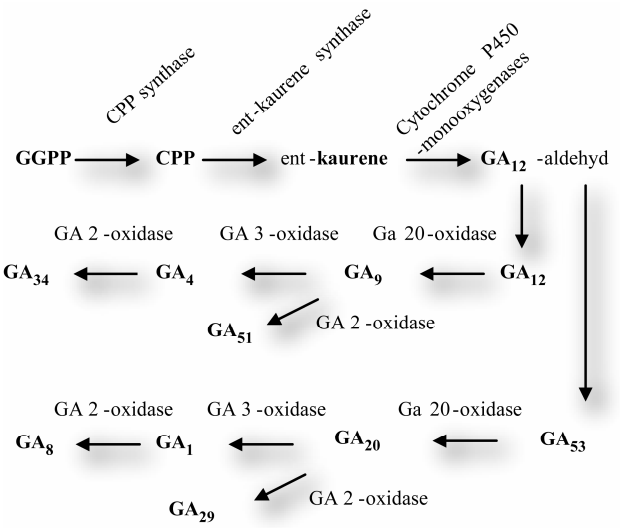

Fig. 4. Schematic representation of the GA biosynthetic pathway. The last steps in the pathway are separated in the non-hydroxylation pathway generating GA_4 as bioactive product and hydroxylation pathway where GA_1 is the bioactive product. In poplar GA_4 is the dominating bioactive GA in stem tissues.

Genes coding for the proteins responsible for the last steps of GA biosynthesis, the GA dioxygenase genes (GA20ox and GA3ox), have been shown to be GA regulated, (reviewed in (Hedden & Phillips, 2000; Valerie, 2001). The feedback mechanism on dioxygenase gene expression is negative when GA concentrations are high. High GA concentrations have also been demonstrated to induce GA2ox, as a mechanism to decrease the concentration of bioactive GAs in *Arabidopsis*, pea and rice (Thomas et al., 1999). When GA concentrations are decreased by chemical inhibition of GA biosynthesis or in GA biosynthesis mutants, feed forward regulation of GA biosynthesis has been observed (Carrera *et al.*, 2000; Cowling *et al.*, 1998; Thomas, Phillips & Hedden, 1999). In *Arabidopsis*, the feedback regulation is, however, only applicable to one of the GA3ox genes (Yamaguchi et al, 1998). Following reduced auxin concentration after decapitation in tobacco and pea, the down-regulation of GA20ox and GA3ox has been shown to be restored by apical addition of auxin (Carla & John, 2001; Ross *et al.*, 2000; Wolbang *et al.*, 2004). This demonstrated that auxin could stimulate GA biosynthesis. GA20ox genes has previously been shown to be differentially regulated in a species dependent manner depending on their different role as rate limiting enzymes (Ross et al., 2001).

Recently, a GA receptor denoted GID1 was identified in rice and in *Arabidopsis* (Griffiths *et al.*, 2006; Ueguchi-Tanaka *et al.*, 2005). The most central player downstream of this GA receptor are the growth repressor proteins in the DELLA family, which consist of 5 members in *Arabidopsis* (Dill & Sun, 2001) reviewed in Olszewski, 2002). The DELLA domain is believed to be indispensable for the repression capacity during GA signalling, where elevated levels of GAs negatively affects DELLA proteins and thereby alleviating growth repression (Fleet & Sun, 2005; Silverstone *et al.*, 2001). The GAI/RGA DELLA -family member have been

shown to be involved in GA-mediated stem elongation in *Arabidopsis*, whereas RGL members, are involved in seed germination (reviewed in (Fleet & Sun, 2005)

Auxin and Gibberellin in wood formation

A role for auxin in cell divisions and xylogenesis in wood formation is well established (Digby & Wareing, 1966; Little & Savidge, 1987; Sundberg, Uggla & Tuominen, 2000). Several studies have shown that exogenously applied auxin to decapitated plants enters the polar transport system, thus mimicking auxin originating from the apical shoot and induce xylem growth in a concentration dependent manner (Gouwentak, 1941; Leitch & Savidge, 1995; Sundberg & Little, 1990). From similar application experiments in *Pinus* using $^{13}C_6$IAA it was concluded that the apical shoot is the major source of auxin (Sundberg & Uggla, 1998).

In experiments where GA has been applied together with auxin, xylem formation was favoured by high ratios of IAA/GA, whereas low ratios induced phloem differentiation (Digby and Wareing, 1966). When GA was applied alone, only cambial cell division, but no xylogenesis was observed. In transgenic poplar trees overproducing GA20ox resulting in increased GA levels, both increased xylem growth and longer xylem fibres were observed (Eriksson et al., 2000). Whether auxin and gibberellin act independently or in synergy have been studied extensively in herbaceous species. Mutants deficient in GA biosynthesis or auxin responses have demonstrated that both hormones can independently stimulate elongation of pea internodes and *Arabidopsis* hypocotyls (Barratt & Davies, 1997; Collett, Harberd & Leyser, 2000; Yang, Davies & Reid, 1996). However, it seems clear that in the intact plant they act in synergy. Such synergy includes stimulation of the GA biosynthetic genes, GA20ox and GA3ox by auxin (Ross*, et al.*, 2000; Wolbang*, et al.*, 2004; Wolbang & Ross, 2001) and polar transport of auxin facilitates GA-induced degradation of DELLA proteins, and is therefore a positive stimulus for GA dependant root elongation (Fu & Harberd, 2003).

Ethylene

Ethylene responses

Ethylene acts as an important gaseous signalling molecule in many organs throughout the life cycle of the plant, *i.e.* in seed germination, flowering, senescence, abscission and ripening of climacteric fruits (Abeles FB, 1992; Dolan, 1997). Ethylene has also been shown to have a central role in numerous stress responses in plants, *e.g.* in wounding, pathogen invasion, and flooding (Abeles FB, 1992; Bleecker & Kende, 2000; Chen *et al.*, 2002a; Guzman & Ecker, 1990; Kieber & Ecker, 1993; Smalle *et al.*, 1997b). Many studies have demonstrated an inhibitory role of ethylene in vegetative growth. A well-known phenotype caused by exogenous ethylene, enhanced ethylene biosynthesis or plants having constitutive ethylene signalling, is the much-exploited triple response in etiolated *Arabidopsis* seedlings. The triple response involves radial swelling of the hypocotyl, inhibition of both hypocotyl and root elongation and exaggerated

tightening of the apical hook (Guzman & Ecker, 1990; Kieber & Ecker, 1993; Knoester *et al.*, 1997).

There is good evidence, however, that ethylene can stimulate growth responses. In *Arabidopsis*, ethylene was shown to trigger cell expansion and thereby stimulate hypocotyl elongation in light (Smalle *et al.*, 1997a). Similar stimulatory effects by low concentrations of ethylene on stem elongation has also been seen in tobacco and wheat (Pierik *et al.*, 2003; Suge *et al.*, 1997). Using ethylene insensitive tobacco, the stimulatory growth effect of ethylene was shown to be an important response for shade avoidance, and competition for light (Pierik*, et al.*, 2003). Ethylene's promotion of cell elongation was also suggested in cotton, where not only several ACC oxidases were shown to be up-regulated during fibre elongation, but also that application of ethylene resulted in increased fibre length, whereas blocking ethylene biosynthesis with L-(2-aminoethoxyvinyl)-glycine (AVG) reduced fibre elongation (Shi et al., 2006). Normally the stimulatory effects of ethylene are under influence of low concentrations of ethylene, but in species such as rice, *Rumex palustris* and *Ranunculus sceleratus* that often grow in flooded habitats, high concentrations of ethylene stimulates internode and petiole elongation (Abeles FB, 1992; Kende, van der Knaap & Cho, 1998; Satler & Kende, 1985; Voesenek *et al.*, 1997). Whether ethylene stimulates or inhibits cell elongation, seems to be species and concentration dependent (Pierik et al., 2006). Most effects of ethylene on growth are related to cell expansion, but interestingly transient ethylene exposure in cucumber (*Cucumis sativus*) hypocotyls was shown to induce cell division in trichomes and guard cells as well as enhanced endoreduplication in hypocotyl epidermis cells (Dan et al., 2003; Kazama et al., 2004).

Ethylene biosynthesis and signal perception

The biosynthetic pathway of ethylene is well studied in higher plants. Ethylene is produced from L-methionine via the intermediates S-adenosylmethionine (AdoMet) and 1-aminocycloporpane-1-carboxylic acid (ACC). These last two steps are catalyzed by the enzymes ACC synthase (ACS) and ACC oxidase, of which there are 8 functional ACC synthases and 6 ACO family members in *Arabidopsis* (Arabidopsis Genome, 2000; Yang & Hoffman, 1984). In the *Populus trichocarpa* genome, 8 ACS and 7 ACO putative protein coding genes have been identified (Tuskan et al., 2006). Both ACS (An *et al.*, 2006; Li, Qu & Li, 2005; Tsuchisaka & Theologis, 2004) and ACO (Barry *et al.*, 1996; Blume & Grierson, 1997; Botella, Arteca & Frangos, 1995; Dunlap & Robacker, 1994; Hudgins JW, 2006; Kende, 1993; Ververidis & John, 1991) are differentially regulated, and may therefore be important as regulatory steps in ethylene biosynthesis (Figure 5). ACS expression has been shown to be regulated both translationally and post-translationally in several studies (Chae, Faure & Kieber, 2003; Fluhr & Mattoo, 1996; Johnson & Ecker, 1998; Wang, Li & Ecker, 2002a). The post-translational regulation, mainly using the ethylene overproducing eto3 and eto2 mutants (corresponding to mutations in *ACS5* and *ACS9* respectively) in *Arabidopsis* has attracted much interest (Chae, Faure & Kieber, 2003). The increased ethylene production in these mutants is a result of increased ACS stabilization, which is possibly caused by loss of phosphorylation capacity in their C-terminal domains

(Tatsuki & Mori, 2001b). Besides its conversion to ethylene by ACO, ACC can also be conjugated to malonyl-ACC (MACC) (Hoffman, Yang & McKeon, 1982) and to glutamyl-ACC (GACC) (Martin, Cohen & Saftner, 1995). ACC conjugation is thought to decrease the ACC pool irreversibly (Abeles FB, 1992) (Fig. 5)

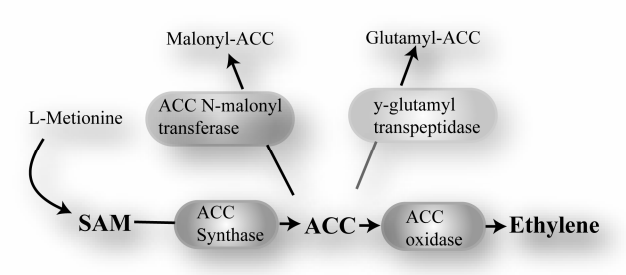

Fig. 5. The biosynthetic pathway of Ethylene. The main regulatory steps are ACC synthase (ACS) and ACC oxidase (ACO) enzymes. SAM: S-adenosyl-1-Metionine, ACC: 1-aminocyclopropane-1-carboxylic acid.

Ethylene in perceived by a family of membrane bound receptors similar to two component regulators in bacteria (Kieber et al., 2002) (Fig. 6). In *Arabidopsis*, five ethylene receptors have been identified; ETR1, ETR2, ERS1, ERS2 and EIN4. ETR1 has been localized to the endoplasmic reticulum (ER) membrane in *Arabidopsis* (Chen *et al.*, 2002c). In poplar, 7 putative ethylene receptors have been predicted from the genome sequence (Tuskan*, et al.*, 2006). Upon ethylene binding to the receptors, the activity of the receptors is repressed and the downstream signalling is activated by a conformational change in the serine/threonine protein kinase, constitutive triple response 1 (CTR1) (Hua & Meyerowitz, 1998) (Figure 6). CTR1 acts as a negative regulator of downstream ethylene signalling, possibly by inactivation of a MAPK cascade (Guzman & Ecker, 1990; Huang *et al.*, 2003; Kieber *et al.*, 1993). The dominant negative mutation in ETR1 (*Atetr1-1*) disrupts ethylene binding, resulting in constitutive signalling of the receptor which creates an ethylene insensitive phenotype (Bleecker, 1988; Guzman & Ecker, 1990; Sakai *et al.*, 1998; Vahala *et al.*, 2003). Downstream of CTR1 are the positive regulators EIN2 (Alonso et al., 1999) and EIN3 (Chao et al., 1997). In the presence of ethylene, EIN3 proteolysis is inhibited, extending its half-life and thereby creates a positive regulatory mechanism of transcription (Chao*, et al.*, 1997; Chuanzao *et al.*, 2006; Guo & Ecker, 2003). The regulation of EIN3 is a post-transcriptional mechanism, where two F-box proteins, EBF1 and EBF2, target degradation of EIN3 for the 26S proteosome in the absence of ethylene (Potuschak *et al.*, 2003; Rieu, Mariani & Weterings, 2003). In the presence of ethylene, EIN3 has been shown to specifically bind to a primary ethylene response element (PERE) sequence in the promoter of an ethylene response factor, ERF (Solano et al., 1998), of which there are 122 in *Arabidopsis* and 139 in rice (Nakano et al., 2006). ERFs are members of the APETALA2-domain-containing transcription factors (AP2) which are known to specifically bind to a *cis*-acting GCC-box, with a consensus sequence of TAAGCCGCC, present in the promoters of many ethylene inducible genes (Meller et al., 1993). The ERF family contain one AP2/ERF domain, consisting of

60-70 amino acids (Nakano*, et al.*, 2006; Sakuma *et al.*, 2002). AtERFs have been classified into three groups based on amino acid sequence (Fujimoto et al., 2000). Class I and III exert transactivation of genes containing the GCC box, whereas class II are actively suppressing transcription in presence of ethylene or ACC addition (Fujimoto*, et al.*, 2000; Ohta *et al.*, 2001; Toshitsugu *et al.*, 2006).

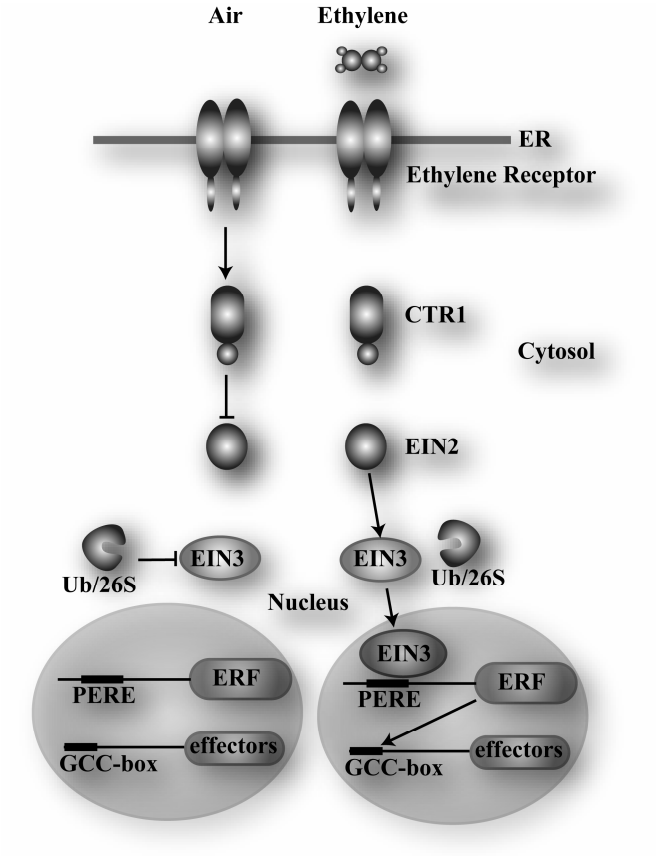

Fig. 6. A model of ethylene signal transduction. Upon presence and binding of ethylene to the receptors, which have been localized to the endoplasmic retilicum (ER), the repression of CTR1 is de-activated. After such relief of repression, the downstream components EIN2 and EIN3 are activated. EIN3 stability is required for proper ethylene response and is targeted for 26S proteosome degradation by SCF complex in the absence of ethylene. In the absence of ethylene, EIN3 is rapidly broken down and no further signalling perceived. EIN3 binds to the primary ethylene response element (PERE), which are driving a number of AP2/ERF domain containing ethylene response factors (ERF), which in turn bind to the GCC-box within the promoters of effector genes involved in ethylene responses. (Modified from Guo and Ecker, 2004)

Modifications of ethylene biosynthesis, perception and signal transduction

Many of the components of ethylene biosynthesis and signalling have been identified. Since ethylene is an important player in many physiological plant

responses of economic interest, several experiments aiming to improve plant performance by modifying ethylene biosynthesis and/or perception have been performed. A number of trials have targeted economically important species where ethylene is known to be an important stimulator. Examples of important aspects for ethylene action are: fruit- and floral development, ozone tolerance and resistance to abiotic stresses (Czarny JC, 2006; Grierson & Fray, 1994; Kende, 1993). In contrast, few workers have modified ethylene biosynthesis or perception in trees. Of the few examples, introducing *Atetr1-1,* reduced ethylene sensitivity and resulted in less sensitivity to ozone stress in birch (Vahala*, et al.*, 2003).

Since the broad approach of increasing or decreasing ethylene production can give unwanted effects, it is favourable to target more specific genes downstream of perception. Candidates for such modifications are members of the ERF family of transcription factors which are known to be involved in transcriptional regulation of growth and development, as well as in responses to environmental stimuli *e.g.* flower development (Elliott et al., 1996), meristem determination (Chuck, Meeley & Hake, 1998), embryo development (Boutilier et al., 2002) and leaf epidermal cell identity (Moose & Sisco, 1996). Many ERFs have also been directly linked to hormonal signal transduction (Ohme-Takagi & Shinshi, 1995), others are responsive to abiotic stresses (Dubouzet *et al.*, 2003; Liu, White & MacRae, 1999; Stockinger, Gilmour & Thomashow, 1997) and biotic stresses (Gu *et al.*, 2000; Yamamoto, Suzuki & Shinshi, 1999). Since many ERFs and DREBs are stress induced, modifications in their expression may potentially be a way of generating more stress tolerant plants in the future (Berrocal-Lobo, Molina & Solano, 2002; Chen *et al.*, 2002b; Pradeep *et al.*, 2006).

Ethylene and wood secondary development

Application experiments have demonstrated a potential role of ethylene in secondary growth in stem tissues. For example, application of the ethylene generating substance, etherel (2-chloroethylphosphonic acid), has been shown to be a positive stimulator of cambial cell division in both gymnosperm and angiosperm trees (Eklund & Little, 1996; Yamamoto, Angeles & Kozlowski, 1987; Yamamoto & Kozlowski, 1987a). Moreover, applied ethylene increased cell wall thickness, and decreased fibre and vessel length in poplar (Junghans et al., 2004), and decreased vessel diameter in *Acer negundo*, *Acer platanoides* and *Ulmus americana* (Savidge, 1988). There are also indications that etherel applications increase cellulose deposition in stems of Norway spruce seedlings (*Picea Abies* L.) (Ingemarsson, Eklund & Eliasson, 1991). Moreover, in Scots pine the onset of cambial activity has been observed to coincide with an increase of ethylene emissions from wood forming tissues, and the peak of ethylene emission corresponded with the period of maximum cambial activity (Ingmarsson BSM, 1991; Klintborg A, 2002). There are however no studies that conclusively demonstrate a role for endogenous ethylene in wood forming tissues.

To summarize, during wood formation, exogenous ethylene has been shown to modify cell division, cell elongation/expansion, cell wall thickness and chemistry. Further, elevated ethylene levels have been observed during the period of most active growth.

Reaction Wood

The asymmetric primary growth in roots and shoots in response to gravity, has been studied for 100 years (Darwin, 1888). Reaction wood (RW) is a secondary growth response to gravity in woody perennial species induced in leaning stems by *e.g.* wind or snow load (Timell, 1969). The most recognized responses in RW are increased cambial growth and alterations in fibre morphology and chemistry. Growth on the opposite side of a leant stem is often reduced. The growth stimuli in RW and the reduced growth on the opposite side create the eccentric growth patterns seen in leaned trees. In gymnosperms, RW is formed on the lower side of the leaned stem or branch and is called compression wood (CW). In angiosperm trees the opposite growth pattern is seen, where RW, called tension wood (TW), is formed on the upper side of the leant stem or branch. CW is pushing, and TW is pulling, the stem to achieve the desired change in growth orientation. This can be a vertical position for stems, or upward bending of a side branch to replace the loss of a leader. Reaction wood can therefore be considered as a response mechanism to maintain an optimal position of stems and branches.

Tension Wood

Tension wood has several characteristics that distinguish it from normal wood. In TW, there is a shift towards more fibres and less vessels (Huges, 1965). The most outstanding characteristic in TW, however, is the change in cell wall composition. An inner cell wall layer (the G-layer) composed of highly crystalline cellulose is replacing S3 and part of the S2 layer (Fig. 2). The G-layer is almost unlignified and is loosely attached to the surrounding cell wall layers (Timell, 1969). In mature wood, the different physical properties of G and S2-layers of TW fibres creates a different longitudinal shrinkage. Such shrinking has been suggested to create a pulling pressure on the on the surrounding cells (Archer, 1987). This process has been suggested to create the physical forces which enables the straightening of the stem after leaning (Hejnowicz, 1997; Norberg & Meier, 1966; Plomion, Leprovost & Stokes, 2001). Such process have in hardwood species been observed to create up to 20 fold higher tensile stress than the OW or NW (Clair et al., 2006a; Clair et al., 2006b)

The strongest evidence indicating RW as gravity response, and not a response to mechanical forces, comes from experiments where bent stems or branches are rotated on a clinostat to eliminate gravitational sensing. In these experiments, RW does not form (Jourez, Vaianopoulos & Hebert, 2003; Timell, 1986). Two major hypotheses related to gravitational sensing in plant cells have emerged. The gravitational-pressure hypothesis (Staves, 1997) is a model where cells are believed to sense their relative buoyancy to the surrounding medium. The other hypothesis, the starch statolith hypothesis (Sack, 1997), involves the sedimentation of starch accumulating amyloplasts in the gravitational downward direction within specialized statocyte cells. One can only speculate which hypothesis is more correct, but the starch statolith hypothesis is currently the most favoured (Morita & Tasaka, 2004). In an attempt to study the role of gravity in RW wood formation, bent seedlings of Loblolly pine were grown at microgravity. Since the growth conditions were sub-optimal the results are not conclusive, but they indicated that during microgravity conditions, RW can still form as a response to mechanical

stress (Kwon et al., 2001). Thus, there are different opinions regarding whether or not RW is due to gravitational or mechanical response. Unquestionable, RW formation is a complex process, and yet no conclusive evidence has been presented to rule out any of the hypothesis about its induction.

Ethylene, gravitropism and Reaction Wood

The idea that ethylene has a role in gravitropic responses had already been in the beginning of the 20th century (Neljubov D, 1901). Many studies demonstrate that the horizontal growth of the apical hook in dark grown *Arabidopsis* seedlings is exaggeration in its curvature after ethylene treatment (Bleeker et al., 1988, Guzuran and Ecker, 1990). By blocking ethylene biosynthesis and sensing, delay in gravitropism has been observed in cocklebur, tomato and cast bean stems (Raymond et al., 1981; Madlung et al., 1999). From analysis of mutants showing agravitropic behaviour or aberrant ethylene physiology, it appears that a complex interplay exists between ethylene and other signalling molecules, in particular with auxin (Muday *et al.*, 2006; Roman *et al.*, 1995).

In trees, enhanced ethylene evolution has been observed to coincide with the known inducers of RW, such as; bending (Blake TJ, 1980; Brown KM, 1973; Leopold AC, 1972; Nelson ND, 1978; Robitaille & Leopold, 1974), shaking (Rinne, 1990; Telewski FW, 1990; Telewski FW, 1986) and leaning (Little & Eklund, 1999; Yamamoto & Kozlowski, 1987b). Recently, experiments that are more extensive showed strong correlations between ethylene evolution and RW formation. In *Aesculus turbinate,* localized ethylene evolution and a high cambial activity was observed at the gravitationally upper side of leaned stems. When the stems where rotated, the site of ethylene evolution was still found to be at the upper side of the stem (Du & Yamamoto, 2003). In poplar, a strong up regulation of *ACO1* accompanied with a depletion of the ACC pool was seen in TW forming tissues, whereas the *ACO1* expression in the OW was unaffected. (Andersson-Gunneras et al., 2003). These studies are, however, descriptive, showing increased ethylene evolution during physical stress, but not that ethylene is necessarily critical for the gravitropic RW formation. Moreover, applied ethylene has never been observed to induce G-layer biosynthesis indicating the involvement of other factors in the RW response.

Some studies have shown a reduction in stem diameter in both angiosperm and gymnosperm trees after leaning when the RW forming tissues has been treated with ethylene (Yamamoto, Angeles & Kozlowski, 1987; Yamamoto & Kozlowski, 1987a). Such reduction can be due to supra-optimal ethylene concentrations that will act to inhibit growth, as recently described by Pierik and co-workers (Pierik*, et al.*, 2006).

Possible Hormonal Crosstalk of Auxin, Gibberellin and Ethylene in RW

There are several lines of evidence suggesting crosstalk between auxin, gibberellin and ethylene. Auxin-ethylene interactions are apparent during the biosynthesis of ethylene and transport and transport of auxin. Auxin stimulates expression of *ACS* which thereby enhances ethylene biosynthesis (Peck & Kende, 1995; Peck,

Pawlowski & Kende, 1998; Rodrigues-Pousada *et al.*, 1999; Tsuchisaka & Theologis, 2004; Woeste, Ye & Kieber, 1999; Yang & Hoffman, 1984), whereas nothing is known about ethylene's possible regulatory role in auxin biosynthesis. However, recent results indicate that downstream of auxin perception, some auxin response factors (ARF), are stimulated by ethylene (Harper *et al.*, 2000; Li, Dai & Zhao, 2006). Ethylene has also been shown to affect auxin transport by restrain the expression of the auxin efflux carriers (Chilley *et al.*, 2006; Eklund & Little, 1995; Prayitno, Rolfe & Mathesius, 2006; Suttle, 1988). Such inhibition could lead to modified auxin distribution in a stem that undergoes elevated ethylene concentration, for example in TW, where induction of ethylene biosynthesis and ethylene evolution is known to occur. Another possible effect of auxin and ethylene interaction has been through auxin sensitivity, which can change in elevated ethylene concentrations (Visser et al., 1996). As mentioned above, an example of auxin-gibberellin interaction is the stimulation of polar auxin transport by GA and auxin affecting GA biosynthesis (Ross*, et al.*, 2000; Wolbang*, et al.*, 2004; Wolbang & Ross, 2001). The known repressor of GA mediated growth responses, the DELLA protein, is known to targeted for destruction in high GA presence, a breakdown that has been demonstrated to be delayed by ethylene (Achard *et al.*, 2006; Achard *et al.*, 2003).

Applied auxin, gibberellin and ethylene have been demonstrated to affect aspects of the RW response. There is, however, no conclusive evidence demonstrating crosstalk between these hormones mediating the RW response. An inhibitory role of auxin in TW formation was suggested from the observation that TW was inhibited after auxin application to the stem surface in leaned poplar and maple stems (Cronshaw, 1965; Necesany, 1958). Contradicting this result, auxin application to one side of upright poplar stems was shown to induce TW on the opposite side to the application (Blum, 1971). In more recent studies, both auxin and gibberellin were shown to induce TW in *Fraxinus mandshurica* and Japanese Cherry (*Prunus spachiana*) (Baba et al., 1995; Jiang et al., 2006). As suggested by Timell (1986), TW is induced either by IAA deficiency or at a position around the stem where IAA concentration is lowest. Such a hypothesis does not however agree with the recent result in poplar, where the endogenous levels of auxin in TW did not change compared to normal wood and the lowest IAA concentration was observed in OW after bending (Hellgren, Olofsson & Sundberg, 2004).

Hormonal crosstalk is a complex phenomenon. An approach to elucidate hormone interactions would be to reduce the levels of the hormones individually, in combination, and then test RW responsiveness. However, since viability of plants often relies on a certain critical hormone levels, reduction of individual hormone levels would probably be lethal. Alternatively, identifying critical transcription factors and/or hormone response genes critical in the RW response, and investigating their regulation by different hormones may bear fruit.

Methodological overview

The methods used throughout this work are described in detail in paper I to IV. Here I will provide a brief overview and some reflections concerning the experimental plant materials and gene expression analysis that had been central for the thesis. I will also provide some methodological details about few matters that are not presented in the papers.

The model species during this thesis work is a male hybrid between *Populus tremula* and *Populus tremuloides*, denoted hybrid aspen (*Populus tremula x tremuloides*) and to a minor extent *Populus tremula L*. Hybrid aspen is a fast growing hardwood species, which is easy to genetically transform using the Agrobacterium transformation system and to regenerate from tissue culture. Hybrid aspen has undergone a large EST sequencing project, where about 120.000 ESTs from 19 different cDNA libraries have been sequenced (Sterky*, et al.*, 2004). From these EST libraries several microarrays have been produced. In this study the 14k and 25k arrays have been used (Andersson*, et al.*, 2004; Hertzberg*, et al.*, 2001a). The close relative, *Populus trichocarpa*, was recently fully sequenced, a work achieved by the joint Genome Institute US Department of Energy, http://www.jgi.doe.gov (Tuskan*, et al.*, 2006). The sequencing project revealed that the poplar have a genome size of 500MB contained in 19 chromosomes, which represents approximately 45k gene models. Even though the poplar genome is four times as large as the *Arabidopsis* genome, it is 50 times smaller than pine and therefore makes it suitable for molecular studies of wood (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html). Some of the work in this thesis was focused on TW formation, which is specific growth response to leaning in hardwood species, such as poplar (Huges, 1965).

Construction of transgenic trees

In this work, transgenic trees with modified ethylene sensing and ethylene biosynthesis were generated and used in papers III and IV. For constitutive expression the 35s promoter was utilized, and to more specifically direct expression to specific stem tissues one promoter with its major expression in cambium/phloem tissues (denoted LMP1) and one in developing xylem tissues (denoted LMX5) were used. The tissue "specific" promoters were identified from a microarray experiment visualizing gene expression across wood forming tissues (Hertzberg *et al.*, 2001b). The corresponding genes for these promoters are for LMP1: EST A001P79U (Genebank number AI161513), and for LMX5: EST A055P19U, (Genebank number AI164126). Transgenic trees expressing the gene coding for β-glucuronidase, *uidA*, from Escheriachia coli, driven by the two promoters were constructed to visualise expression pattern of these promoters (Paper III, figure 1). For generation of transgenic trees with reduced ethylene sensing, the genomic sequence of the mutated ethylene receptor, *Atetr1-1*, driven by 35s, LMP1 and LMX5 promoters was inserted into WT poplars. For increased and decreased ethylene biosynthesis, Ptt*ACO1* was expressed in 35s:*ACO1* sense and anti sense constructs. The reason for selecting Ptt*ACO1* for this purpose, was because this ACC oxidase is the most strongly expressed and induced ACO in the

poplar stem (Andersson-Gunneras*, et al.*, 2003). In general, 10-15 lines of each promoter construct were screened for ethylene insensitivity or changed ACO activity.

Plant materials

Throughout this work tree different kinds of poplar plant materials were used; field grown material from naturally generated stands of aspen (*Populus tremula* L.), greenhouse- and *in vitro* cultured material of the lab clone T89 (*Populus tremula x tremuloides* Michx.). An obstacle working on trees is the space and relative long time required to raise the experimental material. This is particularly a matter for the vegetative propagated transgenic materials, where material ready for screening is not necessarily synchronised in time. Therefore, we established an *in vitro* tree growth system for semi high-throughput experiments to study the effect of ethylene on wood formation (paper III). This system is space saving, eliminates tedious green-house culturing and provides stable environmental conditions making experiments comparable independent of season. Plants were grown *in vivo* in autoclaved Murashige and Skoog , (Saibo *et al.,* 2003) media together with the solidifying agent, phytogel, in $1dm^3$ containers. This experimental system was used to screen for ethylene effect on wood formation, because it allows for comparable ACC applications to the media. These experiments commenced once the plants had established a root system and grown to a height of about 50 mm. At this point, a complete vascular cambium had just formed in the fourth internode below the apex. This internode was used as a reference internode and at harvest (after twelve days of treatment) xylem and phloem formed in this internode were considered to have been under the influence of the treatments.

Greenhouse culture provides a semi-controlled environment. In this condition, trees were grown to a height of about 1.5 meters before harvested. The greenhouse enables us to monitor growth of transgenic lines for a longer time and in a more natural environment where the plants have to adapt to shifting light intensities and variations in temperature. Growth in greenhouse was measured during one month. The most comparable growth rate was seen during the last 14 days of growth, and therefore the growth rate is calculated as growth per day during the last two weeks. Another advantage with greenhouse grown trees compared to *in vitro* is their larger size, hence large amount of material can be sampled from the cambial region tissues and/or from defined position. In this thesis developing xylem and phloem/cambium tissues were collected for gene transcript profiles and enzyme activity (paper III), and stem samples from defined positions (paper I). Field grown trees provide even more materials and are suitable for micro-dissection using tangential microtom (Uggla *et al.,* 1996). This material was used for high resolution profiling of ACC across cambial region tissues (paper II). In some experiments, tension wood was induced by leaning (Figs 9-11 in this thesis and in papers II and III). Greenhouse grown trees were leant at an angle of about 30°. In field-grown trees, TW was induced by bending and tying the tree with a string for about 14 days. In vitro cultured trees were induced to form tension wood by leaning the container for 14 days at an angle of 30°.

Ethylene related pharmacological treatments

In paper III and IV, stem material exposed to ACC, ethylene, or ethylene inhibiting substances were used. 1-aminocyclopropane-1-carboxylate (ACC) treatments in the in vitro culture were delivered in 200µl aliquots directly to the growing media. To test diffusion capacity in the media, alacian blue stain where applied and it showed diffusion in the whole media already after 1 hour. To block ethylene perception chemically, 1-monocyclopropene (1-MCP) (SmartfreshTM) addition was performed twice daily at 30 ppm commencing 5 days prior to ACC or H2O of leaning treatments. Ethylene gas treatments to greenhouse grown poplars were delivered to a cuvette at a constant flow as a mixture of 2ppm ethylene and 250ppm carbon dioxide. Detailed information about ACC, 1-MCP and ethylene treatments is described in detail in paper III.

Quantification of gene transcript levels

Since the discovery of DNA and transcriptional regulation of specific genes, there has been a great interest in quantifying gene transcripts and thereby get indications of possible up or down-regulation of specific proteins. The path from DNA to protein is however not straightforward and involves posttranscriptional regulation. Therefore, one should not draw too strong conclusions between gene transcript and protein abundance, but transcript analysis is still an informative analysis. Methods of gene transcript quantification include the older northern blot analysis to the more recent methods with varieties of quantitative PCR methods and microarray analysis.

QPCR

For high-resolution gene quantification, real time PCR, has been used throughout this work. This is a quantitative, gene specific detection method originating from medical research that enables monitoring of very low expressed levels of genes with high accuracy (Bieche et al., 1998; Raggi et al., 1999). In contrast to conventional PCR where only the end product is determined (Freeman, Walker & Vrana, 1999), real time tracks changes in PCR products during the PCR reaction, and the quantification of gene transcripts are made at a threshold cycle (Ct), which is the cycle number just before exponential growth starts. Low Ct value indicate high initial target amount and high values the opposite. In order to compare gene expression in different samples, a standard gene, which should not be variable, is used. There is an ongoing debate of which standard gene to use, since different tissues have been proven to differentially express many of the common normalisation genes. In these thesis, the ribosomal 18s and actin genes have been used. A detailed description of real time PCR setup can be found in paper II, III and IV. Primers used are described in the separate papers except for *PttACS* and *PttACO* primers that are presented in the results part of this thesis (ACS and ACO primers were generated by Jorma Vahala).

PttACS1*F**:TGGAAAGCCTATGACACTGAT,PttACS1*R**:GGAGGCTTTTGGATTCTTCTT,***PttACS2*F**:GATGGATGGAAAGCTTACGAT,**ACS2**R:ATGGAGGCTTTGGGATGTT,***PttACS3*F**:GAGTCATGGCTGGCTAAGAA,***PttACS3*R**:TTCTGCCATGAATTCTACCAA,***PttACS4*F**:TGGCTTGCTAATAACCAGGA,***PttACS4*R**:AGCCATGAATTCCACCAA,***PttACS5***F:GCTGGAAGGCACACGAT,***PttAC***

S5*R**:GCTTCTGAATTCTGTTCCAAGTA,PttACS6*F2**:TCATCTAATCCATCAGGAGTCAT,***PttACS6*R3**:CTAAACCCTGGCGATCCTTT,***PttACS7*F**:GCTGGAAAGCCTATGACAGTA,***PttACS7*R**:GGAGGCTTTTGGGTTGTT,***PttACS12*F2**:GAATCCATATCACGATGTGCAT,***PttACS12*R2**:CACCATTCTTCTTCAACCCAA,***PttACO1*F2**:GATAACGAGTGGCCATCAA,***PttACO1*R2**:CCTTCTCCATCATTGAATGCTT,***PttACO2*F**:TTGCGACATCTCCCCAT,***PttACO2*R**:GACTACCGCTAGATCCACAGAA,***PttACO3*F**:CGCCATCTCCCTAAGTCAA,***PttACO3*R**:GGACTACCGTTTGATCCATAGAA,***PttACO4*F**:TTGTCATCAATACCGGTGAT,***Ptt*ACO4R**:TGCTGGTGCAGGAGCTA,***PttACO5*F2**:CCAACATCCAACATCAATGAA,***PttACO5*R2**:ATGTAATCCTTGTCCAGTCCAA,***PttACO6*F:**CAACCATGGAGGTGATCAA,***PttACO6*R**:TGCTAGCCACCATCTCCTT,***PttACO7*F**:CCAACCATGGAGAAGATTGAA,***PttACO7*R**:CCACCATTTCCTTGAACCTT

Microarray analysis

Microarray analysis is a relative new technique that allows global gene expression analysis of numerous genes in one experiment. In 1995 when this technique first was published, 45 genes were spotted on the microarray chip (Schena *et al.,* 1995). After that the technique continued to evolve and today the microarray chips can contains a major part of the genes in the genome. The poplar microarray used in these studies have 14k and 25k cDNAs corresponding to about 9.000 and 16.000 gene models (Andersson*, et al.*, 2004; Sterky*, et al.*, 2004). These poplar microarrays have been produced by spotting cDNA sequences obtained from 19 different EST libraries described in Sterky *et al*. (2004). In this thesis microarray analysis was used in paper I (14k array) and IV (25k array). For microarray analysis, RNA was extracted from the samples of interest, reverse transcribed and labelled with fluorescent dyes separately. On the slides, the samples labelled with their respective dye were allowed to hybridize to the spotted cDNA to form duplexes with their respective cDNA. The more transcripts of each gene in the samples, the stronger the signal. The signal strength of the fluorescent hybridized samples were thereafter detected by laser scanning and quantified by image analysis software. Signal intensity was normalised to remove systematic variation and to give the global transcript profile. The microarray technique generates copious amount of data, so therefore it is important to carefully evaluate the obtained data. In both paper II and IV, where hormone feedings affected thousands of genes, a stringent statistical criteria was used to exclude genes that were not trustfully affected. To identify differentially expressed genes, B-statistics with a false discovery rate was applied in both experiments by using the R plugin in UPSC base (Lonnstedt & Speed, 2002; Sjodin *et al.*, 2006). Since many gene-models are represented by several PU numbers (spot identity in the poplar microarrays), it is questionable if a gene should be considered significant different if just, for example, 1 out of 2 PUs pass the statistical filter. An average calculation of these PUs could generate more trustfully differentially expressed gene models, but could also result in increased number of false positives. To get the maximum number of putative affected genes, we choose not to calculate average expression levels of the gene-models that were represented by more than one PU. It must however be pointed out, that whatever approach is used the microarray analysis should be considered as a screening technology and conclusive information about gene expression must for each gene be confirmed with more accurate methods such as qPCR.

FT-IR

FT-IR microspectroscopy together with multivariate data analysis provides an excellent tool for non-destructive *in situ* chemical imagining of wood samples with good spatial resolution, sensitivity and reliability. Alterations in the chemical composition can be located and assigned to different tissues, cell types or even to various parts of a cell. Moreover, the nature of the altered chemistry can be deduced and specified. This is of great importance for a better understanding of plant cell wall structure, organisation and functionality as well as gene functions.

Spectra were recorded on a Bruker Equinox 55 spectrometer equipped with a microscopy accessory and a 64 x 64 FPA (Hyperion 3000) detector, providing a maximum spatial resolution of about 5 x 5 µm. Visual photographs for spectral overlay were snapshots of live images taken with a Sony Exwave HAD colour digital video camera mounted on the top of the microscope. The sample tray was boxed and the box was continuously purged with N2 or dry air. The 20-µm thick sections were mounted between two glass slides or two BaF_2 crystals for drying. Spectra were recorded either with the top crystal removed or with that left in place (where it did not disturb, in order to avoid curling) in transmission mode. Spectral range was 850 to 3850 cm^{-1} with and the resolution was 4 cm^{-1}. For each sample, 64 interferograms were co-added to obtain high signal to noise ratios. Background spectra were recorded for each sample at a nearby empty spot on the BaF_2 crystal, prior to sample measurement, with the same number of scans (64). Spectra were 2-point baseline corrected and area normalised prior to multivariate analysis. The spectral region of 900 to 1900 cm^{-1} was used for multivariate data analysis.

For multivariate analysis, the SIMCA-P software was used (version 11.0.0.0, Umetrics AB,with built in options). The initial PCA analysis (Wold, 1976) was done with 355 observations and 1556 variables on UV scaled pre-treated spectra. After excluding outliers and limiting the spectral area to relevant regions, OPLS analyses (Johan Trygg, 2002) were performed. Generally, models were based on about 40 observations and approximately 470 variables from Pareto scaled pre-treated spectra.

Results and Discussion

Wood development follows genetically determined patterns open to adjustments in response to environmental and developmental stimuli. Plant hormones act as information couriers for such signalling. The concentration and localization of the hormones, together with tissue sensitivity determine the growth and/or developmental responses. Crosstalk between hormones has more recently been recognized as a common phenomena (Achard*, et al.*, 2006; Achard*, et al.*, 2003; Nemhauser, Hong & Chory, 2006), however the complexity of these interactions are far from being elucidated. In the first part of my work, the effects of auxin and gibberellin on cambial growth have been monitored and linked to gene transcript

levels. The focus has been to deepen the understanding of the well established synergism between GA and auxin in cambial growth. In the second part, the focus has been on the hormone ethylene. Its involvement in the regulation of the vascular cambium has been demonstrated using both a pharmacological and a transgenic approach. Further, expression analyses of ethylene response factors together with ethylene responsive genes downstream of ethylene perception in wood forming tissues have been monitored.

IAA and GA crosstalk (Paper I)

Paper I describes several aspects of IAA/GA crosstalk in poplar stem tissues. By decapitation and apical feeding of the hormones, independently and in combination, we confirmed earlier observations that both gibberellin and auxin stimulate cell division of the vascular cambium when applied separately, and induce a synergistic growth response when applied together (Paper I, Fig. 2). By GC-MS measurements of the resulting internal hormone concentration after apical feeding we could document the hormonal balance of IAA and GA in the stem tissues, and study how one hormone affected the level of the other. Further, changes of specific and global gene expression as a result of increasing one or both hormones have been monitored.

Expression analysis of GA biosynthesis genes demonstrated that both IAA and GA act on several steps in the GA biosynthesis pathway (Paper I, Fig. 3&4). Expression of *PttCPS*, coding for an early regulatory enzyme in GA formation in the plastids, was stimulated by IAA similar to earlier observations in *Arabidopsis* (Valerie, 2001; Yamaguchi *et al.*, 1998). In line with the findings of Hedden and Philips (2000), we did not see any induction of *PttCPS* by GA. Expression of the multifunctional GA biosynthetic enzymes, *PttGA20ox1* and *PttGA20ox4* that encode enzymes responsible for the stepwise oxidation of both GA_{12} and GA_{53} to GA_9 and $GA_{20,}$ were strongly decreased in auxin depleted stem tissues and stimulated by IAA. This supports previous findings in pea that auxin stimulates *PsGA20ox* expression (Ross*, et al.*, 2000), and that a critical IAA level is necessary for its expression. However, after combined feeding of IAA and GA (where GA level was increased several times compared to WT levels) especially *PttGA20ox1* decreased strongly. This support the idea of a feedback control by active GAs on *PttGA20ox* expression as earlier observed in several species (Eriksson & Moritz, 2002; Hedden & Phillips, 2000; Yamaguchi, 2000). The known regulators of active GAs (*PttGA2ox1 & 2*) encoding GA deactivation enzymes, showed complementary expression patterns. Expression of *PttGA2ox1* was lower, and *PttGA2ox2* was higher, in the auxin depleted decapitated stem than in control trees. After the combined IAA/GA feeding the expression levels of both genes returned to that of control trees. Previous results in *Arabidopsis* by (Thomas, Phillips & Hedden, 1999) showing on *GA2ox* induction after GA application were not detected in our experiment. Taken together, our data show that auxin is stimulating GA biosynthesis, whereas a feedback regulation involving some GA biosynthetic genes is observed under high concentration of bioactive GAs. The requirement of auxin for enhancing gibberellin biosynthesis demonstrates some of the complexity of hormonal regulation in plants.

The DELLA family of proteins have been shown to act as repressors of GA-mediated processes (Olszewski, Sun & Gubler, 2002). In *Arabidopsis*, the transcription of *AttRGA* (a DELLA family member) is stimulated by bioactive GAs, and was suggested to be involved in feedback regulation of both GA biosynthesis and GA response (Silverstone, Ciampaglio & Sun, 1998). A DELLA-like gene was earlier found to be co-expressed with *PttGA20ox1* in the expansion zone of developing xylem of poplar (Israelsson *et al*. 2005). We found that both GA and IAA induced this DELLA-like transcript. Moreover, the combined IAA and GA application further enhanced its expression, suggesting a synergistic response of this genes, and a possible role in feedback regulation of GA/IAA induced cell expansion (Paper I, figure 3,4). Another DELLA-like gene, *PttRGA2*, was down-regulated in the presence of high auxin concentration (Paper I, Tables S2 and S4). In *Arabidopsis* roots, polarly transported auxin was suggested to enhance the GA induced degradation of DELLA proteins, thus required for the GA induced root growth (Fu & Harberd, 2003). It can be speculated that the auxin induced down-regulation of *PttRGA2*-like gene in a similar way enhance the GA response in the poplar stem as a part of the synergistic GA/IAA response. Nevertheless, the impact of auxin on DELLA-like genes highlights the complex biology of GA/IAA interactions.

Interestingly, the concentration of IAA in the stem tissues was higher when GA was applied in combination with auxin compared to when the same auxin concentration was applied alone (Paper I, Fig. 3A). By using [$^{13}C_6$]IAA as an apical auxin source, we were able to distinguish between transported IAA from endogenous [$^{12}C_6$]IAA (Paper I, Fig. 3B). It could be concluded that the higher auxin concentration measured after the combined GA/[$^{13}C_6$]IAA feeding was a result of an increase in polarly transported IAA, and not a result of increased IAA biosynthesis in the stem. The enhanced auxin transport is possibly linked to the GA_4 induced expression of the auxin efflux carrier *PttPIN1* (Paper I, Fig. 4). In Pea, a similar induction of *PsPIN* expression has been demonstrated after GA application (Chawla & DeMason, 2004). PIN proteins are critical for polar active auxin transport (Petrasek*, et al.*, 2006), and the observed induction of PIN genes in both poplar and pea by GA is likely to be important for polar auxin transport.

The access to stem tissues with different concentrations of IAA and GA provided us with the possibility to monitor changes in transcript profiles at physiological relevant hormone balances after 24 hours of treatment. The poplar 14k cDNA microarray was used to analyse the gene expression in the same samples that were used for hormone measurements and transcript analysis of specific genes. Partial least square discriminant analysis, PLS-DA (Trygg and Wold, 2002), was used to have an overview of the dataset. All treated trees separated from the intact controls in the first component indicating that decapitation and thereby the removal of an important source of IAA, has a major impact on gene expression (Paper I, Fig. 6A). The hormone treated samples separated in the second component. No separation could be seen between the IAA and IAA/GA treated samples until in the fourth component (Paper I, Fig. 6B), suggesting that GA does not induce major transcriptional changes if a high IAA concentration is present. This notion was further supported *per se* by comparing

the list of genes significantly affected by IAA and GA. From this, we observed that of the 14k ESTs spotted on the microarray, representing almost 10k unique gene models, 85% of the GA affected genes were also IAA affected (Paper I, Fig. 7). Such overlap between GA and IAA induced genes suggests that very few genes are responding exclusively to GA. In the combinatory treatment, resulting in high GA and IAA content, there seems, however, to be a synergistic response between the two hormones on a smaller number of genes (Paper I, S4). However, since we demonstrate that GA stimulates auxin transport, it cannot be excluded that the increased auxin concentration in the combinatory treatment may be the trigger for enhanced expression of some genes.

The synergistic growth response after GA/IAA feeding may be a result of additive effects of the two hormones, since both are independently stimulating cambial cell divisions. It can also be an effect of the increased IAA concentrations due to GA stimulated IAA transport. It should also be noted that in the intact trees bioactive GAs are mainly confined to expanding xylem tissue and the concentration in the vascular cambium is low (Paper 1, Fig 8). Therefore, GA effects on cambial growth observed after apical feeding may be a result of a shift in GA localisation to cambial tissues, rather than increased GA concentrations.

Ethylene and wood formation (paper II and III)

Paper II is focused on measuring endogenous levels of ACC, and expression analysis of *pttACO,* during TW formation. In paper III, we have evaluated the importance of ethylene as a stimulator of the vascular meristem.

Applied ethylene is affecting wood anatomy and xylem growth

We aimed to evaluate the role of ethylene on cambial growth, and for this purpose we established a system where ethylene responses readily could be screened. We required an experimental system that increased throughput capacity, and avoided seasonal influences. Further, since ethylene itself is a gas that complicates feeding procedures, we decided to use the ethylene precursor 1-aminocycloporpane-1-carboxylic acid (ACC) as an ethylene source. Therefore, an *in vitro* plant growth system was established were ACC could be fed to the growth medium, and ethylene effects could be evaluated after 2 weeks of treatment (described in paper III). To confirm that the applied ACC was taken up by the roots and transported in the xylem stream, ^{14}C-ACC was applied to the growth media and measured in the stem tissues. Indeed, already after 2 hours, ^{14}C-ACC was detected in the stem (data not shown). Initially, we performed a dose response experiment to establish dose optima. For this purpose height and xylem growth were assayed (Paper III, Fig. 2). Height growth was inhibited progressively with increasing ACC concentration, whereas xylem growth was stimulated progressively up to 100μM of ACC, and inhibited at the highest concentration tested (1mM). Similar stimulation and inhibition of xylem growth was also seen in dose response experiments with *Ulmus Americana* seedlings where etherel was applied to stem tissues (Yamamoto, Angeles & Kozlowski, 1987). The stimulation of xylem growth in our experiment was caused by increased cell production rather than an

increase in radial cell size. This was deduced by measuring vessel and fibre diameters, which showed that vessel diameters were in fact significantly smaller after ACC treatment as compared to control trees (Paper III, Fig. 5). The effect of ACC on vessel diameter could also be observed on transverse sections, which also revealed a lower vessel frequency (paper III, Fig. 3A). All these alteration in wood development is consistent to previous observations with applications of the ethylene generating substance, ethephon, to various woody species including poplar (Junghans*, et al.*, 2004; Little & Pharis, 1995). As a positive control we also established a protocol where 1-methylcyclopropene, 1-MCP, an inhibitor of ethylene perception (Sisler & Serek, 1997), could be applied to the plants. 1-MCP treatments were observed to nullify the effects of ACC on height- and xylem growth, suggesting that the ACC treatments were a result of ethylene action (Paper III, Fig 3D & 4).

To avoid possible unwanted effects from whole plant ACC treatment, and to confirm that ethylene is affecting the stem in the same way as ACC, we treated isolated parts of the stem with ethylene. For this purpose, a cuvette system was fixed to the stems of greenhouse grown poplar trees and exposed to a continuous gas flow. Treatment with a gas mixture of ethylene (2ppm) and carbon dioxide continuously for 18 days induced radial swelling caused by an enhanced production of xylem and phloem (Paper III, Fig. 6). Similar to ACC treatments, vessel size and frequency in the xylem was reduced (Paper III, Fig 6). Thus, all effects observed after ethylene treatment were consistent with the effects observed after ACC treatments in tissue culture. This allowed us to conclude that the more convenient *in vitro* system was relevant for studies of ethylene effects on stem growth.

In summary, it appears that ethylene can act as an important mediator in the plasticity of wood formation at different steps throughout its development, *i.e.* cambial cell division, vessel element differentiation and vessel and fibre expansion.

Construction and evaluation of ethylene insensitive trees

From the application experiments with ethylene and ACC some of the potential of ethylene to regulate xylem growth and development was defined. But such experiments do not provide evidence for a role of endogenous ethylene in wood formation. To answer this question, we heterologously expressed a dysfunctional ethylene receptor from *Arabidopsis* to produce trees that were insensitive to ethylene. Ethylene receptors actively repress ethylene signalling in the absence of ethylene, and upon ethylene binding, they switch to a inactive signalling state, which then leads to the downstream activation of ethylene responses (Hua *et al.,* 1998). When one of these receptors is mutated so that ethylene will not bind to the receptor and inactivate its repression, plants carrying a mutation in binding domain of the receptor, for example *Atetr1-1,* should not respond to ethylene treatment (Bleecker *et al.,* 1988). As dominant negative controls for ethylene responses in our system, we have been using poplar lines expressing *Atetr1-1*, driven by the constitutive, 35s promoter (Angenent et al., 1994), as well as two different tissue

specific promoters; LMP1 and LMX5, which are not ACC induced (Paper III, Tab. 1). The tissue specific promoters were originally identified from a high resolution transcript profiling experiment across the wood forming tissues (Hertzberg, *et al.,* 2001a). GUS reporter constructs show expression of LMP1 mainly in the cambium and developing phloem and of LMX5 in the cambium and developing xylem (Paper III, Fig. 1). We screened for reduced ethylene sensitivity by monitoring height growth and xylem anatomy after ACC treatment in the in vitro system. From 10-15 generated lines per construct, two representative lines were selected for detailed analysis. The lines expressing *Atetr1-1* showed varying degrees of reduced ethylene sensitivity with different promoters (Paper III, Fig. 3 & 4). After ACC treatment, *35s:etr1-1* lines were almost non-responsive regarding height and xylem growth. The *LMP1:etr1-1* lines were ethylene insensitive with regards to height growth, but were somewhat leaky in their xylem response. The *LMX5:etr1-1* lines showed the reversed response to ACC, a leakiness with regard to height growth, but insensitive to ACC stimulation of xylem growth. Ethylene insensitivity of the *35s:etr1-1* plants were confirmed by exposing two different lines (1E and 3A) to ethylene gas using the cuvette system. Both lines were much less responsive to ethylene in comparison to WT as judged from the effects on xylem growth and anatomy (Paper III, Fig. 6). We also observed that some of the lines expressing *Atetr1-1* showed a reduction in xylem growth during untreated conditions (Paper III, Fig. 4). Such response suggests that ethylene has a role in the regulation of cambial growth also under the near optimal growth conditions in vitro. Similar reduction in stem diameter has previously been shown in ethylene insensitive birches during untreated conditions (Ruonala *et al.,* 2006). It cannot, however be excluded, that the reduction in growth is a response to ethylene accumulation in our in vitro culture system. This would enhance growth in WT plants but not in ethylene insensitive lines expressing *Atetr1-1*. When the ethylene insensitive trees were grown under greenhouse conditions, no clear difference in growth was observed. The only exception was a significantly shorter height growth in some lines (Fig. 7). Trees from these lines also could wilt and die unexpectedly, most likely due to a decrease in ethylene dependent defence systems against pathogens. Most lines also grew more reddish leaves compared to WT (data not shown), which may suggest that the reduced ethylene sensing restricted the adaptation to various stresses.

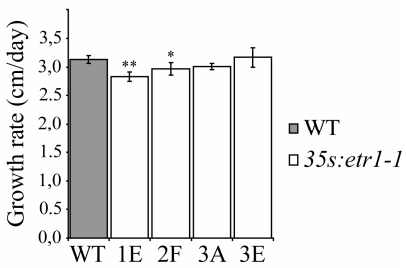

Fig. 7. Growth rate of greenhouse-grown wild type and *35s:etr1-1* poplar lines. Growth rate is calculated over a 14 d period. Mean±s.d. n=5. Asterisks indicate statistically significant differences (t-tests) compared to WT. *P < 0.05, **P < 0.01

Ethylene affects cell wall chemistry of xylem fibres

There are few examples in the literature where the effect of ethylene on wood chemistry has been demonstrated. In *Picea abies* etherel treatments resulted in increased cellulose deposition (Ingemarson, Eklund & Eliasson, 1991). It has also been suggested that ethylene may increase lignification in the secondary xylem of tobacco (Sitbon et al. 1999). To investigate possible ethylene effects on wood fibres in poplar, we used Fourier Transformed Infrared Spectroscopy (FT-IR). FT-IR is a rapid assay of wood chemistry that provides spectra indicating the relative abundance of functional groups characteristic of different wall components. The significant difference in absorbance peaks between spectra from two samples can be identified using OPLS analysis (Trygg & Wold, 2002). We assayed fibre cell walls of plants treated with water and ACC in the *in vitro* system. Using PLS analysis, clear separation in spectra between ACC treated and water controls was observed. (Fig. 8A). OPLS analysis showed that the spectral band explaining most of the difference was an increase in wavelength 1195 cm^{-1}, and a decrease in wavelength 1595 cm^{-1} indicative of respectively: glycosidic links (C-O-C) common in sugar units such as cellulose and hemicelluloses, and aromatic bonds present in lignin (Fig. 8B). The degree of difference in FT-IR spectra between fibre walls formed under ACC and water treatment was much less in ethylene insensitive trees (Fig. 8C). Thus, we can conclude that the observed modifications of ACC on cell wall composition are true ethylene effects. The observation that ethylene and ACC reduces the relative lignin content contradicts a previous suggestion concerning gymnosperm trees (Hudgins *et al.*, 2006; Ralph *et al.*, 2007).

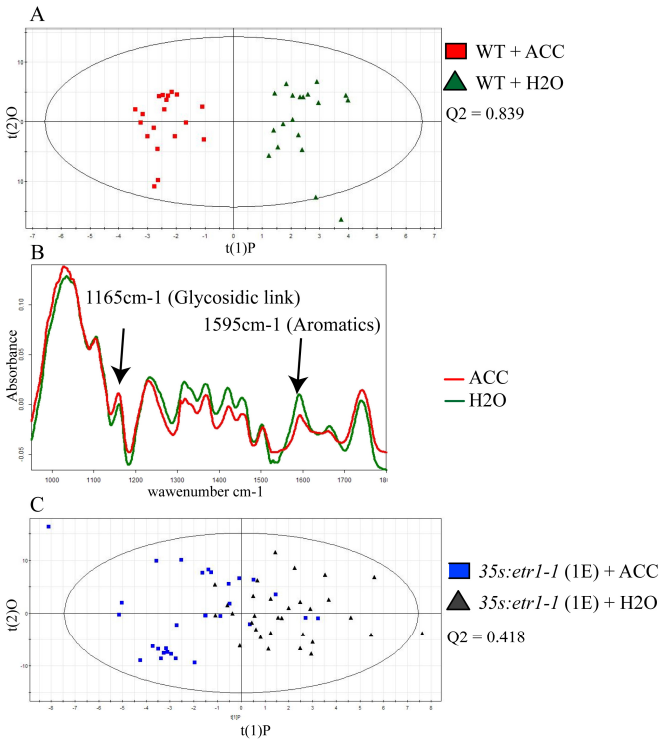

Fig. 8. Effects of ACC treatment on poplar fibre cell walls in WT and *35s:etr1-1* trees. (A) Transverse sections from ACC and water treated WT analysed with FT-IR. OPLS analysis of 20 spectra from each treatment showing a clear separation in component 1 between water and ACC treatment. (B) The average spectra from water and ACC treated WT trees. The main separating factors as judged from OPLS analysis are 1165 cm-1 (glycosidic links) and 1595cm-1 (aromatic vibrations). ACC treatment results in a higher glycosidic link to aromatic bond ratio. (C) OPLS analysis of spectra from ACC and water treated *35s:etr1-1* line 1E. The effect of ACC is much less than in WT plants (A) indicated by the lower Q value. Q: Value of relative separation

Endogenous ethylene stimulates cambial cell division during the TW response

The application experiments previously described, have demonstrated the potential of exogenously ethylene to modify several aspects of wood formation. That raised the question about the function of endogenous ethylene in cambial growth. TW formation has been associated with the evolution of endogenous ethylene, a phenomena shown in several angiosperm trees such as poplar and chestnut (Andersson-Gunneras*, et al.*, 2003; Du & Yamamoto, 2003). Ethylene or ACC applications to poplar stems induce cambial cell divisions with differentiation of smaller and less frequent vessels, similar to the TW response. Such application studies have however not mimicked the induction of G-fibres that are characteristic in TW (Junghans*, et al.*, 2004; Yamamoto, Angeles & Kozlowski, 1987).

The suggested role for ethylene in TW response was investigated by leaning ethylene insensitive trees. As a positive control, we treated leaned trees with 1-MCP. It was found that blocking ethylene sensing did not hinder G-layer formation, which is characteristic in TW formation. However, it was evident that reduced ethylene sensitivity resulted in a significant reduction in the amount of tension wood formed (Paper III, Fig. 7). From this observation, it can be concluded that endogenous ethylene stimulates cambial cell division during TW formation, and thereby is part of the mechanism controlling the asymmetric growth pattern observed after leaning. This is the first conclusive evidence that endogenous ethylene has a function in wood formation in trees and that it stimulates meristematic activity in plants.

Since hormone interactions are common in plants, ethylene itself may not be the only hormone causing the responses previously discussed. For example, polar transport of IAA has previously been shown to be restricted by ethylene in a dose dependant manner (Suttle, 1988). Exactly how this interaction occurs is not fully understood, perhaps there is a crosstalk between ethylene and auxin transport downstream of ethylene perception in the signal transduction pathway. In the literature (Baba*, et al.*, 1995; Cronshaw, 1965; Jiang*, et al.*, 2006; Necesany, 1958; Timell, 1986) there is evidence for both inhibitory and stimulatory effects of IAA upon TW formation, suggesting that auxin is involved in regulating TW formation. Our results are, however, showing a minor decrease of IAA in TW (Paper II. Fig. 4). This was confirmed in detail by (Hellgren, Olofsson & Sundberg, 2004). It has been speculated that ethylene may change auxin

sensitivity (Visser*, et al.*, 1996), so the minor concentration differences of auxin during TW formation may still be important if the auxin sensitivity has changed.

Over-expression of PttACO1 demonstrate a regulatory role of ACC oxidase in ethylene biosynthesis

The general idea that ACS is the limiting and regulating step for ethylene production in plants (An*, et al.*, 2006; Li, Qu & Li, 2005; Tsuchisaka & Theologis, 2004), was challenged by the finding in paper II, (Fig 6C). These data demonstrate a strong increase of ACC in leaning trees. However, whereas ACC accumulated at the opposite side, an enhanced expression of *PttACO1* and low ACC levels was observed at the TW side, suggesting ACO to be limiting for ethylene production. To further explore this hypothesis, we were interested in the complete expression pattern of ethylene biosynthesis genes in stem tissues in response to leaning. QPCR analysis of poplar *PttACS* and *PttACO,* in upright and leaning trees confirmed the northern blot analysis in paper II that *PttACO1* is the main *PttACO* induced during TW formation. The qPCR also demonstrated an induction of *PttACO3* and *PttACO4,* although the abundance of these transcripts is much lower than *PttACO1* in TW (Fig 9B). Interestingly, *PttACO1*, *PttACO3* and *PttACO4* were down-regulated in OW, compared to NW. Whereas ACO genes were mainly expressed in developing xylem tissues (Fig. 9A&C), *PttACS* transcripts where mostly present in cambium/phloem tissues, with *PttACS1* being the most abundant (Fig. 9B). The abundance of ACS transcripts increased in phloem tissue in leaning trees, both on the upper and lower side (Fig. 9B&C). Because ACS activity is mainly determined at post transcriptional level (Chae, Faure & Kieber, 2003; Tatsuki & Mori, 2001a), it is not possible from these data to judge if this increase in *PttACS* expression can explain the large increase in ACC observed in leaning poplar trees (Paper II , Fig. 4).

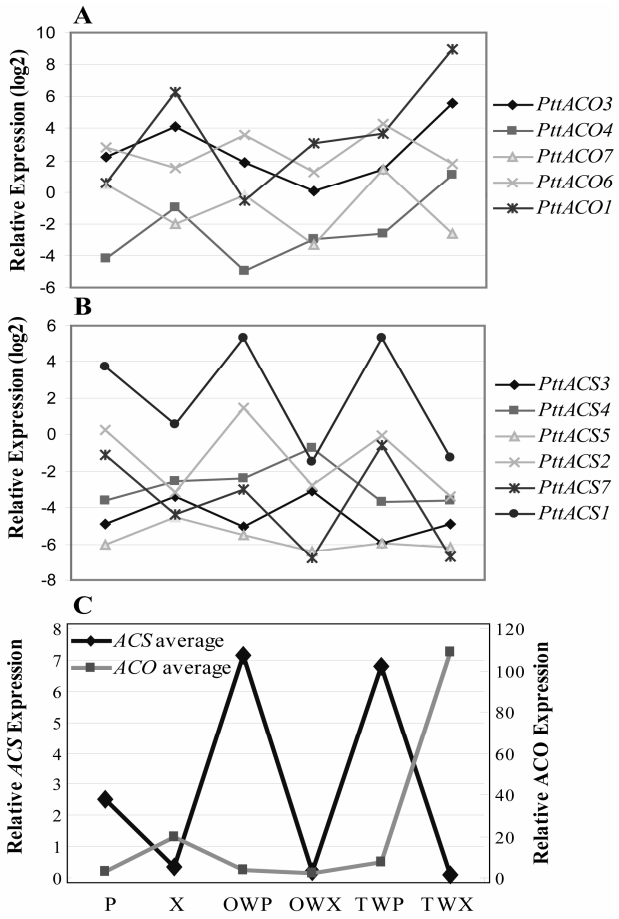

Fig. 9. QPCR analysis of transcript abundance of the most important *PttACOs* and *PttACSs* in upright and leaned greenhouse grown poplars. Tissues are separated in developing xylem and cambium/phloem tissues, and for the leaned stems also between the upper and lower side. (A) Relative expression of 5 *PttACOs*. (B) Relative expression of 6 *PttACSs*. (C) Average relative expression of all *PttACS* and *PttACOs*. Average expression is calculated from three technical replicates of real time PCR from 5 pooled biological replicates. The ribosomal *18s* gene has been used as internal control. *ACS*: *ACC synthase*, *ACO*: *ACC oxidase*

To further investigate the role of ACC oxidase in ethylene biosynthesis, *PttACO1* was both over-expressed and down-regulated in transgenic trees expressing a *35s:ACO1* sense (S) and antisense (AS) construct. The *35s:ACO1* S lines showed both enhanced *ACO1* expression and increased ACO enzyme activity, whereas the AS lines showed a lower *ACO1* expression and lower enzyme activity (Fig. 10 A&B). Interestingly, an increase in *ACO1* expression resulted in reduced height growth and a stimulation of radial growth as measured in the *in vitro* system (Paper III, Fig. 4), indicating that ethylene biosynthesis was increased to a biological significant level in these lines. This was further supported by the observation that the inhibitory and stimulatory effects on height and xylem

growth, respectively, in the *35s:ACO1* lines were reversed by treatment with 1-MCP. When ACO1 sense (S) and antisense (AS) lines were grown in the greenhouse, the *35s:ACO1* S lines showed an average of 16% reduced height growth compared to WT trees, whereas the AS lines where in average 10% higher than WT trees (Fig. 10 C).

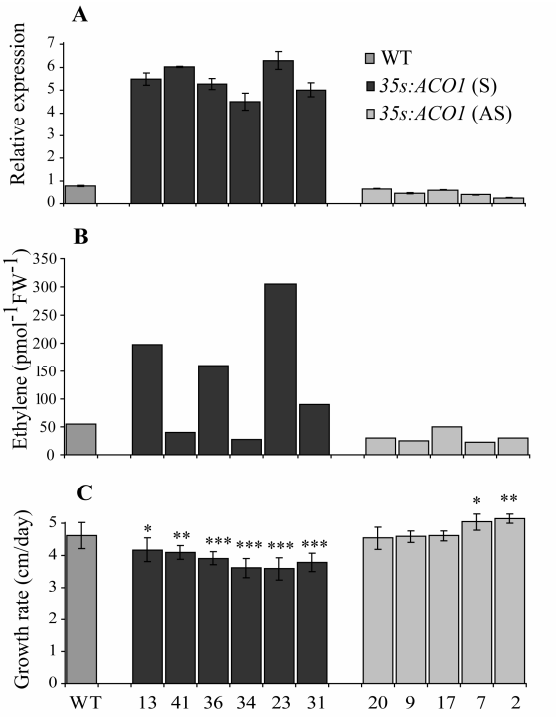

Fig. 10. 35s:ACO1 lines grown in greenhouse and characterised for growth, gene expression and enzyme activity. (A) QPCR analysis of *PttACO1* in xylem tissue. Mean±s.d. of three technical replicates. Each sample was pooled from 5 trees. Ribosomal 18s gene has been used as an internal control. (B) Enzyme activity of ACO. Activity was measured as ethylene evolution after adding ACC to crude protein extracts from xylem tissue from 5 pooled trees. (C) Growth rate calculated during a 14 days period. Mean±s.d. N=5. Asterisks indicate statistically significant differences (t-tests) compared to WT. *P < 0.05, **P < 0.01, ***P < 0.001. S: *35s:ACO1* sense, AS: *35s:ACO1* anti-sense.

Hypothetically, if ethylene evolution in poplar is acting as a stimulatory agent of cambial cell division, an increased ACO expression in OW should increase ethylene evolution in this tissue and thereby increase cambial growth. This hypothesis was tested by leaning two selected *35s:ACO1* S lines (13 and 23). Both *PttACO1* expression and ACO enzyme activity in the *35s:ACO1* S lines were increased in phloem and xylem tissues of upright trees as well as at the opposite side of leaning trees (Fig. 11). The higher expression of *ACO1* in the OW of the transgenic lines resulted in increased xylem growth (Paper III, Fig. 4). This finding further support the results described above that ACO, in addition to ACS (Wang, Li & Ecker, 2002b), is an important enzyme in controlling ethylene biosynthesis in plants. No change in cambial growth was however observed at the

TW side after leaning the ACO overproducing trees. Considering the large induction of ACO activity that is observed in TW of WT trees, additional activity provided by transgenic activity may not generate ethylene of any significant biological difference. Taken together, we conclude that ACO, in addition to ACS, must be considered as a limiting and controlling step in ethylene biosynthesis. To our knowledge, this is the first evidence providing direct evidence supporting active control of ethylene biosynthesis by ACC oxidase.

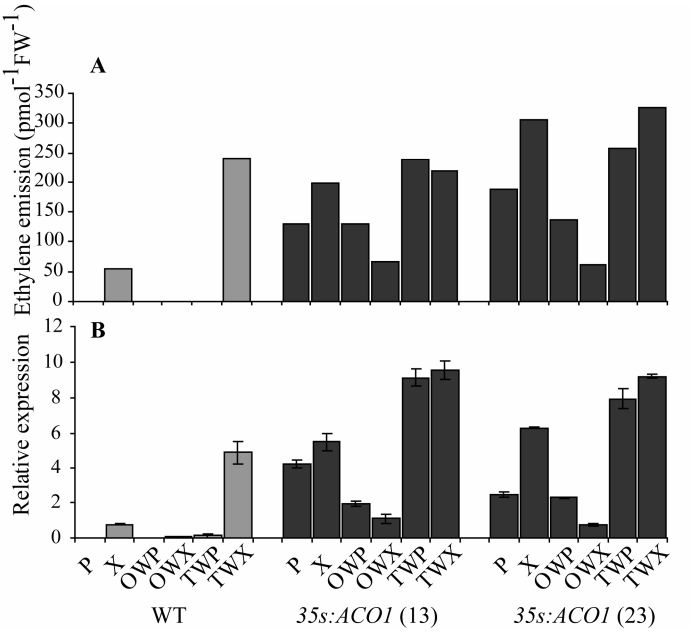

Fig. 11. PttACO1 expression and ACO enzyme activity in upright and leaned greenhouse grown WT and *35s:ACO1* S trees. (A) Enzyme activity of ACO. Activity was measured as ethylene evolution after adding ACC to crude protein extracts from xylem tissue. Each sample represents tissue from 5 pooled trees. (B) QPCR analysis of *PttACO1* expression using ribosomal *18s* gene as internal control. Mean±s.d of 3 technical replicates from 5 pooled trees. P: phloem from upright trees, X: xylem from upright trees, OWP: opposite wood phloem, OWX: opposite wood xylem, TWP: tension wood phloem, TWX: tension wood xylem.

Ethylene response factors and ethylene responsive genes in wood forming tissues (Paper IV)

Ethylene responses are believed to be mediated via the ethylene response factors (ERFs). In the sequenced genome of Populus trichocarpa, we found 173 putitative ERFs, which are divided in the ERF and the DREB subfamilies (Paper IV, Suppl. Fig. 2A&B). In Arabidopsis and rice the number of ERFs are much lower (122 and 139) (Nakano et al., 2006), possibly indicating wood specific ERFs in poplar. For example, the Arabidopsis orthologues At1g80580 and At5g19790 are represented by 13 poplar ERFs (Paper IV, Fig. 5). By qPCR, the expression of all

173 ERFs in response to ACC and ethylene treatment have been monitored. Whole plants were treated with with ACC for 10 hours in the in vitro culture system, and stem specific ethylene treatment for 24 hours and 14 days was done on greenhouse grown trees. Already after 10 hours of ACC treatment, around 50 percent of the ERF or DREBs were responding by changing their expression level more than twofold. The majority of these ERFs and DREBs show an enhanced expression (Paper IV. Fig. 1C&D). Especially interesting is the up-regulation of a few DREBs, since DREBs have not previously been associated with wood formation. The longer duration ethylene treatments resulted in fewer stimulated ERFs and DREBS, but a large increase in down-regulated genes. In particular, many members of the DREB subfamily were seen to be negatively affected by ethylene. Both the stimulated and decreased expression of ERF and DREBs are generally less significant over time, demonstrating the fast response to ethylene of this family of transcription factors. Possibly the observed short boost of the ERFs is enough to activate downstream genes which gives the characteristic observed ethylene phenotypes (described in detail in Paper III).

For an overview of ethylene responsive genes, which are putatively involved in modifications of wood development after ethylene treatment, we analysed global gene expression in woody internodes of in vitro cultured poplar trees treated with ACC for 2 and 10 hours by using the poplar 25k microarray (representing *ca.* 16.000 unique gene-models). Genes responding to ACC, which are downstream of the ethylene receptors, were identified by equivalent treatment of two transgenic lines expressing *Atetr1-1* (line 1E and 3A). ACC affected a large number of genes, of which only a minor part (13 percent) was significantly inhibited in the transgenic plants with reduced ethylene sensing. In total, about four percent of the unique gene-models on the array were shown to be ACC affected mediated via the ethylene receptors, which is an equivalent percentage of ethylene affected genes found in a similar study using ethylene insensetive Arabidopsis (De Paepe et al., 2004). The ACC response of some of the ERFs that were induced in the qPCR analysis were not only confirmed in the array experiment, but also demonstrated to be downstream of the ethylene receptors (Paper IV, Fig. 2). The aim with the microarray experiment was not to make a complete and comprehensive analysis of ethylene responsive genes, but rather to provide a valuable framework for future identification of ERFs and genes downstream of ethylene perception with the potential to modify development and properties of wood.

Conclusions and Future Perspectives

In the first part of this thesis, I show a regulating role of bioactive GAs on polar auxin transport, possible via the induction of an auxin efflux carrier (*PttPIN1*). Further, global gene transcript analysis of GA and IAA regulated genes showed that most of the GA induced genes are also induced by auxin, with only few genes identified to be GA specific. This suggests the potential of extensive crosstalk between the two hormones *in planta*.

In the second part of the thesis, a prominent role for the plant hormone ethylene in wood formation is demonstrated. Trees with reduced ethylene sensing were constructed and used as a tool to demonstrate the potential of ethylene in wood development. In greenhouse and *in vitro* culture experiments, ethylene and ACC were demonstrated to affect cell division, cell differentiation, and cell wall chemistry in poplar stems. Ethylene's regulatory role in a gravity response was also demonstrated using trees with reduced ethylene sensing, which revealed that ethylene is partly responsible for the stimulation of radial growth creating the characteristic eccentric growth pattern during reaction wood formation in poplar. Increased expression of Ptt*ACO1* in transgenic trees demonstrated the regulatory role of ACO in ethylene biosynthesis and further that the normally reduced growth in opposite wood during leaning is partly restored under higher *PttACO1* expression. In the cell wall chemistry part of this project, possible effects of endogenous ethylene in fibre cell wall chemistry will be tested by analysing TW fibres of leaning trees expressing *Atetr1-1* compared to those in WT plants.

Out of the large ERF/DREB family of transcription factors that were identified in the poplar genome, fast inductions by ethylene of many genes in especially the ERF sub-family in wood forming tissues were seen. Future studies will involve high-resolution transcript mapping of ERFs in the wood forming tissues and generation of transgenic lines over and under-expressing some interesting ERFs. By changing the expression of wood specific ERFs, we are interested to see if some wood responses can be modified via specific ethylene response factors.

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