Ethylene and Auxin in the Control of Wood Formation

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Abstract

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This thesis considers aspects of the regulation of growth rate and fibre properties in forest trees. These properties are both genetically determined and influenced by environmental stimuli. Induction of reaction wood is an environmentally induced process involving changes in growth rate and fibre properties that can be readily studied. Plant hormones are signalling agents that play important roles in the initiation and coordination of wood formation; in this thesis the plant hormones auxin and ethylene were investigated using gas chromatography/mass spectrometry (GC/MS).

A novel MS technique for measuring the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) in minute amounts of plant tissue was developed. Ethylene is often connected to stress responses in plants, and ethylene evolution is increased when reaction wood is formed. Here it is demonstrated that this increase is regulated by ACC oxidase, the enzyme catalysing the last step in the ethylene biosynthetic pathway. This is in contrast to most of the earlier findings that tended to indicate that ethylene production directly reflects the availability of ACC. Although ethylene is strongly up-regulated during reaction wood formation, its role in modulating the growth rate and fibre properties remains unknown.

Further, it is demonstrated that reaction wood in both poplar (*Populus tremula* L.) and pine (*Pinus sylvestris* L.) is formed without changes in auxin concentration in the cambial tissues. This suggests that the previously held assumption that the difference in auxin concentration is key factor in the induction of reaction wood is unsound. Further, auxin concentrations were compared in hybrid aspen trees (*Populus tremula* L. x *tremuloides* Michx.) growing vertically at different growth rates. These trees showed good correlations between auxin levels and growth rates. The growth rate was mediated by increases in the cell cycling rate rather than in the width of the cell division zone. Thus, the growth rate in poplar was correlated to auxin levels in normal wood formation, but not during reaction wood formation.

Key words: auxin, ethylene, 1-aminocyclopropane-1-carboxylic acid, wood formation, tension wood, compression wood, mass spectrometry

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Contents

Appendix

List of papers

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

I. Hellgren, J.M., Moritz, T. & Sundberg, B. A simplified GC/MS method for determining the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) in milligram amounts of plant tissue. *Submitted*.

II. Gunneråas-Andersson, S.,* Hellgren, J.M.,* Björklund, S., Moritz, T., Regan, S. & Sundberg, B. Asymmetric expression of a poplar ACC oxidase controls ethylene production during gravitational induction of tension wood. *Plant Journal*, Accepted.

III. Hellgren, J.M., Olofsson, K. & Sundberg B. Patterns of auxin distribution during gravitational induction of reaction wood in poplar and pine. *Submitted*.

IV. Hellgren, J.M., Puech, L., Barlow, P., Fink, S., Mellerowicz, E.J. & Sundberg, B. Auxin and cambial growth rate in poplar. *Manuscript*.

*To be considered joint first authors

Introduction

Many raw materials, such as steel and plastics, are homogenous and therefore result in products with uniform and predictable properties. Wood, on the other hand, is a variable material composed of a number of cell types that are optimised in relation to the tree's growth, survival and reproductive strategies. Most of the wood and fibre properties are determined when the wood is formed. The process of wood formation follows a genetically determined pattern, which can be influenced and modified by environmental factors such as water and nutrient availability, wind, load and photoperiod (Zobel & van Buijtenen, 1989). There are large differences in wood structure not only between hardwoods and softwoods and between different tree species, but individual trees also exhibit huge variations in their wood properties. This variation within trees includes juvenile and mature wood, early- and latewood, reaction wood, sapwood and heartwood (Haygreen & Bowyer, 1996; Mellerowicz *et al.*, 2001; Plomion *et al.*, 2001).

The use of wood

By providing the raw material for sawn timber, boards, pulp and paper, forestry plays a major role in the Swedish economy. Today, 65% of the Swedish land area is covered by forest, and forest products account for about 50% of the total value of Swedish industrial exports (Skogsstyrelsen, 1997). The difference in wood and fibre properties between softwoods and hardwoods is important for the end use of the material. Softwood has a simple structure. It is straight-grained and light in weight, so it is preferred for construction lumber and plywood, and its long fibres yield paper with high strength. Hardwoods, which have a more variable structure, are preferred for furniture and decoration, and their slender fibres are suitable for fine paper for use in offices and printing (Haygreen & Bowyer, 1996).

To take full advantage of wood as a raw material we need to understand the physiological and molecular principles of wood formation, and apply this basic knowledge in silviculture and tree breeding.

Wood formation

The following brief summary of wood development is based on information from Barnett (1981), Mellerowicz *et al.* (2001) and Plomion *et al.* (2001). Wood originates from the dividing cells in the vascular cambium (the cambial zone), which forms a cylindrical sheet in the tree stem between the phloem (bark), and the xylem (wood) (Figure 1A). In the vascular cambium there are two different types of initials: the fusiform initials giving rise to the axial wood elements, such as vessels, fibres and parenchyma cells in hardwoods and tracheids in softwoods, and the ray initials giving rise to horizontally oriented ray cells. Cambial derivatives develop via a sequence of division, expansion and secondary wall formation. Some of the cell types ultimately undergo programmed cell death. The sequence of wood formation is well co-ordinated between the radial files, and the different developmental phases give rise to easily recognized zones (Figure 1B).

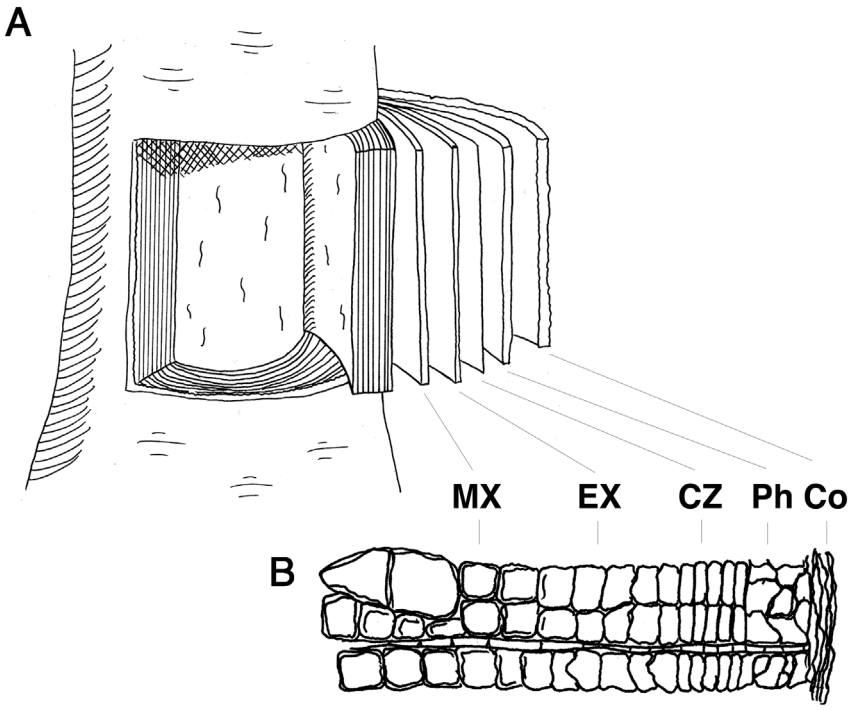

Figure 1. A, Location of different developmental zones in a tree trunk and B, cells in different phases of development and their location within the cambial region tissues. Co=cortex, Ph=phloem, CZ=cambial zone, EX=expanding xylem, MX=maturing and mature xylem

Fibres and axial parenchyma cells in hardwoods expand in the radial direction and undergo elongation by intrusive tip growth. Tracheids in softwoods also expand in the radial direction, but only elongate 5-10% compared to the initial. Vessels expand both radially and tangentially by expansion of the radial wall, and ray cells elongate radially. Once expansion is complete, the xylem elements start to form the secondary cell wall that gives the plant cell physical stability. The secondary wall is a multi-layered structure built up by a network of cellulose microfibrils embedded in a hydrated amorphus network of hemicelluloses and a substantial amount of hydrophobic lignin. Approximately 40 to 50 % of the cell wall consists of cellulose, 25% consists of hemicellulose and the remaining 25 to 35% of lignin. The secondary cell wall also contains small amounts of cell wall proteins and pectins. Lignification is initiated in the middle lamella and continues throughout the wall layers towards the cell lumen. Following lignification the vessels and fibres in hardwoods and the tracheids in softwoods undergo autolysis by hydrolysis of the protoplast to produce the mature functional xylem elements.

Reaction wood

Reaction wood is modified in its anatomical and chemical properties and is formed in stems and branches of many tree species (Scurfield, 1973; Timell, 1986; 1969; Westing, 1965; Wilson & Archer, 1977). In addition to the changes in wood properties, reaction wood formation is often paralleled by asymmetric radial growth. Reaction wood can be induced by various factors, e.g. strong winds or loads, that cause the stems to bend or lean. The modification in wood structure induces physical strains in the wood that force the stem/branch back towards its original position in space. Reaction wood can also form during the course of normal tree development, for example it may form on one side of branches that have been horizontally displaced and thus restore their orientation. In addition, it can form in branches of the uppermost whorl after removal of the apical shoot, thereby causing a side shoot to bend upwards and replace the lost leader. In these cases, the process is induced by internal control mechanisms rather than by environmental induction (Fisher & Stevenson, 1981; Timell, 1986; Westing, 1965, Wilson & Archer, 1983).

It is of great interest to explore the physiological and molecular control of reaction wood formation because a natural developmental sequence is involved that results in wood with altered properties and increased growth rates. Environmental stimuli that induce reaction wood are similar in hardwoods and softwoods, but interestingly the chemical and anatomical characteristics of the wood are very different between these wood types (Haygreen & Bowyer, 1996; Scurfield, 1973; Wilson & Archer, 1977).

Tension wood

Reaction wood in hardwood trees is called tension wood and is formed on the upper side of the leaning stem. The most obvious characteristic of tension wood is an inner cell wall layer of the fibres that consists of almost pure, and highly crystalline cellulose, in which the microfibrils are arranged nearly parallel to the fibre axis (Jourez *et al.*, 2001; Norberg & Meier, 1966; Timell, 1969). Under the microscope, this wall layer has a gelatinous appearance. For this reason, it is named the gelatinous layer (G-layer), and the fibres are called gelatinous fibres (G-fibres). The G-layer is loosely attached to the other cell wall layers and the surface of a cut stem with tension wood can therefore have a woolly appearance (Haygreen & Bowyer, 1996). Tension wood also has fewer and smaller vessels, and fewer rays compared to normal wood. The growth rate is normally increased on the tension wood side of the stem. On the opposite side, growth is decreased or inhibited. These changes cause stems forming tension wood to have an eccentric shape (Jourez *et al.*, 2001; Scurfield, 1973; Timell, 1986).

Compression wood

In softwoods, the reaction wood is called compression wood and is formed on the lower side of the leaning stem. The characteristics and formation of compression wood is reviewed in detail by Westing (1965; 1968) and Timell (1986). The most characteristic feature of compression wood is the rounded shape of the tracheids, resulting in intercellular spaces. Compression wood tracheids are shorter than

normal tracheids and have an increased microfibril angle. The cell walls are thick and have a high proportion of lignin. Like tension wood, growth is often stimulated on the side forming compression wood, causing the stem to be eccentric.

Plant hormones in wood formation

Auxins, cytokinins, gibberellins, abscisic acid and ethylene are the five "classical" groups of plant hormones (Kende & Zeewart, 1997). The term "hormone" is defined as an organic compound produced by one tissue in an organism and transported to another tissue, where it induces a specific physiological response (Lawrence, 1995). The term was originally defined for hormones in animal systems, but was later also applied to plants. Plant hormones can be synthesised in most organs and may have a function in another part of the plant, or within the cell in which they are produced. Plant hormones play important roles during wood formation, (for reviews, see Little & Savidge, 1987; Little & Pharis, 1995). Ideas about their function have evolved from numerous experiments in which applications of the hormones have shown they can affect cell division in the vascular cambium, cell expansion and control of differentiation into different types of cambial derivatives (Mellerowicz *et al.*, 2001). However, recent developments in analytical techniques have further increased our knowledge of the endogenous balance of plant hormones in cambial tissues, and their role in wood formation is becoming clearer (see, for instance, Moritz & Sundberg, 1996; Eklund & Tiltu, 1999; Eriksson *et al.*, 2000; Sundberg *et al.*, 2000; Klintborg *et al.*, 2002).

Ethylene and auxin are the main hormones considered in this thesis. They both stimulate cambial cell division and diameter growth, they have profound effects on wood development, and have suggested involvement in the control of reaction wood (Timell, 1986; Little & Savidge, 1987; Savidge, 1988; Little & Pharis, 1995; Kalev & Aloni, 1999; Little and Eklund, 1999).

Ethylene biosynthesis

The ethylene biosynthetic pathway is well understood in higher plants (reviewed by Yang & Hoffman, 1984; Kende, 1993; Mc Keon *et al.*, 1995; Srivastava, 2002). Ethylene is produced from L-methionine via the intermediates S-adenosylmethionine (AdoMet) and 1-aminocyclopropane-1-carboxylic acid (ACC). The enzymes ACC synthase and ACC oxidase are responsible for catalysis of the last two steps, and for regulating the rate of ethylene biosynthesis. ACC can also be conjugated to form malonyl-ACC (MACC), by ACC N-malonyltransferase (Hoffman *et al.*, 1982; Abeles *et al.*, 1992; Martin & Saftner, 1995). Although some studies have indicated that MACC can be converted to ACC (Jiao *et al.*, 1986), the conjugation of ACC to MACC is generally considered to be irreversible. Another ACC-conjugate, GACC, has been identified in tomato fruit (Martin *et al.*, 1995), but only appears to be present in minor amounts compared to MACC (Peiser & Yang, 1998). Ethylene, ACC and hydrolysable ACC conjugates are all present in the cambial region tissues (Savidge *et al.*, 1983; Eklund, 1991*a*; Klintborg *et al.*, 2002).

Ethylene and wood formation

It is not known whether ethylene is an essential factor for any of the cellular processes of wood formation. However, it is well established that applications of ethylene (in the form of the ethylene-releasing compound ethrel, 2-chloroethylphosphonic acid, for example) increases cambial cell division and, hence, tracheid production in softwood stems (Brown & Leopold, 1973; Barker, 1979; Eklund & Litte, 1996; 1998; Eklund & Tiltu, 1999; Kalev & Aloni, 1999). In hardwood species the results of ethrel application tend to be less conclusive. In some species, such applications result in stimulation, and in other species inhibition, of cambial cell division and diameter growth (Little & Savidge, 1987).

The hypothesis that ethylene may have a role in diameter growth has been supported by seasonal studies of ethylene in softwood stems. For example, higher rates of ethylene evolution have been detected from active trees compared to dormant trees (Eklund, 1990; Ingemarsson *et al.*, 1991*a*; Eklund & Tiltu, 1999; Klintborg *et al.*, 2002). Further, the highest rate of ethylene evolution was correlated to the highest rate of tracheid production (Klintborg *et al.*, 2002). Ethylene has also been suggested to affect cell wall composition by altering deposition of polysaccarides in softwood cell walls (Eklund, 1991*b*; Ingemarsson *et al.*, 1991*b*) and lignification by inducing the activity of enzymes involved in lignin synthesis and polymerisation (Roberts & Miller, 1983; Miller *et al.*, 1985; Abeles *et al.*, 1992).

Auxin homeostasis in the vascular cambium

Several substances with auxin activity have been identified in plants. Indole-3-acetic acid (IAA) is both the most abundant endogenous auxin, and the most widely studied (Davies, 1995). Recently, it was shown that all parts of young *Arabidopsis* plants have the ability to produce IAA (Ljung *et al.*, 2002), but it is believed that the major sites of IAA biosynthesis in older plants are apical shoots and young leaves (Davies, 1995). IAA is transported through plant tissues in a polar fashion, but can also be transported via the phloem (Baker, 2000; Swarup *et al.*, 2001). In the case of tree stems, the main pathway for polar IAA transport is the cambial zone and its most recent derivatives (Sundberg *et al.*, 2000). The polar transport is slow, about 5-20 mm h^{-1} (Srivastava, 2002), and mediated by specific influx and efflux carriers located in the plasma membrane of the cells (Muday & De Long, 2001). Auxin is important in controlling the regulation of many different cellular and developmental processes in plants. This control is mediated not only through changes in the cellular auxin concentration, but also through changes in tissue sensitivity towards auxin and changes in the polar auxin transport (Davies, 1995; Muday & De Long, 2001, Moyle *et al.*, 2002).

IAA is distributed in a steep radial gradient across the cambial region tissues, with the highest concentration in the cambial zone and decreasing concentrations towards the maturing xylem and the phloem (Sundberg *et al.*, 2000). Since the rate of polar transport is slow and the distance from the apical shoot to the basal part of the stem can be considerable in a large tree, additional IAA sources are likely to contribute to maintaining the IAA pool in the cambium. Both *de novo* synthesis of

IAA in the cambium and addition of IAA from mature leaves have been proposed as additional sources of the IAA present in the cambial region (Uggla *et al.*, 1998). Another putative source of IAA is hydrolysis of auxin stored as amide- and ester-conjugates. Most plant organs contain significant pools of hydrolysable IAA conjugates, but in wood-forming tissues of softwoods and hardwoods only trace amounts have been found (Sundberg *et al.*, 1990; Tuominen *et al.*, 1995). The concentration gradient of IAA across the cambial region tissues may be maintained by lateral diffusion followed by removal of IAA when reaching the xylem and phloem transport systems, where endogenous IAA has been found (Crozier *et al.*, 1980; Hoad, 1995).

Auxin and wood formation

It is well established that IAA is an important regulator of cambial growth (for reviews, see Little & Savidge 1987; Little & Pharis 1995; Sundberg *et al.*, 2000). In decapitated stems, apically applied IAA enters the polar transport system and mimics the IAA supply from the apical shoot by inducing cambial cell division and xylem production in a dose-dependent manner (Sundberg & Little, 1990; Leitch & Savidge, 1995). Exogenous IAA also affects the radial diameter of xylem elements (Kutschera, 1994) and induces tracheary differentiation in callus and cell suspension cultures (Roberts, 1988).

The xylem growth rate in a tree is determined by the number of dividing cells and the rate of cell cycling in the cambium (Wilson, 1964; Gregory & Wilson, 1968; Gregory, 1971). Similarly, the final radial size and cell wall thickness of xylem cell elements depend on the rate and duration of the processes of expansion and wall thickening. The duration of the processes of division, expansion and wall formation is partly dependent on the width of the respective developmental zones. The radial IAA gradient across the cambial region tissues is suggested to provide the positional information necessary to establish the width of these zones (Sundberg *et al.*, 2000).

The role of ethylene and auxin in the tension wood response

Application of ethrel to *Acer plataniodes* seedlings did not result in formation of tension wood in a study by Yamamoto & Kozlowski (1987*a*), but increases in ethylene evolution following bending or tilting hardwood stems have been observed in *Malus domestica* (Robitaille & Leopold, 1974; Robitaille, 1975), *Betula* (Rinne, 1990), *Acer plataniodes* (Yamamoto & Kozlowski, 1987*a*), *Pyrus malus* and *Prunus persica* (Leopold *et al*., 1972) and *Eucalyptus gomphocephala* (Nelson and Hillis, 1978). Nelson & Hillis (1978) found ethylene production increased during tension wood formation and proposed that the hormone is involved in the induction of the process.

Many different experiments have indicated that IAA also has a role in tension wood formation (Little & Savidge, 1987). Application of IAA to one side of upright stems induces tension wood on the opposite side of the application site in *Populus tremula* (Blum, 1971) and *Aesculus hippocastanum* (Casperson, 1965). Ringing stems with the IAA transport inhibitor TIBA (triiodobenzoic acid) causes tension wood to form below the point of application in *Acer rubrum* (Cronshaw &

Morey, 1965) and *Ulmus americana* (Kennedy & Farrar, 1965). Further, tension wood formation has been suppressed by IAA application to the upper side of inclined stems of *Populus monilifera* (Necesany, 1958), *Aesculus hippocastanum* (Casperson, 1965) and *Acer rubrum* (Cronshaw & Morey, 1968). These observations collectively suggest that tension wood is induced either by IAA deficiency or at a position around the stem where the IAA concentration is lowest (Timell, 1986).

The role of ethylene and auxin in the compression wood response

Several lines of evidence support the hypothesis that ethylene production is increased in association with compression wood formation. Increases in ethylene evolution and tracheid production have been found on the lower (compression wood forming) side of tilted stems in *Abies balsamea* (Little & Eklund, 1999). The ethylene precursor ACC has been detected on the lower, but not on the upper, side of tilted *Pinus contorta* stems (Savidge *et al.*, 1983), and an ACC oxidase has been identified among up-regulated proteins in compression wood forming tissue of *Pinus pinaster* (Plomion *et al.*, 2000). However, as for hardwoods, application of ethrel to different softwood species did not appear to induce formation of compression wood tracheids in several studies (Eklund & Little, 1996; Yamamoto & Kozlowski, 1987*b*; Little & Eklund, 1999), although Barker (1979) found tracheids with modified properties resembling compression wood following applications of the compound ethrel to *Pinus radiata*.

High concentrations of IAA have consistently been demonstrated to induce the formation of compression wood in upright softwood stems at the site of application (Little & Savidge, 1987). Moreover, application of the IAA transport inhibitor NPA (*N*-1-naphthylphthalamic acid) to upright stems induces compression wood formation above the site of application (Yamaguchi *et al.*, 1980, Sundberg *et al.*, 1994). These application experiments indicated that compression wood is induced by high levels of IAA. However, measurements of endogenous IAA concentrations showed that IAA-levels were not increased above the site of application of IAA transport inhibitors in a study by Sundberg *et al.* (1994).

Reliable measurements of endogenous hormone levels during tension wood and compression wood formation are scarce and, so far, the results have been inconsistent and prone to high levels of uncertainty due to limited sampling (Savidge *et al.*, 1983; Wilson *et al.*, 1989; Funada *et al.*, 1990; Moyle *et al.*, 2002). Consequently, most current ideas are based on the results of application studies.

Studying hormones in plants

To better understand the different processes of growth and development in plants, it is of great importance to learn more about the endogenous balance of plant hormones. Early application experiments provided us with ideas about the relationship between hormones and various physiological processes, but for correct interpretation of such experiments we need to relate the results to endogenous hormone levels. Since hormone levels are strictly regulated in time

and space (Davies, 1995), and may differ greatly in their concentration across a short distance, it is also of interest to develop sensitive analytical techniques in order to measure hormones in small amounts of plant tissues.

Quantification of plant hormones

Plant extracts are complex, multi-component mixtures, and analysing specific substances present in low concentration in the matrix can be a difficult task (Crozier, 1987). Therefore, analytical techniques with high selectivity and sensitivity are required for accurate quantification of trace amounts of plant hormones (Horgan *et al.*, 1995).

A mass spectrometer is a highly selective tool for quantitative and qualitative analysis, and is often used together with gas or liquid chromatography. There are several different types of mass analysers, for example magnetic sector, quadrupole and ion trap instruments. For analysis of plant hormones, GC/MS has often been used. When the efflux from the GC is introduced into the mass spectrometer, the molecules first enter the ionisation chamber, where they may be ionised by a stream of high-energy electrons (in electron impact, or EI, mode). Due to the high energies involved, the ions tend to break further into a variety of fragments. The fragments are separated in the mass analyser according to their mass-to-charge (*m/z*) ratios and measured, thereby generating a mass spectrum showing the relative abundance of the different fragments, which is characteristic of the specific compound and provides valuable structural information. When only a few selected *m/z* ratios are recorded, instead of a whole spectrum, high sensitivity and selectivity can be obtained. This technique is called selected ion monitoring (SIM) and is suitable for quantification of analytes present at low levels, for example hormones in plant extracts. The selectivity can also be increased by using tandem mass spectrometry (MS/MS), including two or more MS steps in series.

A technique related to SIM that is used in double focusing magnetic sector instruments is selected reaction monitoring (SRM) (Gaskell & Millington, 1978). Here, only the metastable decomposition of specifically selected ions are recorded, which further increases the selectivity for the given analyte. Using GC/MS-SRM techniques, picogram amounts of IAA (Edlund *et al.*, 1995) and gibberellins (Moritz & Olsen, 1995) have been determined. The recent development of interfaces between LC and mass spectrometers have opened new possibilities for analysis of non-volatile compounds that were previously difficult to study. Cytokinins, for example, have been successfully analysed using LC/MS (Prinsen *et al.*, 1995; Åstot *et al.*, 1998)

Analysis of ethylene and ACC

Ethylene differs from the other “classical” plant hormones by being a gas. Analysis of ethylene is rapid and simple to perform with gas chromatography coupled to a suitable detector, such as a flame ionisation, thermal conductivity, photo-ionisation or photoacoustic laser detector (Abeles *et al.*, 1992; Hedden, 1993). No sample purification is needed, as there are few interfering substances in the samples. However, tissue-specific analysis of endogenous ethylene is complicated. The well-known function of ethylene as a stress-induced hormone

limits the scope for leaving dissected plant parts in containers to produce ethylene (Abeles *et al.*, 1992). Thus, since ACC availability has been considered as rate limiting for ethylene biosynthesis, ACC is sometimes extracted from plant tissues and measured as an indicator of ethylene production (for a review, see Salvetit & Yang, 1987). This assumption has been challenged, since several studies have concluded that the conversion of ACC to ethylene is the rate-limiting step in the formation of ethylene, rather than ethylene production (Dunlap & Robacher 1994, Vriezen *et al.*, 1999). Nevertheless, it is of great interest to quantify endogenous levels of ACC in plants. A method to extract ACC from plant tissues, convert it to ethylene by NaOCl in the presence of Hg^{2+}, and then quantify the ethylene thus produced by gas chromatography (Lizada & Yang, 1979) is widely used. However, the reliability of this indirect method has been criticised since it has been found to give both over and under estimates and is prone to losses of sensitivity caused by various interfering substances (Bufler & Mor, 1980; Coleman, 1991; Chauvaux *et al.*, 1993).

Recently, several different methods for direct analysis of ACC in plant extracts have been published, including techniques based on ACC derivatisation, isotope dilution and GC/MS or LC/MS analysis (Mc Gaw *et al.*, 1985, Hall *et al.*, 1989; Chauvaux *et al.*, 1993; 1997; Petritis *et al.*, 2000). However, these methods for ACC analysis are still not sensitive enough for tissue-specific analysis of small amounts of plant tissue.

Objectives and summary of the papers

The main goal of the work described in this thesis was to extend our understanding of the role of the plant hormones ethylene and indole-3-acetic acid (IAA) in wood formation, with special emphasis on reaction wood formation. The main objectives were to:

- Develop rapid MS techniques for routine analysis of minute amounts of the ethylene precursor ACC in plant tissues
- Apply microanalytical MS techniques to study the distribution pattern of ACC across cambial region tissues
- Investigate endogenous IAA patterns in poplars with different growth rates
- Investigate the role of ethylene and IAA in reaction wood formation in poplar and pine

The research resulted in the following papers:

Paper I presents a new method for the analysis of ACC in plants using isotope dilution and GC Selected Reaction Monitoring MS, and demonstrates its application in the analysis of different tissues from *Arabidopsis* and poplar.

Paper II is a study of the dynamics of ACC and ACC oxidase in poplar trees. A novel ACC oxidase and its expression pattern are described, and the distribution of ACC across the cambial region tissues is visualised. ACC and ACC oxidase were studied during tension wood formation. It was found that large amounts of ACC and its conjugates are present on the opposite wood side, whereas the expression and activity of ACC oxidase is upregulated on the tension wood side. These observations led to the conclusion that ACC oxidase activity, rather than ACC availability, limits ethylene production during tension wood formation in poplar trees.

Paper III provides a detailed study of IAA-dynamics during tension wood formation in poplar trees and compression wood formation in pine trees. It was demonstrated that both tension wood and compression wood were formed without any significant changes in IAA concentration or distribution patterns across the cambial tissues. The old model proposing that compression wood is induced by high IAA levels and tension wood by IAA deficiency was thus refuted.

Paper IV describes the radial distribution of IAA across the cambial region in wild type and transgenic hybrid aspen trees expressing bacterial IAA biosynthetic genes. The trees were cultured at different growth rates, and in all tree types and treatments the amount of IAA in the cambial tissues correlated to the xylem growth rate. The different growth rates in wildtype poplars could be explained by the rate of cell cycling, and related to an increased concentration of IAA in the cambial meristem. In the transgenic trees, the difference in growth rate was explained from a different number of cambial zone cells.

Experimental

Plant material

Field-grown *Populus tremula* (L.) trees were selected from a natural stand near Umeå, Sweden (63°50' N, 20°20' E) (**II** and **III**) and field-grown Scots pine (*Pinus sylvestris* L.) from an experimental forest site at Kulbäcksliden, Sweden (64°14' N, 19°46' E) (**III**). Reaction wood was induced by bending the stem to an angle of approximately 45° and fixing it in position with string. This was done during the most active period of cambial growth and trees were left to form reaction wood for different times. For the experiments in paper **IV**, greenhouse-grown hybrid aspen (*Populus tremula* L. × *tremuloides* Michx.) of wild type line T89 and the transgenic line G1'2'D were used. The transgenic line expresses the bacterial IAA biosynthetic genes *iaaM* and *iaaH* from the mannopine synthase (mas) 2' and 1' promoters, (Tuominen *et al.*, 1995), respectively. Plants from sterile tissue culture were potted in mineral wool and cultivated under controlled conditions in a greenhouse. The temperature was 22/17°C (day/night) and the photoperiod 18 h. The plants were treated with a nutrient solution with an N-content corresponding to 100 mg N l^{-1} or 12.5 mg N l^{-1}

Sample preparation

Cambial-region tissues were collected by peeling the bark and scraping the exposed surfaces with a scalpel. The tissues were homogenised in liquid nitrogen using a mortar and pestle. For analysis of hormones across the cambial-region tissues, frozen samples were trimmed into blocks about 2 mm (tangential) × 10 mm (radial) × 15 mm (vertical), consisting of phloem, cambium and xylem. Samples for analysis consisted of 30 µm consecutive tangential sections obtained from the blocks using a HM 505E cryomicrotome (Microm Laborgeräte, Walldorf, Germany) at -20°C according to Uggla & Sundberg (2001). The radial position of the tangential sections was determined in cross sections sampled after every third tangential section.

Xylem sap was collected by placing stem pieces vertically and connecting them to rubber tubes filled with coloured water. This created pressure that forced the water through the xylem, thereby displacing the xylem sap and allowing it to be collected. Sampling was terminated when the coloured water had passed through the stem sample.

Anatomical investigations

Stem pieces were fixed in cold 4% glutaraldehyde in 25 mM phosphate buffer, pH 7.2. The samples were then dehydrated in an ascending series of acetone concentrations and embedded in a methacrylate resin. Sections were cut with a HM 505E microtome (Micron Laborgeräte, Walldorf, Germany), stained with toluidine blue O and studied under a light microscope (Zeiss, Axioplan, Oberkochen, Germany).

Plant hormone analysis

Analysis of ACC with GC/MS

A flow diagram of the protocol is shown in Figure 2. After homogenisation of the plant tissue and addition of [$^2H_4$]ACC (1-amino-[2,2,3,3-$^2H_4$]cyclopropane-1-carboxylic acid; Sigma, St. Louis, MO, USA) as an internal standard, samples were extracted in 80% methanol. The extracts were purified by SCX-cation exchange chromatography and ACC was converted to the *N*-benzoyl *n*-propyl derivative by propylation and benzoylation (Figure 3). Samples intended for analysis of ACC conjugates were hydrolysed in 4M HCl for 1 h at 100°C prior to purification. After derivatisation the samples were further purified on C_{18}-solid phase cartridges. The samples were analysed by GC/MS-SRM using a JEOL MStation mass spectrometer (JEOL, Tokyo, Japan) by recording the reactions *m/z* 247.1204 to *m/z* 187.0663 and *m/z* 251.1456 to *m/z* 191.0884. All data were processed by the JEOL MS-MP 9021D data system. This method was used for all ACC measurements in paper **II**.

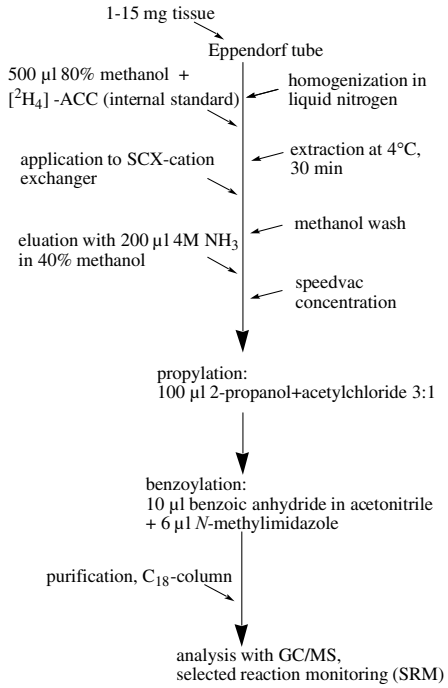

Figure 2. Protocol for the extraction and purification of ACC from milligram amounts of plant tissue and analysis with GC/MS-SRM

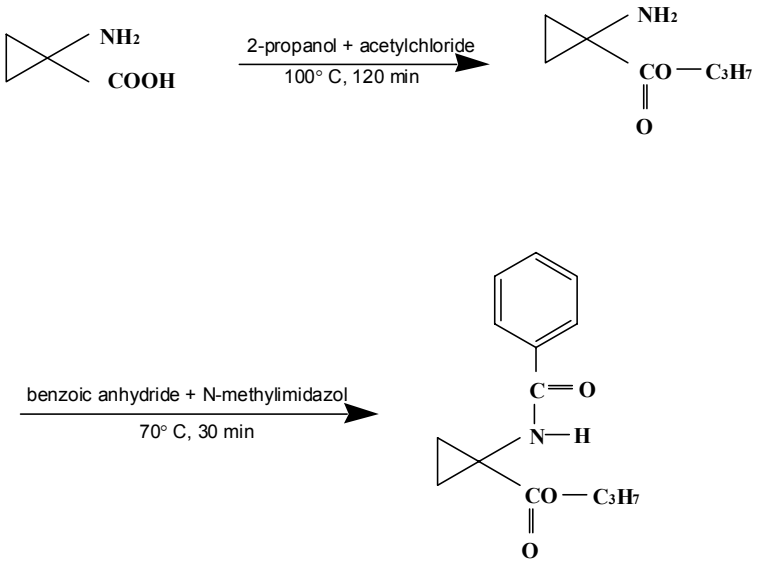

Figure 3. Two-stage derivatisation of ACC to form *N*-benzoyl *n*-propyl ACC

Analysis of ACC with LC/MS

A method for LC/MS analysis of ACC (Figure 4) was developed, in which ACC is extracted from plant tissue as described earlier (**I**) and purified by application to an Oasis MCX dual mode SPE column (Waters, Massachusetts, US). After methanol wash, ACC is eluted with 0.10 M NH_3 in 40% methanol, the sample is taken to dryness and benzoylated with 10 µl of a solution consisting of 100 mg benzoic anhydride in acetonitrile and 6 µl *N*-methylimidazole at 70°C for 30 minutes. In our analyses, samples were then injected into a capillary-LC system (Micromass International, Manchester, UK) connected to a Quatro Ultima tandem mass spectrometer (Micromass) equipped with an electrospray interface working in positive ESI mode. The capillary LC system was equipped with a 10 × 300 µm i.d. C_{18} analytical column (LC packings, Amsterdam, The Netherlands) and the flow was kept at 4 µl/min. The column was eluted with an exponential gradient from 10% to 90% (v/v) acetonitrile/1% (v/v) formic acid over 20 minutes and the effluent was introduced into the ESI ion source held at 90°C. The capillary voltage was 3.02 kV, cone voltage was 50V and dissolvation gas temperature was 150°C. The mass spectrometer was operated in the multi reaction monitoring (MRM) mode, with the collision energy set to 23 eV. The reactions *m/z* 206 to *m/z* 105 and *m/z* 210 to *m/z* 105 were monitored. Data obtained were processed with MassLynx 3.5 software (Micromass).

Analysis of IAA with GC/MS

Quantitative analysis of IAA in studies **II**, **III** and **IV** was performed by an isotope-dilution GC/MS-SRM technique previously described by Edlund *et al.,* 1995. After homogenisation of tissue in liquid nitrogen and addition of [$^{13}C_6$]IAA (Cambridge Isotope Laboratories, Wobourn, MA) as an internal standard, IAA was extracted in 0.01 M phosphate buffer with antioxidant. Samples were purified by addition of XAD-7 ion-exchange resin (Serva, Heidelberg, Germany) and

eluted in dichloromethane. The samples were methylated and trimethylsilylated before analysis by GC/MS-SRM using a JEOL MStation mass spectrometer (JEOL, Tokyo, Japan).

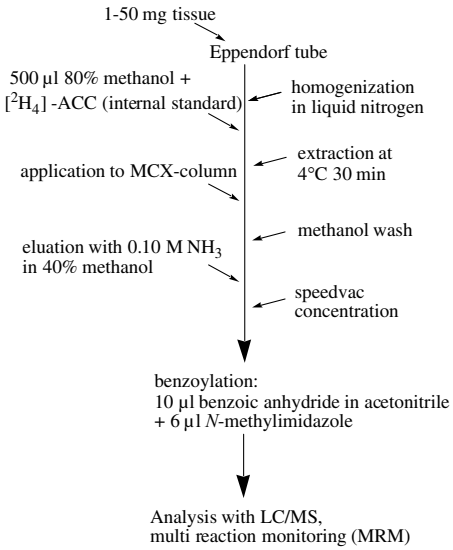

Figure 4. Protocol for the extraction and purification of ACC from milligram amounts of plant tissue and analysis with LC/MS-MRM

Results and discussion

Wood development in hardwood and softwood trees follows genetically determined patterns that are modified by environmental stimuli. Plant hormones have multiple roles in these processes, being involved not only in controlling and maintaining basic patterns of development, but also serving as signals in the transduction of environmental stimuli. However, the exact roles of plant hormones *in planta* are far from known. One useful approach for increasing our understanding of hormonal control is to elucidate how changes in their endogenous balance are related to growth and development. This was the strategy that was followed in the studies outlined in this thesis.

Analysis of ACC

Accurate determinations of ACC could greatly help our understanding of the function of ethylene in different physiological processes in plants. Therefore, a highly sensitive method for GC/MS analysis of ACC was developed, allowing tissue specific analysis of the compound (**I**). The technique allows sample throughput rates of up to 60 samples in three days, the detection limit is 2 pg ACC and samples as small as one mg of non-stressed plant tissue can be analysed. This is at least a 50-fold improvement compared to previous methods (e.g. Hall *et al.*, 1989; Chauvaux *et al.*, 1995; 1997). The method is easy to use and levels of ACC were successfully determined in a variety of different plant tissues (**I, II**). However, we wanted to reach even lower detection limits than those achieved using GC/MS, and attempts to find a suitable protocol for measuring ACC using LC/MS were made. This work has not been presented in any of the following papers and will be discussed here.

LC/MS analysis of ACC requires derivatisation since underivatised ACC will not be retained (at least not on a LC C_{18}-column using water/methanol as a mobile phase), but will pass straight through together with many other polar compounds of low-molecular weight. Thus, interference will be high, and the sensitivity of the LC/MS analysis low. Petritis *et al.* (2000) used this approach to analyse ACC with LC ion spray MS/MS, reporting detection limits as low as 2 ng. Derivatisation of ACC prior to analysis with LC/MS is therefore necessary, and a closer study of different purification and derivatisation protocols was made.

To find a simple purification protocol, three types of solid-phase extraction (SPE) columns were compared: the SCX cation exchange columns used in the previous GC/MS protocol (**I**), a C_{18} column in series with a SCX column and, finally, a MCX column containing a stationary phase of combined SCX and C_{18} material. The recoveries were comparable: 78%, 76% and 81%, respectively. The MCX column was finally chosen since we found it gave a higher grade of purification compared to the SCX column. It was also easier to handle compared to the combination of the C_{18} and SCX columns. In the developed protocol, ACC is applied to the MCX column in the extraction medium (80% methanol), the column is washed with 3 × 3 ml methanol and ACC is finally eluted from the column with 0.10 M NH_3 (Figure 4).

Using HPLC, radio labelled ACC and a radio-activity detector, several different derivatisation protocols were tested: (i) ACC without derivatisation, (ii) propylation (derivatisation step 1 described in Hall *et al.*, 1989; paper **I**; Figure 3), (iii) benzoylation (derivatisation step 2 described in Hall *et al.*, 1989; paper **I**; Figure 3), (iv) *N*-benzoyl *n*-propyl derivative (derivatisation described in Hall *et al.*, 1989; paper **I**; Figure 3), (v) propionylation (Åstot *et al.*, 1998) and (vi) PITH (phenyl isothiocyanate; derviatisation described by Chauvaux *et al.*, 1993; 1997). The aim was to find a suitable derivative for LC/MS analysis that is easier to handle and more stable than the one described in paper **I**. Procedure (v) gave a good recovery, but it did not increase the molecular weight or change the molecular structure sufficiently to avoid the previously described problems associated with underivatised ACC. The PITH reaction in (vi) did not work at all. Of the remaining reactions, alternative (iii), the benzoylation step from the derivatisation described in Hall *et al.*, 1989; paper **I**; Figure 3), was chosen. Settings for the LC/MS analysis were optimised and set to those described above. A calibration curve was recorded and the limit of determination was estimated to 1 pg ACC. A chromatogram of ACC is shown in Figure 5. The method has not yet been validated and additional work is in progress.

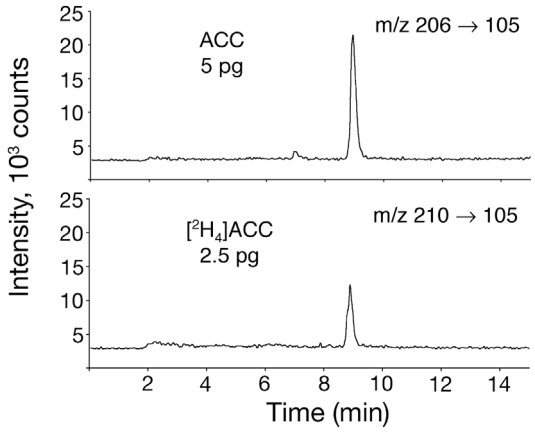

Figure 5. Chromatogram of benzoyl-ACC

In summary, the method developed for GC/MS analysis of ACC is suitable for routine analysis and can be further automated using a sample preparation robot. Since ACC is a charged molecule, it is likely to be suitable for LC-electrospray-MS. Further, using LC/MS, the whole sample can be injected, thereby lowering the detection limits. These first attempts to find a suitable method for LC/MS analysis of ACC are promising, and with further optimisation of the method and/or experiments with different derivatisation protocols, e.g. benzylation, lower detection limits are likely to be achieved.

Ethylene and IAA in wood formation

Ethylene in wood formation

Ethylene is present in low concentrations in non-stressed vegetative tissues (including wood-forming tissues). Under these conditions it has a suggested

involvement in the regulation of basic processes of wood development such as ray initiation, lignification and seasonal growth patterns (Barker 1979; Roberts & Miller, 1983; Little & Savidge, 1987; Yamamoto & Kozlowski, 1987*b*; Ingemarsson *et al.*, 1991*b*; Abeles *et al*., 1992; Eklund & Little 1995). Knowledge about ethylene homeostasis in wood-forming tissues is limited, but important for understanding ethylene's role in wood formation. Since ACC is normally limiting for ethylene biosynthesis in vegetative tissues (Wang *et al.*, 2002), it is of interest to document its presence in the cambial region. Further, it is of particular interest to investigate if ACC is enriched in specific tissues within the cambial region. The method developed for measuring small amounts of ACC (**I**) was used in combination with tangential cryosectioning (Uggla & Sundberg, 2001) and allowed the ACC distribution across the cambial region to be mapped in poplar trees. It was demonstrated that ACC levels did not vary much across these tissues (**II**). The symplast/apoplast compartmentalization of ACC is unknown, but the uniform distribution pattern across the wood-forming tissues suggests there may be a high proportion of apoplastic ACC that easily diffuses across cambial tissues.

EST sequencing (http://poppel.fysbot.umu.se/) detected three different ACC oxidases in wood-forming tissues of poplar. The most abundant (*PttACO1*) was cloned and found to be specifically expressed in the developing xylem tissues and, to some extent, in root tips (**II**). Moreover, tissue-specific dot-blot analysis (Moyle *et al.*, 2002) showed that *PttACO1* was highly up-regulated at the stage of secondary wall formation (**II**). The tissue-specific expression of *PttACO1* corresponded well with measurements of ACC oxidase activity, which was higher in developing xylem than in cambium/phloem fractions (**II**). A similar observation was made in Scots pine (Klintborg *et al.*, 2002). Despite a lack of knowledge about the expression pattern of the other ACC oxidases in cambial tissues of poplar, available data on ACC oxidase activity indicate that the conversion of ACC to ethylene is increased towards developing xylem. It is not known if the increased ACC oxidase activity in developing xylem is reflected in higher ethylene concentrations in these tissues. Measurement of the endogenous distribution of ethylene across cambial tissues is difficult, even with modern techniques such as laser detection that have been successfully used in more accessible tissues like leaves (Voesenek *et al.*, 1997). However, using molecular markers for ethylene (Peck *et al.*, 1998) could be a way to obtain such data in the future.

The presence of significant amounts of hydrolysable ACC conjugates in cambial region tissues of both poplar (**II**) and pine (Klintborg *et al.*, 2002) indicates that there is an excess of ACC in the cambial region. The ACC present in the cambial region tissues may originate from *de novo* synthesis in the stem itself, or from production in another part of the tree and transport to the cambial region via the xylem or the phloem sap (Bradford & Yang, 1980; Amrhein *et al.*, 1982; Morris & Larcombe 1995).

During the EST sequencing of cambial region cDNAs (Sterky *et al.*, 1998) one ACC synthase homologue was identified. Its expression was too weak to be detected by northern blot analysis, but RT-PCR confirmed its presence in cortex, phloem and developing xylem tissues (Björklund, personal communication). Klintborg *et al.* (2002) also detected low levels of ACC synthase activity in

cambial region tissues of Scots pine. Interestingly, in the studies of both poplar and Scots pine, ACC synthase expression/activity was increased towards the outer phloem/cortex tissues compared to the developing xylem tissues. The presence of both ACC synthase transcript and activity indicate that *de novo* synthesis of ACC occurs in the stem. The observed expression of ACC synthase in xylogenetic cultures of *Zinnia* cells provides further evidence for ethylene production during xylogenesis (Demura *et al.*, 2002). It should be noted that ACC is also present in xylem sap of tomato (Bradford & Yang, 1980; Hall *et al.*, 1993) and sunflower seedlings (Finlayson *et al.*, 1991) as well as in poplar stems (Figure 6). The possibility cannot be excluded that ACC transported in the xylem stream may provide an additional supply of ACC for cambial tissues.

Despite the evidence for ethylene production in the cambial region tissues, the role of ethylene in wood development remains to be unequivocally established. No clear requirement for ethylene has been shown for wood formation in any experimental system. Future characterisation of poplar trees after knocking out specific genes in ethylene biosynthesis/perception should help resolve this issue. In addition, *Arabidopsis* has recently become a candidate as a model system for studying wood formation (Little *et al.*, 2002), and characterisation of single or multiple mutants deficient in genes involved in ethylene biosynthesis and/or perception is another potentially interesting approach.

IAA distribution across wood-forming tissues

IAA is required for cell division in the vascular cambium and differentiation of cambial derivatives (Little & Savidge 1987; Aloni, 1991; Sundberg *et al.*, 2000). The precise roles of endogenous IAA in regulating either genetically determined or environmentally induced variations of wood are less well established, although recent data from conifers have shed some light on these issues (Sundberg *et al.*, 2000). However, for hardwood trees, reliable measurements of endogenous IAA using physiochemical methods are limited. Moreover, the distribution of IAA as a radial concentration gradient across cambial region tissues, peaking in the dividing cells, makes IAA measurements in samples containing combined tissues of the cambial region difficult to interpret (see Uggla *et al.*, 2001).

In paper **IV**, the radial distribution of IAA across the cambial region in young poplar stems was shown to exhibit two peaks, one associated with the cambial meristem and the other with the early stages of secondary wall formation. This pattern is different from that observed in older stems of Scots pine, where the distribution was more symmetric around a peak in the cambial meristem. However, the asymmetric patterns observed here are consistent with earlier observations in young internodes of poplar (Tuominen *et al.*, 2000), and the second peak could also be detected in older internodes of poplar stems (Tuominen *et al.*, 1997; **III**). These findings demonstrate that cambial derivatives in these internodes are exposed to similar IAA concentrations at different stages of their development, suggesting that other signals in addition to auxin are required for positional signalling in wood development. Such signals could include the sucrose to auxin ratio, which putatively determines xylem/phloem patterns (Uggla *et al.*, 1998). Positional signalling could also involve other plant hormones, such as

gibberellins and cytokinins. In the near future, tangential cryosectioning combined with sensitive analysis should provide valuable data not only for establishing the distribution of these hormones, but also for determining global profiles of gene expression (Hertzberg *et al.*, 2001), metabolites and proteins. These datasets should, in turn, reveal many secrets concerning the control of wood development.

Impact of nitrogen availability on growth rate and IAA

To determine the relationship between endogenous IAA and the rate of xylem growth, IAA was measured across the cambial tissues in poplars cultured at different growth rates by providing different concentrations of nitrogen. Both wild type trees and transgenic trees expressing bacterial IAA biosynthetic genes were included in the study. Rates of xylem production were found to be positively correlated to both the total amount of IAA and the concentration of IAA in the cambial meristem (**IV**). In wild-type trees, the high IAA concentration could be related to a high rate of cell cycling, in contrast to earlier results from mature Scots pine trees (Uggla *et al.*, 1998), in which no relation was found between IAA concentration in the cambial meristem and xylem production. Instead, xylem production was related to the size of the cambial meristem, which correlated well with the width of the auxin distribution pattern. Considering all these findings, the possibility cannot be excluded that auxin may increase xylem production by stimulating both the size of the meristem and the rate of cell cycling.

Reaction-wood formation

The genetically determined pattern of wood formation can be modified both temporally and spatially by the influence of environmental factors such as water stress, temperature, nutrient availability, photoperiod and gravitational stimulation. The most dramatic type of modification is probably induced by gravitational stimulation, which results in reaction wood formation. When reaction wood is formed, wood formation is modified at all developmental stages, including events in the cambial meristem affecting the frequency of cell divisions and cell fate decisions leading to vessels and fibres (in hardwoods), cell expansion and thus the shape of tracheary elements (in softwoods), and in secondary cell wall biosynthesis affecting the structure and chemistry of the cell wall (Haygreen & Bowyer, 1996). Reaction wood is easy inducible by bending or leaning the stem and has been the focus in two of the studies in this thesis (**II, III**). Bending treatments cause both mechanical and gravitational forces to act upon the stem. However, the primary inductive response causing reaction wood formation is believed to be of a gravitational nature. The major evidence for this conclusion includes the results of clinostat experiments and the fact that reaction wood is induced unilaterally in bending experiments. This unilateral induction is dependent on the direction of bending rather than on compression/tension stress in the tissue (Timell, 1986).

Ethylene in tension wood formation

Bending poplar stems induced a slight increase in ACC on the tension wood side of the stem, whereas the opposite wood side accumulated high levels of both ACC

and ACC conjugates (**II**). However, *PttACO1* was highly up-regulated on the tension wood side. This was accompanied by increases in ACC oxidase activity. Thus, the low ACC level at the tension wood side probably reflects its conversion to ethylene, implying that the limiting step of ethylene production associated with tension wood formation is the step catalysed by ACC oxidase rather than ACC synthase. If so, the ACC levels do not directly reflect ethylene production in this system.

Ethylene evolution from the upper side of tension wood forming stems in *Malus* trees was observed by Nelson and Hillis (1978). Other studies using tilted/bent shoots have found higher rates of ethylene evolution from the lower (opposite) side (Robitaille 1975; Yamamoto & Kozlowski, 1987*a*; Rinne, 1991), but it should be noted that the formation of tension wood was confirmed only one of these cases, the Yamamoto & Kozlowski study. In general, measurements of ethylene evolution from wounded plant tissues should be considered with caution. They may not reflect the true ethylene concentrations *in planta*, as the wounding (required to expose cambial tissues) is known to induce ethylene biosynthesis. Moreover, spontaneous conversion of ACC to ethylene may occur after wounding. In our preliminary experiments, we did not find any difference in ethylene evolution between the tension wood and opposite sides. However, considering the very high concentration of ACC at the opposite side there is a strong possibility that some of the recorded ethylene originated from the very high concentration of ACC detected at the opposite side.

The distribution of ACC across the cambial region tissues was mapped during tension and opposite wood formation. The distribution was quite uniform across the tissues and similar to that found in normal wood (**II**). This is surprising considering the tissue-specific increases seen in ACC oxidase transcript levels and activity on the xylem side. However, the uniformity may reflect high mobility of ACC across cambial tissues, which would even out any concentration differences. The presence of ACC in the xylem sap (Figure 6) and its induction after bending also reflect its presence and mobility in the apoplast.

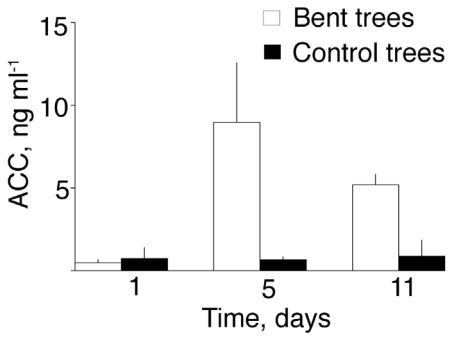

Figure 6. ACC in the xylem sap from poplar trees bent to form tension wood (white bars) and from control trees (black bars). Mean ± s.d., n=3.

The cellular localisation of ACC-oxidase action and ethylene production have not yet been clearly established. Attempts to immunolocalise the ACC oxidase protein have provided conflicting results. Some studies have found the protein to be located in the cytoplasm (Reinhardt *et al.*, 1994; Chung *et al.*, 2002), while others have reported an extracellular localisation, either in the cell wall (Rombaldi *et al.*, 1994), or by the plasma membrane (Ramassamy *et al.*, 1998).

Ethylene responses to gravitational stimuli vary greatly in their timing. Robitaille & Leopold (1974) measured ethylene evolution from bent apple stems over a period of 38 days and found that levels peaked after two days. After three weeks of bending, ethylene evolution had returned to control levels. Gravitational induction of ethylene in association with gravitropic bending of primary tissues, on the other hand, is often observed within hours (Clifford *et al.*, 1983; Philosoph-Hadas *et al.*, 1996; Madlung *et al.*, 1999). In poplar, increases in ACC and ACC oxidase could be detected after 24 hours of bending, but it took three days for bending-induced changes to be clearly established (**II**). This time course shows that the "ethylene-burst" in woody tissues induced by gravity is a slow response. Thus, it seems unlikely that ethylene is important in any primary gravity sensing mechanism. It is rather a downstream component of the tree's adaptive response to the gravitational stimuli. The increased ACC levels were maintained throughout the experimental period of 26 days. In the xylem sap of bent trees, the level of ACC was unchanged after one day, peaked after five days, and had decreased again after 11 days of bending (Figure 6). A closer study with more intense sampling and isotope feeding is needed to elucidate the importance of the ACC in the xylem sap for ethylene production during tension wood formation.

IAA in tension wood formation

The previously described distribution of IAA in upright poplar trees, i.e. a peak concentration in the cambial zone and steep gradients towards the xylem and phloem (Tuominen *et al.*, 1997; Sundberg *et al.*, 2000) was also found on both the tension wood and opposite wood side in bent poplars (**III**). Moreover, the content of IAA in the cambial region tissues on the tension wood side was similar to that in upright control trees. Although a slight decrease in IAA concentration was observed in the tension wood forming tissues, the results do not support the hypothesis that tension wood is induced by a deficiency of IAA. A major reason for this conclusion is that the IAA concentration decreased to a much greater extent on the opposite side. In addition, it can be concluded that the increase in xylem growth rate associated with the induction of tension wood in the poplar trees was not caused by an increase in IAA. The decrease in growth on the opposite side, on the other hand, was correlated with a decrease in IAA concentration. Whether this was a cause or a result of the reduction in cambial growth remains to be established.

The decrease of IAA on the opposite wood side of bent stems was slow, and there were no indications of the process within the first 24 hours of treatment (**III**). Within a similar time frame, Moyle *et al.* (2002) found increased expression of an auxin response gene, *PttIAA7*, at the tension wood forming side of poplar stems. Furthermore, *PttIAA7* was specifically expressed in the cortex, cambium and

developing xylem. However, the first responses to the bending in this study were evident after just six hours of gravistimulation, including down-regulation of two other auxin response genes on the tension wood forming side. Further, no major change in IAA concentration was seen during the experimental period.

Collectively, our results demonstrate that tension wood is not induced by changes in the IAA balance contrary to earlier suggestions prompted by the results of various application experiments. Nevertheless, the possibility cannot be excluded that changes in tissue sensitivity towards IAA or the polar transport of IAA form part of the tension wood response.

Ethylene in compression wood formation

ACC was measured in the cambial region tissues of pine trees after 21 days of bending, when the stems were actively forming compression wood (Figure 7). The results show there was a four-fold increase in ACC on the compression wood forming side compared to both the opposite side and to upright control trees. This is consistent with the results of Savidge *et al.* (1983). In their study, ACC was detected on the compression wood forming side, but not on the opposite side of plagiotropically growing branches of *Pinus contorta*. Moreover, an ACC oxidase was found to be up-regulated in compression wood forming tissues of *Pinus pinaster* (Plomion *et al.*, 2000). Increased ACC levels and up-regulated ACC oxidase activity on the lower side of compression wood forming stems and branches are also reflected in the higher rates of ethylene evolution detected in several studies from these tissues compared to the opposite side (Blake *et al.*, 1980; Little and Eklund, 1999). The absence of a decrease in ACC at the opposite side is a marked difference between compression wood and tension wood. This suggests that different mechanisms of the induction of ACC synthesis during reaction wood formation in softwoods and hardwoods. Future identification and characterisation of ACC synthase genes should shed more light on this issue.

The exact role of ethylene during compression wood formation is unknown, and whether it can only be speculated if it is involved in changing the growth rate or affects tracheid differentiation are purely speculative issues. The pine trees in our experiments had increased ACC levels, but showed no increase in growth rate (**III**).

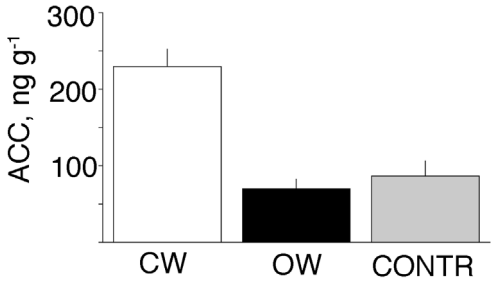

Figure 7. ACC on the compression wood side (white bar), and the opposite side (black bar) of pine trees bent for 21 days to form compression wood, and in unbent control trees (grey bar). Mean ± s.d., n=3.

Little & Eklund (1999) found the ethylene evolution to be positively correlated to the stimulation in growth only during compression wood formation. Both of these pieces of evidence suggest that ethylene plays a role in the differentiation of compression wood tracheids rather than in the growth response. These results are intriguing since applied ethylene precursors are known to stimulate growth in a variety of softwood species (Little & Savidge, 1987), but have never been observed to induce compression wood.

IAA in compression wood formation

After the induction of compression wood in Scots pine by bending, the typical pattern of IAA distribution across the cambial region tissues in the tissues (as earlier described for upright trees by Uggla *et al*., 1996) was unaffected on both the compression wood and opposite wood sides (**III**). Moreover, a time course experiment demonstrated that the IAA content in the cambial region tissues was not affected in the bent trees. Thus, cambial derivatives in developing compression wood are exposed to similar IAA concentrations as those that develop into normal wood in upright trees. The induction of compression wood tracheids cannot, therefore, be explained by alterations in IAA levels.

Often, but not always, compression wood formation is associated with higher xylem growth rates (Timell, 1996) and considering the stimulating effects of IAA on cambial growth, it seems likely that IAA mediates this growth stimulation. In our time course experiment, IAA levels and xylem growth rate were both found to be unaffected. Funada *et al.* (1990) found higher amounts of IAA and increased growth rates in the compression wood forming side of inclined *Cryptomeria japonica* stems. However, in a study of the induction of compression wood in branches of Douglas fir (Wilson, *et al.*, 1989) the IAA concentration was shown to be equal or even lower in the tissues forming compression wood, despite an increase in xylem production. However, in this experiment, the IAA in the cambial meristem was not included in the analysed tissue. With the limited data available, it is not possible to draw any firm conclusions about the role of IAA in the growth stimulation normally associated with compression wood formation. But, as in poplar trees it is possible that mechanisms that do not involve increases in IAA levels are responsible for this stress induced growth stimulation.

Parallels between gravitropism and reaction wood formation

Gravitropism in primary roots and shoots, as well as reaction wood formation in secondary tissues of trees are induced by displacement of the organ in relation to the gravitational forces. In all cases, this results in differential growth responses. Gravitropism has been intensively studied in coleoptiles, roots and shoots (Kaufmann *et al.*, 1995) and, as in reaction wood induction, both IAA and ethylene have been considered as key signals in the process (Philosoph-Hadas *et al.*, 1996; Chen *et al.*, 1999; Maldung *et al.*, 1999; Philippar *et al.*, 1999; Friml *et al.*, 2002).

Gravistimulation of primary roots and shoots involves lateral transport of IAA towards the lower side of the organ, thereby stimulating cell expansion (Philippar *et al.*, 1999; Friml *et al.*, 2002). Moreover, analysis of *Arabidopsis* mutants with a

deficiency in the gravitropic response has demonstrated the importance of auxin signalling in this process (Tasaka *et al.*, 2001). Ethylene has also been proposed as a regulator of the gravitropic response, and is induced in the lower portions of gravistimulated above-ground organs, such as flower stalks, hypocotyls, coleoptiles and apical shoots (Clifford *et al.*, 1983; Wheeler *et al.*, 1986; Prasad *et al.*, 1989; Woltering, 1991; Kaufman *et al.*, 1995; Philosoph-Hadas *et al.*, 1996; 2001). However, the general conclusion from studies in which inhibitors of ethylene synthesis or action were applied during gravitropism is that ethylene influences the extent and rate of gravitropic bending rather than being involved in the initiation of the gravitropic response (Wheeler *et al.*, 1986; Woltering, 1991; Philosoph-Hadas *et al.*, 1996).

The reaction wood response does include a redistribution of IAA (in poplar) and an induction of ethylene. However, the roles of IAA and ethylene in the growth response are not clear. A tree that is displaced from its original position displays both the reaction wood response in the stem and a gravitropic response in the primary, current year shoots (Kaufmann *et al.*, 1995). It would be of interest to compare, for example, hormone dynamics and global gene expression using microarray analysis during gravitational stimuli of primary and secondary tissues in the same tree.

Summary of factors inducing reaction wood

We have shown that both tension wood and compression wood are formed without any major changes in cellular auxin content. Given that exogenously applied IAA induces reaction wood, but reaction wood is formed without changes in endogenous IAA content, it can be speculated that auxin signal transduction is involved in the response, and that the gravistimuli affect auxin sensitivity. Another possibility is that experiments involving auxin applications induce artificial responses that are different from those taking place under natural circumstances.

During formation of both tension wood and compression wood, ACC levels are higher at the gravitationally lower side of the bent stem. In softwoods, this seems to be associated with higher ethylene production and compression wood formation, whilst in hardwoods the ethylene production seems to occur at the upper, tension wood forming side. However, since ethylene does not have the ability to induce reaction wood formation it is not likely to be the main triggering factor for the changes in wood anatomy. An important function of the ethylene induced during the reaction wood response may be to stimulate cambial growth, as it is known to do from many application experiments (Little & Savidge, 1987; Little & Pharis 1995)

In many developmental processes, there are interactions between different classes of plant hormones (Mc Court, 1999; Swarup *et al.*, 2002). For example, IAA can induce ethylene synthesis by induction of ACC synthase (Yang & Hoffman, 1984; Peck & Kende, 1995; Rodrigues-Poustada *et al.*, 1999), and ethylene has been reported to affect the metabolism and transport of auxins (Eklund & Little, 1995; 1996; Visser *et al.*, 1996). In the case of reaction wood formation, the stimulation of ethylene production appears to be independent of an increase in auxin content. However, it can be speculated that the increase in ethylene during reaction wood

formation could change the tissue sensitivity towards auxin or in other ways interact with the auxin signal transduction pathways.

Our results indicate that either auxin or ethylene is a primary factor for triggering the differentiation of reaction wood, and the role of other substances needs to be studied. Interesting candidates among the plant hormones for further examination are the gibberellins, which have the ability to induce tension wood characteristics in Japanese cherry (Baba *et al.*, 1995) and other hardwood species (Funada, personal communication).

Conclusions

We have developed methods for analysing the ethylene precursor ACC in small amounts of plant tissue using both GC/MS and LC/MS. The GC/MS method was successfully used for tissue-specific measurements of ACC across the cambial region tissues of poplar trees. The ACC distribution was quite uniform across the tissues, suggesting that high degrees of radial diffusion occur. Further, the specific expression of ACC oxidase in the secondary wall forming xylem cells was not reflected in the ACC levels. During tension wood formation, a gene encoding an ACC oxidase, as well as ACC oxidase activity, were highly up-regulated at the tension wood side, whereas high levels of ACC and its hydrolysable conjugates accumulated on the opposite side. This leads to the conclusion that ACC oxidase regulates the rate of ethylene production during tension wood formation, and that the ACC levels in this case do not directly reflect the ethylene production.

High-resolution measurements of IAA across the cambial region tissues of poplar and pine forming reaction wood revealed IAA patterns similar to those seen in normal wood. Thus, the old model based on the hypothesis that high IAA concentrations induce compression wood formation, and IAA deficiency induces tension wood, has to be refuted. The growth stimulation associated with tension wood formation occurs without changes in IAA concentration, but the inhibition of growth on the opposite side is associated with a decrease in IAA. Further, we showed that in poplar trees growing at different growth rates, but without forming tension wood, IAA concentrations in the cambium are well correlated to the growth rate, and we also obtained data suggesting that IAA can stimulate growth by increasing the rate of cell cycling in the vascular cambium. Thus, IAA is correlated to the growth rate in poplar undergoing “normal growth” but not during tension wood formation.

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Sammanfattning

Den här avhandlingen behandlar reglering av tillväxthastighet och fiberegenskaper hos träd. Många av dessa egenskaper är genetiskt bestämda, men de kan också påverkas av miljöfaktorer. En typ av yttre stress som påverkar både tillväxthastighet och fiberegenskaper är när träd bildar reaktionsved. Detta sker när trädets stam eller grenar rubbas ur sitt vanliga läge till exempel som följd av vind, snölast eller markrörelser. Reaktionsvedens funktion är att återställa stammens eller grenarnas normala läge. När reaktionsved bildas får man en större variation i fiberegenskaperna inom ett och samma träd, vilket gör slutanvändningen av vedråvaran mer komplicerad, både om slutprodukten är papper och om den är sågade trävaror.

Växthormoner är viktiga signaler i både den normala vedbildningen och i de förändringar som sker när reaktionsved bildas. För att förstå mer om hur vedbildningen regleras är det av intresse att kunna mäta halter av växthormoner i de vedbildande vävnaderna under olika utvecklingsstadier. Genom att använda gaskromatografi kopplat till masspektrometri (GC/MS) har vi studerat växthormonerna auxin och etylen, som båda är viktiga signalsubstanser för vedbildningsprocessen.

Vi har visat att reaktionsved, i både tall (*Pinus sylvestris*) och i asp (*Populus tremula*), kan bildas utan förändringar i auxinkoncentrationer. Detta motbevisar tidigare antaganden om att skillnad i auxinkoncentration är en viktig faktor för induktion av reaktionsved. Vidare jämfördes auxinhalter i hybridaspar (*Populus tremula* × *tremuloides*) som växte vid olika hastigheter, men utan att bilda reaktionsved. Det fanns en god korrelation mellan mängden auxin och tillväxthastigheten i dessa träd. Tillväxthastigheten ökade genom en ökad celldelningshastighet, och inte genom att fler celler delade sig samtidigt. Således var tillväxtökningen i asp korrelerad till auxin vid vanlig vedbildning men inte vid bildningen av reaktionsved.

Etylen är ofta kopplat till olika stressresponser i växter. Vid bildning av reaktionsved ökar etylenproduktionen, och vi har visat att denna ökning regleras av ACC-oxidas, som är enzymet som katalyserar sista steget i bildningen av etylen. Detta är i motsats till de flesta hittills kända fall är etylenproduktionen styrs av tillgången på 1-aminocyklopropan-1-karboxylsyra (ACC) som är förstadiet till etylen. Trots att etylenproduktionen ökar vid reaktionsvedsbilding, vet vi inte vilken betydelse detta har för att förändra tillväxten och fiberegenskaperna hos trädet.