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Lignification in Development

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ABSTRACT

Developmental lignification is a process where lignin is deposited to the plant cell wall during the normal cell differentiation. Lignification of cell walls occurs where increased support or water impermeability is needed, such as in tracheary elements and in sclerenchymatic tissues of e.g. fibers and in the endocarp of some fruits. The chemical structure and amount of lignin varies between plant species, cell types, and cell wall layers and as response to environmental conditions. Lignification is apparently regulated by hormonal signaling (e.g. gibberellin) controlling the multiple enzymes via various transcription factors (e.g. MYB and LIM) involved in the monolignol biosynthesis pathway and probably by the level of reactive oxygen species in the apoplast. The most extensively studied area of developmental lignification is the lignification of xylem cells. In this review, two main areas of xylem research are covered: Lignin biosynthesis in xylem cells of trees, and the tracheary element differentiation in the *Zinnia elegans* cell culture. Knowledge on xylem lignification in trees has increased enormously during the recent years due to development e.g. in histological and in molecular biology methods, whereas the *Z. elegans* system presents a well characterized model for studying cellular events in xylem differentiation. In addition, studies on other cases of developmental lignin biosynthesis in particular in monocotyledonous species are included.

Keywords: gibberellin, grass, lignin, LIM, MYB, sclerenchyma, trees, xylem, *Zinnia*
Abbreviations: **ABC**, ATP-binding cassette; **CAD**, cinnamyl alcohol dehydrogenase; **Cald5H**, coniferaldehyde 5-hydroxylase; **CCoAOMT**, caffeoyl coenzyme A 3-*O*-methyltransferase; **CCR**, cinnamoyl-CoA reductase; **C3H**, *p*-coumarate 3-hydroxylase; **C4H**, cinnamate 4-hydroxylase; **4CL**, 4-coumarate coenzyme A:ligase; **COMT**, hydroxyconiferaldehyde 5-*O*-methyltransferase; **F5H**, ferulate coniferaldehyde 5-hydroxylase; **HCT**, hydroxycinnamoyl CoA: quinate/shikimate hydroxycinnamoyl transferase; **PAL**, phenylalanine ammonia-lyase; **POX**, class III peroxidase

CONTENTS

DEVELOPMENTAL CELL WALL LIGNIFICATION IN PLANTS

The arise of terrestrial life forms on earth set new demands to structural features of multi-cellular organisms. In order to increase in size on dry land, plants had to overcome forces of gravity and wind, which demanded additional supporting structures. The first examples of lignified tissues in plants come from the time of emergence of land plants of the early Devonian (Boyce *et al*. 2003). Presence of lignin in the plant cell wall made the structure rigid, more resistant to physical and chemical stress, and in addition, rendered the cells impermeable to water. In early land plants lignin deposition did not occur in water conducting tissues but in supporting tissues of the outer cortical layers. It was only in the development of eutracheophytes that lignified xylem developed to support the increasing plant mass, acting at the same time as water conducting tissue, a crucial step for the spread of plants to different environments (Boyce *et al*. 2003). However, it has been postulated further that the distribution of the hydrophobic lignin polymer and hydrophilic polysaccharides in the cell walls affect water conducting capacity of xylem tissue in such a manner that weak primary cell wall lignification favours high hydraulic conductivity e.g. in ferns and *vice versa* e.g. in conifer tracheids (Boyce *et al*. 2004).

Developmental lignification is a process where lignin is deposited to the plant cell wall during the normal cell differentiation. Those plant cells where extensive cell wall lignification is part of cell differentiation go through programmed cell death (PDC) at the end of the differentiation process. The hollow tubes of tracheary elements and structural fibers and masses of sclerenchymatic tissues e.g. in seeds and in the endocarp of some fruits are all dead cells with

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Invited Review

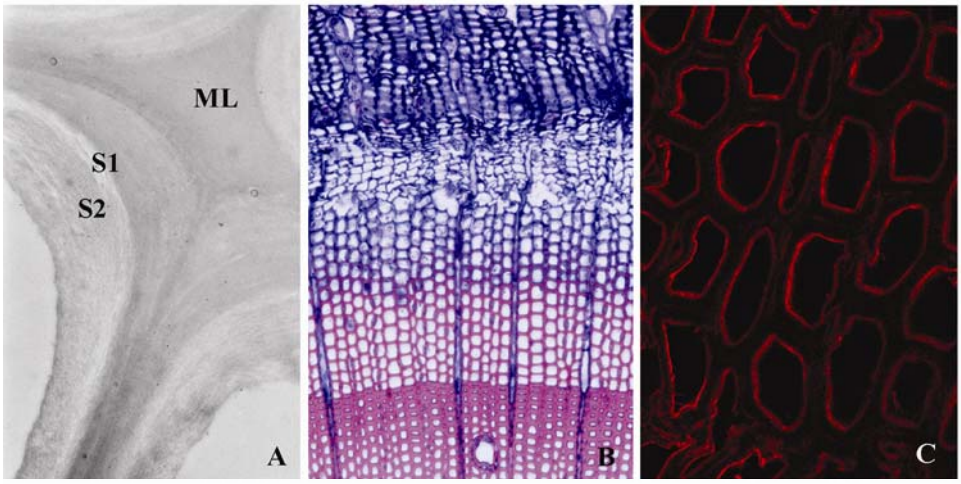

Fig. 1 Secondary cell wall structures and lignification as seen in stem xylem cross sections of Norway spruce (*Picea abies*). (**A**) electron microscopy image of developing secondary cell walls at the cell corner (M, middle lamella; S1, S2, secondary cell wall sublayers); (**B**) light microscopy image of developing xylem stained with safranin for lignin (red) and Alcian blue for cellulose (blue); (**C**) confocal image of developing xylem treated with an antibody against dibenzodioxocin (a lignin substructure) with Alexa Fluor 642 detection (red).

highly lignified cell walls, which are required for their biological functions in the plants. In this review, recent achievements in research of developmental lignification in xylem and some other tissues are described, with emphasis on the controlling factors of lignin deposition during tissue development. It is becoming more and more obvious that the amount of regulatory factors in developmental lignification is large, thus allowing both tight control and sensitive fine-tuning of lignification patterns during plant life.

LIGNIN STRUCTURES AND DEPOSITION

Lignin is a branched polymer of lignin monomers, mainly hydroxycinnamyl alcohols coniferyl, sinapyl and *p*-coumaryl alcohol (monolignols). The chemical structure of lignin varies between plant species. Generally, in gymnosperms lignin consists mainly of guaiacyl units derived from coniferyl alcohol while in angiosperms lignin is a co-polymer of sinapyl and coniferyl alcohols (syringyl (S) and guaiacyl (G) units). *p*-Coumaryl alcohol derived lignin is relatively high in monocots (H-type lignin) but it is present in both angiosperms and gymnosperms in small amounts. Lignin amount and composition is also different in different cell types and cell wall layers (Terashima *et al*. 1986), suggesting that lignins are deposited in a highly regulated manner for different biological functions.

Most of the information on proceeding of cell wall lignification comes from the studies on cell wall development in xylem cells. At the early stage of cell wall formation middle lamella and cell corner areas are porous containing cellulose and hemicelluloses embedded in a hydrophilic pectin gel (Carbita and Gibeault 1993). The secondary cell walls of xylem cells consist of three sublayers (S1, S2, and S3), which are formed at consecutive periods during cell differentiation (**Fig. 1A**). The carbohydrate matrix and the orientation of microfibrils influence lignin deposition (Taylor *et al*. 1992). In the middle lamella and the primary wall, lignin is spherically formed while in the secondary wall lignin forms lamellae that follows the orientation of the cellulose microfibrils (Carbita and Gibeault 1993).

Cell wall lignification initiates from cell corners and middle lamella, proceeding towards inner parts of the cell wall along with cell wall thickening (**Fig. 1B**). In lignin polymerization process monolignol units are linked together via radical coupling reactions either by carbon-oxygen (ether bond) or carbon-carbon bonds linkages. The most frequent inter-unit linkage is the β-*O*-4 (β-aryl ether) linkage, which is easily cleaved chemically for example in pulping (non-condensed lignins). Lignins containing mainly G units, such as in conifers, have high amounts of more resistant β-5 and 5-5 linkages (condensed lignins) than lignins incorporating S units, because of the availability of the C5 position for coupling (Boerjan *et al*. 2003). However, cell walls of vessel elements contain more G-units and consequently condensed lignin than fibre walls also in broadleaf trees (Grünwald *et al*. 2002). The dibenzodioxocin (5-5-*O*-4) substructure is frequently found from the S3 layer of conifers and broadleaf trees, and its formation could be related to the termination of lignin deposition (**Fig. 1C**; Kukkola *et al*. 2004).

During first stages of lignification, both in conifers and broad leaf trees relatively high amounts of H units are deposited to middle lamella. Condensed G in conifers and condensed G and GS lignin subunits in cell corner and intercorner middle lamella regions are also deposited prior to S1 formation (Terashima *et al*. 1998; Grünwald *et al*. 2002). Simultaneously with the completion of S1 polysaccharide deposition, non-condensed lignin subunits are deposited into S1 layer while S2 polysaccharide deposition initiates in outer secondary wall regions near cell corners. With on-going polysaccharide deposition in S2 layer, non-condensed lignification extends from the S1 to the outer part of S2 layer and subsequently towards the inner S2 after the start of S3 formation (Terashima *et al*. 1998; Grünwald *et al*. 2002).

LIGNIN BIOSYNTHESIS

The monolignols are synthesized in the cytosol, transported to the developing cell wall and polymerized by radical coupling (**Fig. 2**). Phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H) and 4-coumarate coenzyme A:ligase (4CL) catalyze the reactions initiating from phenylalanine and leading to synthesis of *p*-coumaryl CoA, which is the precursor of various secondary metabolites in plants, including the lignin monomers, the monolignols. PAL, C4H and 4CL exist as small gene families in various plants, and it has been found that different members of these gene families function in the synthesis of different secondary metabolites (Kao *et al*. 2002; Hamberger and Hahlbrock 2004; Lu *et al*. 2006). In *Pinus taeda* cell culture, the transcript levels of several genes involved in monolignol biosynthesis were up-regulated and the amounts of coniferyl and *p*-coumaryl alcohols were increased by providing the cultures with saturating levels of phenylalanine, showing that amount of phenylalanine is one of the controlling factors of monolignol biosynthesis (Anterola *et al*. 2002). Down-regulation of C4H causes decreased PAL expression in transgenic tobacco, which indicates feedback regulation of PAL by cinnamate (Blount *et al*. 2000). PAL1 and C4H co-localise in the ER membrane at least in tobacco, which enables efficient metabolic channeling between the two enzymes and thus may control the feedback inhibition of PAL by reducing the size of cellular cinnamate pool (Achnine *et al*. 2004).

Monolignols are synthesized from *p*-coumaryl CoA through multiple enzymatic reactions (**Fig. 2**). The common enzymes in the synthesis all the three monolignols, *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol, are cinnamoyl-CoA reductases (CCR) and cinnamyl alcohol dehydrogenases (CAD). These enzymes exist as small gene fa-

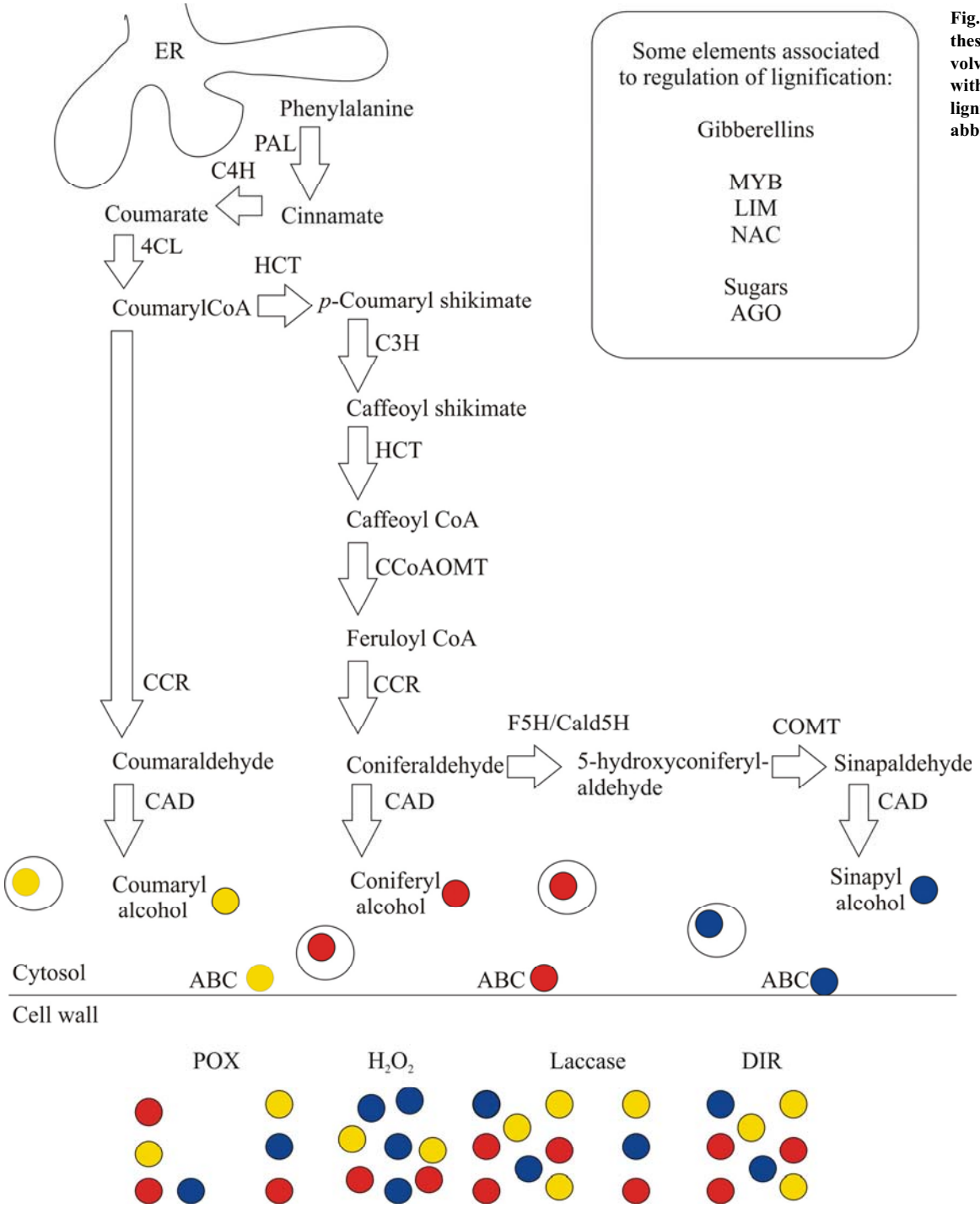

Fig. 2 Lignin biosynthesis. Enzymes involved in/associated with biosynthesis of lignin (see text for abbreviations).

milies and due to their expression profiles can be divided into those assigned for developmental lignification and others which are defence related (reviewed by Boudet *et al*. 2004). According to the current view of monolignol biosynthesis, *p*-coumarate 3-hydroxylases (C3H), hydroxycinnamoyl CoA: quinate/shikimate hydroxycinnamoyl transferase (HCT) and caffeoyl coenzyme A 3-*O*-methyltransferase (CCoAOMT) are involved in the synthesis of both coniferyl and sinapyl alcohols, whereas ferulate or coniferaldehyde 5-hydroxylases (F5H or Cald5H) and hydroxyconiferaldehyde 5-*O*-methyltransferases (COMT or AldOMT) are required for the synthesis of sinapyl alcohol (Boerjan *et al*. 2003). HCT is located in branching point of synthesis of monolignols and other products of phenyl propanoid pathway, and recently, it has been shown that silencing of HCT causes not only decreased lignification but also accumulation of flavonoids and as consequence of that, inhibited auxin transport and reduced growth in transgenic *Arabidopsis* plants (Besseau *et al*. 2007). On the other hand, a recent study on genetically modified alfalfa, where several monolignol biosynthetic genes were down-regulated independently, questioned the role of CCoAOMT in the synthesis of sinapyl alcohol (Chen *et al*. 2006), suggesting that re-evaluation of routes for synthesis of different types of lignin may be needed.

While the amount of unclear points in the monolignol biosynthetic pathway is decreasing, much less is known about the final transport and polymerization of the monolignols. Monolignols are found in plants either as free monolignols or as monolignol glucosides. It is still unclear whether the monolignol glucosides are a transport form, an intermediate or a storage form (Tsuji and Fukushima 2004). Transport of monolignols/monolignol glucosides to the apoplast is thought to occur by vesicle transport or via specific ATP-binding cassette (ABC) transporters. In high resolution examination of lodgepole pine cambial and xylem sections, Samuels *et al*. (2002) detected dark staining Golgi vesicles in developing xylem cells in osmiatic samples, indicating

phenolic, possibly monolignol content. On the other hand, in global transcript profiling of *Arabidopsis* stems, seven genes coding ABC transporters have shown similar expression profiles to known monolignol biosynthetic genes (Ehlting *et al*. 2005). Recently, it has been suggested even that the hydroxylation level of monolignols would allow their passage through the plasma membrane by diffusion. This view is supported by the observation that the partition of dilignols into liposomes resembled the partitioning of membrane-penetrating pharmaceutical drugs (Boija and Johansson 2006).

The final polymerization of the monolignols still awaits conclusive results. The oxidation of monolignols, as well as the oxidation of the existing lignin polymer, is required for the polymerization to proceed. Class III plant peroxidases (POXs) and laccases and other phenol oxidases are the enzyme groups considered to be responsible for the oxidative reactions in lignin polymerization (**Fig. 2**). Both POXs and laccases exist as multigene families apparently in all land plants (e.g. in *Arabidopsis*, 73 POXs (Welinder *et al*. 2002) and 17 laccases (McCaig *et al*. 2005) and they show wide functional diversity (Mayer and Staples 2002; Passardi *et al*. 2005), which makes their functional determination difficult. Laccases are expressed in lignifying tissues in many plant species (Bao *et al*. 1993; Ranocha *et al*. 1999), and recently it has been shown that lignin amounts are decreased in seeds of *Arabidopsis thaliana* plants with a mutation in a laccase gene (Liang *et al*. 2006). Both anionic and cationic POXs have been implicated in lignin polymerization e.g. due to their expression profiles, catalytic properties and impacts of their down-regulation in transgenic plants (Østergaard *et al*. 2000; Li *et al*. 2003; López-Serrano *et al*. 2004). However, there is no evidence that monolignol polymerization would be denoted to a single enzyme and it is more likely that it is done by co-operation of several enzymes *in vivo*.

In addition to oxidative enzymes, involvement of dirigent proteins (DIR) in the polymerization of lignin has been debated. Dirigent proteins (Latin: *dirigere*, to align or guide) are cell wall proteins, which themselves lack oxidative capability, but are able to bind and orientate monolignol radicals thereby promoting stereoselective radical coupling (Davin *et al*. 1997). They have been shown to guide the coupling of monolignol radicals in the formation of lignans, monolignol dimers with an important role in defense reactions and it has been postulated that dirigent proteins would direct monolignol polymerization also in lignin synthesis, which is thought to explain e.g. the structural variation between lignins in different cell wall layers (Davin *et al*. 1997; Burlat *et al*. 2001).

PLAYERS IN THE REGULATION OF DEVELOPMENTAL LIGNIFICATION

Over a decade ago, Aloni *et al*. (1990) showed that auxin and gibberellin have an impact on lignin composition and yield in phloem fibers and secondary xylem in stems of *Coleus blumei*. Since then, intensive studies have produced a lot of information on the function of auxin, in addition to cytokinin, in xylem development, while only little is known about the mechanisms of hormonal regulation of lignin biosynthesis. However, recently Biemelt *et al*. (2004) have shown that in transgenic tobacco plants with reduced amounts of gibberellin, expression of monolignol biosynthetic genes decreased and the amount of lignification was reduced when compared to wild type. In addition, short term feeding of GA_3 to these gibberellin deficient plants resulted in increased lignin accumulation without transcriptional activation of monolignol biosynthesis, suggesting a role for gibberellin also in the polymerization of monolignols (Biemelt *et al*. 2004). Similarly, ectopic expression of BnET, a repressor of gibberellin mediated processes from *Brassica napus*, in *Arabidopsis* and tobacco plants, also caused reduced xylem lignification (Ellerström *et al*. 2005), indicating again that gibberellin responses are needed in the normal developmental lignification in xylem tissue. Expression levels of the genes involved in monolignol biosynthesis vary according to the circadian rhythm, and are controlled by light and carbon availability (Rogers *et al*. 2005). Feeding experiments of dark-grown *Arabidopsis* plants also suggested that sugars work not only as carbon sources but also as signals for transcriptional enhancement of lignin biosynthesis (Rogers *et al*. 2005).

Promoters of several genes encoding enzymes involved in monolignol biosynthesis (PAL, C4H, COMT, CCoAOMT, 4CL, CCR and CAD) contain common conserved elements rich in cytosine and adenosine called AC elements (or H-boxes or PAL boxes) (Logemann *et al*. 1995; Bell-Lelong *et al*. 1997; Chen *et al*. 2000; Lacombe *et al*. 2000; Lauvergeat *et al*. 2002). LIM and MYB type transcription factors can bind to the AC elements and control the expression of lignin biosynthetic genes in transgenic tobacco plants (Tamagnone 1998; Kawaoka *et al*. 2000; Kawaoka and Ebinuma 2001). Genome-wide analysis of lignification related genes in *Arabidopsis* has shown that in seven of the lignin biosynthesis related gene families (PAL, 4CL, HCT, C3H, CCoAOMT, CCR and CAD) at least one member of the family has AC elements in the promoter region (Raes *et al*. 2003). The gene families lacking AC-elements in their promoters (C4H, F5H and COMT), except C4H, were specifically involved in sinapyl alcohol synthesis. From this the authors concluded that AC-elements could be involved especially in controlling the synthesis of G-type lignin in *Arabidopsis* (Raes *et al*. 2003).

Recently, involvement of NAC domain transcription factors in regulation of secondary wall formation and lignification has been demonstrated (Mitsuda *et al*. 2007; Zhong *et al*. 2007). Mitsuda *et al*. (2007) have shown that in double knock-out *Arabidopsis* plants lacking two NAC domain transcription factors, NST1 and NST3, the lignified secondary cell wall thickenings in interfascicular stem fibers were completely suppressed. Similarly, simultaneous inhibition of expression of NAC transcription factor SND1 and NST1, caused loss of all the major secondary cell wall components, cellulose, xylan and lignin, in *Arabidopsis* stem fibers (Zhong *et al*. 2007). Inhibition of NST1/NST3 or SND1/NST1 had no impact on vessel walls, and inhibition of none of the transcription factors alone were able to create the defective fiber wall phenotype, indicating that these two transcription factors control secondary cell wall formation specifically in fibers redundantly (Mitsuda *et al*. 2007; Zhong *et al*. 2007). Transcriptional analysis of the NST1/NST3 and SND1/NST1 inhibited lines revealed reduced expression of genes involved in synthesis of secondary wall components, including genes coding for enzymes involved in lignin biosynthesis (Mitsuda *et al*. 2007; Zhong *et al*. 2007). Interestingly, several transcription factor genes, including three MYB and two other NAC genes, were also down-regulated in SND1/NST1 inhibited plants, indicating that they may function in regulation of genes involved in synthesis of secondary cell wall components, e.g. lignin (Zhong *et al*. 2007). On the other hand, over-expression of *Arabidopsis* MYB26 gene, involved in regulation of secondary thickening formation in anther endothecium, increased expression of two NAC-domain transcription factors, NST1 and NST2, and induced ectopic secondary thickening and lignification especially in epidermal tissues of transgenic *Arabidopsis* and tobacco plants (Yang *et al*. 2007).

LIGNIFICATION IN DEVELOPMENT: CASE STUDIES

Lignification during xylem development

Mature tracheary elements (TEs), tracheids and vessel elements in xylem tissue are hollow dead cells, joined together either with masses of ring pores (tracheids) or with openings at the vertical ends of the cells (vessels). During differentiation, the developing tracheary elements deposit highly lignified secondary cell walls, with annular, spiral, reticular, or

pitted thickening patterns. In addition to TEs, xylem tissue contains parenchymatic cells, the cell walls of which may become partly lignified and are involved TE development. In this review, recent achievements in two important areas of xylem research are covered: the TE lignification in the *Zinnia elegans* cell culture system and xylem lignification in trees. The *Zinnia* cell culture system is a well characterized model system for studying xylogenesis *in vitro*, whereas trees, being the major carbon stores both in forms of cellulose and lignin, are invaluable ecologically, economically and culturally.

Studies on tracheary element lignification in the *Zinnia* cell culture

Zinnia elegans suspension cultured cells have been used successfully in the study of the regulation of tracheary element (TE) differentiation. When supplied with appropriate amounts auxin and cytokinin, in this culture system (up to 96 hours) the isolated *Zinnia* mesophyll cells transdifferentiate into TEs (Fukuda 1997). Being a model for xylogenesis, the *Zinnia* system is also a valuable tool for studying lignification during xylem development. Pesquet *et al*. (2005) have demonstrated that relative amounts of auxin and cytokinin in *Zinnia* culture media have an impact on CCoAOMT and COMT expression patterns in differentiating TEs: in the presence of auxin CCoAOMT shows two expression peaks during TE development, whereas when cytokinin alone is present, CCoAOMT expression remains at the elevated level once induced. The supplying of gibberellin in the culture media, and on the other hand, inhibition of endogenous gibberellin synthesis, have impacts on the degree of TE lignification (Tokunaga *et al*. 2006), suggesting that gibberellins may function in the downstream hormonal regulation of lignification during TE development. Gibberellin treatment also caused reduction of lignin precursors in culture media indicating again that gibberellin enhances lignin polymerization (Tokunaga *et al*. 2006).

Microarray analysis of different stages of TE development have revealed that while genes coding for lignin polymerizing enzymes (six laccases and one peroxidase) were specifically expressed in lignifying TEs, the monolignol biosynthetic genes were expressed apparently both in lignifying TEs and in non-lignifying cells (Demura *et al*. 2002). This is in accordance with the findings of Hosokawa *et al*. (2001), who demonstrated that in the *Zinnia* system monolignols polymerized to lignin are supplied by not only the TEs but also by the parenchymatic cells in the medium. Interestingly, oligolignol feeding studies with *Zinnia* cultures suggest that lignin polymerization in TEs can proceed by incorporating also preformed dilignols into the lignin polymer (Tokunaga *et al*. 2005). The transcriptional analysis of *Zinnia* TEs by Demura *et al*. (2002) suggested the involvement of several laccases to TE lignification. However, there is also convincing evidence for the participation of cationic POXs to monolignol polymerization in *Zinnia* TEs, as they localize correctly spatially and temporally and catalyze the oxidation of both sinapyl and coniferyl alcohols efficiently (Gabaldón *et al*. 2005; Sato *et al*. 2006).

In accordance to the importance of peroxidases in TE lignification, Gabaldón *et al*. (2006) have demonstrated that the availability of hydrogen peroxide is a restricting factor in monolignol polymerization in *Zinnia* cells. Hydrogen peroxide is provided by both the differentiating tracheary elements and the parenchymatic cells, and it has been noticed that hydrogen peroxide levels are high in *Zinnia* cell cultures during the entire culture period, in the order of 1-3 mM, and localize especially in the areas of the developing cell wall where cell wall thickenings and lignin deposition are found (Gómez Ros *et al*. 2006). The H_2O_2 level depends on its production rate (by NADPH oxidase, polyamine oxidases and oxalate oxidases (Allan and Fluhr 1997) and consumption by various chemical and enzymatic events such as reactions with intercellular ascorbic acid (Baker and Mock 2004). Furthermore, a rise in H_2O_2 levels has been observed at the late phase of tracheary element differentiation in the *Zinnia* system and this coincides with the expression of a cationic peroxidase apparently responsible for monolignol polymerization (López-Serrano *et al*. 2004), occurring at the same time as lignin polymerization begins (Hosokawa *et al*. 2001).

Xylem lignification in trees

Trees produce large amounts of lignified xylem during secondary growth. In fact, with their enormous height and durability they can be considered as the ultimate example of extensive lignin deposition in the plant kingdom. Because of their woody characteristics, trees have been rather difficult to study: they are too large, too difficult to handle and the time between generations is too long. However, the recent development in histological, biochemical and molecular biology methods has produced large amounts of new information about the lignification and its regulation during xylem formation in trees. In the first completely sequenced genome of a tree species, *Populus trichocarpa* (Tuskan *et al*. 2006), 34 phenylpropanoid and lignin biosynthetic genes have been found. It has been discovered also that most of the lignin biosynthetic gene families are larger in *Populus* than in the first completely sequenced dicot species, *Arabidopsis*. The only exception is CAD, which in *Populus* is present as only one copy, in comparison to two in *Arabidopsis* (Tuskan *et al*. 2006).

Modification of lignin structures and amount through genetic manipulation of monolignol biosynthesis route has obtained not only scientific but also economic interest, since these factors affect the cost of wood processing e.g. in pulp and paper industry (Halpin and Boerjan 2003). The greatest reduction in total lignin amount has been achieved in transgenic trees where the targets of genetic modification have been genes involved in synthesis of apparently all monolignols (e.g. 4CL, CCoAOMT), whereas alteration of expression of genes specific for sinapyl alcohol synthesis (e.g. COMT, CAld5H) have had a strong impact on S/G lignin ratio (Lapierre *et al*. 1999; Jouanin *et al*. 2000; Zhong *et al*. 2000; Li *et al*. 2003).

Multiple POXs and phenol oxidases, possibly involved in lignin polymerization, are found in the xylem of gymnosperm and angiosperm trees during developmental lignification. Laccases have been associated with lignification in trees since they have correct spatiotemporal localization and biochemical properties (Bao *et al*. 1993; Ranocha *et al*. 1999; Sato *et al*. 2001, 2006). However, the structural changes in secondary cell walls of transgenic poplars expressing antisense laccases suggest an important role for laccase in cell wall attachment, but not in lignification (Ranocha *et al*. 2002). Quantitative RT-PCR results of Koutaniemi *et al*. (unpublished) have revealed that at least 13 different POX genes with varying transcript levels are expressed in Norway spruce developing xylem. Christensen *et al*. (1998) have characterized an anionic syringaldazine oxidizing POX in poplar xylem apparently involved in syringyl lignin synthesis, and Li *et al*. (2003) have shown that down-regulation of an anionic POX alters lignin content and composition in hybrid aspen. Cationic POXs are dominant in Norway spruce xylem protein extracts through the period of annual growth (Marjamaa *et al*. 2003) and are able to catalyze the oxidation of coniferyl alcohol (Marjamaa *et al*. 2006a). At least one of the cationic spruce POXs, *PaPx1*, is specifically expressed in lignifying tracheids in spruce seedlings (Marjamaa *et al*. 2006b). Recently, Sasaki *et al*. (2004) have shown that a cationic POX from poplar callus cultures (*Populus alba* L.) is able to catalyze the oxidation of polymeric lignin. The gene coding for this cationic POX is constitutively expressed in developing xylem and the enzyme localizes to the middle lamella, cell corners and secondary cell walls of lignifying fibers in poplar, further suggesting that this enzyme is involved in lignin formation (Sasaki *et al*. 2006).

In the lignin forming tissue culture system of Norway

spruce, inhibition of peroxidase activity through H_2O_2 scavenging caused a drastic reduction in lignin formation (Kärkönen *et al*. 2002). Availability of hydrogen peroxide could be one of the controlling factors in lignin polymerization also in trees. Accumulation of hydrogen peroxide in the cell walls is controlled by reducing agents, most importantly ascorbate, the amount of which is apparently reduced to a minimum in lignifying cell walls in trees through ascorbate oxidase activity (Kärkönen *et al*. 2006; Ros-Barceló *et al*. 2006). Lignification was also induced and the expression of lignin biosynthetic genes was increased in transgenic hybrid aspen trees with elevated extracellular hydrogen peroxide concentrations due to expression of antisense superoxide dismutase (Srivastava *et al*. 2007).

Not much is known about hormonal control of xylem lignification in trees. The role of gibberellin in the regulation of lignin synthesis has been studied in trees. In transgenic hybrid aspen with elevated gibberellin levels especially the expression levels of genes involved in sinapyl alcohol synthesis were increased, resulting in increased syringyl content in xylem lignin (Israelsson *et al*. 2003).

R2R3-MYB family transcription factors are involved in transcriptional regulation of monolignol biosynthetic genes during xylem lignification in trees, apparently working as inducers or repressors of gene expression. In angiosperm trees, MYB transcription factors can regulate the lignin amount and syringyl versus guaiacyl lignin content, although results here have been somewhat controversial. Over-expression of R2R3-MYB transcription factor from *Eucalyptus*, EgMYB2, in transgenic tobacco plants increased expression the genes specific for monolignol synthesis (Goicoechea *et al*. 2005). The cell walls of xylem cells of the transgenic plants were thicker and showed more intense staining with lignin dyes such as phloroglucinol-HCl and Mäule reagent. Thioacidolysis analysis showed that transgenic tobacco lines also had relatively higher syringyl lignin content than control plants, apparently depending upon the strong increase in COMT expression, indicating such a role for EgMYB2 in the regulation of lignin composition (Goicoechea *et al*. 2005). On the other hand, the transcriptional and promoter analysis of C4H genes in *Populus* showed that the C4H genes without AC-elements in promoters are expressed in areas with high syringyl lignin content, suggesting again a varying role for MYB binding AC elements in the synthesis of different types of lignin (Lu *et al*. 2006). On the other hand, antisense expression of one of the hybrid aspen MYB transcription factors, PttMYB21a resulted in increased lignification and transcription of CCoAOMT in transgenic aspen, indicating that this MYB transcription factor acts as a transcriptional repressor of lignin biosynthesis (Karpinska *et al*. 2004).

Pinus taeda R2R3-MYB transcription factors PtMYB1 and PtMYB4 are both expressed in the developing xylem and are able to bind to AC elements (Patzlaff *et al*. 2003). PtMYB4 is specifically expressed in lignifying pine cells and lead to increased lignin deposition in transgenic tobacco plants through activation of the transcription of monolignol biosynthetic genes (Patzlaff *et al*. 2003). Interestingly, both PtMYB1 and PtMYB4 have been found to be able to activate the promoter of *Pinus sylvestris* glutamine synthase gene *PsGS1b* (Gómez-Maldonado *et al*. 2004). Nitrogen recycling has been shown to occur in lignifying *Pinus taeda* cell cultures and glutamine synthases are proposed to be important enzymes involved in nitrogen recycling (van Heerdent *et al*. 1996). The co-regulation of genes involved in nitrogen recycling and monolignol biosynthesis provides a mechanism for coordination of lignin synthesis and reassimilation of the remarkable amounts of ammonium released during the phenylpropanoid metabolism (Gómez-Maldonado *et al*. 2004).

As long living plant individuals, trees have developed mechanisms to maintain their structural organization despite changes in the environment. When the existing tree trunk is bent, so called reaction wood is formed in order to resume the original alignment. In conifers, reaction wood (compression wood) appears on the lower side of bent structure, while in angiosperms reaction wood (tension wood) appears on the upper side of e.g. branches. Both xylem structure and lignification patterns are altered in the reaction woods. In fibres of tension wood, S3 layer in secondary cell wall is replaced with gelatinous G layer composed mainly of crystalline cellulose. Gierlinger and Schwanninger (2006) detected a small (0.5 mm) lignified border toward the lumen in the gelatinous layer of poplar tension wood by confocal Raman microscopy, which can impede the degradation of the cellulosic G-layer and thus may act as a defense line against microorganisms. Compression wood is characterized with higher lignin content and higher amounts H-type lignin compared to normal wood (Önnerud and Gellerstedt 2003). In severe compression wood tracheid cross-sections are round, with large intercellular spaces, and S3 layer of secondary cell wall is absent.

The reduced lignin content in tension wood and the increased lignin amounts in compression wood are apparently caused by changes in transcription levels of monolignol biosynthetic genes (Zhang and Chiang 1997; Andersson-Gunnerås *et al*. 2006; Koutaniemi *et al*. unpublished), but how this is regulated is still poorly understood. The MYB transcription factor PttMYB21a with an ability to repress the expression of monolignol biosynthetic genes (Karpinska *et al*. 2004) was induced in compression wood in *Populus*, indicating again its role in down-regulation of lignin biosynthesis (Andersson-Gunnerås *et al*. 2006). On the other hand, Lu *et al*. (2005) have shown that novel microRNAs in poplar xylem are regulated by mechanical stresses and thus may function in the controlling the stress wood formation by selective gene silencing.

Some other cases of developmental lignification

In addition to conductive tissues in xylem, developmental lignification occurs in several other cell types where additional support, strength or impermeability is needed. Lignified sclerenchymal cells, sclereids and structural fibers form supporting and strengthening structures in plant tissues e.g. in seed coats and stems of many monocot species. When mature, their live protoplasts have disappeared and the secondary cell walls are thick and highly lignified and the lumen small in volume. Structural fibers are typically located in xylem and phloem tissues, whereas sclereids are found as layers, small groups or individual cells e.g. in many seeds and fruits. Lignin makes plant tissues less digestible by animals, which can enhance seed dispersal, but in the case of e.g. grass forages, is an unfavorable trait. In roots, apoplastic diffusion of water and nutrients is hindered by deposition of suberin and lignin into the endodermal and to some extent hypodermal cell walls. Here, recent studies on developmental lignification in monocotyledon grass species are reviewed.

Studies on grass lignin

Lignin in grasses is composed of guaiacyl, syringyl, and *p*-hydroxyphenyl units, with incorporation of relatively high amounts of hydroxycinnamyl acids, most of all p-coumarate and ferulate. *p*-Coumarate is one of the intermediate precursors of monolignols synthesized from cinnamate by C4H, and in the cell wall, it is often ester linked to lignin S units (Grabber *et al*. 1996). Recent studies indicate that ferulate is synthesized from coniferylaldehyde by the enzyme aldehyde dehydrogenase (ALDH), and not as previously thought through methylation of caffeic acid (Nair *et al*. 2004). Ferulate can be ester linked to cell wall polysaccharides and/or ether linked to lignin G units, thus cross-linking polysaccharides to lignin (Ralph *et al*. 1998). During tall fescue development, the most remarkable lignification related anatomical change in stems is the formation and development of a sclerenchymal ring (Chen *et al*. 2002). At the same time, the amount of lignin in stems increased ten fold and ruminal degradability decreased from 80% to 30% (Chen *et al*.

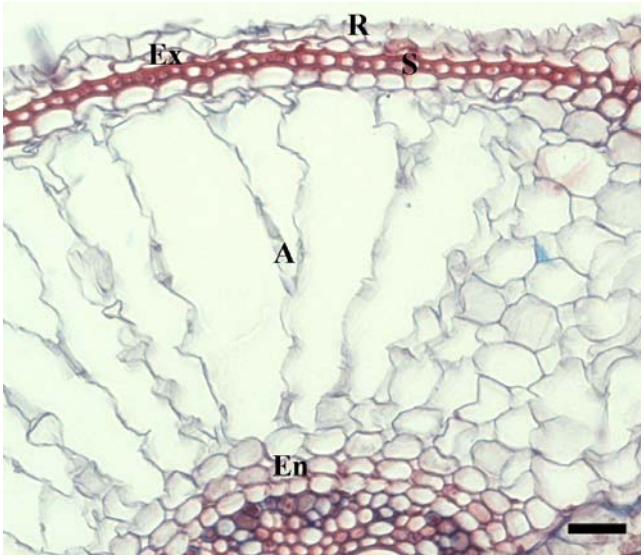

Fig. 3 Lignin deposition in the endodermis and hypodermis in an aerenchymatic rice root. The root section is stained with safranin-Alcian blue for lignin (red) and cellulose (blue), respectively. A, aerenchyma; En, endodermis; Ex, exodermis; R, rhizodermis; S, sclerenchyma; Bar=20 μm.

2002). S/G lignin ratio also increased during stem development in tall fescue, while the amount of H units remained relatively low (Chen *et al*. 2002). The increase in tall fescue lignification was parallel with increased transcription and enzyme activities of COMT and CAD (Chen *et al*. 2002). The large portion of lignin in grass stems deposited to the sclerenchyma could be a suitable target for lignin genetic modification in order to achieve more digestable forage (Chen *et al*. 2002).

Analysis of the chemical composition of endodermal and hypodermal cell walls in roots of several monocotyledonous and dicotyledonous plant species has revealed that while endodermal lignin is mainly composed of syringyl and guaiacyl units, considerable amounts of *p*-hydroxyphenyl units, coumarate and ferulate are incorporated in lignin in the hypodermis, possibly reflecting its role in resisting mechanical stress (Schreiber *et al*. 1999). In roots of many monocotyledonous wetland species or plants suffering from flooding as in rice (**Fig. 3**), the barrier formation is enhanced along with aerenchyma formation in order to prevent radial oxygen loss (McDonald 2002). A study on *OsCAD2* promoter activity in rice leaves, stems and roots has revealed, that while the promoter activity was clearly related to lignifying cells in stems and leaves, in roots the promoter was active in the xylem but not in the lignifying sclerenchyma of the hypodermis (Tobias and Chow 2004). Instead, the *OsCAD2* promoter was active in cortical cells adjacent to the exodermis, indicating again that these cells may supply monolignols to lignifying cells, but also showing differences in the process of lignification in these two cell types (Tobias and Chow 2004).

There are a limited number of studies on the impact of genetic modification of lignin biosynthetic genes to lignin contents and composition in monocots, and until recently, most of the information of lignification genetics have been obtained from naturally occurring mutants. Natural maize mutant lines *bm1*, *bm2*, *bm3* and *bm4* show lower lignin contents, altered lignin compositions and increased digestibility (Barrière *et al*. 2004). The most studied mutant lines are *bm1* and *bm3*, which show reduced CAD and COMT activities, respectively (Barrière *et al*. 2004). The reduction of lignin content is greatest in *bm3* line (up to 40%) with decreased amounts of S units in lignin (Barrière *et al*. 2004). A recent cell wall macro array analysis of the maize mutants has revealed that bm3 mutant phenotype is caused mainly by reduction in a single *COMT* gene expression (Guillaumie *et al*. 2007). In a transgenic maize plant expressing antisense *COMT* under a xylem and sclerenchyma specific promoter, reduced lignin content and decreased S/G lignin ratio especially in sclerenchyma fibers were observed (Piquemal *et al*. 2002). *p*-Coumarate esters were decreased in both *bm1* maize and *COMT* antisense maize, being possibly a consequence from decreased S-unit deposition (Piquemal *et al*. 2002; Barrière *et al*. 2004). The *COMT* deficient maize plants with increased S/G ratio showed also increased ruminal digestibility (Piquemal *et al*. 2002), which is opposite to the results from studies on chemical degradation of tree lignins (Li *et al*. 2003). *CAD*-deficient mutants of maize (*bm1*) and rice (GOLD HULL AND INTERNODE2, Zhang *et al*. 2006) and *CAD* down-regulated transgenic tall fescue (Chen *et al*. 2003) all showed a decrease in total lignin content without affecting relative amounts of different lignin units, differing from e.g. *CAD* down-regulated poplar plants, where increase in cinnamoyl aldehyde content but no impact on total lignin amounts were observed (Baucher *et al*. 1996).

In addition to several *CAD/SAD* gene family members, many other genes coding for proteins involved in phenylpropanoid metabolism and gene regulation were down-regulated in *bm1* maize plants, e.g. chorismate synthase (synthesis of phenyl alanine), PAL, 4CL, several CAD/SAD, peroxidases, laccases, and MYB and HDZIP III transcription factors, indicating that a mutation in *bm1* line affects upstream regulative element of phenylpropanoid metabolism (Guillaumie *et al*. 2007). Interestingly, showing otherwise very similar transcriptome to *bm1*, maize *bm2* have highly decreased transcription in one of the ABC transporter genes. This could, if the transporter is specialized for coniferyl alcohol transport, be at least partly the reason for decreased guaiacyl lignin content detected in *bm2* plants (Guillaumie *et al*. 2007).

Little is known about regulation of lignification in grasses. Down-regulation of MYB and HDZIP III transcription factors in *bm1* maize may indicate their role as transcriptional inducers of expression of lignin synthesis related genes (Guillaumie *et al*. 2007). On the other hand, over-expression of MYB transcription factors in transgenic maize plants caused *COMT* down-regulation and decreased lignin contents (Fornale *et al*. 2006). In both *bm1* and *bm2* plants, several *ARGONAUTE* genes, which may function in the control of post-transcriptional gene silencing of transcription factors, were down-regulated, further increasing the complexity of the regulatory system in lignin formation in grasses (Guillaumie *et al*. 2007).

CONCLUSIONS

Developmental lignification is a complicated process occurring in multiple cell types and it is influenced by activities of several enzymes and regulatory factors, as well as by other cell wall components and by environmental changes. Recent years have produced huge amount of information on the different components in lignin synthesis leaving still a lot of fascinating questions to be answered in the future. Among the unanswered questions are e.g. the process of monolignol transport and polymerization in the cell wall, possible differences in lignin biosynthesis pathways between monocotyledonous and dicotyledonous plant species and with a huge question mark, functioning of the network of regulatory elements underlying lignin biosynthesis. Once these are revealed, we can truly start to understand how lignin is produced under normal developmental events, and on the other hand, as a response to various environmental cues.

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REFERENCES

Achine L, Blancaflor EB, Rasmussen S, Dixon RA (2004) Colocalization of L-phenylalanine ammonia-lyase and cinnamate 4-hydroxylase for metabolic channeling in phenylpropanoid biosynthesis. *Plant Cell* **16**, 3098-3109

Allan AC, Fluhr R (1997) Two distinct sources of elicited reactive oxygen species in tobacco epidermal cells. *Plant Cell* **9**, 1559-1572

Aloni R, Tollier MT, Monties B (1990) The role of auxin and gibberellin in controlling lignin formation in primary phloem fibers and in xylem of *Coleus blumei* stems. *Plant Physiology* **94**, 1743-1747

Andersson-Gunneråas S, Mellerowicz EJ, Love J, Segerman B, Ohmiya Y, Coutinho PM, Nilsson P, Henrissat B, Moritz T, Sundberg B (2006) Biosynthesis of cellulose-enriched tension wood in *Populus*: global analysis of transcripts and metabolites identifies biochemical and developmental regulators in secondary wall biosynthesis. *The Plant Journal* **45**, 144-165

Anterola AM, Jeon J-H, Davin LB, Lewis NG (2002) Transcriptional control of monolignol biosynthesis in *Pinus taeda*. *The Journal of Biological Chemistry* **277**, 18272-18280

Baker CJ, Mock NM (2002) A method to detect oxidative stress by monitoring changes in the extracellular antioxidant capacity in plant suspension cells. *Physiological and Molecular Plant Pathology* **64**, 255-261

Bao W, O'Malley DM, Whetten R, Sederoff RR (1993) A laccase associated with lignification in loblolly pine xylem. *Science* **260**, 672-74

Barrière Y, Ralph J, Méchin V, Guillaumie S, Grabber JH, Argillier O, Chabbert B, Lapierre C (2004) Genetic and molecular basis of grass cell wall biosynthesis and degradability. II. Lessons from brown-midrib mutants. *Comptes Rendues Biologies* **327**, 847-860

Baucher M, Chabbert B, Pilate G, Van Doorsselaere J, Tollier MT, Petit-Conil M, Cornu D, Monties B, Van Montagu M, Inzé D, Jouanin L, Boerjan W (1996) Red xylem and higher lignin extractability by down-regulating a cinnamyl alcohol dehydrogenase in poplar. *Plant Physiology* **112**, 1479-1490

Bell-Lelong DA, Cusumano JC, Meyer K, Chapple C (1997) Cinnamate-4-hydroxylase expression in *Arabidopsis*. Regulation in response to development and the environment. *Plant Physiology* **113**, 729-738

Besseau S, Hoffmann L, Geoffroy P, Lapierre C, Pollet B, Legrand M (2007) Flavonoid accumulation in *Arabidopsis* repressed in lignin synthesis affects auxin transport and plant growth. *Plant Cell* **19**, 148-162

Biemelt S, Tschiersch H, Sonnewald U (2004) Impact of altered gibberellin metabolism on biomass accumulation, lignin biosynthesis and photosynthesis in transgenic tobacco plants. *Plant Physiology* **135**, 254-265

Blount JW, Korth KL, Masoud SA, Rasmussen S, Lamb C, Dixon RA (2000) Altering expression of cinnamic acid 4-hydroxylase in transgenic plants provides evidence for a feedback loop at entry point into phenylpropanoid pathway. *Plant Physiology* **122**, 107-116

Boerjan W, Ralph J, Baucher M (2003) Lignin biosynthesis. *Annual Reviews in Plant Biology* **54**, 519-546

Boija E, Johansson G (2006) Interactions between model membranes and lignin-related compounds studied by immobilized liposome chromatography. *Biochimica et Biophysica Acta* **1758**, 620-626

Boudet AM, Hawkins S, Rochange S (2004) The polymorphism of the genes/enzymes involved in the last two reductive steps of monolignol synthesis: what is the functional significance? *Comptes Rendus Biologies* **327**, 837-65

Boyce CK, Cody GD, Fogel ML, Hazen RM, Alexander CMO'D, Knoll AH (2003) Chemical evidence for cell wall lignification and the evolution of tracheids in early devonian plants. *International Journal of Plant Science* **164**, 691-702

Boyce CK, Zwieniecki MA, Cody GD, Jacobsen C, Wirick S, Knoll AH, Holbrook NM (2004) Evolution of xylem lignification and hydrogel transport regulation. *Proceedings of the National Academy of Sciences USA* **101**, 17555-17558

Burlat V, Kwon M, Davin LB, Lewis NG (2001) Dirigent proteins and dirigent sites in lignifying tissues. *Phytochemistry* **57**, 883-897

Carbita NC, Gibeaut DM (1993) Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *The Plant Journal* **3**, 1-30

Chen C, Meyermans H, Burggraeve B, de Rycke RM, Inoue K, de Vleesschauwer V, Steenackers M, van Montagu MC, Engler GJ, Boerjan WA (2000) Cell-specific and conditional expression of caffeoyl-coenzyme A-3-*O*-methyltransferase in poplar. *Plant Physiology* **123**, 853-867

Chen L, Auh C, Chen F, Cheng X, Aljoe H, Dixon RA, Wang Z (2002) Lignin deposition and associated changes in anatomy, enzyme activity, gene expression, and ruminal degradability in stems of tall fescue at different developmental stages. *Journal of Agricultural and Food Chemistry* **50**, 5558-5565

Chen F, Srinivasa Reddy MS, Temple S, Jackson L, Shadle G, Dixon RA (2006) Multi-site modulation of monolignol biosynthesis suggests new routes for formation of syringyl lignin and wall-bound ferulic acid in alfalfa (*Medicago sativa* L.) *Plant Journal* **48**, 113-124

Christensen JH, Bauw G, Welinder KG, van Montagu M, Boerjan W (1998) Purification and characterization of peroxidases correlated with lignification in poplar xylem. *Plant Physiology* **118**, 125-135

Demura T, Tashiro G, Horiguchi G, Kishimoto N, Kubo M, Matsuoka N, Minami A, Nagata-Hiwatashi M, Nakamura K, Okamura Y, Sassa N, Suzuki S, Yazaki J, Kikuchi S, Fukuda H (2002) Visualization by comprehensive microarray analysis of gene expression programs during transdifferentiation of mesophyll cells into xylem cells. *Proceedings of the National Academy of Sciences USA* **99**, 15794-15799

Davin LB, Wang HB, Crowell AL, Bedgar DL, Martin DM, Sarkanen S, Lewis NG (1997) Stereoselective biomolecular phenoxy radical coupling by an auxillary (Dirigent) protein without an active center. *Science* **275**, 362-366

Ehlting J, Mattheus N, Aeschliman DS, Li E, Hamberger B, Cullis IF, Zhuang J, Kaneda M, Mansfield SD, Samuels L, Ritland K, Ellis BE, Bohlmann J, Douglas CJ (2005) Global transcript profiling of primary stems from *Arabidopsis thaliana* identifies candidate genes for missing links in lignin biosynthesis and transcriptional regulators of fiber differentiation. *The Plant Journal* **42**, 618-640

Ellerström M, Reidt W, Ivanov R, Tiedemann J, Melzer M, Tewes A, Moritz T, Mock HP, Sitbon F, Rask L, Baumlein H (2005) Ectopic expression of EFFECTOR OF TRANSCRIPTION perturbs gibberellin-mediated plant developmental processes. *Plant Molecular Biology* **59**, 663-681

Fornale S, Sonbol F-M, Maes T, Capellades M, Puigdoménech P, Rigau J, Caparrós-Ruiz D (2006) Down-regulation of the maize and *Arabidopsis thaliana* caffeic acid *O*-methyl-transferase genes by two new maize R2R3-MYB transcription factors. *Plant Molecular Biology* **62**, 809-823

Fukuda H (1997) Tracheary element differentiation. *Plant Cell* **9**, 1147-1156

Gabaldón C, López-Serrano M, Pedreño MA, Ros-Barceló A (2005) Cloning and molecular characterization of the basic peroxidase isoenzyme from *Zinnia elegans*, an enzyme involved in lignin biosynthesis. *Plant Physiology* **139**, 1138-1154

Gabaldón C, López-Serrano M, Pomar F, Merino F, Cuello J, Pedreño MA, Ros-Barceló A (2006) Characterization of the last step of lignin biosynthesis in *Zinnia elegans* suspension cell cultures. *FEBS Letters* **580**, 4311-4316

Gierlinger N, Schwanninger M (2006) Chemical imaging of poplar wood cell walls by confocal Raman microscopy. *Plant Physiology* **140**, 1246-1254

Goicoechea M, Lacombe E, Legay S, Mihaljevic S, Rech P, Jauneau A, Lapierre C, Pollet B, Verhaegen D, Chaubet-Gigot N, Grima-Pettenati J (2005) EgMYB2, a new transcriptional activator from *Eucalyptus* xylem, regulates secondary cell wall formation and lignin biosynthesis. *The Plant Journal* **43**, 553-567

Gómez-Maldonado J, Avila C, Torre F, Canas R, Canovas FM, Campbell MM (2004) Functional interactions between a glutamine synthetase promoter and MYB proteins. *The Plant Journal* **39**, 513-526

Gómez-Ros LV, Paradiso A, Gabaldón MA, de Gara L, Ros-Barceló A (2006) Two distinct cell sources of H_2O_2 in the lignifying *Zinnia elegans* cell culture system. *Protoplasma* **227**, 175-183

Grabber JH, Quideau S, Ralph J (1996) *p*-Coumaroylated syringyl units in maize lignin; implications for β-ether cleavage by thioacidolysis. *Phytochemistry* **43**, 1189-1194

Grünwald C, Ruel K, Schmitt U (2002) On the cytochemistry of cell wall formation in poplar trees. *Plant Biology* **4**, 13-21

Guillaumie S, Pichon M, Martinant JP, Bosio M, Goffner D, Barrière Y (2007) Differential expression of phenylpropanoid and related genes in brown-midrib *bm1*, *bm2*, *bm3*, and *bm4* young near-isogenic maize plants. *Planta* **226**, 235-250

Halpin C, Boerjan W (2003) Stacking transgenes in forest trees. *Trends in Plant Science* **8**, 363-365

Hamberger B, Hahlbrock K (2004) The 4-coumarate:CoA ligase gene family in *Arabidopsis thaliana* comprises one rare, sinapate-activating and three commonly occurring isoenzymes. *Proceedings of National Academy of Sciences USA* **101**, 2209-2214

Hosokawa M, Suzuki S, Umezawa T, Sato Y (2001) Progress of lignification mediated by intercellular transportation of monolignols during tracheary element differentiation in isolated *Zinnia* mesophyll cells. *Plant and Cell Physiology* **42**, 959-968

Hu WJ, Kawaoka A, Tsai CJ, Lung J, Osakabe K, Ebinuma H, Chiang VL (1998) Compartmentalized expression of two structurally and functionally distinct 4-coumarate:CoA ligase genes in aspen (*Populus tremuloides*). *Proceedings of the National Academy Sciences USA* **95**, 5407-5412

Israelsson M, Eriksson ME, Hertzberg M, Aspeborg H, Nilsson P, Moritz T (2003) Changes in gene-expression in wood-forming tissue of transgenic hybrid aspen with increased secondary growth. *Plant Molecular Biology* **52**, 893-903

Jouanin L, Goujon T, de Nadai V, Martin MT, Mila I, Vallet C, Pollet B, Yoshinaga A, Chabbert B, Petit-Conil M, Lapierre C (2000) Lignification in transgenic poplars with extremely reduced caffeic acid *O*-methyltransferase activity. *Plant Physiology* **123**, 1363-1374

Kao YY, Harding SA, Tsai CJ (2002) Differential expression of two distinct phenylalanine ammonia-lyase genes in condensed tannin-accumulating and lignifying cells of quaking aspen. *Plant Physiology* **130**, 796-807

Karpinska B, Karlsson M, Srivastava M, Stenberg A, Schrader J, Sterky F, Bhalerao R, Wingsle G (2004) MYB transcription factors are differentially expressed and regulated during secondary vascular tissue development in hybrid aspen. *Plant Molecular Biology* **56**, 255-270

Kawaoka A, Kaothien P, Yoshida K, Endo S, Yamada K, Ebinuma H (2000) Functional analysis of tobacco LIM protein Ntlim1 involved in lignin biosyn-

thesis. *Plant Journal* **22**, 289-301

Kawaoka A, Ebinuma H (2001) Transcriptional control of lignin biosynthesis by tobacco LIM protein. *Phytochemistry* **57**, 1149-1157

Kukkola EM, Koutaniemi S, Pöllänen E, Gustafsson M, Karhunen P, Lundell TK, Saranpää P, Kilpeläinen I, Teeri TH, Fagerstedt KV (2004) The dibenzodioxocin lignin substructure is abundant in the inner part of the secondary wall in Norway spruce and silver birch xylem. *Planta* **218**, 497-500

Kärkonen A, Koutaniemi S, Mustonen M, Syrjanen K, Brunow G, Kilpelainen I, Teeri TH, Simola LK (2002) Lignification related enzymes in *Picea abies* suspension cultures. *Physiologia Plantarum* **114**, 343-353

Kärkonen A, Fry SC (2006) Effect of ascorbate and its oxidation products on H_2O_2 production in cell-suspension cultures of *Picea abies* and in the absence of cells. *Journal of Experimental Botany* **57**, 1633-1644

Lacombe E, van Doorsselaere J, Boerjan W, Boudet AM, Grima-Pettenati J (2000) Characterization of *cis*-elements required for vascular expression of the cinnamoyl CoA reductase gene and for protein-DNA complex formation. *The Plant Journal* **23**, 663-676

Lapierre C, Pollet B, Petit-Conil M, Toval G, Romero J, Pilate G, Leple JC, Boerjan W, Ferret VV, de Nadai V, Jouanin L (1999) Structural alterations of lignins in transgenic poplars with depressed cinnamyl alcohol dehydrogenase or caffeic acid *O*-methyltransferase activity have an opposite impact on the efficiency of industrial kraft pulping. *Plant Physiology* **119**, 153-164

Lauvergeat V, Rech P, Jauneau A, Guez C, Coutos-Thevenot P, Grima-Pettenati J (2002) The vascular expression pattern directed by the *Eucalyptus gunnii* cinnamyl alcohol dehydrogenase *EgCAD2* promoter is conserved among woody and herbaceous plant species. *Plant Molecular Biology* **50**, 497-509

Li Y, Kajita S, Kawai S, Katayama Y, Morohoshi N (2003) Down-regulation of an anionic peroxidase in transgenic aspen and its effect on lignin composition. *Journal of Plant Research* **116**, 175-182

Li L, Zhou Y, Cheng X, Sun J, Marita JM, Ralph J, Chiang VL (2003) Combinatorial modification of multiple lignin traits in trees through multigene co-transformation. *Proceedings of National Academy of Sciences USA* **100**, 4939-4944

Liang M, Davis E, Gardner D, Cai X, Wu Y (2006) Involvement of *AtLAC15* in lignin synthesis in seeds and in root elongation of *Arabidopsis*. *Planta* **224**, 1185-1196

Logemann E, Parniske M, Hahlbrock K (1995) Modes of expression and common structural features of the complete phenylalanine ammonia-lyase gene family in parsley. *Proceedings of National Academy of Sciences USA* **92**, 5905-5909

López-Serrano M, Fernándes MD, Pomar F, Pedreño MA, Ros-Barceló A (2004) *Zinnia elegans* uses the same peroxidase isozyme complement for cell wall lignification in both single-cell tracheary elements and xylem vessels. *Journal of Experimental Botany* **55**, 423-431

Lu S, Zhou Y, Li L, Chiang VL (2006) Distinct roles of cinnamate 4-hydroxylase genes in *Populus*. *Plant and Cell Physiology* **47**, 905-914

Lu S, Sun YH, Shi R, Clark C, Li L, Chiang VL (2005) Novel and mechanical stress-responsive microRNAs in *Populus trichocarpa* that are absent from *Arabidopsis*. *Plant Cell* **17**, 2186-2203

Marjamaa K, Lehtonen M, Lundell T, Toikka M, Saranpaa P, Fagerstedt KV (2003) Developmental lignification and seasonal variation in β-glucosidase and peroxidase activities in xylem of Scots pine, Norway spruce and silver birch. *Tree Physiology* **23**, 977-986

Marjamaa K, Kukkola E, Lundell T, Karhunen P, Saranpaa P, Fagerstedt KV (2006a) Monolignol oxidation by xylem peroxidase isoforms of Norway spruce (*Picea abies*) and silver birch (*Betula pendula*). *Tree Physiology* **26**, 605-611

Marjamaa K, Hildén K, Kukkola E, Lehtonen M, Holkeri H, Haapaniemi P, Teeri T, Koutaniemi S, Teeri TH, Fagerstedt K, Lundell T (2006b) Cloning, characterization and localisation of three novel class III peroxidases in lignifying xylem of Norway spruce (*Picea abies*). *Plant Molecular Biology* **61**, 719-732

Mayer AM, Staples RC (2002) Laccase: new functions for an old enzyme. *Phytochemistry* **60**, 551-565

McCaig BC, Meagher RB, Dean JF (2005) Gene structure and molecular analysis of the laccase-like multicopper oxidase (LMCO) gene family in *Arabidopsis thaliana*. *Planta* **221**, 619-636

McDonald MP, Galwey NW, Colmer TD (2002) Similarity and diversity in adventitious root anatomy as related to root aeration among a range of wetland and dryland grass species. *Plant, Cell and Environment* **25**, 441-451

Mitsuda N, Iwase A, Yamamoto H, Yoshida M, Seki M, Shinozaki K, Ohme-Takagi M (2007) NAC transcription factors, NST1 and NST3, are key regulators of the formation of secondary walls in woody tissues *Arabidopsis*. *Plant Cell* **19**, 270-280

Nair RB, Bastress KL, Ruegger MO, Denault JW, Chapple C (2004) The *Arabidopsis thaliana* REDUCED EPIDERMAL FLUORESCENCE1 gene encodes an aldehyde dehydrogenase involved in ferulic acid and sinapic acid biosynthesis. *Plant Cell* **16**, 544-554

Önnerud H, Gellerstedt G (2003) Inhomogenities in the chemical structure of spruce lignin. *Holzforschung* **57**, 165-170

Østergaard L, Teilum K, Mirza O, Mattsson O, Petersen M, Welinder KG, Mundy J, Gajhede M, Henriksen A (2000) *Arabidopsis* ATP A2 peroxidase. Expression and high-resolution structure of a plant peroxidase with implications for lignification. *Plant Molecular Biology* **44**, 231-243

Passardi F, Cosio C, Penel C, Dunand C (2005) Peroxidases have more functions than a Swiss army knife. *Plant Cell Reports* **24**, 255-265

Pesquet E, Ranocha P, Legay S, Digonnet C, Barbier O, Pichon M, Goffner D (2005) Novel markers of xylogenesis in zinnia are differentially regulated by auxin and cytokinin. *Plant Physiology* **139**, 1821-1839

Piquemal J, Chamayou S, Nadaud I, Beckert M, Barrière Y, Mila I, Lapierre C, Rigau J, Puigdomenech P, Jauneau A, Digonnet C, Boudet A-M, Goffner D, Pichon M (2002) Down-regulation of caffeic acid *O*-methyltransferase in maize revisited using a transgenic approach. *Plant Physiology* **130**, 1675-1685

Prassinos C, Ko J-H, Yang J, Han K-H (2005) Transcriptome profiling of vertical stem segments provides insights into the genetic regulation of hybrid aspen trees. *Plant and Cell Physiology* **46**, 1213-1225

Patzlaff A, McInnis S, Courtney A, Surman C, Newman LJ, Smith C, Bevan MW, Mansfield S, Whetten, RW, Sederof RR, Campbell MM (2003) Characterization of a pine MYB that regulates lignification. *The Plant Journal* **36**, 743-754

Raes J, Rohde A, Christensen JH, Peer YV, Boerjan W (2003) Genome-wide characterization of the lignification toolbox in *Arabidopsis*. *Plant Physiology* **133**, 1051-1071

Ralph J, Hatfield RD, Grabber JH, Jung HG, Quideau S, Helm RF (1998) Cell wall cross-linking in grasses by ferulates and diferulates. In: Lewis NG, Sarkanen S (Eds) *Lignin and Lignan Biosynthesis*, American Chemical Society, Washington DC, pp 209-236

Ranocha P, McDougal G, Hawkins S, Sterijiades R, Borderies G, Steward D, Cabanes-Machetau M, Boudet A-M, Goffner D (1999) Biochemical characterization, molecular cloning and expression of laccases – divergent gene family – in poplar. *European Journal of Biochemistry* **259**, 485-495

Ranocha P, Chabannes M, Chamayou S, Danoun S, Jauneau A, Boudet AM, Goffner D (2002) Laccase down-regulation causes alterations in phenolic metabolism and cell wall structure in poplar. *Plant Physiology* **129**, 145-155

Rogers LA, Dubos C, Cullis IF, Surman C, Poole M, Willment J, Mansfield SD, Campbell MM (2005b) Light, the circadian clock, and sugar perception in the control of lignin biosynthesis. *Journal of Experimental Botany* **56**, 1651-1663

Ros-Barceló A, Gómez-Ros LV, Ferrer MA, Hernández X (2006) The apoplastic antioxidant enzymatic system in the wood-forming tissues of trees. *Trees* **20**, 145-156

Samuels AL, Rensing KH, Douglas CJ, Mansfiel SD, Dharmawardhana DP, Ellis BE (2002) Cellular machinery of wood production: differentiation of secondary xylem in *Pinus contorta* var. *latifolia*. *Planta* **216**, 72-82

Sasaki S, Nishida T, Tsutsumi Y, Kondo R (2004) Lignin dehydrogenative polymerization mechanism: a poplar cell wall peroxidase directly oxidizes polymer lignin and produces *in vitro* dehydrogenative polymer rich in β-*O*-4 linkage. *FEBS Letters* **562**, 197-201

Sasaki S, Baba K, Nishida T, Tsutsumi Y, Kondo R (2006) The cationic cell-wall-peroxidase having oxidation ability for polymeric substrate participates in the late stage of lignification of *Populus alba* L. *Plant Molecular Biology* **62**, 797-807

Sato Y, Wuli B, Sederoff R, Whetten R (2001) Molecular cloning and expression of eight laccase cDNAs in loblolly pine (*Pinus taeda*). *Journal of Plant Research* **114**, 147-155

Sato Y, Whetten RW (2006) Characterization of two laccases of loblolly pine (*Pinus taeda*) expressed in tobacco BY-2 cells. *Journal of Plant Research* **119**, 581-588

Sato Y, Demura T, Yamawaki K, Inoue Y, Sato S, Sugiyama M, Fukuda H (2006) Isolation and characterization of a novel peroxidase gene ZPO-C whose expression and function are closely associated with lignification during tracheary element differentiation. *Plant and Cell Physiology* **47**, 493-503

Schreiber L, Hartmann K, Skrabs M, Zeier J (1999) Apoplastic barriers in roots: chemical composition of endodermal and hypodermal cell walls. *Journal of Experimental Botany* **50**, 1267-1280

Srivastava V, Schinkel H, Witzell J, Hertzberg M, Torp M, Srivastava MK, Karpinska B, Melzer M, Wingsle G (2007) Downregulation of high-isoelectric-point extracellular superoxide dismutase mediates alterations in the metabolism of reactive oxygen species and developmental disturbances in hybrid aspen. *The Plant Journal* **49**, 135-148

Tamagnone L, Merida A, Parr A, Mackay S, Culianez-Macia FA, Roberts K, Martin C (1998) The AmMYB308 and AmMYB330 transcription factors from *Antirrhinum* regulate phenylpropanoid and lignin biosynthesis in transgenic tobacco. *Plant Cell* **10**, 135-154

Taylor JG, Owen Jr, TP, Koonce LT, Haigler CH (1992) Dispersed lignin in tracheary elements treated with cellulose synthesis inhibitors provides evidence that molecules of the secondary cell wall mediate wall patterning. *Plant Journal* **2**, 959-970

Terashima N, Fukushima K, Takabe K (1986) Heterogenity in formation of lignin VIII. An autoradiographic study on the formation of guaiacyl and syringyl lignin in *Magnolia kobus* DC. *Holtzforschung* **40**, 101-105

Terashima N, Nakshima J, Takabe K (1998) Proposed structure for protolignin in plant cell walls. In: Lewis NG, Sarkanen S (Eds) *Lignin and Lignan Biosynthesis*, ASC Symposium Series **697**, pp 180-193

Tobias CM, Chow EK (2004) Structure of the cinnamyl-alcohol dehydrogenase gene family in rice and promoter activity of a member associated with lignification. *Planta* **220**, 678-688

Tokunaga N, Sakakibara N, Umezawa T, Ito Y, Fukuda H, Sato Y (2005) Involvement of extracellular dilignols in lignification during tracheary element differentiation of isolated *Zinnia* mesophyll cells. *Plant and Cell Physiology* **46**, 224-232

Tokunaga N, Uchimura N, Sato Y (2006) Involvement of gibberellin in tracheary element differentiation and lignification in *Zinnia elegans* xylogenic culture. *Protoplasma* **228**, 179-187

Tuskan GA, Difazio S, Jansson S, Bohlmann J, Grigoriev I, Hellsten U, Putnam N, Ralph S, Rombauts S, Salamov A, Schein J, Sterck L, Aerts A, Bhalerao RR, Bhalerao RP, Blaudez D, Boerjan W, Brun A, Brunner A, Busov V, Campbell M, Carlson J, Chalot M, Chapman J, Chen GL, Cooper D, Coutinho PM, Couturier J, Covert S, Cronk Q, Cunningham R, Davis J, Degroeve S, Dejardin A, Depamphilis C, Detter J, Dirks B, Dubchak I, Duplessis S, Ehlting J, Ellis B, Gendler K, Goodstein D, Gribskov M, Grimwood J, Groover A, Gunter L, Hamberger B, Heinze B, Helariutta Y, Henrissat B, Holligan D, Holt R, Huang W, Islam-Faridi N, Jones S, Jones-Rhoades M, Jorgensen R, Joshi C, Kangasjarvi J, Karlsson J, Kelleher C, Kirkpatrick R, Kirst M, Kohler A, Kalluri U, Larimer F, Leebens-Mack J, Leple JC, Locascio P, Lou Y, Lucas S, Martin F, Montanini B, Napoli C, Nelson DR, Nelson C, Nieminen K, Nilsson O, Pereda V, Peter G, Philippe R, Pilate G, Poliakov A, Razumovskaya J, Richardson P, Rinaldi C, Ritland K, Rouze P, Ryaboy D, Schmutz J, Schrader J, Segerman B, Shin H, Siddiqui A, Sterky F, Terry A, Tsai CJ, Uberbacher E, Unneberg P, Vahala J, Wall K, Wessler S, Yang G, Yin T, Douglas C, Marra M, Sandberg G, van de Peer Y, Rokhsar D (2006) The genome of black cottonwood, *Populus trichocarpa* (Torr. & Gray). *Science* **313**, 1596-1604

Tsuji Y, Fukushima K (2004) Behavior of monolignol glucosides in angiosperms. *Journal of Agricultural and Food Chemistry* **52**, 7651-7659

van Heerden PS, Towers GHN, Lewis NG (1996) Nitrogen metabolism in lignifying *Pinus taeda* cell cultures. *The Journal of Biological Chemistry* **271**, 12350-12355

Welinder KG, Justesen AF, Kjaersgård IVH, Jensen RB, Rasmussen SK, Jespersen HM, Duroux L (2002) Structural diversity and transcription of class III peroxidases from *Arabidopsis thaliana*. *European Journal of Biochemistry* **269**, 6063-6081

Yang C, Xu Z, Song J, Conner K, Vizcay Barrena G, Wilson ZA (2007) *Arabidopsis MYB26/MALE STERILE35* regulates secondary thickening in endothecium and is essential for anther dehiscence. *Plant Cell* **19**, 534-548

Zeier J, Goll A, Yokoyama M, Karahara I, Schreiber L (1999) Structure and chemical composition of endodermal and rhizodermal/hypodermal walls of several species. *Plant, Cell and Environment* **22**, 271-279

Zhang X-H, Chiang VL (1997) Molecular cloning of 4-coumarate: Coenzyme A ligase in loblolly pine and roles of this enzyme in the biosynthesis of lignin in compression wood. *Plant Physiology* **113**, 65-74

Zhang K, Qian Q, Huang Z, Wang Y, Li M, Hong L, Zeng D, Gu M, Chu C, Cheng Z (2006) GOLD HULL AND INTERNODE2 encodes a primarily multifunctional cinnamyl-alcohol dehydrogenase in rice. *Plant Physiology* **140**, 972-983

Zhong R, Morrison WH 3rd, Himmelsbach DS, Poole FL 2nd, Ye ZH (2000) Essential role of caffeoyl coenzyme A O-methyltransferase in lignin biosynthesis in woody poplar plants. *Plant Physiology* **124**, 563-578

Zhong R, Richardson EA, Ye Z-H (2007) Two NAC domain transcription factors, SND1 and NST1,function redundantly in regulation of secondary wall synthesis in fibers of *Arabidopsis*. *Planta* **225**, 1603-1611