Molecular Factors Involved in the Formation of Secondary Vascular Tissues and Lignification in Higher Plants

Studies of CuZn-SOD and Members of MYB and Zinc-finger Transcription Factor Families

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Abstract

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The formation of secondary vascular tissues involves complex processes and many steps, a number of which have been examined in detail in this study. A novel CuZn-SOD, with a high pI and thus denoted hipI-SOD, was identified and characterized in *Pinus sylvestris*. Results from immunolocalisation analyses indicated that it is localised in lignified structures, suggesting that SOD might participate in the formation of secondary cell walls and lignification. To further investigate its role in these processes, a *Zinnia* mesophyll cell system was set up. This enabled us to follow the differentiation from mesophyll cell to tracheary element. Various inhibitors against SOD and H_2O_2-production were applied. The results suggested that hipI-SOD might have a novel and important function in secondary cell wall formation and lignification processes. The expression pattern and localization of the protein during formation of tracheary elements support this assumption.

The other part of this study involved analysis of transcription factors and their regulation, especially in secondary vascular tissues. The genes encoding three MYB-transcription factors and one novel Zinc-finger transcription factor were found in an EST-library from the cambial region of hybrid aspen (*Populus tremula* L. x *tremuloides* Michx.). The genes were cloned and characterized and their regulation by hormones, sucrose and gravity was investigated. The genes were found to be under hormone and sucrose control, and their expression altered during tension wood formation. Transgenic plants were constructed, carrying one of two antisense constructs of *MYB*-genes, *PttMYB46* or *PttMYB76*, which were strongly expressed in lignified tissues. Analysis of plants with either of these constructs displayed a complex phenotype, including reduced growth, increased concentration of some phenolic acids and changes in lignin composition. Some of the phenotypic traits were indicative of strong investment in defensive characters.

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Till minne av Janne, Clay och alla andra fyrbenta vänner jag har haft förmånen av att ha i mitt liv.

Contents

Appendix

List of papers

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

I. Karpinska, B., Karlsson, M., Schinkel, H., Streller, S., Süss, K-H., Melzer, M. & Wingsle, G. (2001) A novel superoxide dismutase with a high isoelectric point in higher plants. Expression, regulation, and protein localization. *Plant Physiology* 126: 1668-1677.

II. Karlsson, M., Melzer, M., Prokhorenko, I., Johansson, T. & Wingsle, G. HipI-Superoxide dismutase, a possible regulator of hydrogen peroxide in the lignification of tracheary elements in *Zinnia elegans* L. *Submitted.*

III. Karlsson, M., Stenberg, A., Schrader, J., Sterky, F., Bhalerao, R., Wingsle, G. & Karpinska, B. MYB transcription factors in the secondary vascular tissues of hybrid aspen and their regulation by hormones, sucrose and gravity. *Manuscript*.

IV. Karlsson, M., Witzell, J., Srivastava, M., Wiklund, S., Rodriguez-Buey, M., Edlund, U., Karpinska, B., Mellerowicz, E.J. & Wingsle, G. Morphological, anatomical and chemical changes in hybrid aspen carrying antisense constructs of a MYB gene. *Manuscript*.

Introduction

Plants acquired the ability to occupy terrestrial environments approximately 400 to 430 million years ago (Kenrick & Crane, 1997). The development of the first terrestrial plants coincided with the evolution of a vascular system that could both transport water and solutes from the roots to the aerial tissues, and provide structural support. The vascular and conductive tissue upon which this system is based is known as xylem. It has been postulated that the evolution of xylem was an absolute requirement for plants to occupy terrestrial habitats (Kenrick & Crane, 1991; Cook & Friedman, 1998).

The formation of wood (xylem), and phloem, involves complex processes consisting of many steps, some of which have been closely examined in the studies outlined here. To set the context for these investigations, this introductory section will briefly discuss secondary vascular tissues, but mostly wood, its formation and components. Especially SOD and transcription factors will be discussed.

Wood formation

The following summary of wood formation is based on information from Mellerowicz *et al*., (2001) and Plomion *et al*., (2001). Wood is derived from dividing cells in the vascular cambium (the cambial zone), which is positioned between the phloem (living inner bark) and the xylem (wood) of the stem (Figure 1A). The vascular cambium is a secondary meristem that plays a major role in the radial growth of gymnosperm and angiosperm stems and roots. Cambial activity ensures the perennial life of trees through the regular renewal of functional xylem and phloem. The cambial zone includes the cambium, which is composed of juvenile cells, called initials, and the phloem and xylem mother cells, both of which are produced by the dividing cambial initials.

Xylem mother cells always divide more frequently than phloem mother cells, which explains the disproportion in size between phloem and xylem tissues.
In the vascular cambium there are two different types of initial cells. One type consists of the fusiform initials that produce secondary vascular tissues in a position-dependent manner (Figure 1B). On the inner side fusiform initials produce xylem elements (tracheids in gymnosperms, vessels and fibres in angiosperms) and axial parenchyma cells. On the outer side, they produce phloem cells (sieve tubes in angiosperms, sieve cells in ferns and gymnosperms) along with companion cells, fibres, and axial parenchyma. The other type of initial cell is comprised of the ray initials, which give rise to the horizontally oriented ray cells that are essential to the translocation of nutrients between the phloem and xylem.

The daughter cells produced by the cambial initials give rise to a variety of wood cells. The differentiation of xylem elements, i.e. tracheids, vessels and fibres, involves four major steps: cell expansion, followed by the ordered deposition of a thick multi-layered secondary cell wall, lignification and finally cell death.

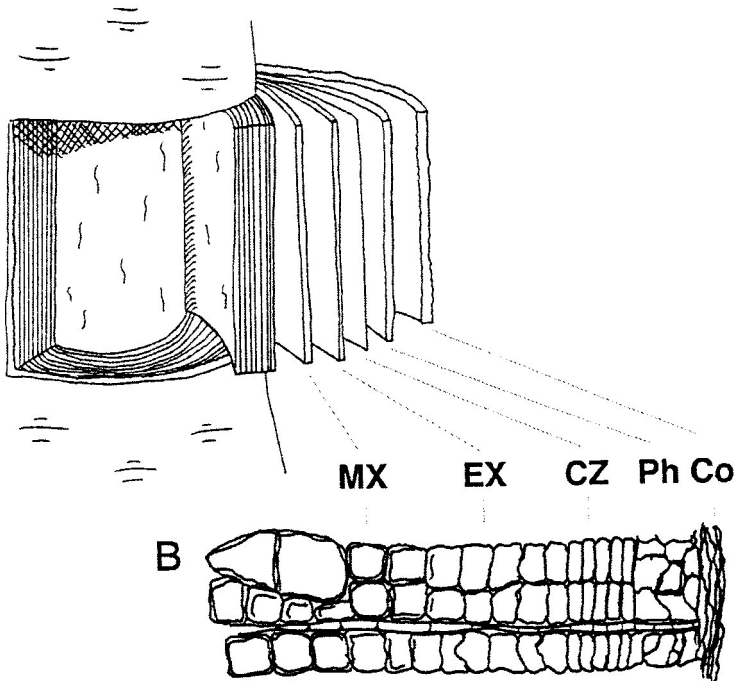


Figure 1. (A) Tree trunk, showing different developmental zones. (B) Cells of different developmental phases and their location within the cambial region tissues. Co=cortex, Ph=phloem, CZ=cambial zone, EX=expanding xylem, MX=maturing and mature xylem (Hellgren, 2003). (The figure is reproduced by permission of the person concerned).

Derivative cells expand longitudinally and radially to reach their final size during the formation of the primary wall. Once expansion is complete, the formation of the secondary cell wall begins, involving the synthesis and assembly of four major classes of compounds: polysaccharides (cellulose, hemicelluloses), lignins, cell wall proteins and other minor compounds, some of which are soluble (stilbenes, flavoloids, tannins and terpenoids) and some insoluble (pectins and cell wall proteins) in a neutral solvent.

Wood consists of 40-50% cellulose. The fundamental structural units are the microfibrils, which consist of different chains of b-linked glucose residues. The water-insoluble cellulose microfibrils are associated with hemicelluloses: soluble non-cellulosic polysaccharides that account for about 25% of the dry weight of wood. Generally they occur as heteropolymers such as glucomannans, galactoglucomannan, arabinogalacatan and glucuronoxylan, or as homopolymers like galactan, arabinan and b-1,3-glucan.

The third main component of wood (25%-35%) is lignin, a phenolic polymer which will be described in more detail later in this introduction. Lignin embeds the

polysaccharide matrix and gives rigidity and cohesiveness to the wood tissue (Figure 2). After completion of lignification, vessel elements undergo programmed cell death, which involves the hydrolysis of the protoplast.

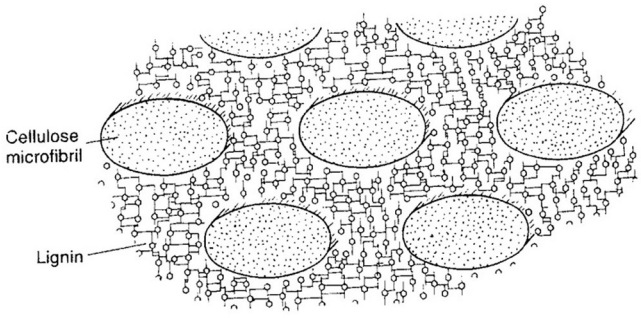


Figure 2. Model of the cell wall showing the way in which lignin phenolics fill spaces between cellulose microfibrils. A rigid, impermeable cell wall is thus created (Brett and Waldron, 1996).

Lignin polymerisation

Lignin is considered an undesirable factor in paper-making as it limits accessibility to cellulose. Lignin that remains after the pulping process also reduces paper quality. Removal and bleaching of lignin is expensive, and is associated with the release of toxic pollutants. In addition, a high lignin content limits the digestibility of fodder crops (Jung *et al*., 1993). However, it can be beneficial when it comes to using wood as a fuel (Baucher *et al*., 1998). Lignin holds more potential energy than any of the other major wood components, so the energy content of the wood can be enhanced by increased lignin levels. For all these reasons, there are major economic interests in the potential to modify lignin composition and content in plants.

Lignin is a heterogenous phenolic polymer that is mainly present in the secondary cell walls of specialized cells, including those of xylem, sclerenchyma, phloem fibres and periderm tissues (Campbell & Sederoff, 1996). Lignin is essential for mechanical support, it gives rigidity to cell walls, it provides hydrophobicity and it makes tracheary elements impermeable, thereby allowing the transport of water and solutes through the vascular system. In addition, lignin also plays a role in plant defence by providing a physicochemical barrier against pathogens (Vance *et al*., 1980).

The synthesis of the lignin precursors proceeds through the phenylpropanoid pathway, starting with the deamination of phenylalanine to cinnamic acid (Figure 3A). The lignin polymer is mainly derived from the dehydrogenative polymerisation of three different hydroxycinnamyl alcohols (or monolignols): p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. These alcohols give rise to the hydroxyphenyl-(H-), guaiacyl- (G-) and syringyl- (S-) units of the lignin polymer, respectively, and differ from each other only in their degree of

methoxylation (Figure 3B). Lignins with high proportions of S-units are more efficiently extracted during the kraft pulping process (Chiang *et al*., 1998).

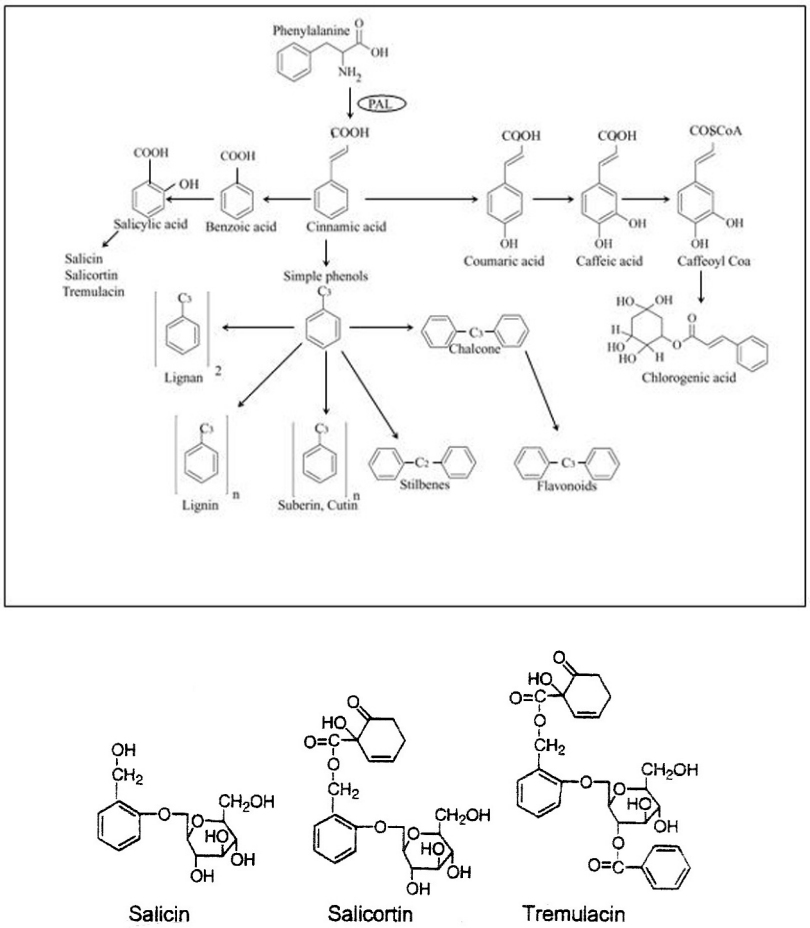


Figure 3. (A) A highly simplified diagram illustrating different steps and products in the phenylpropanoid pathway (PAL=phenyl alanine ammonia lyase). Three phenolic glycosides, salicin, salicortin and tremulacin, are displayed separately.

The content and composition of lignin are known to vary among taxa (for example, no S units are detected in gymnosperms, but they are found in angiosperms), tissues, cell types and cell wall layers and to depend both on the developmental stage of the plant and environmental conditions (Cote, 1977; He & Terashima, 1991; Campbell & Sederoff, 1996; Joseleau & Ruel, 1997; Monties, 1998). Today it is possible to modify lignin content and composition by genetically modifying the expression of the relevant genes in the phenyl propanoid pathway. Genes and cDNAs encoding most of the known enzymes of the monolignol biosynthesis pathway have been cloned (reviewed by Baucher *et al*., 1998; Christensen *et al*., 2000) and ESTs for all of these enzymes have been found in xylem and cambium libraries of hybrid aspen (Sterky *et al*.,1998). The

results obtained have made it possible to redraw the lignin biosynthesis pathway, which had remained constant for many years in biochemistry textbooks (for recent literature on the subject see Baucher *et al*., 1998; Whetten *et al*., 1998; Grima-Pettenati & Goffner, 1999; Boudet, 2000; Anterola & Lewis, 2002; Li *et al*., 2001).

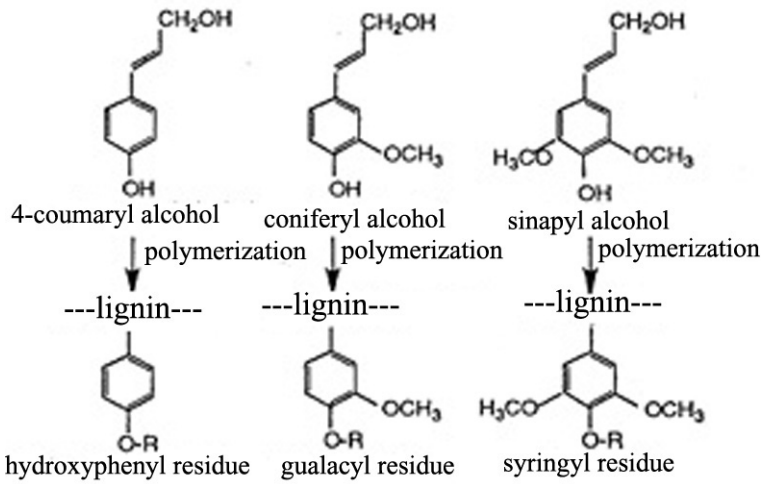


Figure 3. (B) The three hydroxycinnamyl alcohols which give rise to three different units of the lignin polymer. They differ from each other only in their degree of methoxylation.

Which enzymes are really involved in the oxidation of monolignols?

Radicals produced through the single-electron oxidation of monolignols are responsible for the coupling of lignin (Boudet, 2000). The nature of the enzymes catalysing the oxidative polymerisation of monolignols is still a matter of debate. Until approximately ten years ago, peroxidases had been considered the only class of enzyme involved in the final step of lignification. However, convincing evidence has been published suggesting that other oxidases, particularly laccases, may be involved in lignification (for reviews: O'Malley *et al*., 1993; Dean & Eriksson,1994). Many purified enzymes are capable of oxidizing monolignols in vitro, but no unequivocal proof has been obtained for the role of any particular oxidase in lignification through loss-of-function experiments in transgenic plants. The very high redundancy of different oxidases and the broad spectrum of substrates utilized by their different isoforms are the major reasons it has been difficult to assign a specific function to any particular oxidase (Grima-Pettenati & Goffner, 1999; Boudet, 2000).

Peroxidases use hydrogen peroxide (H_2O_2) as a substrate. The presence of endogenous H_2O_2 in lignifying cells has been shown by several groups (Olson & Varner 1993; Czaninski *et al*., 1993; Schopfer, 1994; Ros-Barcelo *et al*., 2002). However, the mechanisms of its generation are still a matter of debate. The

possible involvement of SOD in lignification as a producer of H_2O_2 has been discussed by Ogawa *et al*. (1996), Ogawa *et al*. (1997) (Figure 4).

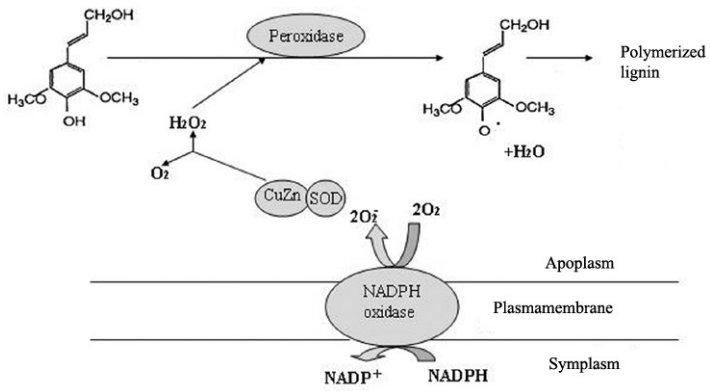


Figure 4. Proposed model of different participants in the lignification process (after Ogawa et al., 1997),

Laccase catalyses the oxidation of phenolic substrates using molecular oxygen as the electron acceptor. A laccase has been shown to polymerise monolignols in vitro (Sterjiades *et al*., 1992). Laccase activity has been found in cell wall preparations (Davin *et al*., 1992) and localisation studies have shown quite specific occurrence of laccase in differentiating xylem (Driouich *et al*., 1992; Bao *et al*., 1993).

In addition to laccases, in conifers another H_2O_2-independent phenoloxidase, coniferyl alcohol oxidase, has been proposed to participate in lignification (Savidge *et al*., 1992, 1998). It is possible that different plant species use any one of the enzymes mentioned above, or combinations of them to varying degrees.
The last step in the process of lignification is the enzymatic oxidative polymerisation of the cinnamyl alcohols that form the lignin (Boudet *et al*., 1995). The actual cross-linking was long supposed to be a random process. However, another possibility has been postulated recently (and much debated): that dirigent proteins coordinate the formation of the lignin polymer (Davin & Lewis 2000).

Superoxide dismutases

A family of metalloenzymes, the superoxide dismutases (SODs; superoxide:superoxide oxidoreductases, EC 1.15.1.1), catalyse the disproportionation of superoxide anion radicals to yield molecular oxygen and H_2O_2 (McCord & Fridovich 1969).

$O_2^- + O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$

The main function of SOD is to scavenge O_2^- radicals generated in various physiological processes, and thus prevent the oxidation of biological molecules either by the radicals themselves or by their derivatives (Liochev & Fridovich, 1994; Fridovich, 1995). Different kinds of environmental stresses can promote the production of O_2^- within plant tissues, and plants are believed to rely on the enzyme SOD to detoxify this reactive oxygen species.

SODs are among the fastest enzymes known, with a Vmax of $2x10^9$ $M^{-1}s^{-1}$. They occur in three different forms, with Mn, Fe or Cu and Zn as prosthetic metals (Fridovich, 1986). CuZn-SOD is the most abundant form in plants (Asada *et al*., 1980) and is the form of SOD that will be discussed in this thesis.

CuZn-SOD

The Cu_2^+ in CuZn-SODs active site is the catalytic agent, first accepting an electron from one O_2^- radical and then donating it to a second catalytic agent to produce, together with two protons, H_2O_2 (Fielden *et al*., 1974). The Zn_2^+ stabilises the structure of the active site (Fridovich, 1986).
CuZn-SODs are generally homodimers with a molecular weight of around 32 kD, although there are exceptions to this rule.

Distribution of CuZn-SOD in different compartments

All plant SODs that have been investigated genetically are encoded in the nucleus, in spite of their different locations. Isoforms of CuZn-SOD are found in the cytosol (Perl-Treves *et al*., 1988), chloroplasts (Scioli & Zilinskas, 1988, peroxisomes (Sandalio & del Rio, 1987; Bueno *et al*., 1995) nucleus (Ogawa *et al*., 1996) and apoplast (Streller & Wingsle, 1994). Apoplasmic SOD activity was first detected in spruce needles (Castillo *et al*., 1987). More detailed experiments have proven the existence of extra-cellularly localised CuZn-SODs (EC-SODs) in pine needles by isolating and purifying one of the isoforms and analysing its N-terminal amino acid sequence (Streller & Wingsle 1994). In addition to the traditional role of SOD as part of the defence against active oxygen species, EC-SODs could also be involved in nitric oxide metabolism, pathogen defence (Schinkel *et al*., 1998) and in lignification (Ogawa, 1997; Schinkel, 2001) (Figure 4).

Factors regulating vascular tissue formation and lignification

Not much is known about the cellular, molecular and developmental factors that regulate secondary vascular tissue formation and lignification. The processes are controlled by a wide variety of factors, both exogenous (e.g. photoperiod and temperature) and endogenous (e.g. phytohormones) and by interaction between them (Plomion *et al*., 2001). Hormones and sucrose are known to be molecular transducers of positional and environmental information for vascular tissue formation in trees (Mellerowicz *et al*., 2001; Uggla *et al*., 2001) and will be discussed in more detail.

Plant hormones

Plant hormones are important regulators of plant development and morphology, and environment stimuli often act on plant growth by modulating the hormonal balance. The definition of the term ”hormone” is an organic compound produced by one tissue in an organism and transported to another tissue, where it induces a specific physiological response (Lawrence, 1995). When hormones are applied exogenously they have been observed to affect most aspects of cambial growth, such as cell division, cell expansion, final cell morphology, the induction of differentiation into different cell types and cell wall chemistry (Little & Savidge, 1987; Aloni, 1991; Little & Pharis, 1995; Sundberg *et al*., 2000).

Some of the ”classical” groups of plant hormones (Kende & Zeevaart, 1997) will be discussed in more detail. Auxin seems to be a key signal in xylogenesis. In contrast to other plant hormones, it is sufficient, on its own, to induce differentiation of vascular elements when applied to plant tissues (Roberts, 1988). Auxin together with cytokinin can induce tracheary element differentiation when applied to *Zinnia* cultures (Fukuda & Komamine, 1980). Numerous experiments have also demonstrated that the application of auxin to cambial tissues stimulates cambial cell division (Sundberg *et al*., 2000). In high-resolution analyses, endogenous auxin concentration peaked in the cambium and its most recent derivatives, but declined to low levels in maturing xylem and phloem, suggesting that auxin modifies cambial growth by influencing developmental patterns (Uggla *et al*., 1996; Tuominen *et al*., 1997; Sundberg *et al*., 2000). However, auxin is not the only agent that provides positional signals for the cambial meristem. Cells at the phloem side of the meristem remain meristematic at a lower auxin concentration than cells at the xylem side. Hence, the auxin gradient does not by itself provide enough information for positioning of the different initials.

Gibberellins (GA:s) stimulate meristematic activity and xylem fibre elongation when applied together with auxin (Digby & Wareing, 1966). The role of GA:s in wood formation has recently been demonstrated by over-expression of a GA-20 oxidase in hybrid aspen (Eriksson *et al*., 2000), which resulted in a 20-fold increase in levels of the biologically active GA1 and GA4. The transgenic trees exhibited increased longitudinal and radial growth, as well as increased xylem fibre length, which is consistent with data from exogenous applications. Cytokinins have a well-established function in cell division, but their role in cambial growth is far from clear (Little & Savidge, 1987; Little & Pharis, 1995). Studies on the vascular cambium have detected no difference in cytokinin concentrations between dormant and actively dividing cambial cells (Moritz & Sundberg, 1996). Application of exogenous ethylene stimulates cambial cell division, possibly by increasing auxin levels through interaction with auxin transport (Eklund & Little, 1996).

Sugars

Sugars, like sucrose, glucose and fructose, provide the building blocks for essentially all compounds present in plant tissues. They play important roles in

intermediary and respiratory metabolism, and are precursors in the synthesis of complex carbohydrates such as starch and cellulose. These metabolic processes have been studied in depth for a long time (Smeekens, 2000). However, another aspect of sugars has recently become the focus of intense research efforts in plant science: their signalling functions. Sugars, as such, can induce alterations in gene expression, in a similar fashion to hormones. Both accumulation and depletion of carbohydrates can enhance or repress the expression of genes (Koch, 1996).
Sugars as signalling compounds have profound effects in all stages of the plant's life cycle from germination through vegetative growth to reproductive development and seed formation (Lu *et al*., 2002). Sugar-signalling pathways do not operate in isolation, but form parts of broader cellular regulatory networks, and recent results have clearly shown that cross talk occurs between different signalling systems, especially those of sugars, phytohormones, and light (Smeekens, 2000).

The demonstration of steep concentration gradients of soluble carbohydrates across the cambium (Uggla *et al*., 2001), together with accumulating data suggesting that plants can sense sugars (Sheen *et al*., 1999) provide substantial evidence for the concept that auxin/sucrose ratios determine the positioning of the cambium (Warren Wilson & Warren Wilson, 1984).

Cytokinin and sugar signals have overlapping functions in the transcriptional regulation of a number of genes (Cheng *et al*.,1992; Vincentz *et al*., 1993; Crowell & Amasino, 1994). They can also affect the cell cycle (Jacqmard *et al*., 1994; Lindsey & Yeoman, 1985) and auxin antagonism (Mok, 1994), as well as an array of morphological changes (Mok, 1994). Sugars are known to repress some hormone signalling pathways, especially those involving gibberellins (Perata *et al*., 1997).

Reaction wood formation: A tree response to stem displacement

In these studies, tension wood formation was used to examine gene regulation in wood undergoing alterations in its anatomical and chemical properties.

Reaction wood is generally formed in response to a non-vertical orientation of the stem caused by winds, snow, slope or asymmetric crown shape. In hardwood species, reaction wood is called tension wood and is formed on the upper side of a leaning stem or branch. The overall lignin content of tension wood is lower and the cellulose content higher than in normal wood (Timell, 1969). A characteristic of tension wood is the presence of an inner cell wall layer in the fibres that consists of almost pure, and highly crystalline cellulose: the G-layer (Norberg and Meier, 1966; Timell, 1969; Jourez *et al*.,2001). Tension wood also has fewer and smaller vessels, as well as fewer rays compared to normal wood, and the growth rate is usually increased on the tension side of the stem (Scurfield, 1973; Timell, 1986; Jourez *et al*., 2001). The signalling pathway that controls reaction wood formation is still poorly understood, but it may be essentially a gravitropic response of the tree, related to intrinsic growth direction and phytohormone

distribution and interactions, especially interactions between ethylene and auxin (Timell, 1986; Sundberg *et al*., 1994; Little & Eklund, 1999).

The importance of transcription

The fundamental dogma of molecular biology is that DNA produces RNA, which in turn produces protein. Hence, if the genetic information that each individual inherits as DNA (the genotype) is to be converted into proteins that produce the corresponding characteristics of the individual (the phenotype), it must first be converted into an RNA product. The process of transcription, whereby an RNA product is produced from DNA, is therefore an essential element in gene expression. If this process fails in some respect, it will affect all the other steps that follow the production of the initial RNA transcript in eukaryotes, such as RNA splicing, transport to the cytoplasm or translation into protein (for reviews of these stages, see Nevins, 1983; Latchman, 1998).

The central role of transcription makes it an attractive control point for regulating the expression of genes in particular cell types or in response to specific signals. Today it is evident that, in most cases, where a particular protein is produced only in a specific tissue or in response to a particular signal, the specificity is achieved by control processes that ensure that its corresponding gene is transcribed only in that tissue or in response to such a signal (for reviews, See Darnell, 1982; Latchman, 1998).

Modulation of transcriptional activity is fundamental to the regulation of gene expression. It is associated with most biological phenomena and is largely mediated through proteins that interact directly or indirectly with specific DNA sequences (cis elements) in the promoter region of genes (Ferl & Paul, 2000). In recent years a large amount of information about eukaryotic transcription factors has become available. This includes information on their biological roles, interactions with DNA sequences and other regulatory proteins, and three-dimensional structures. In general, transcription factors have modular structures composed of a few functional domains for binding to target DNAs, interaction with other proteins including other transcription factors and components of basic transcriptional machinery (Ferl & Paul, 2000). The categorization of transcription factors is based on certain structural motifs, which are conserved among species. These can be either in the DNA-binding domain or in the functional domain of the protein and fall into four major categories: helix-turn-helix motifs, basic leucine zippers, zinc fingers, and high-mobility group (HMG) box motifs (Ferl & Paul, 2000). Transcription factors containing the helix-turn-helix motif or the zinc finger motif will be discussed in more detail in this thesis.

Transcriptional regulation of the formation of secondary vascular tissues

Developmental processes like the formation of secondary vascular tissues, depend upon the regulated expression of many thousands of genes (Goldberg *et al*., 1978; Kamalay & Goldberg, 1980; Kamalay & Goldberg, 1984; Doebley & Lukens,

1998). Even if the intervening signal transduction steps remain mysterious, it can be assumed that these signalling inputs alter patterns of gene transcription, which in turn requires the activity of specific transcription factors. Very little is known about the transcriptional regulatory mechanisms involved in the formation of xylem and phloem. However, considerable progress has been made in understanding the roles of transcription factors in controlling lignification. Analyses of lignification genes have shown the presence in the promoter of conserved motifs that are important in xylem localized gene expression (Lacombe *et al*., 2000). Proteins that can bind to this motif and activate the transcription belong to the MYB family. In addition, MYB genes preferentially expressed in Pinus taeda xylem have been proposed to be involved in regulating transcription during xylogenesis (Newman & Campbell, 2000).

MYB transcription factors

Members of the MYB family of transcription factors have been found in nearly all eukaryotes. The first to be identified was the v-myb oncogene from the *Avian myeloblastosis* virus, which causes leukaemia in chickens (Lipsick, 1996). MYB transcription factors can be structurally dissected into a highly conserved N-terminal DNA-binding domain and a C-terminal transcriptional activation domain. The DNA-binding domain consists of a region of 50-53 amino acids that binds to DNA in a sequence-specific manner (Lipsick, 1996). In the vertebrate cellular (c) –MYB, this domain is, with a few exceptions, repeated three times. Each repeat adopts a helix-turn-helix conformation, allowing it to intercalate in the major groove of the target DNA. Three regularly-spaced tryptophan residues, which form a tryptophan cluster in the three-dimensional helix-turn-helix structure, are characteristic of a MYB repeat. These tryptophans play a role in the formation of the hydrophobic core of the MYB domain required for the helix-turn-helix fold (Klempnauer & Sippel, 1987).

Members of the MYB family often activate their target genes in close cooperation with DNA-binding proteins of other classes. The highly conserved MYB DNA-binding domain serves a complex role as both a DNA- and a protein-binding interface (Ness, 1999).

The C-terminal domain is highly variable among all plant MYB transcription factors. In plants, it has been estimated that there might be over 100 different MYB loci in each species (Martin & Paz-Ares, 1997), in contrast to only three *MYB* genes in vertebrates (Rushton *et al*., 2001). This is supported by the finding of 125 different genes encoding R2R3-MYB proteins alone in *Arabidopsis thaliana* (Stracke *et al*., 2001).

Plant MYB transcription factors are classified into three subfamilies, called MYB1R factors, R2R3-type MYB factors and MYB3R factors (Stracke *et al*., 2001). The classification depends on the number of imperfect repeats, R1, R2 and R3, found in the DNA-binding domain.

MYB transcription factors with a single repeat are fairly divergent, and include factors that bind the consensus sequence of plant telomeric DNA. It has also been shown that MYB1R factors can act as transcriptional activators (Baranowskij *et al*., 1994) and some are associated with the activity of the circadian clock (Schaffer *et al*., 2001). In addition, it has recently been shown that plant MYB3R factors similar to MYB proteins in animals are involved in controlling the cell cycle (Ito *et al*., 2001), indicating that there may be considerable degrees of functional conservation among MYB3R genes from plants and animals.

MYB genes containing two repeats (i.e. R2R3-MYBs) comprise the largest *MYB* gene family in plants. For most of the 125 R2R3-type MYB genes found in *A. thaliana* no functional data are available. However, R2R3-type *MYB* genes have been shown to regulate phenylpropanoid metabolism in *A. thaliana* (Borevitz *et al*., 2000). Analysis has also shown that R2R3-type MYB factors can act as transcriptional activators as well as repressors (Jin *et al*., 2000). Bender *et al*. (1998) have shown the involvement of a MYB-related protein in tryptophan biosynthesis, which demonstrates that the pathways controlled by such factors are not limited to secondary metabolism.

Another important function for R2R3-type MYB transcription factors is the control of development and determination of cell fate/differentiation and identity (Oppenheimer *et al*., 1991; Lee *et al*., 1999). R2R3-type MYB transcription factors also participate in plant responses to environmental factors and in mediating hormone actions: examples are discussed in Jin and Martin (1999). In other cases, MYB genes have been correlated with cell death during the hypersensitive response upon pathogen attack or elicitor treatment (Daniel, 1999; Sugimoto, 2000; Lee, 2001). Overall, the findings suggest that R2R3-type *MYB* genes are involved predominantly in controlling ”plant-specific” processes (Martin & Paz-Ares, 1997). This observation is especially interesting since MYB genes of the R2R3 type are, at least as far as we know today, only present in plants (Riechmann, 2000).

Zinc-finger transcription factors

The term “zinc finger” refers to the sequence motifs in which cysteines and/or histidines coordinate (a) zinc atom(s) to form local peptide structures that are required for their specific functions. The zinc-finger motifs, which are classified according to the arrangement of the zinc-binding amino acids, are present in a number of transcription factors and play critical roles in interactions with other molecules. Some classes of zinc-finger motifs (e.g. the TFIIIA and GATA types) are, in most cases, part of the DNA-binding domains of transcription factors and have been shown to be directly involved in the recognition of specific DNA sequences (Takatsuji, 1998). Other classes (e.g. LIM and RING-finger types) are mostly implicated in protein-protein interactions. Most of the eukaryotic zinc-finger motifs have been found in plants. In addition, some new types of zinc fingers have been identified (e.g. WRKY and Dof motifs) that have only been identified in plants to date (Takatsuji, 1998).

Recent results indicate that a member of the LIM class of zinc fingers is involved in the regulation of phenylalanine ammonia-lyase (PAL) (Kawaoka & Ebinuma, 2001). Transgenic tobacco plants with antisense Ntlim1 have shown low levels of transcripts from some key phenylpropanoid pathway genes encoding enzymes such as phenylalanine ammonia-lyase, hydroxycinnamate CoA ligase and cinnamyl alcohol dehydrogenase. Furthermore, a more than 20% reduction in lignin content has been observed in transgenic tobacco expressing antisense Ntlim1 (Kawaoka & Ebinuma, 2001).

Objectives

The objective of this study was to investigate molecular factors involved in secondary cell wall formation and lignification. A novel CuZn-SOD with a high isoelectric point (pI) and thus denoted hipI was characterized in pine. Its localisation in lignified tissues prompted interest in its role in secondary cell wall formation and lignification. In order to study the expression, activity and possible function of hipI SOD during these processes, a *Zinnia* mesophyll cell system was set up and the enzyme was studied during the trans-differentiation from mesophyll cell to a hollow tracheary element with a lignified secondary cell wall.
Secondary cell wall formation and lignification involves complex structures and are intricate subjects, so there was interest in approaching them from additional angles. Regulated gene expression is bound to play an important role in secondary vascular tissue formation, and the involvement of transcription factors is an important aspect of regulated gene expression. However, the mechanisms whereby transcription factors regulate wood formation are still poorly understood. Therefore, transcription factors from the MYB family and Zn-finger family from the cambial region of hybrid aspen were characterized and their regulation was studied. In addition, to further investigate the function of some MYB-related transcription factors, transgenic hybrid aspen plants expressing antisense constructs of the factors were produced and investigated.

Methodological considerations

Techniques used

General techniques

The studies presented in this thesis relied on standard molecular biology techniques, such as gene cloning, gene expression studies, PCR-based methods and fundamental biochemical methods. Here some of the methods that were used are elaborated, because they were important elements of the work, and the reader may not be fully familiar with them.

The Zinnia *cell culture system*

To study different processes involved in the formation of secondary cell walls and lignification an obvious tool to use was the well-characterised *Zinnia* cell culture system (Kolenbach & Schmidt, 1975; Fukuda & Komamine, 1980; Sato *et al*., 1993; Milioni *et al*., 2001). For this, mesophyll cells from *Zinnia elegans* L. were isolated mechanically and incubated in an inductive media containing auxin and cytokinin. After 72-96 h the cells trans-differentiated into tracheary elements, i.e. dead, hollow cells with lignified secondary cell walls **(II)** (Figure 5).
To be successful with this technique the growth conditions for the plants used to supply the cells seem to be crucial. They must not be put under any kind of stress, such as drought or pathogens. It should also be noted that the *Zinnia* cell culture system is not a completely synchronised system since the different cells do not differentiate simultaneously. Furthermore, every cell culture is unique and it should be stressed that the number of tracheids that are differentiated in one cell culture may differ considerably from the number differentiated in another culture. Thus, careful interpretation of the results from different experiments is necessary.

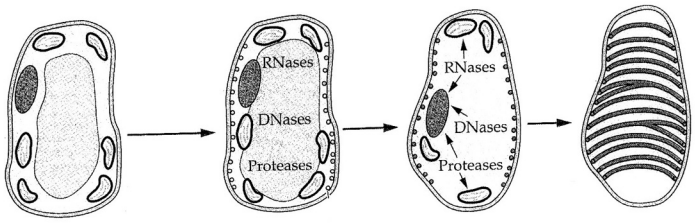


Figure 5. Photosynthesising mesophyll cells from the first true leaves of *Zinnia elegans* were isolated mechanically and incubated in an inductive media. About 6 h after the start of secondary cell wall synthesis the central vacuole collapses and hydrolytic enzymes are released. Within a few hours after the collapse of the vacuole degradation of most cellular content has occurred. After 72-96 hours the cells has trans-differentiated into dead, hollow cells with lignified secondary cell walls, i.e., tracheary elements. (Dangl et al., 2000).

Relative quantitative RT-PCR

In order to characterize the very weakly expressed *MYB* genes, relative quantitative RT-PCR seemed a good choice of method **(III)**. RT-PCR overcomes the limitations imposed by low numbers of transcripts. Relative quantitative RT-PCR was performed using 18S internal standards. The majority of RNA consists of rRNA, the level of which remains essentially constant from sample to sample. Since an endogenous control must be in the same linear range as the RNA of interest, the abundance of rRNA is a problem. In the protocol adopted in this study (Ambion), the limitation of using rRNA as an endogenous control was solved by using competitive primers to modulate the amplification efficiency of a PCR template without affecting the performance of other targets in a multiplex PCR. In each PCR reaction two products were obtained: the gene-specific product and the 18S standard. Thus, there was an internal control to confirm that the PCR reaction had worked. It was also possible to relate the expression of the gene-specific product to that of the 18S standard.

Relative quantitative RT-PCR is considered to be a rapid method for estimating transcriptional levels of genes in plants but it can be extremely time consuming under certain circumstances, e.g. optimisation of the PCR-reactions can be laborious. The problems faced with RT-PCR can be caused by factors such as poor cDNA quality, e.g. RNA may be left in the cDNA sample and interfere in the PCR reaction. Problems can, of course, be caused by bad primer design, or the PCR machines may not heat and/or chill the samples efficiently (for various reasons) causing uneven results.

Plant transformation

The transgenic plants described in this thesis and in paper **IV** were generated by transforming cuttings of hybrid aspen (*P. tremula* L. x *tremuloides* Michx.) clone T89, cultured in vitro, with *Agrobacterium tumefaciens* strains carrying the gene construct of interest according to a protocol developed by Nilsson *et al*., (1992). Cells that incorporated the construct also incorporated resistance to certain antibiotics. A nutritionally complete plant medium (Murashige and Skoog, MS) containing mineral nutrients and supplemented with plant hormones, sucrose and appropriate antibiotics was used to select for the transformed cells. Only transformed cells could develop into plants in the presence of the antibiotic. Antibiotic resistant plants were further grown on an elongation-inducing medium without antibiotics. Cuttings from these elongated plantlets were taken for rooting on a half-strength MS medium without plant hormones and sucrose. Cuttings of elongated, rooted transformed plants were transferred to the hormone-free medium on a regular basis. The plants were rooted and maintained in vitro or planted in soil. In this thesis only work with primary transformants is discussed, since the regeneration time of hybrid aspen is at least approximately 10 years. The slow growth and long regeneration time of a tree system such as hybrid aspen are major drawbacks. It takes a significant amount of time for plants to become big enough to be analysed, and the fact that homozygous plants cannot be obtained by crossing, due to the long regeneration time required, will unfortunately cause the loss of information. A homozygous plant would provide valuable information concerning the phenotype that would result from the gene being down-regulated on both chromosomes of a chromosome pair of the diploid hybrid aspen plants. However, hybrid aspen has the big advantage of being a tree and as such forming secondary vascular tissues.

Antisense techniques

In one of the studies this thesis is based upon **(IV)**, the promoter from Cauliflower Mosaic Virus (CaMV) 35S was used. Nilsson *et al*. (1992) have investigated the expression pattern of this promoter in hybrid aspen, and shown it to be fairly ubiquitous. The promoter was used to obtain the antisense plants generated and examined in the presented experiments **(IV)**.

Antisense techniques can be used to down-regulate both the transcription of the corresponding gene, and the levels of corresponding proteins. This enables the functionality of specific genes to be eliminated in plants where screening for

mutants is difficult. The antisense effect can be caused by transcribing the target gene in the reverse of its wildtype direction (i.e. from its end to its start). The gene fragment in antisense orientation is inserted behind a strong promoter such as CaMV 35S followed by a poly-adenylation site. The gene product obtained from a construction like this is believed to hybridise with the endogenous copy and affect RNA stability, transcription and/or translation directly, or to generate a signal that induces gene silencing and pathogen responses (Fagard & Vaucheret, 2000). This will normally result in a reduction or lack of transcript of the target gene. To succeed with this technique it is important to use a gene or piece of a gene that has very high homology to the corresponding gene.

Results and discussion

The main objective of the studies described in this thesis were to closely examine some of the complex processes involved in the formation of secondary vascular tissues and lignification (see the Introduction of this thesis for a brief discussion of these processes).

The expression, localisation and function of a novel hipI-SOD

Traditionally, a great deal of research has been performed on pine due to its economic importance for generating timber and pulp. This study involved investigation of the gene expression and localisation of a novel CuZn-SOD in pine. In addition, its potential functions in lignification and secondary cell wall formation were investigated in an *in vitro* system: the *Zinnia* cell culture system.

HipI-superoxide dismutase in Scots pine: purification, cloning, antibody production and localisation

SODs with high pI:s represent a separate group of SOD isoforms that migrate on IEF gels to > pI 7, which makes them easily distinguishable from cytosolic (cyt) and chloroplastic (cp) SODs which are detected at pI 5.5 (Schinkel *et al*.,1998). A cDNA corresponding to one of these isoforms was isolated from homogenates of Scots pine needles. Both the chromatographic and gel electrophoretic properties of the protein indicate that this SOD is a dimeric enzyme. The final preparation contained a single protein band at a position corresponding to a size of 16kD. The pI of the active enzyme, as determined by IEF on a pH gradient gel of 6.5 to 10.5, was about 10.2 and it was named high pI-SOD (hipI-SOD) **(I)**. The protein represents a CuZn-type of SOD enzyme. To characterize the expression of hipI-SOD in different organs and tissues of Scots pine, poly(A+) RNA isolated from the stem and needles was analysed by northern hybridisation. A hipI-SOD transcript of about 1,000 bp was found in samples isolated from stem tissues and in both primary and secondary needles, with higher expression in secondary needles. Among stem tissues the highest transcript level of hipI-SOD was found in phloem. The same northern blot was used for hybridisation with two other probes

containing cyt- and cp-SOD cDNAs, and it was found that their expression patterns differed **(I)**.
Polyclonal antibodies were raised against purified hipI-SOD protein and were used for immunohistochemistry experiments. The protein was localised in the sieve elements, secondary cell walls (sCW) of the xylem, bordered pits of xylem elements and intercellular spaces. The localisation of the protein in lignified structures like the cell walls of tracheids and their bordered pits, structures known to be highly lignified, indicated that SOD might participate in the lignification process by generating H_2O_2 **(I)**. The contribution of SOD to lignification has been suggested previously on the basis of experiments showing the co-localization of "cytosolic" CuZn-SOD in vascular tissues of spinach hypocotyls and sites of H_2O_2 production (Ogawa *et al*., 1996; Ogawa *et al*., 1997).

HipI-SOD, H_2O_2 and Zinnia

To further investigate the potential role of H_2O_2 and hipI SOD in the process of lignification, the *Zinnia* mesophyll cell culture system seemed to be a good model system (Kolenbach and Schmidt, 1975; Fukuda and Komamine 1980). The amount of hipI-SOD present was studied during the differentiation process from mesophyll cell to tracheary element using a polyclonal antibody obtained from *P. sylvestris* **(I)**, together with isoelectric focusing gels and SDS gels. The protein was only weakly expressed during the first 48 h, when no tracheary elements (TEs) were visible, but it was present at progressively higher levels as the number of TEs in the cell culture increased **(II)**.

The subcellular distribution of hipI-SOD was investigated by immunogold electron microscopy. After 48-72 h, in cells before and during the first appearance of secondary cell wall (sCW) formation, strong hipI-SOD labelling was observed in Golgi bodies and at the plasmalemma in close vicinity to these organelles. After 120-144 h the secondary cell walls of mature TE:s showed strong labelling of hipI-SOD.

To study levels of H_2O_2 in the cells during TE formation, two techniques were used: fluorescence activated cell sorting (FACS) and fluorescence microscopy, using DCFH-DA as a probe. DCFH-DA is a flurochrome that is commonly used for detecting reactive oxygen species, especially H_2O_2 (Oyama *et al*.,1994). At the time when sCW develops, small scavenger molecules of H_2O_2, inhibitors of NADPH oxidase and CuZn-SOD and a peroxidase inhibitor were applied. In all these cases, a dramatic or moderate reduction of fluorescence was observed, reflecting a decline in H_2O_2 levels in the developing tracheary elements. Addition of SOD increased the intensity of the fluorescence, indicating a rise in the production of H_2O_2.

The experiments described above showed H_2O_2 to be present in the sCW during the development of TEs in the *Zinnia* cell culture system, and that NADPH-oxidase and SOD might be involved during this stage of development **(II)**.
The first sign of sCW formation in differentiating TEs was accompanied by lignification. If scavengers of H_2O_2 were applied at the time when the sCW started

to develop, the amount of lignin in the mature TEs was reduced. Similarly, if production of reactive oxygen species (ROS), such as O_2^-. and H_2O_2, was inhibited by inhibitors of NADPH oxidase and SOD, reductions in lignin content were observed. Peroxidase inhibitors also reduced the lignin content (Figure 8). The results suggested that H_2O_2 plays a key role in the lignification process and supports a previous report suggesting that peroxidases oxidise monolignols to radicals, thereby initiating the polymerisation of monolignols to lignin (Boudet *et al*., 1995; Østergaard *et al*., 2000; Ros-Barcelo *et al*., 2002).

If cells after 48 h, before the initiation of TE formation, were treated with inhibitors of NADPH oxidase, (CuZn-SOD, peroxidase and H_2O_2, a very low frequency of TE:s developed, supporting reports concerning the necessity of H_2O_2 for the differentiation of sCW:s (Potikha *et al*.,1999) **(II)**.

However, a number of questions concerning hipI-SOD remain to be resolved. For instance, immunohistochemical experiments showed hipI-SOD to be localised in the Golgi apparatus and extracellular compartments like the secondary thickenings in the mature xylem **(I, II)**. These results suggest that the hipI-SOD gene should include a leader sequence (Galili *et al*., 1998). Leader sequences encode signal peptides that are responsible for the import of the protein into the ER so that it can be transported out of the cell in the ER/Golgi vesicle system. No such leader sequence has been identified in either the Scots pine **(I)** or hybrid aspen transcripts (Schinkel, 2001). However, although most proteins that are exported out of the cell have such leader sequences, there are exceptions (Ye *et al*., 1988; Revest *et al*., 2000). Some proteins contain internal signal sequences that are not cleaved off, and this may be the case for hipI-SOD (Schinkel, 2001).

Furthermore, it should be noted that since hybrid aspen contains at least three different genes encoding hipI-SOD (Schinkel *et al*., 2001), it probably comprises a small gene family. It is not clear why there are so many virtually identical isoforms, or if they have different functions and/or localisations. Additionally, the specificity of the different inhibitors applied on the *Zinnia* mesophyll cell system **(II)** can be criticized. For example, Bolwell *et al*. (1998) demonstrates that DPI inhibits peroxidase-mediated generation of H_2O_2 as well as NAD(P)H-oxidase mediated generation of O_2^-.

Transcriptional regulation of secondary vascular tissues and the phenylpropanoid pathway

Not much is known about the transcriptional events that regulate xylem and phloem formation. However, transcriptional control is bound to play a significant role in developmental processes like the formation of secondary vascular tissues as shown by Hertzberg *et al*. (2001).

MYB transcription factors comprise the largest family of transcription factors in plants. They have been shown to be involved in a wide spectrum of plant-specific processes, as well as processes that are common to both plants and other

organisms. Studies on the function of MYB transcription factors has been performed in species as *Arabidopsis*, *Antirrhinum* and *Nicotiana tabacum* L., but very little work have been performed in a tree model system, which is an essential complement to work done on other species. In contrast to herbaceous species such as *Arabidopsis*, trees produce large quantities of secondary xylem (wood), and improvement of wood is an important goal for the forest industry.

Characterisation and regulation of MYB-related transcription factors in hybrid aspen

Today, more than 95 000 poplar ESTs (expressed sequence tags) have been sequenced from over 20 different cDNA libraries by the Swedish Centre for Tree Functional Genomics. The libraries have been constructed from different tissues at various developmental stages (F. Sterky, pers. comm.). Libraries of this EST collection were screened to identify genes encoding MYB-related transcription factors involved in vascular tissue formation **(III)**. Ten ESTs encoding proteins homologous to MYB-related transcription factors were present in libraries from activated cambium (AC), general vascular tissues including xylem and phloem (VT), cells undergoing cell death in their final stage of differentiation (WZ) and tissues with developed tension wood (TW). Three MYB genes from the cambial region in hybrid aspen (*Populus tremula* L. x *tremuloides* Michx.) were isolated and cloned, two of which (*PttMYB46* and *PttMYB76*) were only found in the VT-library. The third gene (*PttMYB75*) was found in two additional libraries, TW and WZ as well as the VT library. Two of the genes, *PttMYB75* and *PttMYB76*, belonged to the R2R3-type of MYB transcription factors, while the third gene was a MYB3R transcription factor. The gene expression patterns were studied in different organs and tissues **(III)**. Two of the genes, *PttMYB46* and *PttMYB75*, had ubiquitous patterns of expression, while *PttMYB76* showed very strong expression in xylem, but was hardly detectable in the other tissues. The pattern of expression of the three MYB genes in the stem of hybrid aspen was investigated at higher resolution by dot blot assays. *PttMYB46* was most strongly expressed in the secondary cell wall formation zone, but low levels of the transcript were also detected in other vascular fractions. Transcript levels of *PttMYB75* were high in the cortex, lower in phloem fibres and the zone of secondary cell wall formation, and hardly detectable in the remaining layers. *PttMYB76* was strongly expressed in the zone of secondary cell wall formation, less strongly in phloem fibres and very weakly in remaining sections **(III)**.

Plant hormones seem to be important developmental regulators of the secondary vascular tissues (reviewed by Mellerowicz *et al*., 2001) and since the gene expression studies suggested that the three MYB-genes might also be involved in the formation of vascular tissues it was of interest to analyse if they were subject to hormonal regulation and/or sugar regulation, since sugars can act as signalling molecules, controlling gene expression and developmental processes in plants in a similar manner to classical plant hormones (Sheen *et al*.,1999). Therefore, stem segments of hybrid aspen were subjected to various hormones, auxin (IAA), cytokinin (BAP), auxin + cytokinin (I+B), gibberellin (GA)) and sucrose

treatments. The results indicated that the genes were under hormonal regulation. The results of the sucrose treatment showed that *PttMYB75* and *PttMYB76* also were under sucrose regulation. In addition, the results suggested that the genes were under tissue-specific control since their expression levels differed between phloem and xylem **(III)**. Tension wood provides a system with altered anatomical and chemical properties, so studies of gene expression during tension wood formation may provide valuable information about the function of genes involved in developmental processes. Therefore, expression of the genes was studied in tissues from the tension side of trees that were bent for different lengths of time **(III)**, and the results suggested that *PttMYB46* and *PttMYB76* may play roles in tension wood formation. A gradual reduction in the amount of *PttMYB46* transcript was observed, indicating that it may be involved in cell cycle regulation. The lower amount of *PttMYB75* and *PttMYB76* transcripts in phloem six hours after induction coincides with the down-regulation of PAL and CAD transcripts observed by Stenberg (2000) in the same experimental material.

Characterisation of MYB antisense plants

In order to explore further the role of MYB in hybrid aspen, transgenic plants expressing the 3' part of the cDNA of *PttMYB76* or *PttMYB46* in antisense orientation were constructed.

Analysis of PttMYB76 antisense plants

Out of twelve independently obtained transgenic lines, four were selected for growth analysis. All of these transgenic lines grew more slowly than WT, and one line (76V) exhibited clear phenotypic divergence from WT, being stunted, with fewer and shorter internodes **(IV)**. It also displayed reduced radial stem growth compared to the WT. The other transformed lines selected for growth analysis (76III, 76VI, 76VIII) showed phenotypic traits that resembled those of 76V, although the deviations from WT were weaker **(IV)**. 76V also showed a somewhat altered leaf-shape. The southern blot analysis detected a single copy of the gene in the WT, and a fragment of the same size was found in all transgenic lines investigated. Line 76V had four additional fragments, i.e. four T-DNA inserts, line 76VIII had three inserts, while lines III and VI both had one insert. The severity of the phenotype of the transgenic lines examined may be related to the number of inserts of the antisense fragments.

To characterise the transgenic lines a variety of different methods was applied. For some of the more detailed analyses, in which stem anatomy, HR/MAS NMR spectra, PAL activity and lignin composition were examined, only the most severely affected line (76V) and WT controls were included. In the remaining experiments, in which phenolic concentrations, lignin and carbohydrate concentrations, and resistance to infection were assessed, line 76III was analysed as well. This line contained only one insert, but exhibited a similar phenotype to line 76V, having a harder stem than WT, for instance.

Analysis of the stem anatomy in line 76V and WT revealed strong anatomical alterations in the antisense line, e.g. reduction of vascular bundles in young internodes, reduction of phloem fibres and abnormal vessel differentiation **(IV)**.
The severe phenotypical alterations observed in lines carrying the antisense construct of *PttMYB76* prompted us to study how the transgenic lines and WT differed metabolically. NMR analyses (Claridge, 1999) combined with multivariate statistical treatments of the data, including PCA and PLS (Martens & Neas, 1994), showed that stems from WT and line 76V have different chemical profiles **(IV)**. Additionally, earlier results in *Antirrhinum* demonstrate that overexpression of two different *MYB* genes represses phenolic acid metabolism and lignin biosynthesis (Tamagnone *et al*., 1998). To further evaluate the chemical differences between WT and transgenic plants the lignin and carbohydrate concentrations, and also the lignin composition, in the wood and bark of WT and *PttMYB76* antisense plants were studied. No significant differences in lignin and carbohydrate concentration were observed in the wood between the WT and transgenic lines **(IV)**. However, an 18% increase in the syringyl-/guiacyl lignin (S/G) ratio in the stem was found in line 76V compared to WT **(IV)**. Interestingly, lower concentrations of carbohydrates and higher concentrations of acid soluble lignin, i.e., phenolic compounds including lignin, in the bark, were found in line 76V compared to WT (Table 1). This indicates that some of the carbon usually allocated to carbohydrates may have been switched to phenolic compounds in line 76V.

Table 1. Lignin and carbohydrate concentration of PttMYB76 antisense plants and WT of hybrid aspen. Lignin and carbohydrate concentration was analysed in bark on 10 weeks old plants (n=4). Values represent mean ± standard deviation for n=4.

	Acid soluble lignin	Acid insoluble lignin	Total lignin	Total carbohydrates	Carbohydrate/ lignin
	% dry wt	% dry wt	% dry wt	% dry wt	
WT	4.15±0.31	24.43±1.91	28.57±2.21	42.79±2.01	1.76±0.18
76 IIIB	4.97±0.11	25.93±1.97	31.13±2.14	38.19±3.69	1.48±0.26
76 V	7.44±0.46	27.70±2.34	36.14±1.47	27.97±3.59	1.02±0.21

The phenolic concentrations in leaves of both young plants grown *in vitro* and six-week-old potted plants were examined and compared in lines 76III, 76V, and WT **(IV)**. In potted plants, the levels of some individual phenolic acid derivatives of cinnamic acid and chlorogenic acid were markedly higher in the transgenic lines compared to WT. The high levels of cinnamic acid derivatives in line 76V, in which the composition of lignin was altered may reflect the involvement of the analysed cinnamic acid derivatives in lignin metabolism (MacAdam & Grabber 2002). Interestingly, the results revealed differences in the levels of some phenolic compounds between sterile-cultivated and potted seedlings. These changes may be due to differences in the ontogenic phase between the two types of plants (Bryant & Julkunen-Tiitto, 1995) or the different growth conditions **(IV)**. The

concentrations of salicylates were also investigated. Higher than WT levels of salicin and salicortin were found in line 76V, but not in the other transgenic line. The observed growth reduction in transgenic plants as shown in paper **IV** could be related to the metabolic costs of synthesis and maintenance of a higher level of phenolics. In several woody plants a negative relationship between carbon-based defensive secondary metabolites and growth has been observed (Ruuhola, 2001). Phenylalanine ammonia lyase (PAL) is a major control point of the phenylpropanoid pathway (Bate *et al*. 1994), and MYB-related proteins are believed to be involved in its regulation (Jackson *et al*. 1991; Grotewold *et al*., 1994; Sablowski *et al*., 1994; Sablowski *et al*., 1995). Thus the activity of PAL was assayed. In the youngest leaves of line 76V the PAL activity was reduced compared to WT. In more mature leaves, like the first fully expanded leaf (comparable to the leaves sampled for phenolic analysis), the differences were not significant. These results indicate that the down-regulation of PttMYB76 had no effect on, or only slightly reduced, PAL activity in the leaves. Therefore, the changes in phenolic contents induced by the antisense PttMYB76 construct presumably mainly originate from alterations in other steps of the phenylpropanoid pathway, or perhaps in the regulation of the amino acid phenylalanine. The accumulation of phenolics in the transgenic plants may also be due to slower catabolism or turnover of these compounds, or translocation from other plant parts (Wiermann, 1981). However, the reduced activity of PAL may also be an effect of feed back inhibition. If phenolic compounds accumulate in excess in the transgenic lines they may inhibit their corresponding biosynthetic enzymes, such as PAL. In the stem the PAL activity was lower compared to WT in all internodes examined **(IV)**. This may be related to the chemical deviations observed in this line compared to WT.

One of the most striking features of *PttMYB76* antisense plants was the increased density of hairs on their leaves and stem **(IV)**. Leaf trichomes are known to be important structural features of plant defences against herbivores. At least two members of the MYB family, *GLABROUS1* (*AtMYBGl1*) and *MIXTA* (*AmMYBMx*), influence the differentiation of trichomes (Oppenheimer *et al*., 1991; Noda *et al*., 1994; Glover *et al*., 1998), acting as positive regulators of trichome initiation. The hybrid aspen gene *PttMYB76* seemed to be a negative regulator of trichome differentiation because of the much higher trichome density found in the transgenic lines compared to WT.

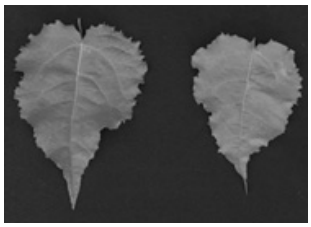
Line 76V seemed to be relatively resistant to insect attacks in the greenhouse and climate chambers (personal observations M. Karlsson). To study the defensive potential of transgenic lines in a more controlled manner, we compared the resistance of WT, line 76III and line 76V to an isolate of a common fungal pathogen of poplar, *Venturia tremulae*. Because of the higher levels of potential defences, i.e. phenolics and trichomes, in lines 76III and 76V, the expectation was that the transgenic lines would be better defended against the pathogen than the WT. However, no difference in resistance to this specific pathogen was observed between WT and the transgenic lines.

Analysis of PttMYB46 antisense plants

Of eight transgenic lines recovered, one (46III) showed a strongly altered phenotype with reduced length and stem diameter, together with fewer and shorter internodes compared to the WT (Figure 6 and 7A-C). The growth of this, and two other independent lines, was also monitored more intensively. One of the other lines (46II) showed a similar growth pattern to 46III, but the other line (46IV) did not show any significant growth alterations compared to the WT (Figure 7A-C). However, 46IV shared other traits with 46III, e.g., harder stem, especially in plants grown *in vitro* (personal observations M. Karlsson).

To verify the presence of *PttMYB46* antisense T-DNA in the transformed plants, they were checked by PCR, using a gene-specific forward primer and a forward primer designed from the 35S promoter. The plasmid containing the construct was used as a positive control and DNA from the WT was used as a negative control. Of ten independent lines, two did not contain the antisense construct.
In the following analysis, lines 46III and 46IV were included, since they showed altered traits at an early stage compared to WT *in vitro*.

A.



B.

Figure 6. Phenotypes of *PttMYB46* antisense line 46III and WT hybrid aspen plants. (A) 10-week-old plants, and (B) eighth leaf, counting from the first unfolded leaf from the top of ten-week-old plants. WT (left) and 46III (right) in both cases.

Lignin composition was analysed and compared in wood from one individual each of 46III and WT as in paper **IV**. The syringyl/guiacyl (S/G) ratio was increased by 15% in the transgenic plant (Table 2A). A lignin and carbohydrate concentration analysis was performed on four individuals each of WT and the transgenic lines 46III and 46IV. No significant differences were observed regarding lignin concentration in the wood, but a slight decrease in total carbohydrates was observed in the bark of the transgenic lines (Table 2B).

Phenolic analyses were performed on six-week-old potted plants in the same way as for *PttMYB76* antisense plants **(IV)**. These analyses were carried out on five

individuals each of 46III, 46IV and WT. 46IV showed a strongly reduced concentration of salicin, and slightly reduced levels of salicortin and tremulacin were observed in lines 46III and 46IV (Figure 8A-C).

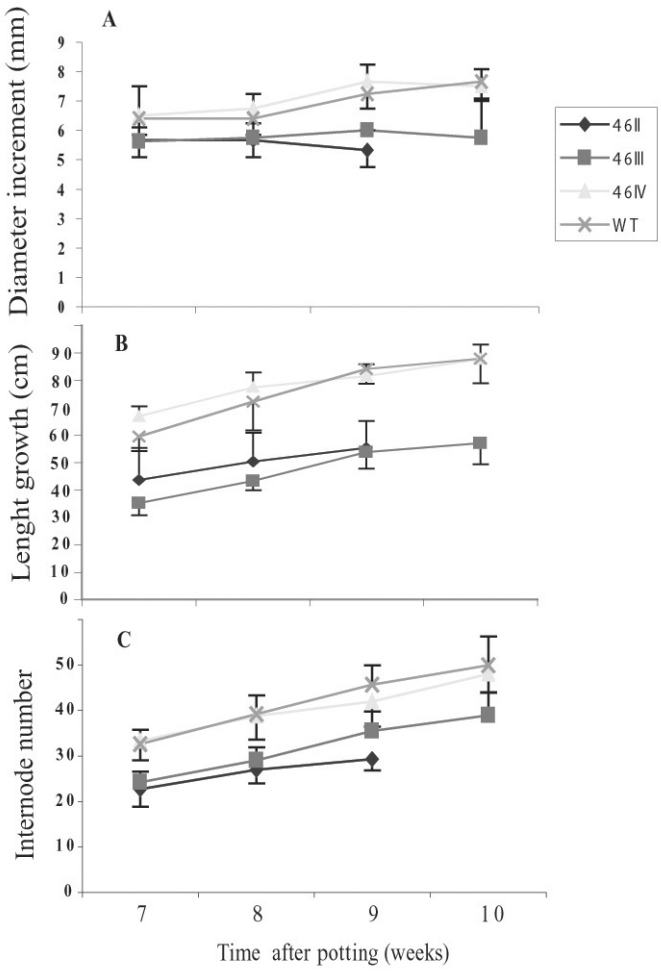


Figure 7. Growth characteristics of *PttMYB46* antisense lines 46II, 46III, 46IV, and WT hybrid aspen plants. Plants were measured 7, 8, 9 and 10 weeks after they were potted in soil. (A) Length growth (cm). (B) Diameter increment (mm). (C) Internode number. The error bars represent standard deviations (n=4) for the WT and transgenic lines. Exceptions: line 46II, week 7-10, n=3; line 46III, week 8, n=3; line 46IV week 9, n=3; and WT, week 10, n=3.

Table 2. (A) Lignin and carbohydrate concentrations and lignin composition of *PttMYB46* antisense plants and WT of hybrid aspen. Lignin and carbohydrate concentration was analysed in wood on 10 weeks old plants (n=4). The S/G ratio was analysed on whole stem (bark + wood) on approximately one year old plants of WT and line 46III (n=1). Values represent + standard deviation for (n=4).

	Acid soluble lignin % dry wt	Acid insoluble lignin % dry wt	Total lignin % dry wt	Total carbo-hydrates % dry wt	Carbo-hydrate/ lignin	S/G ratio*
WT	2.69±0.08	24.65±1.29	27.33±1.37	50.68±1.80	2.06±0.16	100%
46III	2.71±0.11	22.93±0.12	25.64±0.21	50.55±3.94	2.21±0.17	115%
46IV	2.56±0.15	24.46±1.35	27.01±1.45	48.08±3.55	1.97±0.18	n.d

Table 2 (B) Lignin and carbohydrate concentration was analysed in bark on 10 weeks old plants (n=4) of PttMYB46 antisense plants and WT of hybrid aspen.

	Acid soluble lignin % dry wt	Acid insoluble lignin % dry wt	Total lignin % dry wt	Total carbo-hydrates % dry wt	Carbo-hydrate/ lignin
WT	4.15±0.31	24.43±1.91	28.57±2.21	42.79±2.01	1.76±0.18
46III	4.13±0.37	25.17±2.68	29.30±2.90	36.23±2.24	1.46±0.22
46IV	3.48±0.06	25.53±3.26	29.01±3.32	37.36±0.57	1.49±0.21

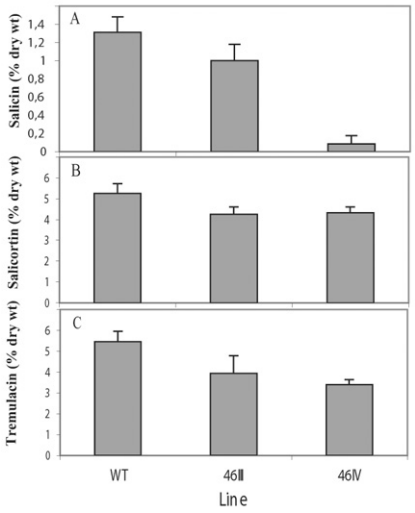


Figure 8. Concentrations (% of dry weight, salicin equivalents) of salicylates in six-week-old potted plants of *PttMYB46* antisense lines 46III, 46IV, and WT hybrid aspen: (A) salicin, (B) salicortin and (C) tremulacin. The first fully expanded leaf was collected (approximately leaf number 7, counting from the first unfolded leaf) and tested. Error bars represent standard errors (n=5).

The concentrations of three different chlorogenic acids and two different cinnamic acids were dramatically increased in both 46III and 46IV compared to WT (Figure 9A-E). Chlorogenic acid, 5-CQA is recognized as an antioxidant (Nardini *et*

al.,1995), a scavenger of reactive species of oxygen and nitrogen (Kono *et al*., 1997), and an inhibitor of the formation of conjugated diene from linoleic acid oxidation (Morishita & Kido, 1995).

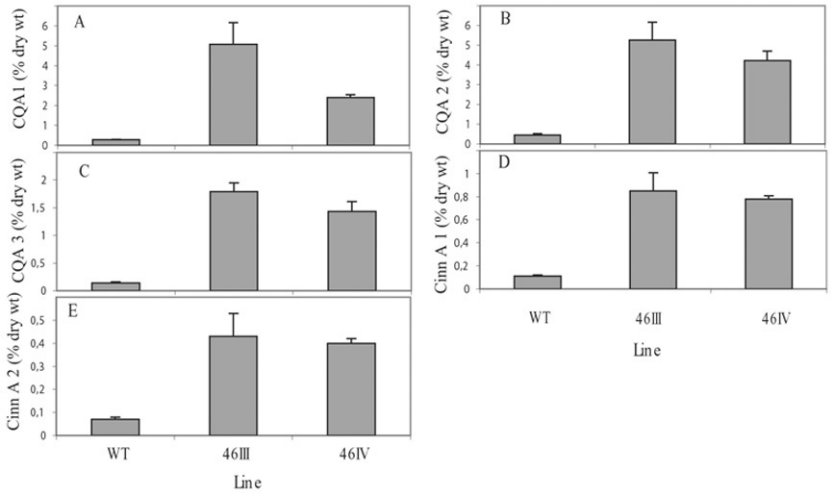


Figure 9. Concentrations of phenolic acids. % of dry weight, chlorogenic acid (CQA), A-C, or cinnamic acid (Cinn A), D-E, equivalents, in six-week-old potted plants of *PttMYB46* antisense lines 46III, 46IV and WT hybrid aspen. The first fully expanded leaf was collected (approximately leaf number 7, counting from the first unfolded leaf) and tested. Error bars represent standard errors (n=5).

The observed changes in the levels of phenolic acids in the transgenic plants indicate that PttMYB46 may have a role in the phenylpropanoid pathway at an early stage.

Infection experiments were performed with conidia of *Pollacia radiosa* (an anamorph of *Venturia tremulae*) on six-week-old potted plants of 46III, 46IV and WT, as earlier described above and in detail in paper **IV**. The results showed that the infection in the transgenic plants started two days earlier than infection in the WT (Figure 10A, B), and a larger percentage of the leaf area was infected in the transgenic lines compared to WT. It seems likely that the pathogen was able to use the phenolics as a carbon source in a similar way as in the infection experiments performed on antisense plants of *PttMYB76* **(IV)**.

As postulated for plants of line 76V, the reduction in growth in 46III plants may be related to the metabolic costs of synthesis and maintenance of a higher level of phenolics. Thus, the traits observed in these plants may, as discussed for the *PttMYB76* antisense lines **(IV)**, reflect a trade-off between investment in phenolics and biomass (Ruuhola, 2001).

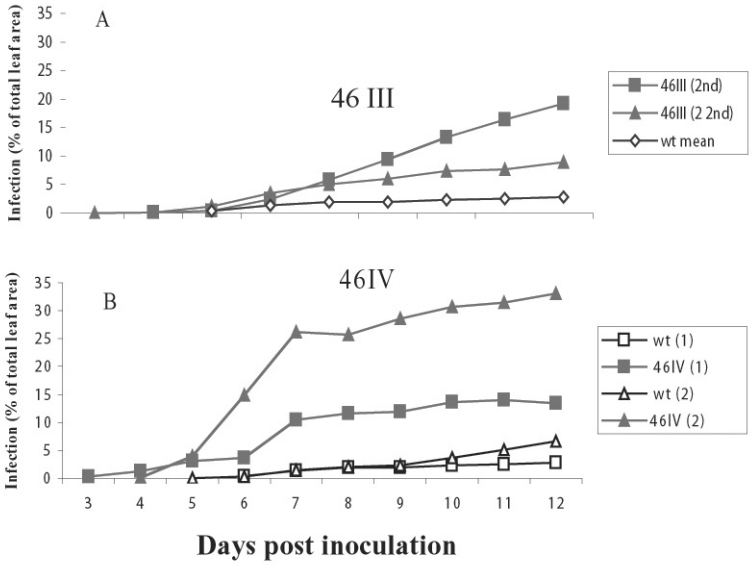


Figure 10. Percentage necrotic area of leaves from plants of *PttMYB46* antisense lines 46 III and 46 IV and WT hybrid aspen infected with *Venturia tremulae* as described in (IV). Results of experiments, repeated twice. (A) Infection experiments on six-week-old potted plants of WT and line 46III. (B) Infection experiments on six-week-old potted plants of WT and line 46 IV.

A novel Zinc-finger transcription factor in the cambial region of hybrid aspen: Its characterisation and regulation

The MYB family consists of a large group of transcription factors that are known to be involved in a wide spectrum of different processes. However, other groups of transcription factors are also of interest, e.g. those containing the zinc finger motif (the so-called "Zinc fingers"). Many of the (putative) zinc-finger transcription factors have been implicated in important biological processes (Takatsuji, 1997). For this reason, a novel zinc-finger was selected from a cambial EST-library of hybrid aspen (Sterky *et al*., 1998). The gene was cloned, and its predicted amino acid sequence was compared with sequences of proteins from *Arabidopsis thaliana* L. The protein with highest similarity, protein id: At2G16050, a putative protein deduced from its gene sequence, showed 58% identity to the hybrid aspen Zn-finger transcription factor. A CHP-rich zinc finger putative protein, protein id: At2g37820.1, showed 36% identity. The low

homology to sequences in *Arabidopsis* suggest that this is a novel zinc-finger transcription factor that may also be tree-specific.

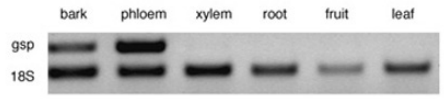


Figure 11. Relative expression levels of the zinc-finger gene in different organs and tissues quantified by RT-PCR. Total RNAs were prepared and subjected to RT-PCR as described in paper **(III)**. Gel bands obtained in a representative experiment corresponding to both PttMYB (gene specific=gsp) and 18S messages are shown. All samples were collected from hybrid aspen except fruits, which originated from aspen. Pooled samples from several individuals were used.

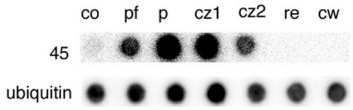
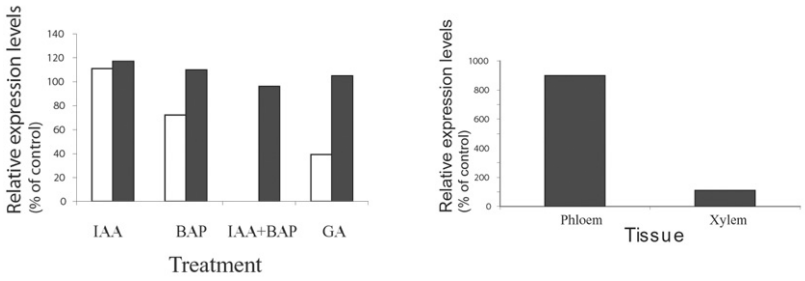


Figure 12. mRNA accumulation in developmental zones obtained from hybrid aspen stems, as revealed by dot-blot hybridisation of poplar cDNAs with a gene-specific probe for the zinc-finger gene. Polyubiquitin was used as a control. Abbreviations correspond to the following subsections of wood: cortex (co); phloem fibre (pf); phloem (p); cambial zone of phloem (cz1); cambial zone of xylem (cz2); radial expansion zone (re); and secondary wall formation zone (sw).

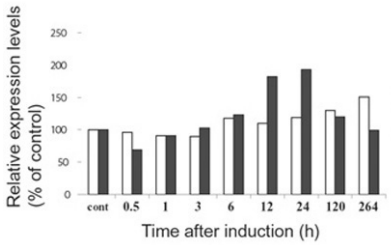
Distribution of the transcript of the novel gene was analysed in different organs and tissues by RT-PCR as earlier described in paper **III**. The expression of the gene was strong in phloem, somewhat weaker in bark, and not detectable or hardly detectable in the remaining tissues (Figure 11). Its expression was also analysed at higher resolution in stem by dot blot assay, as earlier described **(III)**, which showed that the expression was strongest in phloem and cambial zone1 (cz1), and somewhat weaker in cambial zone 2 (cz2) and phloem fibres (Figure 12). The expression analysis in the stem indicated that the gene is involved in cambial activities, where plant hormones and sugars seem to have important roles as regulators (Mellerowicz *et al.*, 2001, Uggla *et al*., 2001). Therefore, it was of interest to analyse whether the gene was subject to hormone and sugar regulation.

Hormone and sucrose treatment experiments were performed as earlier described in paper **III**. In the phloem sample treated with GA the transcript was significantly reduced, and in the phloem sample treated with IAA + BAP no transcript of the novel Zinc-finger transcription factor was detected. In the IAA and BAP samples no major changes in gene expression were detected in phloem. In xylem no major

changes were observed in gene expression after any of the hormonal treatments (Figure 13A).



A. B.



C.

Figure 13. (A) Tissue-specific relative expression levels (%) of the zinc-finger gene after treatment with different hormones. Stem segments of two individuals of hybrid aspen were treated with MS + sucrose (0.1 M) solution containing the following hormones: auxin (IAA) (500 µM), cytokinin (BAP) (10 µM) and gibberellic acid (GA) (5 µM) for 12 h. Total RNAs were prepared from pooled tissues of xylem or phloem and subjected to RT-PCR as described in (III). Stem segments subjected to MS + sucrose (MS + S) treatment served as controls and the expression level for each tissue in the controls was set to 100%. Open bars represent phloem and closed bars represent xylem. Abbreviation: nd, not detected.
(B) Tissue-specific relative expression levels (%) of the zinc-finger gene after treatment with sucrose. Stem segments of two hybrid aspen individuals were separately treated with sucrose (0.1M) dissolved in MS for 12 h. Total RNAs were prepared from pooled tissues of xylem or phloem and subjected to RT-PCR as described in (III). The control (MS) level for each tissue was set to 100%. Open bars represent phloem and closed bars represent xylem.
(C) Tissue-specific relative expression levels (%) of the zinc-finger gene of aspen after bending treatment. Unbent trees served as controls. Xylem (closed bars) and phloem (open bars) were isolated from the upper, tension wood forming side. Total RNAs were isolated from pooled tissues of three individuals for each time point and subjected to RT-PCR, as described in (III). Relative specific expression levels after different durations of bending are represented as a histogram. The control levels were set to 100 % expression.

The gene showed very strong induction in phloem after sucrose treatment (Figure 13B). The results suggest that this gene ,encoding a novel zinc-finger transcription, factor is under tissue-specific control since its responses in phloem and xylem to the hormone and sucrose treatments differed.
During tension wood induction the gene showed stable expression in both xylem and phloem in the first few hours after the bending had started. At 12 and 24 hours after induction the expression had increased by approximately 75% in the xylem sample. After five and eleven days the gene expression had returned to the same level as in the control. In the phloem samples, strong induction was seen after eleven days (Figure 13C).

Conclusions

A novel superoxide dismutase (SOD) with a high pI (hipI-SOD) was isolated and characterized in pine. The results of immunolocalisation experiments indicated that the protein was present in lignified tissues, suggesting that HipI-SOD may be involved in the lignification process. To further study this possibility, a *Zinnia* mesophyll cell system was set up and various inhibitors against hipI-SOD and H_2O_2-production were applied. In essence, it was found that reduction of H_2O_2 levels, and/or inhibition of SOD, reduced lignin formation in *Zinnia* tracheary elements (TE:s). Thus, hipI-SOD may have a novel and important function in the secondary cell wall formation and lignification processes as a regulator of H_2O_2. The expression pattern and localization of a hipI-SOD isoform during the TE differentiation support this assumption.

The genes encoding three MYB transcription factors and one novel zinc-finger transcription factor were isolated from a cambial EST library of hybrid aspen. Their expression patterns were studied in various organs and tissues. The gene expressions were also analysed after treatment with hormones, sucrose and bending.

The expression analysis of *PttMYB46* and *PttMYB76* indicated that they are involved in secondary cell wall biosynthesis, lignification or cell death. The results of *PttMYB46* suggests that a MYB3R factor can be involved in plant-specific processes besides cell cycle regulation. *PttMYB75* showed a high expression in tissues where there is little lignification which may indicate a function as a suppressor in the phenylpropanoid pathway. The gene expression of the novel zinc-finger transcription factor indicated a role in cell cycle regulation. This was supported by the increase in transcript observed during tension wood formation. Furthermore, the expressions of all investigated genes altered as tension wood was formed. Additionally, the results of the hormone and sucrose treatments indicate that the genes investigated are under hormone and sucrose control. The expression of the novel zinc-finger transcription factor and low homology to proteins in the herbaceous species *Arabidopsis thaliana* suggest that it is tree-specific.

To further evaluate the role of the genes, hybrid aspen plants carrying antisense constructs of *PttMYB76* or *PttMYB46* were generated. They showed a complex phenotype, including reduced growth and a dramatic increase in some phenolics. These results suggest that the plants invest more heavily than WT in carbon–based secondary metabolites, at the cost of reduced growth. Alterations in lignin composition, an increase in phenolic concentration and reduction in the carbohydrate concentration in bark in line 76V compared to WT were also observed. Finally, the transgenic lines, 76III and 76V also displayed an increased density of trichomes. The high concentrations of some phenolics, and the high trichome density in line 76V, are indicative of heavy investments in defensive characters in this line.

Infection experiments performed with the fungal pathogen *Venturia tremula* showed that in contrast to theoretical expectations, the transgenic lines were more susceptible to infection than the WT (especially lines carrying the *PttMYB46* antisense construct). This indicates that the increased concentration of phenolics may support the pathogen by providing an additional carbon source rather than promoting resistance. However, infection experiments ought to be performed with other kinds of pathogens and herbivores to evaluate the effectiveness of phenolics as plant defenses against pathogens.

Future plans

To further evaluate the function of hipI-SOD, overexpression in hybrid aspen and/or *Arabidopsis* may give valuable information.
One of the most obvious tasks for the future is to analyse whether the respective gene transcripts in plants carrying the *PttMYB76* and *PttMYB46* antisense constructs are down-regulated. For plants with the *PttMYB76* antisense construct, at least one more line should be analysed anatomically, chemically (HR/MAS NMR), and with respect to PAL activity and lignin composition. To complement the analysis of PAL activity, the activity of other enzymes in the phenylpropanoid pathway should be assayed. Micro-array experiments may provide additional information about the transgenic plants.
When it comes to plants carrying the *PttMYB46* antisense construct, the number of t-DNA inserts should be investigated. Stem anatomy should also be examined in at least two different lines.
Further studies of phenolic contents, but in the stem, of both *PttMYB46* and *PttMYB76* antisense plants should be interesting, to see if the results are consistent with the data obtained from leaves. To further investigate the defensive traits in the *PttMYB76* and *PttMYB46* antisense plants, studies involving wounding and infection with herbivorous organisms will be performed.
It may also be of interest to over-express the two genes in both *Arabidopsis* and hybrid aspen. Finally, the novel Zinc-finger gene has a very interesting expression pattern, and over-expressing it in *Arabidopsis* may give valuable insights into its functions.

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Sammanfattning

Bildningen av vaskulära vävnader (xylem och floem) är en komplex process som består av många steg, varav några studeras närmare i den här avhandlingen. Den första delen av detta projekt involverar identifieringen och karaktäriseringen av ett hittills okänt CuZn-superoxiddismutas i tall (*Pinus sylvestris*). Proteinet hade en ovanligt hög isoelektrisk punkt (pI) och fick därför namnet hipI-SOD (high pI-SOD). Immunolokaliseringsexperiment lokaliserade proteinet till lignifierade strukturer vilket antydde att detta specifika SOD protein kan vara delaktigt i bildningen av sekundära cellväggar och lignifiering. För att närmare undersöka proteinets roll i dessa processer startades cellkulturer upp bestående av mesofyll-celler från trädgårdsväxten *Zinnia elegans.* Dessa cellkulturer inkuberas i ett induktivt medium och efter 72-96 timmar har cellerna ombildats till döda, ihåliga celler med lignifierad sekundär cellvägg, så kallade trakeidala element. Detta gjorde det möjligt att följa uttrycket av hipI-SOD under bildningen av sekundära cellväggar och lignin. Olika inhibitorer mot SOD och H_2O_2 applicerades på cellkulturerna och resultaten indikerade att HipI-SOD kunde ha en hittills okänd och viktig funktion i bildning av sekundära cellväggar och lignifiering. Uttrycksmönstret och lokaliseringen av proteinet under bildningen av trakeidala element stödde detta antagande.

Den andra delen av projektet behandlar analysen av transkriptionsfaktorer och deras reglering i xylem och floem. Gener som kodar för tre MYB transkriptions-faktorer och en hittills okänd Zink-finger transkriptions-faktor plockades upp från ett kambialt EST-bibliotek i hybridasp (*Populus tremula x tremoloides*). Generna klonades och karaktäriserades och deras reglering efter påverkan av hormoner, sukros och gravitation undersöktes. Resultaten tyder på att generna regleras av hormoner och sukros. Genuttrycken påverkades också under dragvedsbildning. Nästa steg blev att göra transgena hybridaspar. Två av MYB-generna, som visade starkt uttryck i lignifierade vävnader, (*PttMYB46* och *PttMYB76*) nedreglerades. De transgena växterna uppvisade en komplex fenotyp. De växte långsammare och innehöll ökade koncentrationer av fenoler och ändrad lignin-komposition. Vissa av deras fenotypiska drag indikerade att växterna har investerat mycket i sitt försvar mot patogener, t ex skadeinsekter.