Critical Reviews in Biochemistry and Molecular Biology, 38:305–350, 2003

ISSN: 1040-9238
DOI: 10.1080/10409230390242443

Lignin: Genetic Engineering and Impact on Pulping

Marie Baucher,[1,4] Claire Halpin,[2] Michel Petit-Conil,[3] and Wout Boerjan[4]*

[1]Laboratoire de Biotechnologie Végétale, Université Libre de Bruxelles, Brussels, Belgium; [2]Plant Research Unit, School of Life Sciences, University of Dundee at the Scottish Crop Research Institute, Invergowrie, Dundee, UK; [3]Centre Technique du Papier, Grenoble Cédex 9, France; [4]Department of Plant Systems Biology, Flanders Interuniversity Institute for Biotechnology (VIB), Ghent University, Gent, Belgium

* Address correspondence to: Wout Boerjan, Department of Plant Systems Biology, Flanders Interuniversity Institute for Biotechnology (VIB), Ghent University, Technologiepark 927, B-9052 Gent, Belgium; Tel.: 32-9-3313881; Fax: 32-9-3313809; E-mail: wout.boerjan@psb.ugent.be.

ABSTRACT: Lignin is a major component of wood, the most widely used raw material for the production of pulp and paper. Although the biochemistry and molecular biology underpinning lignin production are better understood than they are for the other wood components, recent work has prompted a number of re-evaluations of the lignin biosynthetic pathway. Some of the work on which these revisions have been based involved the investigation of transgenic plants with modified lignin biosynthesis. In addition to their value in elucidating the lignin biosynthetic pathway, such transgenic plants are also being produced with the aim of improving plant raw materials for pulp and paper production. This review describes how genetic engineering has yielded new insights into how the lignin biosynthetic pathway operates and demonstrates that lignin can be improved to facilitate pulping. The current technologies used to produce paper are presented in this review, followed by a discussion of the impact of lignin modification on pulp production. Fine-tuned modification of lignin content, composition, or both is now achievable and could have important economic and environmental benefits.

KEYWORDS: Chemical pulp, monolignol biosynthetic pathway, pulp and paper, transgenic trees, wood

TABLE OF CONTENTS

ABBREVIATIONS: **CAD:** cinnamyl alcohol dehydrogenase; **CCoAOMT:** caffeoyl-CoA *O*-methyltransferase; **CCR:** cinnamoyl-CoA reductase; **C4H:** cinnamic acid 4-hydroxylase; **C3H:** *p*-coumarate 3-hydroxylase; **4CL:** 4-coumarate:CoA ligase; **CMP:** chemimechanical pulp; **COMT:** caffeic acid/5-hydroxyferulic acid *O*-methyltransferase; **CTMP:** chemithermomechanical pulp; **CWR:** cell wall residue; **DFRC:** derivatization followed by reductive cleavage; **DP:** degree of polymerization; **DRIFT:** diffuse reflectance infrared Fourier transform; **ECF:** elemental chlorine free; **F5H:** ferulate 5-hydroxylase; **FTIR:** Fourier transform infrared; **G:** guaiacyl; **GC-MS:** gas chromatography-mass spectrometry; **GM:** genetically modified; **H:** *p*-hydroxyphenyl; **HCT:** hydroxycinnamoyl-CoA:shikimate/quinate hydroxycinnamoyltransferase; **HPLC:** high-pressure liquid chromatography; **LAC:** laccase; **5OHG:** 5-hydroxyguaiacyl; **NMR:** nuclear magnetic resonance; **PAL:** phenylalanine ammonia-lyase; **PGW:** pressure groundwood; **POX:** peroxidase; **RMP:** refiner mechanical pulp; **S:** syringyl; **SAD:** sinapyl alcohol dehydrogenase; **Sin:** sinapaldehyde; **Sy:** sum of syringaldehyde and syringic acid; **Syr:** syringaldehyde; **TCF:** totally chlorine free; **TMP:** thermomechanical pulp; **SGW:** stone groundwood; **V:** sum of vanillin and vanillic acid; **Van:** vanillin; ***:** see glossary.

I. INTRODUCTION

Lignin, along with cellulose, is a major constituent of wood and one of the most abundant biopolymers on Earth. Despite the importance of wood as an industrial raw material and a renewable energy resource, investigation into the biochemistry and molecular biology of wood formation is still in its infancy. Relatively little is known about the detailed biochemistry underlying the production of cellulose and hemicellulose wood components, and genes involved in these processes have only recently been cloned. Lignin biochemistry, on the contrary, has matured over 40 years of research and much of the molecular biology of lignification has been elucidated over the past decade. The rapid advance in lignin research over that of other wood components has been partly due to the relative tractability of the problem, but has also significantly benefited from the interest in, and knowledge of, lignin chemistry gained from the pulp and paper industry. During the manufacture of high-quality paper, lignin is chemically separated from the polysaccharide components of wood during pulping and bleaching reactions. Lignin extraction consumes large quantities of chemicals and energy leading to a poor environmental image for the industry (Higuchi, 1985; Odendahl, 1994; Biermann, 1996). The benefits of removing as much lignin as possible in order to avoid residual lignin, which causes discoloration and reduces paper brightness (Chiang *et al*., 1988), have to be balanced against the loss of pulp quality and strength that result when cellulose is significantly degraded. For these reasons, new biological approaches to pulping are continuously being researched. One approach has been to pre-treat wood chips with enzymes or fungi that degrade lignin (Messner & Srebotnik, 1994; Viikari *et al*., 1994; Paice *et al*., 1995; Akhtar *et al*., 1998a, 1998b) and at least one such biopulping application is nearing commercialization (Kirk & Akhtar, 2000). An alternative, but complementary, idea has been to modify lignin content or structure in genetically engineered trees to reduce lignin production or to make lignin easier to extract. Clearly, achieving this goal requires a profound understanding of lignin biosynthesis at the biochemical and molecular levels. Therefore, recent research into lignin biosynthesis has been motivated by the concerns and interests of the pulping industry. This research has equally been invaluable in yielding fundamentally new and unexpected insights into the basic biochemistry and molecular biology of lignification. In some cases, the most useful result of genetic engineering experiments aimed at improving pulping has been the realization that the *traditional* scheme of the lignin pathway is wrong in some respects, prompting renewed investigation of the pathway by classical biochemical and enzymological approaches. In other cases, modification of lignin biosynthetic genes has indeed led to the production of plants with improved pulping characteristics, but the results have not been wholly intuitive. The ability to predict how specific genetic modifications might influence pulping is still a significant challenge to our limited understanding of the chemistry and biochemistry of the plant cell walls that constitute wood.

This review presents the current status of our understanding of the reactions and genes involved in lignin biosynthesis and the impact these have in determining lignin quality for industrial applications, primarily wood pulping. It provides an introduction to wood and lignin structure and to the lignin biosynthetic pathway before focusing on the large body of information that has recently been gained by modifying the expression of lignin biosynthetic genes *in planta*. Finally, following a brief overview of different pulping processes, we evaluate the potential impact of changes in wood biochemistry, achieved by genetic engineering of lignin biosynthesis, to current pulping practices.

II. LIGNIN

A. Wood—The Raw Material

Wood is the major raw material for the production of pulp and paper (Food and Agriculture Organization, 2001a). Wood characteristics vary in different types of plant; for instance, conifers (gymnosperms) produce softwood whereas angiosperms produce hardwoods. Softwoods are mainly composed of three cell types, tracheids (which play a role both in rigidity and conduction), and axial and ray parenchyma cells. Hardwoods are mainly made of fibers, vessels, and axial and ray parenchyma cells. Vessels transport water and solutes through the vascular system while fibers provide rigidity, and ray cells facilitate centripetal nutrition (Higuchi, 1997). Tracheids, vessels, and fibers vary in shape and size (Table 1). The dimensions and chemical composition of the different cell types of wood depend on genetic and developmental factors, but are also influenced by environmental conditions, such as the climate and soil type (Vallette & de Choudens,

TABLE 1
Dimensions of the Different Cell Types of Softwoods and Hardwoods (Fengel & Wegener, 1984)

Cell Type	Character	Cell Dimensions
Softwood (*Picea abies*)		
Tracheids	Diameter	20–40 μm
	Wall thickness	2.1–4.3μm
	Length	1.7–3.7 mm
Hardwood (*Fagus* sp.)		
Fibers	Diameter	15–20 μm
	Wall thickness	5 μm
	Length	0.6–1.3 mm
Vessels	Diameter	5–100 μm
	Wall thickness	1 μm
	Length	0.3–0.7 mm

1992). Softwoods and hardwoods differ in their pulping characteristics. Indeed, the individual cell types within wood differ in their chemical characteristics, reflecting the underlying differences in biochemistry and molecular biology that are only beginning to be appreciated.

The three major components of wood cell walls are cellulose, hemicellulose, and lignin (Table 2). Long molecules of cellulose provide the skeleton of the walls. Linear cellulose chains are aligned together in structures known as 'elementary fibrils' or 'protofibrils' that, in turn, associate into more complex structures called microfibrils (Figure 1). Microfibrils are highly organized and form distinct fibrillar cell wall layers (Delmer & Amor, 1995).

TABLE 2
Chemical Components of Softwoods and Hardwoods (Aitken *et al*., 1988)

Components	Type	Softwoods	Hardwoods
Cellulose		42–44%	43–47%
Lignin		25–30%	17–26%
Hemicelluloses	Hexosans	±15%	5–8%
	Pentosans	10–15%	15–35%
Waxes, resins, fats		1–10%	0.5–2%
Mineral substances		<1%	<1%

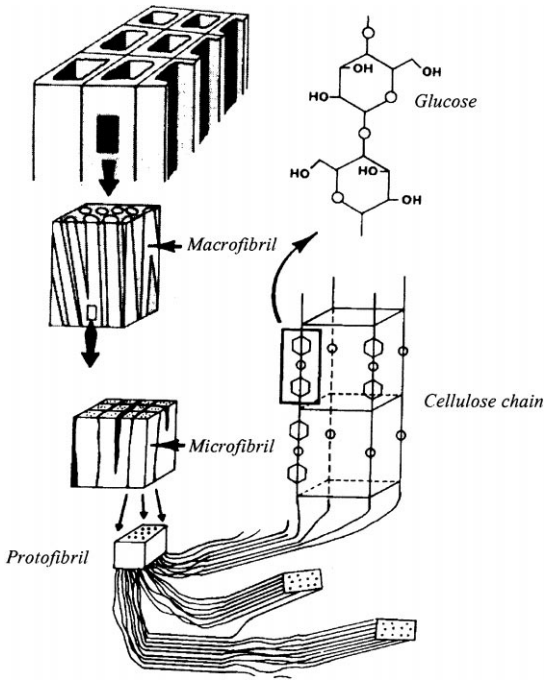

FIGURE 1. Organization of the cellulose skeletons in the fiber wall (Parham, 1987).

Hemicelluloses and other carbohydrates provide the matrix of the cell wall, whereas lignin, a heterogenous hydrophobic phenolic polymer, encrusts the other wall components to waterproof and strengthen the wall.

In a transverse plane, the parietal structure of wood cells is made of a primary and a secondary wall, the latter consisting of two or three layers, designated S1, S2, and S3 (Figure 2). Outside the primary wall, the middle lamella connects adjacent cells. The various cell wall layers differ in chemical composition (Mellerowicz *et al*., 2001). Studies on the distribution of lignin by UV microscopy show

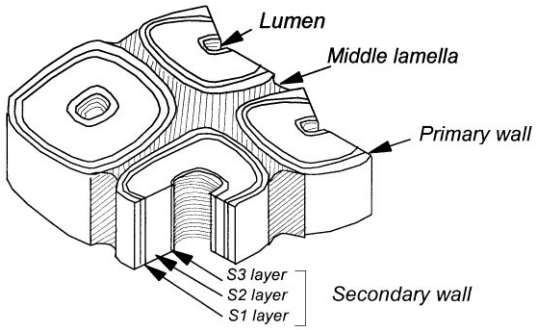

FIGURE 2. Structural organization of the cell walls of fibers (Petit-Conil, 1995).

that the middle lamella and the primary wall of secondary thickened cells are rich in lignin (70–80% lignin, which is approximately 15–20% of the total lignin in the cell wall). In contrast, the secondary walls are composed of only 20–25% lignin, but contain 80% of the total lignin because they constitute the largest part of the total cell wall (Fergus *et al*., 1969).

B. Structure of Lignin

The lignin polymer is produced by the dehydrogenative polymerization of essentially three different cinnamyl alcohols (*p*-coumaryl, coniferyl, and sinapyl alcohol) that differ in the degree of methoxylation at the C_3 and C_5 positions of the aromatic ring (Figure 3). When incorporated into lignin, these alcohols are called the *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units of the polymer, respectively. In addition to the three main monolignols, lignin contains traces of units from incomplete monolignol biosynthesis and incorporates various other phenylpropanoid units, such as hydroxycinnamyl aldehydes, acetates, *p*-coumarates, *p*-hydroxybenzoates, and tyramine ferulate (Sederoff *et al*., 1999; Boerjan *et al*., 2003). A variety of chemical linkages, including ether and carbon-carbon bonds, connect the units in lignin (Figure 4; Higuchi, 1990; Ralph *et al*., 1998; Boerjan *et al*., 2003). The complexity and heterogeneity of the polymer depend on the relative proportions of the three principal monolignol units as well as the different types of interunit linkages (Campbell & Sederoff, 1996; Monties, 1998). For example, lignin from gymnosperms consists mainly of G units and low levels of H units, whereas lignin from angiosperms is predominantly made up of both G and S units along with traces of H units. Lignin from grasses incorporates G and S units at comparable levels and more H units than dicots. Lignin rich in G units, such as gymnosperm lignin, has relatively more carbon-carbon bonds than lignin rich in S units because the aromatic C_5 position of G units is free to make linkages. As a consequence, wood lignins essentially made of G units (softwoods) are less susceptible to Kraft delignification than lignins made of G and S units (hardwoods) (Chiang & Funaoka, 1990; Ona *et al*., 1996; Lapierre *et al*., 1999) because the targets of the chemical Kraft delignification process are the non-condensed ether β-O-4-linkages in lignin (see section IV.C.1.a), whereas the carbon-carbon bonds (β-β, β-1, β-5, and 5-5) are more resistant to chemical degradation.

Determining the amount, structure, or monomeric composition of lignin in a plant is extremely difficult because of the heterogeneity of the polymer and the high proportion of covalent bonds linking different monomers. Moreover, during isolation, lignin undergoes secondary modifications, such as condensation, oxidation, addition, or substitution. Therefore, a combination of several methods has to be used to obtain reliable information on lignin structure. Different histochemical, chemical, and spectroscopic methods have been developed to investigate lignin content and composition (Monties, 1989; Lapierre, 1993; Dean, 1997; Lu & Ralph, 1997). Each method has its own limitations (for discussion, see Anterola & Lewis, 2002) and apparent discrepancies in the literature can often be attributed to the different techniques used by different laboratories. Therefore, it is important that the techniques are specified when, for example, lignin content or structure evaluations from transgenic plants are compared. In this review, lignin methods, whether specifically referred to or not, will be indicated by superscript letters and defined in the glossary. Lignin can be quantified either by removing all the cell wall constituents, with the exception of lignin (for example, Klason[e] lignin determination) or by extracting the lignin component from the cell wall (e.g., with thioglycolic acid[h] or acetyl bromide[a]). The main methods used to determine lignin structure and composition are based on the analysis of the degradation products of lignin [by pyrolysis gas chromatography-mass spectrometry (pyrolysis GC-MS)[g], alkaline nitrobenzene oxidation[b], thioacidolysis[i], derivatization followed by reductive cleavage (DFRC)[c], or Fourier transform infrared (FTIR) spectroscopy[d]]. The targets of these techniques are essentially the β-O-4 linkages that are the most abundant linkages in lignin. Physical methods, such as UV and IR spectroscopy, allow the identification of particular structures that absorb light at specific wavelengths. Thanks to recently developed methods, such as NMR spectroscopy[f], a more detailed picture of lignin structure has been obtained, especially clarifying the different chemicals bonds in the polymers (Ralph *et al*., 1998, 1999a).

C. Biosynthesis of Lignin

Lignin monomers, or monolignols, are produced intracellularly, then exported to the cell wall, and subsequently polymerized. The monolignols are

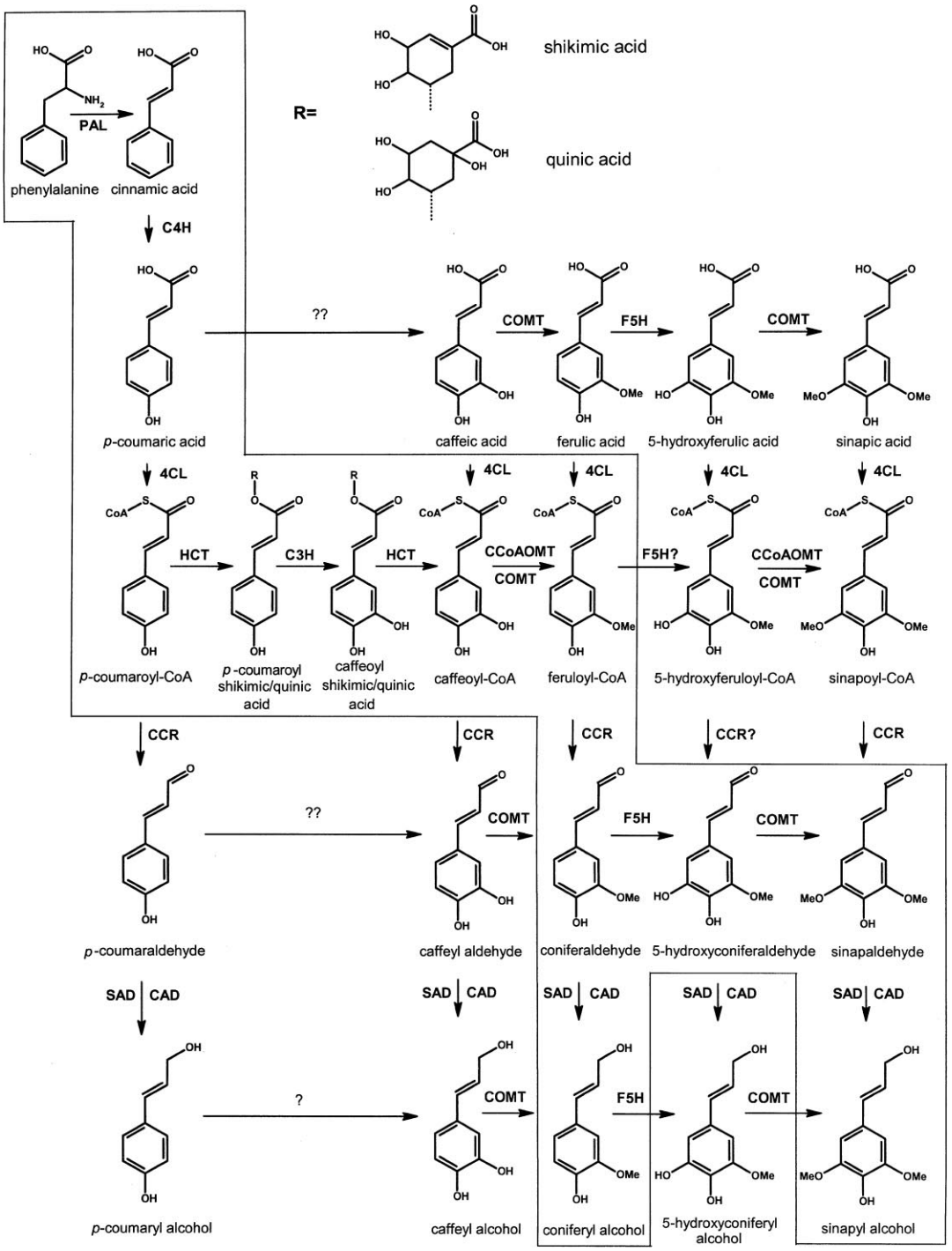

FIGURE 3. Phenylpropanoid and monolignol biosynthesis pathways. CAD, cinnamyl alcohol dehydrogenase; 4CL, 4-coumarate:CoA ligase; C3H, *p*-coumarate 3-hydroxylase; C4H, cinnamate 4-hydroxylase; CCoAOMT, caffeoyl-CoA *O*-methyltransferase; CCR, cinnamoyl-CoA reductase; COMT, caffeic acid *O*-methyltransferase; HCT, *p*-hydroxycinnamoyl-CoA:shikimate/quinate *p*-hydroxycinnamoyltransferase; F5H, ferulate 5-hydroxylase; PAL, phenylalanine ammonia-lyase; SAD, sinapyl alcohol dehydrogenase. The boxed area represents the most likely pathway to the production of monolignols. All enzymatic reactions shown have been demonstrated by *in vitro* assays and do not necessarily occur *in vivo*. F5H? and CCR?, substrate not tested; ?, conversion demonstrated but enzyme unknown; ??, direct conversion not convincingly demonstrated.

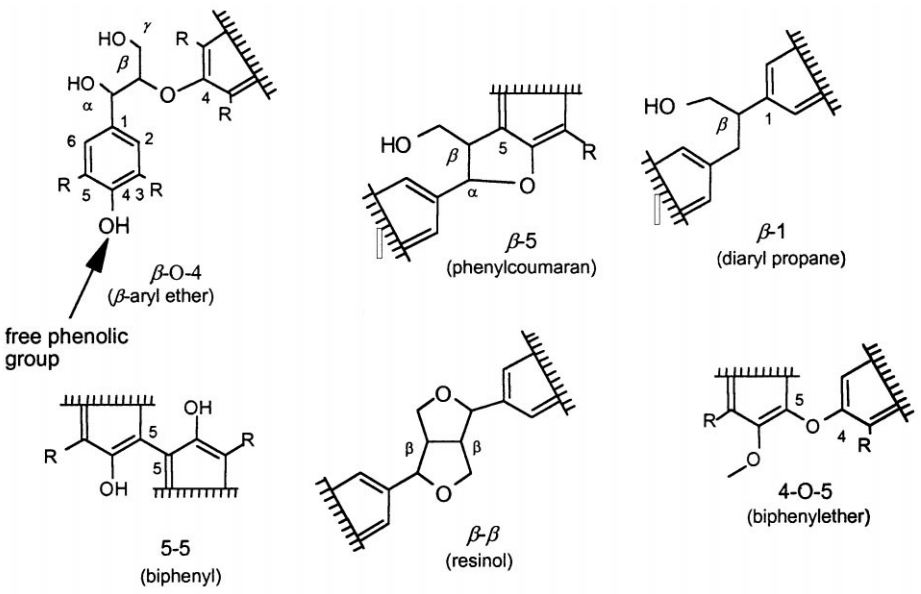

FIGURE 4. Possible intermonomeric linkages in lignin (Ralph *et al*., 1998; Lapierre *et al*., 1999; Boerjan *et al*., 2003). *p*-hydroxyphenyl (H), R=H; guaiacyl (G), R=OCH_3 at C-3 and R=H at C-5; syringyl (S), R=OCH_3.

products of the phenylpropanoid pathway, starting from phenylalanine (Figure 3), and most of the genes involved in monolignol production have been cloned or are present in expressed sequence tag/genomic databases (for a review, see Christensen *et al*., 2000; Boerjan *et al*., 2003; Raes *et al*., 2003). The lignin biosynthetic pathway has been the subject of many recent reviews (Baucher *et al*., 1998; Whetten *et al*., 1998; Grima-Pettenati & Goffner, 1999; Lewis, 1999; Boudet, 2000; Dixon *et al*., 2001; Humphreys & Chapple, 2002; Anterola & Lewis, 2002; Boerjan *et al*., 2003), but is still being continually revised and updated to incorporate the results of new research. Because of the already extensive coverage of the pathway in the literature, only the most important and most recent revisions to the traditional pathway will be described here.

The hydroxylation and methylation reactions that ultimately determine the monomeric composition of lignin (because the three monolignols differ only in their degree of methoxylation) have long been considered to occur at the level of the cinnamic acids (Higuchi, 1985). Several experiments have demonstrated that the methylation steps can also take place at the hydroxycinnamoyl-CoA level, mediated by either caffeic acid/5-hydroxyferulic acid *O*-methyltransferase (COMT) or caffeoyl-CoA *O*-methyltransferase (CCoAOMT) (Ye *et al*., 1994; Li *et al*., 1997; Inoue *et al*., 1998; Martz *et al*., 1998; Meng & Campbell, 1998; Maury *et al*., 1999). However, recent work based on radiotracer and *in vitro* enzyme assays has shown that the hydroxylation and methylation reactions occur preferentially at the cinnamaldehyde and cinnamyl alcohol level in reactions catalyzed by ferulic acid 5-hydroxylase (F5H; also named coniferaldehyde 5-hydroxylase or Cald5H) and COMT (alternatively called 5-hydroxyconiferaldehyde *O*-methyltransferase or AldOMT) (Matsui *et al*., 1994, 2000; Chen *et al*., 1999; Humphreys *et al*., 1999; Osakabe *et al*., 1999; Maury *et al*., 1999; Li *et al*., 2000; Parvathi *et al*., 2001; Guo *et al*., 2002). COMT preferentially methylates caffeyl aldehyde, 5-hydroxyconiferaldehyde, and 5-hydroxyconiferyl alcohol (Figure 3), although differences exist between the COMTs of different species analyzed (Humphreys *et al*., 1999; Li *et al*., 2000; Parvathi *et al*., 2001; Zubieta *et al*., 2002). These revisions to the role and position of COMT within the lignin pathway help to explain the results of previous studies on COMT-suppressed plants where total lignin contents were found to be maintained despite dramatic reductions in S lignin.

A continuing controversy surrounds the involvement of 4-coumarate:CoA ligase (4CL) and sinapic acid in S lignin biosynthesis. Traditionally, sinapic acid was thought to be a lignin precursor that was converted to sinapoyl-CoA by 4CL. However, results of *in vitro* experiments with 4CLs from different plants throw significant doubt on

this assumption. Whereas 4CL isoforms from some plants have been found to convert sinapic acid to sinapoyl-CoA (Grand *et al*., 1983; Higuchi, 1997; Lindermayr *et al*., 2002a; Yamauchi *et al*., 2003), enzymes from other plants apparently are deficient in this activity (Allina *et al*., 1998; Hu *et al*., 1998; Ehlting *et al*., 1999; Dixon *et al*., 2001; Yamauchi *et al*., 2003). Small differences between 4CL isoforms can significantly influence its activity. Recently, a model based on structural data postulates that the substrate specificity of 4CL is determined by 12 amino acid residues (Schneider *et al*., 2003). Indeed, deletion of a single Val residue in the soybean Gm4CL2 and Gm4CL3 isoforms, which do not normally convert sinapic acid into sinapoyl-CoA, allowed these enzymes to gain this activity (Lindermayr *et al*., 2002b). Similarly, deletion of the amino acid Val-355 or Leu-356 in the *Arabidopsis* At4CL2 confers activity toward sinapic acid (Schneider *et al*., 2003). Interestingly, a novel 4CL isoform lacking the Leu residue has recently been identified in *Arabidopsis* by phylogenetic analysis and this isoform efficiently converts sinapic acid (Schneider *et al*., 2003). These data suggest that the progression into the monolignol biosynthesis pathway in different plants may depend on the specificity of the particular 4CLs present, and probably also on their cell-specific expression patterns.

Perhaps the most significant recent discovery has been the realization, based initially on classical enzyme assays, that *p*-coumaroyl-shikimate and *p*-coumaroyl-quinate (and not *p*-coumaric acid as previously thought) are the preferred substrates for *p*-coumarate 3-hydroxylase (C3H) (Schoch *et al*., 2001). Although recombinant C3H can slowly convert *p*-coumaric acid into caffeic acid in yeast (Nair *et al*., 2002), this reaction is no longer thought to be important in the lignin biosynthetic pathway. Instead, *p*-coumaroyl-CoA is probably converted into shikimate and quinate esters by a reversible hydroxycinnamoyl-CoA:shikimate/quinate hydroxycinnamoyltransferase (HCT) and these compounds subsequently act as preferred substrates for C3H (Schoch *et al*., 2001). Recently, the gene encoding HCT has been cloned (Hoffmann *et al*., 2003). After hydroxylation by C3H, the resulting caffeoyl-CoA esters are converted to caffeoyl-CoA by the same enzyme, HCT, before being methylated by CCoAOMT or COMT, as previously envisaged. Significantly, analysis of *Arabidopsis* mutants in the *C3H* gene provides strong support for the proposed revised role of this enzyme in the lignin biosynthetic pathway (Franke *et al*., 2002a, 2002b).

Another potential revision to the lignin pathway concerns the role of cinnamyl alcohol dehydrogenase (CAD), the enzyme thought to catalyze the reduction of the cinnamaldehydes, coniferyl aldehyde, and sinapyl aldehyde into cinnamyl alcohols. Recently, Li *et al*. (2001) have identified a sinapyl alcohol dehydrogenase (SAD) from poplar that uses sinapaldehyde as the preferred substrate and demonstrated that SAD co-localizes with S lignin formation both temporally and spatially. This suggests that SAD may be the enzyme responsible for the production of sinapyl alcohol, the S lignin precursor, relegating CAD to a role only in the production of the G lignin precursor, coniferyl alcohol (Li *et al*., 2001). The data provided by Li *et al*. (2001) are convincing, but circumstantial. Further work, particularly the production of *sad* mutants or SAD-suppressed transgenic plants, is needed to validate/investigate the role of SAD *in vivo* in a variety of angiosperms.

The final steps in the biosynthesis of lignin are the oxidation of the cinnamyl alcohols to the corresponding radicals in the cell wall and their subsequent polymerization. The possible mechanisms of lignin polymerization have been recently reviewed (Lewis, 1999; Christensen *et al*., 2000; Boerjan *et al*., 2003). A significant body of research over the past decade has proposed that peroxidases, laccases, and other phenol oxidases may be involved in the radical formation process (Savidge & Udagama-Randeniya, 1992; Dean & Eriksson, 1994; McDougall *et al*., 1994; Richardson *et al*., 1997). Recently, Önnerud *et al*. (2002) have suggested that the monolignols are polymerized by a redox shuttle-mediated oxidation. However, the exact role of these different enzymes remains as elusive as ever and little real progress has been made. This is in part due to the multiplicity of these enzymes that exist in plant cells and the probability that there may be some redundancy in function between them. Consequently, determining the role of individual enzymes in a process such as lignification can be difficult. This lack of progress on the mechanisms of lignin polymerization has exacerbated the surprisingly heated controversy that has developed over whether monolignol coupling is a random or a highly orchestrated process. With the discovery of a dirigent protein in *Forsythia*, capable of catalyzing the stereoselective coupling of two coniferyl alcohol radicals into the lignan pinoresinol (Davin *et al*., 1997), it has been suggested that monolignol radical coupling during lignin biosynthesis is also tightly controlled in plants (Gang *et al*., 1999)—a proposition in direct conflict to the well-established and supported random coupling model (Hatfield &

Vermerris, 2001). This controversial suggestion, however, still awaits testing by reverse genetic approaches.

A persistent challenge is to understand how similar enzymes in different plants can give rise to the striking natural heterogeneity that exists in lignin. The content and composition of lignin differs not only among plant taxa, but also between different cell types of a single tissue and even within a single cell wall (Joseleau & Ruel, 1997). Lignin can also be influenced by environmental stress. Current thinking regards lignin heterogeneity as a product of the spatio-temporal and conditional expression of the genes involved in the lignin pathway and of differences in the substrate specificity and kinetics of the enzymes they encode (Campbell & Sederoff, 1996; Weisshaar & Jenkins, 1998; Chen *et al*., 2000; Harding *et al*., 2002; Zubieta *et al*., 2002). These processes may also be regulated by pathway intermediates because the concentration of certain intermediates has been shown to affect enzyme activity and gene expression (Osakabe *et al*., 1999; Blount *et al*., 2000; Li *et al*., 2000; Anterola *et al*., 2002). Despite our increasing knowledge of lignin biosynthesis, major uncertainties remain (Boudet *et al*., 1995; Whetten & Sederoff, 1995; Campbell & Sederoff, 1996; Douglas, 1996; Dixon *et al*., 2001). For instance, little is known about the cell biology of the process. Although the monolignols are assumed to be stored as glucosides before being transported to the cell wall and polymerized into lignin, little hard evidence supports this assumption. Similarly, the precise subcellular location of most of the lignin biosynthetic enzymes is still an open question. Furthermore, as illustrated above, some of the recent revisions to the lignin pathway have been made purely on the basis of classical biochemical experiments to determine the substrate specificity and kinetics of action of particular enzymes. It is crucial to determine whether these experiments, performed *in vitro*, reliably indicate the role of the respective enzymes *in vivo*. In order to gain a fuller understanding of how the lignin biosynthetic pathway operates *in vivo*, many research laboratories have been studying mutant and transgenic plants with altered expression of lignin biosynthetic genes. Both model (*Arabidopsis* and tobacco) and economically important species (alfalfa and poplar) have been genetically engineered, and results obtained have generally been in accordance. This research has revolutionized our understanding of lignin biosynthesis, having prompted and, in some cases, confirmed the revisions to the pathway made on the basis of biochemical data. It has also opened up new research areas, posed new questions, and indicated how lignin could be modified to improve industrial processes, such as pulping.

III. MUTANTS AND TRANSGENIC PLANTS WITH MODIFIED LIGNIN

A. Up- and Down-Regulation of Phenylalanine Ammonia-Lyase (PAL)

Phenylalanine ammonia-lyase (PAL) catalyzes the first step of the phenylpropanoid pathway—the non-oxidative deamination of L-phenylalanine to cinnamic acid (Figure 3). PAL has been suppressed by 85% and >98% in the stems of transgenic plants with resultant 52% (Sewalt *et al*., 1997) and 70% (Korth *et al*., 2001) reduction in Klason[e] lignin content, respectively (Table 3). Lignin monomeric composition, determined by pyrolysis GC-MS[g], was characterized by a lower proportion of G units and a 1.7-fold increase in S/G ratio (Sewalt *et al*., 1997). Similarly, using an independent method[i], Korth *et al*. (2001) determined a 4-fold increase in the S/G ratio caused by a more pronounced reduction in the level of G units than in S units. Because PAL catalyzes the first step of the phenylpropanoid pathway, reduction of its activity results in a wide range of abnormal phenotypes. The transgenic plants were stunted, had curled leaves, and had thinner cell walls in the secondary xylem with less lignin than those of the control (Elkind *et al*., 1990; Bate *et al*., 1994). These plants were also more susceptible to the fungal pathogen *Cercospora nicotianae* (Maher *et al*., 1994). A slight increase in Klason[e] lignin and dry matter content was observed in the stem of PAL-overexpressing plants (Howles *et al*., 1996; Korth *et al*., 2001). Overexpression of PAL did not lead to changes in lignin composition as determined by pyrolysis GC-MS[g] (Sewalt *et al*., 1997), but to a decrease in the amount of S units, yielding a reduction in the S/G ratio when lignin was analyzed by thioacidolysis[i] (Korth *et al*., 2001). The level of chlorogenic acid (3-caffeoylquinic acid) has been correlated with the PAL enzymatic activity in leaves and stems of both PAL-silenced and PAL-overproducing tobacco (Elkind *et al*., 1990; Bate *et al*., 1994; Maher *et al*., 1994; Howles *et al*., 1996; Blount *et al*., 2000; Korth *et al*., 2001).

TABLE 3
Lignin Mutants and Transgenic Plants

Plant Gene	Enzyme Activity In Stems (%)	Lignin Content	Lignin Composition	References
Arabidopsis thaliana				
C3H (*ref8* mutant)	—	Reduced[1,2]	Traces of S and G[5,6,11], principally H[5,6,11]	Franke *et al*. (2002b)
COMT (*Atomt1* mutant)	6.6	Increased[1]	Traces of S, G increased,5OHG[7]	Goujon *et al*. (2003a)
COMT (S)	212	No changes[1]	No Changes[7]	Goujon *et al*. (2003a)
F5H (*fah1* mutant)	—	No changes[1]	Trace levels of Syr[5], Sy[5] and S[8,11], Van[5], V[5] or G[11] increased	Chapple *et al*. (1992), Meyer *et al*. (1998), Marita *et al*. (1999), Sibout *et al*. (2002)
F5H (S)	—	Reduced[1]	Sy[5] or S[8,11] increased, V[5] or G[11] decreased	Meyer *et al*. (1998), Marita *et al*. (1999)
F5H (S)	—	Reduced[1]	S increased[7], G decreased[7]	Sibout *et al*. (2002)
4CL (AS)	8	Reduced[2]	V decreased[5], Sy/V increased[5]	Lee *et al*. (1997)
CCR (AS)	19	Reduced[1]	S/G increased or decreased depending on culture conditions[7]	Goujon *et al*. (2003b)
CCR (*irx4* mutant)	—	Reduced[2,8]	—	Jones *et al*. (2001)
CAD (*Atcad-C* mutant)	38	Reduced[1]	No changes[7]	Sibout *et al*. (2003)
CAD (*Atcad-D* mutant)	6	Reduced[1]	S decreased[7], G increased[7], S/G decreased[7], Sin and Syr increased[7]	Sibout *et al*. (2003)
Liriodendron tulipifera				
LAC (AS)	—	No changes	No changes	Dean *et al*. (1998)
Lycopersicon esculentum				
POX (S)	—	Increased[2]	—	El Mansouri *et al*. (1999)
Medicago sativa				
COMT (AS)	4.5	Reduced[1]	G decreased[7], S/G decreased[7], no S[7], no 5OHG[7]	Guo *et al*. (2001)
COMT (S)	15	Reduced[1]	S[7,8,11] and G[8,11] decreased, S/G decreased[7,8,11], 5OHG[7,8], Syr/V decreased[5]	Guo *et al*. (2001), Marita *et al*. (2003)
CCoAOMT (AS)	4	Reduced[1]	S[11] and G[7,11] decreased, S/G increased[7,8,11], Syr/V increased[5]	Guo *et al*. (2001), Marita *et al*. (2003)
COMT (AS)/ *CCoAOMT* (AS)	12/5	Reduced[1], No changes[4]	S decreased[7], S/G decreased[7]	Guo *et al*. (2001)
COMT (S)/ *CCoAOMT* (S)	11/25	Reduced[1], No changes[4]	S and G decreased[7], S/G decreased[7]	Guo *et al*. (2001)
CAD (AS)	30	No changes[1]	More aldehydes, S/G decreased[7]	Baucher *et al*. (1999)
Nicotiana tabacum				
PAL (S)	2.3	Reduced[1]	S and G decreased[7], S/G increased[7]	Korth *et al*. (2001)
PAL (S)	10	Reduced[1,2,13]	S/G increased[6], G decreased[6]	Elkind *et al*. (1990), Bate *et al*. (1994), Sewalt *et al*. (1997)
PAL (S)	150	Increased[1]	S decreased[7], S/G decreased[7]	Korth *et al*. (2001)

(Continued on next page)

TABLE 3
Lignin Mutants and Transgenic Plants *(Continued)*

Plant Gene	Enzyme Activity In Stems (%)	Lignin Content	Lignin Composition	References
PAL (S)	200	Increased[1]	No changes[6]	Howles *et al*. (1996), Sewalt *et al*. (1997)
C4HI (AS)	20	Reduced[1]	S/G decreased[6]	Sewalt *et al*. (1997)
C4HI (S)	200	No changes[1]	No changes[6]	Sewalt *et al*. (1997)
C4HII (AS and S)	10	Reduced[4]	S/G decreased[7]	Blee *et al*. (2001)
COMT (AS)	42	Reduced[2]	No changes[5]	Ni *et al*. (1994)
COMT (AS)	54	No changes[3]	Sy/V decreased[5], Sy decreased[5]	Dwivedi *et al*. (1994)
COMT (S)	2	No changes[1]	S decreased[7], 5OHG[7]	Atanassova *et al*. (1995)
COMT (S)	322	—	No changes[7]	Atanassova *et al*. (1995)
CCoAOMT (AS)	25	Reduced[1]	S/G increased[6], S and G decreased[7]	Zhong *et al*. (1998)
CCoAOMT (AS)	∼20	Reduced[1]	No changes[7]	Pinçon *et al*. (2001a)
CCoAOMT (AS)/ *COMT* (AS)	8/14	Reduced[1]	S/G decreased[6], S and G decreased[6]	Zhong *et al*. (1998)
CCoAOMT (AS)/ *COMT* (AS)	∼20/∼30	Reduced[1]	S decreased[7], 50 HG[7]	Pinçon *et al*. (2001a)
F5H (S)	—	Reduced[1,2] or lignin more extractable	S/G increased[5], S increased[5,11] and G decreased[5]	Franke *et al*. (2000)
4CL (S)	<1	Reduced[1,4]	Syr/Van decreased or increased[5], less Van[5] and Syr[5,], more *p*-hydroxybenzaldehyde[5], H increased, S and G reduced[15], more HCAs[8]	Kajita *et al*. (1996, 1997)
4CL (AS)	∼20	Reduced[4]	Syr/Van increased, less aldehydes[5]	Kajita *et al*. (1996)
CCR (AS)	30	Reduced[1,4]	S and G decreased[7], S/G increased[7], tyramine ferulate increased[8]	Piquemal *et al*. (1998), Ralph *et al*. (1998)
CCR (S)	1	Reduced[1]	S and G decreased[7], S/G increased[7]	O'Connell *et al*. (2002)
COMT (AS)/ *CCR* (AS)	41/10	Reduced[1]	S/G increased[7]	Pinçon *et al*. (2001b)
CAD2 (AS)	7	No changes[1]	More aldehydes[6], S/G decreased[7]	Halpin *et al*. (1994)
CAD2 (AS)	8	No changes[1,4]	S/G decreased[7], more cinnamaldehydes[8]	Yahiaoui *et al*. (1998), Ralph *et al*. (1998)
CAD2 (AS)	9	No changes[4]	More aldehydes[10]	Stewart *et al*. (1997)
CAD2 (AS)	45	No changes[4]	More aldehydes[7]	Higuchi *et al*. (1994), Hibino *et al*. (1995)
CAD2 (AS)/ *COMT* (AS)	variable/ variable	No changes[1,4]	No changes[7]	Abbott *et al*. (2002)
CAD2 (S)/ *COMT* (S)	15/36	Reduced[1,4]	—	Abbott *et al*. (2002)
CAD2 (AS)/ *CCR* (AS)	12/32	Reduced[1]	S/G increased[7], S and G decreased[7], tyramine ferulate increased[8]	Chabannes *et al*. (2001b)

(Continued on next page)

TABLE 3
Lignin Mutants and Transgenic Plants *(Continued)*

Plant Gene	Enzyme Activity In Stems (%)	Lignin Content	Lignin Composition	References
CAD2 (S)/ *COMT* (S)/ *CCR* (S)	4/24/18	Reduced[1,4]	—	Abbott *et al*. (2002)
POX (S)	250	Increased[2,4]	No changes[7]	Chabbert *et al*. (1992), Lagrimini (1991)
POX (S)	—	No changes[1]	More coniferaldehyde[7]	Elfstrand *et al*. (2002)
POX (AS)	1	No changes[2,4]	No changes[7]	Chabbert *et al*. (1992), Lagrimini *et al*. (1997a)
Pinus taeda				
CAD (*cad-n1* mutant)	1	Reduced[1]	More dihydroconiferyl alcohol[8], more coniferaldehyde[8]	MacKay *et al*. (1997), Ralph *et al*. (1997)
Populus kitakamiensis				
POX (AS)	56	Reduced[4,12]	More β-O-4[7]	Yahong *et al*. (2001)
P. tremula x P. alba				
COMT (AS)	5	No changes[1]	S/G decreased[7], G increased[7], 5OHG[7]	Van Doorsselaere *et al*. (1995), Lapierre *et al*. (1999), Ralph *et al*. (2001a, 2001b), Pilate *et al*. (2002)
COMT (S)	<3	Reduced[1]	S/G decreased[7], 5OHG[7], more coniferaldehyde[7]	Jouanin *et al*. (2000), Ralph *et al*. (2001b)
CCoAOMT (S)	10% protein amount	Reduced[1]	S/G increased[7], S and G decreased[7], more *p*-hydroxybenzoic acid[8]	Meyermans *et al*. (2000)
CCoAOMT (AS)	30	Reduced[1,14]	S and G decreased[6]	Zhong *et al*. (2000)
F5H (S)	—	—	S/G increased[11], Sy[5] or S[11] increased, V decreased[5]	Franke *et al*. (2000)
CCR (S, AS)	—	Reduced[1]	S/G increased[7]	J.-C. Leplé, C. Lapierre and W. Boerjan
CAD2 (AS)	30	Reduced[1]	More syringaldehyde	Baucher *et al*. (1996), Lapierre *et al*. (1999), Pilate *et al*. (2002)
CAD2 (S)	30	No changes[1]	No changes[7]	Baucher *et al*. (1996), Lapierre *et al*. (1999)
POX (S)	800	No changes[1]	No changes[7]	Christensen *et al*. (2001a, 2001b)
LAC (AS)	—	No changes[1,4]	No changes[7]	Ranocha *et al*. (2000, 2002)
P. tremuloides				
COMT (S)	28	No changes[1,4]	S/G decreased[7], 5OHG[7], more coniferaldehyde[7]	Tsai *et al*. (1998)
4CL (AS)	10	Reduced[1]	No changes[7]	Hu *et al*. (1999)
4CL (AS)	10	Reduced[1]	No changes[7,8]	Li *et al*. (2003)
F5H (S)	280	No changes[1]	S/G increased[7,8]	Li *et al*. (2003)
4CL (AS)/ *F5H* (S)	10/110	Reduced[1]	S/G increased[7,8]	Li *et al*. (2003)

(Continued on next page)

TABLE 3
Lignin Mutants and Transgenic Plants *(Continued)*

Plant Gene	Enzyme Activity In Stems (%)	Lignin Content	Lignin Composition	References
Sorghum bicolor				
COMT (*bmr12* and *bmr18* mutants)	—	Reduced[1,4]	S reduced[6,7], 5OHG[6,7], Syr/V decreased[5]	Chabbert *et al*. (1993), Suzuki *et al*. (1997), Bout & Vermerris (2003)
COMT (*bmr26* mutant)	—	—	S reduced[6]	Bout & Vermerris (2003)
CAD? (*bmr6* mutant)	~30	Reduced[1,2]	S and G decreased[6,7], coniferaldehyde increased[6,7], Van and Syr decreased[5], Syr/V decreased[5]	Pillonel *et al*. (1991), Chabbert *et al*. (1993), Suzuki *et al*. (1997)
Zea mays				
COMT (*bm3* mutant)	10	Reduced[1,4]	S[7] and Syr[5] decreased, 5OHG[7]	Kuč *et al*. (1968), Grand *et al*. (1985), Lapierre *et al*. (1988), Chabbert *et al*. (1994a, 1994b), Vignols *et al*. (1995), Suzuki *et al*. (1997).
CAD (*bm1* mutant)	~30	Reduced[1]	S and G decreased[7]	Halpin *et al*. (1998)

Numbers in superscript refer to the method used to analyze lignin content or composition; [1]Klason; [2]thioglycolic acid; [3]sum of Klason and acid-soluble lignin; [4]acetyl bromide; [5]nitrobenzene oxidation; [6]pyrolysis GC-MS; [7]thioacidolysis; [8]NMR; [9]alkaline hydrolysis followed by gas chromatography; [10]FTIR, [11]DFRC; [12]permanganate oxidation; [13]toluidine blue and UV fluorescence; [14]DRIFT; [15]Pyrolysis GC; (AS), transgenic plants with an antisense construct; G, guaiacyl; H, *p*-hydroxyphenyl; HCA, hydroxycinnamic acid; 5OHG, 5-hydroxyguaiacyl; LAC, laccase; POX, peroxidase; S, syringyl; (S), transgenic plants with a sense construct; Sin; sinapaldehyde; Sy, sum of syringaldehyde and syringic acid; Syr, syringaldehyde; V, sum of vanillin and vanillic acid; Van, vanillic acid; —, not determined; ?, gene not identified.

B. Up- and Down-Regulation of Cinnamic Acid 4-Hydroxylase (C4H)

Hydroxylation at the C_4 position of cinnamic acid to *p*-coumaric acid is catalyzed by C4H, a cytochrome P_{450}-linked monooxygenase belonging to the CYP73 subfamily (Teutsch *et al*., 1993; Chapple, 1998). In transgenic tobacco plants, C4H activity was altered by expressing the alfalfa class I C4H (CYP73A3) (Sewalt *et al*., 1997; Blount *et al*., 2000) or the French bean class II C4H (CYP73A15) (Blee *et al*., 2001) genes in sense or antisense orientation (class I and class II C4H share approximately 60% similarity). Overexpression of class I C4H had no effect on Klason[e] lignin, nor on the S/G ratio[g]. In contrast, a 76% reduction in total C4H activity led to a 63% decrease in Klason[e] lignin and a modification of the lignin monomeric composition. The amount of S units[g] was strongly reduced and S/G decreased by over 90% (Sewalt *et al*., 1997). Similarly, a reduction by 90% of C4H activity by down-regulation of the class II C4H resulted in a 27% decreased lignin content[a], and one line had a decreased S/G ratio[i] (Blee *et al*., 2001). These results are hard to reconcile with an increase in S/G when PAL is suppressed (see previous section) because PAL and C4H are presumed to be sequential enzymes in the lignin pathway. Three possible explanations have been proposed by Dixon *et al*. (2001): (i) the pathway to G lignin may somehow bypass C4H; (ii) C4H may catalyze additional reactions in the lignin biosynthetic pathway; and (iii) a specific form of C4H might be organized in a complex with other enzymes or in a metabolic channel committed to S lignin biosynthesis. In support of this last proposition, Rasmussen and Dixon (1999) have shown apparent channeling between PAL and C4H, which was reduced by overproduction of PAL. Other evidence potentially supporting the channeling hypothesis is the appreciation that regulatory mechanisms probably exist to coordinate PAL and C4H expression. For example,

decreased PAL activity has been shown in transgenic tobacco modified to reduce C4H expression (Blount *et al*., 2000). Regulation may be mediated by pathway intermediates; for example, cinnamic acid may act as a feedback regulator of the phenylpropanoid pathway. Nevertheless, given the importance of the suggestion that metabolic channeling may exist on the lignin pathway, it is surprising that little direct proof has yet been provided. The detection of protein:protein complexes, for example, should not be difficult given the sophistication of modern molecular, biochemical, and cell biological methods and will undoubtedly provide a productive area for investigation in the near future.

C. Knock-Out Mutation for *p*-Coumarate 3-Hydroxylase (C3H)

The gene encoding *p*-coumarate 3-hydroxylase (C3H) has only recently been cloned by two independent research groups. Using a functional genomics approach, Schoch *et al*. (2001) identified CYP98A3 as a possible candidate for C3H. CYP98A3 was highly expressed in developing xylem as determined by immunolocalization and had high activity when using 5-*O*-(4-coumaroyl) D-quinate and 5-*O*-(4-coumaroyl) shikimate as substrates and low activity when using *p*-coumaroyl-CoA (Figure 3). In parallel, by screening *Arabidopsis* mutants under UV light, Franke *et al*. (2002a) isolated the reduced epidermal fluorescence 8 (*ref8*) mutant. By positional cloning, the *REF8* gene was identified as the cytochrome P_{450}-dependent monooxygenase *CYP98A3*. The *ref8* mutant had collapsed xylem vessels, a higher cell wall degradability, and a higher susceptibility to fungal colonization (Franke *et al*., 2002b), associated with the accumulation of *p*-coumarate esters instead of sinapoylmalate and with a reduction in lignin content of 60-80%[e,h]. A range of analyses showed that lignin composition was dramatically altered, being almost entirely made of *p*-coumaryl alcohol units[b,c,g] (Franke *et al*., 2002b). This work confirmed that CYP98A3 is C3H and added further support to the idea that the quinate and shikimate esters of *p*-coumaric and caffeic acid are probably important intermediates in lignin biosynthesis.

D. Down-Regulation of Caffeic Acid *O*-Methyltransferase (COMT)

Down-regulation of COMT activity has been achieved using either antisense or sense transgenes in tobacco (Dwivedi *et al*., 1994; Ni *et al*., 1994; Atanassova *et al*., 1995), poplar (Van Doorsselaere *et al*., 1995; Tsai *et al*., 1998; Jouanin *et al*., 2000), and alfalfa (Guo *et al*., 2001). In all three species, drastic reductions in the lignin S/G ratio[c,i] were apparent and an unusual phenolic compound (5-hydroxyguaiacyl[i], 5OHG; Figure 3) was present in the polymer (Atanassova *et al*., 1995; Van Doorsselaere *et al*., 1995; Tsai *et al*., 1998; Lapierre *et al*., 1999; Jouanin *et al*., 2000; Guo *et al*., 2001; Marita *et al*., 2003). 5OHG has been described in the lignin of maize *bm3* (Chabbert *et al*., 1994a, 1994b; Suzuki *et al*., 1997), sorghum *bmr6* and *bmr18* (Suzuki *et al*., 1997), and *Arabidopsis Atcomt1*[i] (Goujon *et al*., 2003a) mutants. In the lignin of the transgenic poplars described by Jouanin *et al*. (2000), the level of 5OHG units even exceeded that of S units, whereas lignin of the *Arabidopsis* mutant totally lacked S units and had increased levels of 5OHG and G units[i]. A novel dimer composed of a G unit linked to a 5OHG unit by an α-β diether bond has previously been identified as a normal, but minor, component of the lignin polymer by thioacidolysis[i] (Figure 5) (Hwang and Sakakibara, 1981). These data, together with NMR[f] experiments showing the presence of benzodioxane units in lignin, demonstrate that 5OHG is incorporated into lignin as a monolignol (Figure 5) (Jouanin *et al*., 2000; Ralph *et al*., 2001a, 2001b;

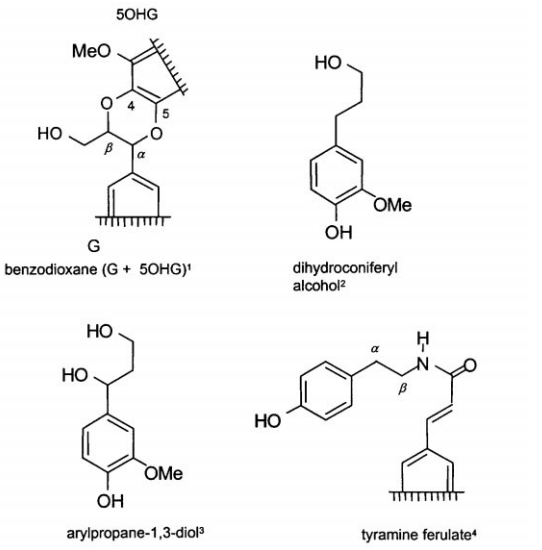

FIGURE 5. Molecules found in altered amounts in the lignin of transgenic plants, as determined by NMR. [1]Ralph *et al*. (2001b); [2]Ralph *et al*. (1997); [3]Ralph *et al*. (1999b); [4]Ralph *et al*. (1998).

Marita *et al*., 2003). In COMT-suppressed transgenic plants, the yield of thioacidolysis[i] products (S+G+5OHG) was lower than in wild-type plants, indicating a reduced proportion of the targets of this degradative procedure, the β-O-4 linkages, and a correspondingly increased proportion of C-C linkages. Indeed, an enrichment in biphenyl (5-5) and phenylcoumaran (β-5) linkages has been detected[i] (Lapierre *et al*., 1999; Jouanin *et al*., 2000; Guo *et al*., 2001). The increased frequency of these dimeric structures corresponds with an increase in the degree of lignin condensation, which makes the lignin more similar to softwood lignin. In COMT-suppressed alfalfa, β-β, β-1, and β-5 linkages involving S units, were absent[i] (Guo *et al*., 2001), whereas in COMT-down-regulated poplar, free phenolic groups in β-O-4-linked G units (Figure 4) were less abundant (Lapierre *et al*., 1999). In poplar, when the bark was removed, the wood of the COMT-suppressed lines had a rose (Van Doorsselaere *et al*., 1995) or red-brown color (Tsai *et al*., 1998; Jouanin *et al*., 2000) compared to the whitish wild-type wood—this coloration has been ascribed to an increased amount of coniferaldehyde (Tsai *et al*., 1998).

In the maize *bm3* and the sorghum *bmr6* and *bmr18* mutants, which have little COMT activity (Kuč *et al*., 1968) because of mutations in the *COMT* gene (Vignols *et al*., 1995; Bout & Vermerris, 2003), lignin content is reduced. In COMT-suppressed alfalfa, Klason[e] determinations showing that lignin content is reduced are at odds with data from acetyl bromide[a] determinations in which no change in lignin amount is found (Guo *et al*., 2001; Marita *et al*., 2003). Similarly, in COMT-suppressed poplar, reduced lignin content[e] has been reported (Jouanin *et al*., 2000) as well as no change in lignin amount in poplar[e] (Van Doorsselaere *et al*., 1995) or aspen[a,e] (Tsai *et al*., 1998). Reports describing COMT-suppression in tobacco also differ on whether lignin content is[h] (Ni *et al*., 1994) or is not[e] (Dwivedi *et al*., 1994; Atanassova *et al*., 1995) reduced. In the *Arabidopsis Atcomt1* mutant, even a slight increase in lignin content[e] was detected (Goujon *et al*., 2003a). The explanation for these discrepancies is not known and may be related to the actual level of COMT suppression achieved in each case. However, these data also highlight the caution that has to be exercised when comparing lignin data from plants grown for different lengths of time under varying environmental conditions and analyzed using different techniques.

Despite these small discrepancies, the data from all of the COMT-suppressed tobacco and poplar plants indicate that COMT plays a predominant role in determining the incorporation of S units into the lignin polymer in these species. These results are consistent with those of Li *et al*. (2000), who showed, for several angiosperm species, that 5-hydroxyconiferaldehyde is the preferred substrate for COMT *in vitro*. In alfalfa, a reduction in COMT activity affected both the content of G and S units (Guo *et al*., 2001; Marita *et al*., 2003), in accordance with the results of Parvathi *et al*. (2001), who found that in alfalfa, COMT is also involved in the methylation of caffeyl aldehyde (Figure 3).

E. Down-Regulation of Caffeoyl-CoA *O*-Methyltransferase (CCoAOMT)

Until recently, the methylation reactions at the C_3 and C_5 hydroxyl functions of the lignin precursors were thought to occur mainly at the cinnamic acid level by bi-functional COMT. However, the association of CCoAOMT expression with lignification (Pakusch *et al*., 1991; Ye *et al*., 1994; Ye & Varner, 1995; Ye, 1997; Martz *et al*., 1998; Chen *et al*., 2000) and the observation that down-regulation of COMT preferentially affected the amount of S units (see section III.D) suggested the existence of an alternative pathway for the methylation of the lignin precursors at the hydroxycinnamoyl-CoA level (Figure 3). Down-regulation of CCoAOMT affected the Klason[e] lignin content by 12–50% in transgenic tobacco (Zhong *et al*., 1998; Pinçon *et al*., 2001a), alfalfa (Guo *et al*., 2001; Marita *et al*., 2003), and poplar (Meyermans *et al*., 2000; Zhong *et al*., 2000). In tobacco and poplar, the decreased lignin content was due to reduction of both G and S units as determined by pyrolysis GC-MS[g] (Zhong *et al*., 1998, 2000) or thioacidolysis[i] (Meyermans *et al*., 2000). Because the decrease in G units was more pronounced, the S/G ratio increased (Zhong *et al*., 1998; Meyermans *et al*., 2000). In contrast, the S unit amount was not reduced in transgenic alfalfa (Guo *et al*., 2001) nor in other, independently produced, tobacco (Pinçon *et al*., 2001a), as determined by thioacidolysis[i]. In addition, Zhong *et al*. (2000) have shown by diffuse reflectance infrared Fourier transform (DRIFT)[d] spectroscopy that the

lignin extracted from wood of the transgenic poplars was less cross-linked than that of the control. In contrast to the transgenic poplars, which were not affected in growth or morphology, the transgenic tobacco plants down-regulated for CCoAOMT had collapsed vessel walls (probably because of the reduced lignin content) and altered growth and flower development (Pinçon *et al*., 2001a). Other consequences of CCoAOMT down-regulation in transgenic poplar were an enhanced fluorescence of the vessel cell walls and the accumulation of *p*-hydroxybenzoic acid esterified to lignin. In addition, increased amounts of methanol-extractable phenolics, namely O^3-β-D-glucopyranosyl-caffeic acid, O^4-β-D-glucopyranosyl-sinapic acid, and O^4-β-D-glucopyranosyl-vanillic acid, were detected in the wood of the transgenic poplars (Meyermans *et al*., 2000). Similarly, soluble caffeoyl glucoside accumulated in stem extracts of transgenic alfalfa (Guo *et al*., 2001). The accumulation of glucosides of caffeic and sinapic acid results most probably from a detoxification of free caffeic and sinapic acid, as indicated by feeding experiments with these two hydroxycinnamic acids (Meyermans *et al*., 2000). The observation that lignin content was reduced in poplars down-regulated for CCoAOMT, whereas O^4-β-D-glucopyranosyl-sinapic acid accumulated, is in agreement with the hypothesis that sinapic acid is not the main precursor for S units *in vivo*. The incorporation in the cell wall of *p*-hydroxybenzoic acid may be responsible for the increased fluorescence (Meyermans *et al*., 2000). A red (Meyermans *et al*., 2000) or a light orange (Zhong *et al*., 2000) color has been noticed in the xylem of the transgenic poplars, but its origin is unknown.

Simultaneous down-regulation of both COMT and CCoAOMT in tobacco (Zhong *et al*., 1998; Pinçon *et al*., 2001a) and alfalfa (Guo *et al*., 2001) resulted in combinatorial and/or additive effects. In comparison with the respective single transformants, a greater reduction in Klason[e] lignin content was measured in tobacco (Pinçon *et al*., 2001a) but not in alfalfa (Guo *et al*., 2001). In both species, the lignin S/G ratio was reduced although in tobacco this was due to decreases in both G and S units[g] (Zhong *et al*., 1998), whereas only S units decreased in alfalfa[i] (Guo *et al*., 2001). To explain the relative preservation of G units in alfalfa, Guo *et al*. (2001) suggest that additional enzymes may be involved in the methylation of the monolignol precursors of G units.

F. Up- and Down-Regulation of Ferulic Acid 5-Hydroxylase (F5H)

For many years, it was generally accepted that the 5-hydroxylation of the monomethoxylated lignin precursor, catalyzed by the enzyme ferulic acid 5-hydroxylase (F5H), would take place at the hydroxycinnamic acid level (Figure 3). However, Osakabe *et al*. (1999) and Humphreys *et al*. (1999) demonstrated that the 5-hydroxylation of the monomethoxylated precursor occurs preferentially at the cinnamaldehyde level, leading to the renaming of the enzyme to coniferaldehyde 5-hydroxylase or Cald5H. A number of reports have indicated that the hydroxylation step at C_5 may also occur at the cinnamyl alcohol level (Matsui *et al*., 1994, 2000; Daubresse *et al*., 1995; Chen *et al*., 1999; Humphreys *et al*., 1999; Parvathi *et al*., 2001).

An *Arabidopsis* mutant deficient in F5H (*fah1*) was described more than 10 years ago. The mutant produced a lignin deficient in S units (Chapple *et al*., 1992) with a consequently increased frequency of phenylcoumaran (β-5) and biphenyl (5-5) linkages (Marita *et al*., 1999) (Figure 4). On the other hand, when *Arabidopsis* F5H was overexpressed from the *C4H* promoter in the mutant, a lignin that was almost entirely composed of S units linked by β-O-4 linkages was produced (Meyer *et al*., 1998; Marita *et al*., 1999). The proportion of S units in the lignin of these plants was the highest ever reported for any plant (Ralph, 1996). Similarly, lignin of tobacco and poplar transformed with the same chimeric gene was enriched in S units (Franke *et al*., 2000). Recently, Li *et al*. (2003) have overexpressed a sweetgum F5H (Cald5H) under the control of a xylem-specific promoter (*Pt4CL1P*) in transgenic aspen and reported a 2.5-fold increase in the S/G ratio[i] and no changes in lignin content[e]. Interestingly, an accelerated maturation/lignification of stem secondary xylem cells was noted in these F5H-overexpressing plants, a phenomenon attributed to the involvement of oligomeric S lignin moieties in signaling mechanisms that promote secondary wall thickening (Li *et al*., 2003). More research is needed to explore and potentially confirm this intriguing possibility.

In F5H-overexpressing *Arabidopsis*, benzodioxane structures were detected in lignin, probably as a consequence of the increased flux to 5-hydroxyconiferaldehyde and 5-hydroxyconiferyl alcohol (Ralph *et al*., 2001b). A 25–35% reduction in Klason[e] lignin content was observed in

F5H-overexpressing *Arabidopsis* (Marita *et al*., 1999) and tobacco (Franke *et al*., 2000). However, the apparent reduction in Klason[e] lignin may be an artefact because S units have fewer radical coupling sites, reducing the possibilities for coupling and branching and thereby making S-rich lignins more easily degraded and extracted (Marita *et al*., 1999; Franke *et al*., 2000). These studies demonstrate that F5H plays a pivotal role in determining lignin monomer composition. Therefore, modifying F5H expression and altering the relative amount of S units may offer opportunities for engineering lignin quality in hardwoods and softwoods.

G. Down-Regulation of 4-Coumarate:CoA Ligase (4CL)

Transgenic plants with reduced 4CL activity have been produced in tobacco (Kajita *et al*., 1996, 1997), *Arabidopsis* (Lee *et al*., 1997), and aspen (Hu *et al*., 1999; Li *et al*., 2003). In tobacco, reduction of 4CL by over 90% resulted in 25% less lignin[a,i]. In poplar and *Arabidopsis* with a >90% reduced 4CL activity, lignin content was reduced by 45–50%[i].

In tobacco, the low 4CL activity was associated with browning of the xylem tissue (Kajita *et al*., 1996). The monomeric composition of lignin[b] was altered and characterized by a 3-fold increase in the amount of *p*-hydroxybenzaldehyde and an 80% and a 67% decrease in the amount of syringaldehyde (Syr) and vanillin (Van), respectively, resulting in a 40% reduction in the Syr/Van ratio (Kajita *et al*., 1997). The amount of the ester- and ether-linked *p*-coumaric, ferulic, and sinapic acids increased dramatically in the brown xylem tissue (as determined by alkaline hydrolysis of the cell walls followed by gas chromatography and NMR[f] of milled wood lignin). In contrast, in transgenic *Arabidopsis*, the Sy/V (Sy is the sum of syringaldehyde and syringic acid and V is the sum of vanillin and vanillic acid) ratio[b] was increased because of a 40% reduction in the amount of V units, suggesting that 4CL is required for the synthesis of G, but not S units, as postulated by Lee *et al*. (1997) and Hu *et al*. (1998). In transgenic aspen down-regulated for 4CL, Hu *et al*. (1999) also detected an increase in non-lignin alkali-extractable wall-bound phenolics (*p*-coumaric acid, caffeic acid, and sinapic acid), and showed by NMR[f] that these acids were not incorporated into the lignin polymer. However, they did not detect any difference in lignin S/G composition using thioacidolysis[i], in contrast to the data obtained for *Arabidopsis* and tobacco. Another discrepancy between the results published by Kajita *et al*. (1997) and Hu *et al*. (1999) is that the transgenic tobacco lines with the most severe reduction in lignin content (25%) were characterized by a collapse of vessel cell walls and reduced growth (Kajita *et al*., 1997), whereas the transgenic poplars with a 45% reduction in lignin content had a normal cell morphology and a higher growth rate than the control (Hu *et al*., 1999). However, the increased growth was probably due to pleiotropic effects caused by the constitutive down-regulation of 4CL governed by the CaMV35S promoter, because it was not observed in the transgenic aspen reported by Li *et al*. (2003), in which the antisense *Pt4CL* was under the control of an aspen xylem-specific promoter, *Pt4CL1P*. The increased level of hydroxycinnamic acids as non-lignin cell wall constituents has been suggested to contribute to the cell wall strength in transgenic poplar (Hu *et al*., 1999). Because several 4CL isozymes exist with different cell-specific expression, down-regulation of several or all isozymes simultaneously may perturb metabolite levels other than those involved in lignin, with a secondary effect on growth as a consequence.

Interestingly, antisense inhibition of 4CL in aspen trees led to a 15% increase in cellulose content. These results suggest that lignin and cellulose deposition are regulated in a compensatory fashion and that a reduced carbon flow toward phenylpropanoid biosynthesis increases the availability of carbon for cellulose biosynthesis (Hu *et al*., 1999; Li *et al*., 2003).

A combinatorial down-regulation of 4CL along with an overexpression of F5H in xylem has been achieved by co-transformation of two *Agrobacterium* strains in aspen (Li *et al*., 2003). Additive effects of independent transformation were observed, in particular a 52% reduction in lignin content associated with a proportional increase in cellulose and a higher S/G ratio. The results show that stacking transgenes allows several beneficial traits to be improved in a single transformation step (Halpin & Boerjan, 2003).

H. Down-Regulation of Cinnamoyl-CoA Reductase (CCR)

CCR catalyzes the reduction of hydroxycinnamoyl-CoA thioesters to the corresponding aldehydes, a reaction considered to be a potential control point that regulates the

overall carbon flux toward lignin (Lacombe *et al*., 1997). Transgenic tobacco (Piquemal *et al*., 1998; Ralph *et al*., 1998; O'Connell *et al*., 2002) and *Arabidopsis* (Goujon *et al*., 2003b) down-regulated for CCR, are characterized by an approximate 50% decrease in Klason[e] lignin. The lignin S/G ratio[i] was increased (mainly because of a decrease in the G unit amount) in transgenic tobacco and variable, depending on the growth conditions, in transgenic *Arabidopsis*. In tobacco, an orange-brown color was observed in the xylem. The presence of unusual phenolics (such as ferulic acid and sinapic acid) in the cell wall may account for this color, because semi-*in vivo* incorporation of these two hydroxycinnamic acids into stem sections resulted in a comparable phenotype (Piquemal *et al*., 1998). A change in the lignin structure was also indicated by the higher amount of alkali-labile material that could be released from the extractive-free lignin polymer of the transgenic lines (O'Connell *et al*., 2002). The transgenic plants with the lowest CCR activity and 50% reduced lignin had abnormal phenotypes, such as collapsed vessels, stunted growth, and abnormal leaf development. Important alterations in the fiber cell walls were observed, such as a loosening in the arrangement of the cellulose microfibrils, that resulted in reduced cell wall cohesion (Pinçon *et al*., 2001b; Goujon *et al*., 2003b). Chabannes *et al*. (2001a) have shown that the reduction of lignin deposition in tobacco was not uniform in the cell wall, but that the S2 and S3 layers of the fibers and the vessels were mainly influenced. Similarly, lignin deposition was mainly affected in the inner S2 layer in CCR down-regulated *Arabidopsis* plants (Goujon *et al*., 2003b). A decrease in thioacidolysis[i] yield was measured in the lignin of the CCR down-regulated tobacco plants, indicating that the remaining lignin was more condensed (Piquemal *et al*., 1998; O'Connell *et al*., 2002). Also an increased amount of tyramine ferulate (Figure 5), an unsual component of tobacco cell walls that is probably a sink for feruloyl-CoA, was incorporated into the lignin of the CCR-down-regulated tobacco plants (Ralph *et al*., 1998). Down-regulation of CCR in transgenic poplar led to an orange coloration of the xylem, a 20% decrease in Klason[e] lignin content, and an increase in the S/G ratio[i] (J.-C. Leplé, C. Lapierre, & W. Boerjan, unpublished results). A *ccr* mutant, designated irregular xylem (*irx4*), has been identified in *Arabidopsis* (Jones *et al*., 2001). Like the CCR-down-regulated tobacco and *Arabidopsis* described above, this mutant is characterized by a 50% reduced lignin content[f,h], a collapse of the vessels, and an altered growth and morphology. Taken together, the results obtained by altering the expression of CCR indicate that this gene controls the quantity of lignin produced, although the adverse phenotypes associated with CCR suppression will limit its use as a target for modifying the lignin content in plants.

By crossing transgenic tobacco down-regulated for COMT (Atanassova *et al*., 1995) with tobacco down-regulated for CCR (Piquemal *et al*., 1998), a simultaneous reduction in COMT and CCR expression was achieved (Pinçon *et al*., 2001b). Progeny were inhibited in both CCR and COMT activities and had intermediate phenotypes. The line with most down-regulated expression of both genes had lignin characteristics similar to those of the CCR-down-regulated parent (low lignin content and high S/G ratio) (Table 3), but characteristics typical of COMT down-regulation, such as a low S/G and the incorporation of 5OHG, were not found (Pinçon *et al*., 2001b). These observations could result from insufficient COMT down-regulation to trigger the phenotype. Alternatively, the down-regulation of CCR, which is upstream in the pathway, may prevent the accumulation of 5OHG derivatives.

I. Down-Regulation of Cinnamyl Alcohol Dehydrogenase (CAD)

CAD catalyzes the last step in the biosynthesis of the monolignols, which is the reduction of cinnamaldehydes to cinnamyl alcohols. Transgenic plants with reduced CAD activity have been produced in tobacco (Halpin *et al*., 1994; Hibino *et al*., 1995; Stewart *et al*., 1997; Yahiaoui *et al*., 1998), poplar (Baucher *et al*., 1996), and alfalfa (Baucher *et al*., 1999), whereas *cad* mutants exist in pine (MacKay *et al*., 1997), maize (Halpin *et al*., 1998), and *Arabidopsis* (Sibout *et al*., 2003). CAD suppression has been associated with a red or red-brown color of the stem xylem. An unusual monomer, dihydroconiferyl alcohol (Figure 5), was shown to be incorporated into the lignin[f] of the pine *cad* mutant and accounted for 30% of the lignin compared to only 3% in wild-type lignin (Ralph *et al*., 1997). Accordingly, higher amounts of arylpropane-1,3-diol structures (Figure 5), arising from dihydroconiferyl alcohol, have been found in the lignin of the pine *cad* mutant by NMR[f] analysis (Ralph *et al*., 1999b, 2001a). In contrast, no dihydroconiferyl alcohol has been found in the lignin of transgenic angiosperms down-regulated for CAD, such as tobacco and poplar (Ralph *et al*., 1998).

A higher amount of cinnamaldehydes has been detected in the lignin of CAD-down-regulated tobacco by pyrolysis GC-MS[g] (Halpin *et al*., 1994) and NMR[f] (Ralph *et al*., 1999a, 2001a), in CAD-down-regulated poplars by thioacidolysis[i] (Kim *et al*., 2002), in the pine *cad* mutant by NMR[f] (Ralph *et al*., 1997), and in the *Arabidopsis Atcad-D* mutant by thioacidolysis[i] (Sibout *et al*., 2003). Using the CAD-down-regulated tobacco, Kim *et al*. (2000) demonstrated by NMR[f] that sinapaldehyde can make β-O-4 linkages with both G and S units, whereas coniferaldehyde cross-couples only with S units in the lignin. The cross-coupling of cinnamaldehydes into the lignin polymer results probably in a more extended conjugated system in the polymer, which might cause the red color of the xylem (Higuchi *et al*., 1994). The lignin of plants with low CAD activity was more extractable in alkali (Halpin *et al*., 1994; Baucher *et al*., 1996; Bernard-Vailhé *et al*., 1996; Yahiaoui *et al*., 1998; MacKay *et al*., 1999). When fed to sheep, transgenic tobacco and alfalfa with suppressed CAD activity had slightly improved *in situ* cell wall degradability, indicating that CAD may be a suitable target for modifying forage digestibility (Bernard-Vailhé *et al*., 1995; Baucher *et al*., 1999).

In contrast to what might be expected, only a slighty lower Klason[e] lignin content was measured in the wood of transgenic poplar lines down-regulated for CAD (Lapierre *et al*., 1999; Pilate *et al*., 2002), in the pine *cad* mutant (MacKay *et al*., 1997), and in the *Arabidopsis Atcad-D* mutant (Sibout *et al*., 2003). These results may be explained by the incorporation into the lignin polymer of other phenolics, such as aldehydes and dihydroconiferyl alcohol, as described above. In transgenic poplar, the S and G unit composition and the percentage of β-O-4 linkages did not differ from those of the control, but the lignin was enriched in free phenolic groups in both S and G units and in diarylpropane (β-1) structures (Lapierre *et al*., 1999) (Figure 4). Similarly, the proportion of G units with free phenolic groups was higher in the *Atcad-D* mutant than in the control (Sibout *et al*., 2003). The relative proportion of the free phenolic groups may be an important parameter in determining the solubility of lignin (Lapierre *et al*., 1999). In contrast, the S/G ratio of the lignin of transgenic tobacco (Ralph *et al*., 1998) and transgenic alfalfa (Baucher *et al*., 1999) was reduced, suggesting that in these plants the uncondensed S structures are more affected than their G analogs. These data are in apparent conflict with the recent proposal that SAD, and not CAD, is involved in S lignin biosynthesis in angiosperms (Li *et al*., 2001).

A simultaneous down-regulation of CAD and CCR has been achieved by crossing homozygous transgenic lines in which either CAD (Halpin *et al*., 1994) or CCR (Piquemal *et al*., 1998) was down-regulated (Chabannes *et al*., 2001b). The lignin content[e] was decreased by approximately 50% in tobacco with 32% of wild-type CCR activity and 12% of wild-type CAD activity, a reduction that is comparable to that in the homozygous, but not in the hemizygous, parental line down-regulated for CCR. This observation suggests that down-regulation of both genes has synergistic effects on the reduction of lignin quantity (Chabannes *et al*., 2001b). The lignin S/G composition of the double transformed lines had increased to a level similar to that of the down-regulated CCR parent, when determined by thioacidolysis[i]. Surprisingly, the NMR[f] spectra showed that the lignin structure of the double transformants was closer to that of wild-type plants than to the CCR- or the CAD-down-regulated parent. The phenotype of the double transformants was normal with only slight alterations in the vessel shape, showing that, similarly to the results of Zhong *et al*. (1998) and Hu *et al*. (1999), plants can tolerate important reductions in lignin content.

A simultaneous suppression of COMT (to 24% of wild-type level), CCR (to 18% of wild-type level), and CAD (to 4% of wild-type level) was achieved in tobacco by a single chimeric construct, consisting of partial sense sequences for the three different genes. The transgenic lines were stunted and had characteristics of COMT, CCR, and CAD suppression in lignin; for example, the xylem was red (indicative of CAD suppression), contained collapsed vessels (indicative of CCR suppression), and had reduced staining for S lignin (indicative of COMT suppression) (Abbott *et al*., 2002). This approach to reducing expression of several genes simultaneously by a single construct was found to be more efficient than crossing different transgenic lines altered in the expression of single genes.

J. Up- and Down-Regulation of Peroxidases

Although peroxidases are believed to catalyze the final condensation of cinnamyl alcohols in the formation of lignin, no definitive proof has been presented yet for the involvement of any specific peroxidase isozyme *in vivo*, mainly because of the high number of genes that encode peroxidases (Tognolli *et al*., 2002) and the typically low substrate specificities of these enzymes.

Both anionic and cationic peroxidases have been implicated in lignification based on their affinity for coniferyl alcohol, their location in the cell wall, and their expression in lignified tissue (Mäder & Füssl, 1982; Lagrimini *et al*., 1987; El Mansouri *et al*., 1999). Nevertheless, no change in lignin content was obvious in transgenic tobacco plants that were deficient in the major anionic peroxidase (Lagrimini *et al*., 1997a). However, transgenic poplar with a 44% reduction in the activity of a stem-specific anionic peroxidase (PRXA3a) had a 21% reduced lignin content and a higher content in β-O-4 linked (uncondensed) structures in lignin[i] (Yahong *et al*., 2001).

The overexpression of peroxidase genes in transgenic poplar (PXP 3-4; Christensen *et al*., 2001a, 2001b) and in tobacco (spi 2; Elfstrand *et al*., 2002) resulted in 800-fold and 5-fold increased total peroxidase activity, respectively. No effects of the genetic modification on the overall phenotype, the Klason[e] lignin content, or the recovery yield of G and S units after thioacidolysis[i] were identified. However, in the transgenic tobacco produced by Elfstrand *et al*. (2002), a higher frequency of coniferaldehyde was detected[i] and reduced stem flexibility was noted, suggesting modifications of the cell wall properties. In addition, the tobacco plants with higher peroxidase activity were more susceptible to *Phytophthora parasitica*, but allowed less growth of *Erwinia carotovora.* In contrast, independently produced transgenic tobacco lines with a 10-fold higher peroxidase activity were characterized by increased lignin content[a,h] in leaves, stems, and roots (Lagrimini, 1991; Chabbert *et al*., 1992). No changes in lignin monomeric composition[i] were detected, but the amount of monomers involved in β-O-4 linkages was lower in the transgenic lines, suggesting that the lignin was more condensed (Chabbert *et al*., 1992). These plants had reduced root mass and fewer root branches, probably because of altered auxin metabolism (Lagrimini *et al*., 1997b) and were characterized by wilting of the leaves and a rapid browning of wounded tissues (Lagrimini *et al*., 1990; Lagrimini, 1991). Similarly, overproduction of an anionic peroxidase in tomato led to an increase in lignin content in leaves and fruit (Lagrimini *et al*., 1993; El Mansouri *et al*., 1999).

K. Down-Regulation of Laccases

The precise role played by laccases in lignification is not yet understood, but there is correlative evidence that laccase and oxygen participate in the polymerization of monolignols. For instance, when peroxidase is inhibited either in the absence of H_2O_2 or in the presence of H_2O_2 scavengers (catalase and superoxide dismutase), coniferyl alcohol is still oxidized and O_2 consumed in tobacco xylem (McDougall *et al*., 1994). Because laccases operate in the absence of toxic H_2O_2, these enzymes could be involved in the early stages of lignification (Sterjiades *et al*., 1993). Five divergent laccase genes have been cloned and characterized from poplar (Ranocha *et al*., 1999, 2000). Transgenic *Liriodendron* (Dean *et al*., 1998) and poplar (Ranocha *et al*., 2000, 2002) down-regulated in laccase had no altered phenotype nor any change in lignin amount[a,e] or S/G composition[i]. However, in transgenic poplar down-regulated for one of the laccase genes (*lac3*), the walls of xylem cells were irregular in contour when compared with the control and had adhesion defects either at the primary cell wall of adjacent cells or within the secondary cell wall of a given cell (Ranocha *et al*., 2002).

As described above, many different transgenic plants and a few mutants are now available with altered lignin content, altered lignin composition/structure, or both. Whether or not these changes in cell wall biochemistry could have advantages in industrial operations, such as pulping, can only be determined experimentally. A few such experiments have been performed. To put the results into context, we will first describe briefly the different methods and parameters important in the production of pulp and paper before reviewing the impact that specific genetic engineering can have on pulping properties.

IV. PAPERMAKING PROCESSES

Two major categories of processes exist for the production of paper pulp: chemical and mechanical. The former process uses chemicals to remove lignin from fiber cell walls to obtain long and flexible fibers that consist of polysaccharides only. The latter pulping process focuses on the mechanical separation of fibers without the removal of lignin. Mechanical pulping gives the highest pulp yield, but the pulp has limited bleachability and can revert in brightness upon exposure to light (the paper becomes yellow as it ages because of the presence of lignin). In contrast, chemical pulping yields individual intact fibers that can, due to the fact that the hydrophobic lignin has been removed, interact with other fibers via hydrogen bonds, making a very strong paper. Nowadays, the Kraft process is the most widely used chemical procedure for the

TABLE 4
Main Wood Pulping Processes

Pulping Process	Chemical Treatment	Mechanical Treatment	T (°C)	pH	Wood*	Cooking Time (h)	Pulp Yield (%)
Mechanical Processes							
Stone groundwood (SGW)	—	Grinder	130–180		S	—	93–95
Pressure groundwood (PGW)	Steam	Grinder	105–125		S	—	93–95
Refiner mechanical (RMP)	—	Disc refiner			S	—	93–95
Thermomechanical (TMP)	Steam	Disc refiner	110–130		S	—	80–90
Chemithermomechanical (CTMP)	Steam+ Na_2SO_3, NaOH	Disc refiner	120–130		S/H	—	80–90
Chemimechanical (CMP)	Na_2SO_3, NaOH	Disc refiner	150–170		S/H	—	80–90
Semimechanical Processes							
Neutral sulfite (NSSC)	Na_2SO_3 and Na_2CO_3 or $NaHCO_3$	Disc refiner	160–185	7–10	H	0.5–3	70–85
Green liquor (GLSC)	Na_2SO_3 and Na_2CO_3	Disc refiner			H		70–85
Non-sulfur	NaOH and Na_2CO_3	Disc refiner			H		70–85
Chemical Processes							
Kraft	NaOH and Na_2S	—	170–175	13–14	S/H	2–5	45–55
Soda	NaOH	—	170–175		H		40–50
Soda-anthraquinone	NaOH and anthraquinone	—			H		45–55
Soda-oxygen	NaOH and oxygen	—			H		45–55
Sulfite or bisulfite	$Ca(HSO_3)_2$, $NaHSO_3$, NH_4HSO_3 or $Mg(HSO_3)_2$ and H_2SO_4	—	120–150	1.5–5	H/S	6–8	45–55

H, hardwood; S, softwood; —, not appropriate; *wood mainly used.

production of paper. However, this process is gradually being replaced by thermomechanical (TMP) and chemithermomechanical (CTMP) pulping methods that give higher pulp yields and consume less water. In 2000, the world wood pulp production has been estimated at 135,852 thousands of metric tons, from which 6.3% are of mechanical pulp, 15.6% TMP pulp, 3.6% semimechanical pulp, and 74.5% chemical pulp (Food and Agriculture Organization, 2001a).

Pulping methods can be classified according to their yield (Table 4) and will be briefly explained below. Examples of properties of pulp obtained by different pulping methods with several wood samples are given in Table 5. Explanations for the terminology used to describe pulping processes or parameters are given in the glossary and are indicated by an asterix* in the text. For a detailed overview on pulp and paper production, we refer the reader to Biermann (1996).

A. Mechanical Pulps

1. Mechanical Processes Based on Stone Grinders

In the stone groundwood (SGW) process, debarked wood logs are submitted to the action of a rotating stone covered with abrasive grains of silicon carbide, aluminum oxide, or quartz in ceramics. Mechanical energy is transformed into heat and steam, inducing a local increase in the contact temperature at the stone/wood interface that reaches the glass transition point of lignin (approximately 140°C) and results in fiber separation. When the fibers are liberated and separated, the pulp is removed from the stone surface by sprinklers and collected in a pit under the grinder. Subsequently, the pulp is screened* to remove the shives (fiber bundles) and fines (fiber fragments or ray cells) that have a negative effect on pulp quality.

In the pressure groundwood (PGW) process, the grinder is pressurized with steam and the wood is heated and softened prior to grinding. The higher contact temperature between the wood log and the stone enhances the fiber separation and reduces the amount of fines generated. The resulting pulp is stronger than that derived from the SGW process, as measured by the tensile*, burst*, and tear indexes* (Table 5).

2. Mechanical Processes Based on Refiners (RMP)

The refiner mechanical pulp (RMP) procedure is based on the use of rotating metal discs with

TABLE 5
Properties of Different Wood Pulps (Data from Holder and Murray, 1970; Leask *et al*., 1987; Vallette & de Choudens, 1992; Petit-Conil, 1995)

Pulping Properties and Wood Type	Drainage Index (ml CSF)	Tensile Index (mN/g)	Burst Index ($kPam^2/g$)	Tear Index (mNm^2/g)	Breaking Length (km)	Scattering Coefficient (m^2/kg)	Brigthness (%ISO)
Spruce							
SGW	100	27	1.10	3.6	—	68	—
PGW	100	32	1.60	4.8	—	68	—
TMP	68	—	2.35	8.6	4.20	43.5	53.0
CTMP	68	—	2.90	8.2	5.30	38.0	57.0
Aspen							
TMP	68	23	0.90	3.1	—	68.0	58.0
CTMP	68	51	2.60	6.2	—	41.0	49.5
Balsam fir							
SGW	68	—	1.90	6.2	3.69	—	—
TMP	72	—	3.00	8.2	4.97	—	—
Black spruce							
SGW	65	—	1.20	5.6	2.90	—	—
TMP	68	—	3.00	9.0	5.40	—	—
Pulps from softwoods							
Kraft	40	—	5.8	12.0	8.9	—	28
Pulps from hardwoods							
Kraft	40	—	4.5	8.0	7.5	—	29
Pulps from maritime pine							
Sulfate	40	—	5.8	12.0	8.9	—	28
Bisulfite	40	—	4.0	7.5	6.5	—	60

CTMP; chemithermomechanical pulp; PGW, pressure groundwood; SGW, stone groundwood; TMP, thermomechanical pulp; —, not determined.

specific bars and grooves that enable separation of the fibers from the wood matrix. The passage of wood particles over the disc bars induces repeated cycles of compression and expansion. When carried out in the presence of water, these cycles generate steam that softens the chips and mechanically ruptures the fibers at the middle lamella. As a result, fewer fines and shives are formed and, therefore, RMP pulps are stronger than SGW pulps.

3. Thermomechanical Pulping Process (TMP)

The TMP process involves a presteaming of the chips prior to two refining stages. The main effect of the preheating is softening of lignin, allowing the recovery of longer fibers with fewer fines and shives than in the RMP process. The first refining stage, called defibering, is carried out at an elevated temperature (generally between 110 and 130˚C) under pressure to promote fiber liberation. The second refining stage is carried out at ambient temperature, either at atmospheric or at elevated pressure.

4. Chemimechanical (CMP) and Chemithermomechanical (CTMP) Pulping Processes

To improve pulp quality for high-quality paper grades, mechanical, thermal, and chemical treatments can be combined. A chemical pretreatment of the wood chips prior to a mechanical treatment enhances defibering and renders the lignin more hydrophilic. After the mechanical treatment, the fibers are longer and more flexible and the properties of the produced paper are better than without chemical

pretreatment (Table 5). Furthermore, these beneficial effects are associated with a higher pulp yield and a decrease in the pulp opacity*.

As with the TMP process, the electrical energy is mainly transformed into steam in the refiner discs. The steam is recovered, cleaned, and used in the drying part of the paper machine. It is worth noting that the different stages of all these mechanical processes (SGW, TMP, CTMP, and CMP) are integrated into a single paper machine.

5. Bleaching of Mechanical Pulps

Bleaching involves treatment of the pulp with chemicals to increase its brightness*, which can be improved in two basic manners. Whereas chemical pulp is bleached by lignin removal, bleaching of mechanical pulp preserves the lignin, but removes the chromophores present in lignin. Therefore, bleaching of mechanical pulps is usually referred to as brightening. Brightness is not permanent because UV light and oxygen create more chromophores and cause yellowing (brightness reversion).

The brown color of mechanical pulp is mainly caused by the presence of lignin. In addition, the high temperature, atmospheric oxidation, and mechanical action during the pulping also favor the formation of the brown color. The chemicals that are generally used to bleach mechanical pulp are oxidizing compounds, such as hydrogen peroxide (H_2O_2), and/or reducing agents, such as dithionite (sodium hydrosulfite [$Na_2S_2O_4$]). Usually, the peroxide bleaching is carried out in a single step after potential pretreatments, such as chelation of metallic cations (e.g. Fe^{2+}, Mn^{2+}, and Cu^{2+}), which decrease the stability of peroxide. However, for the production of printing-writing paper (such as newsprint), high levels of brightness are needed and bleaching sequences of two or three steps that combine both oxidizing and reducing chemicals have to be used.

B. Semi-Chemical Pulps

Semi-chemical pulps are essentially mechanical pulps that have been mildly pretreated with sodium sulfite (Na_2SO_3) and sodium carbonate (Na_2CO_3) to partially remove lignin and hemicelluloses and reduce the energy requirement during the processing. The most common methods in this pulping category are the neutral sulfite semi-chemical (NSSC) and the Kraft semi-chemical methods. Semi-chemical pulps are mostly bleached with H_2O_2.

C. Chemical Pulps

Chemical pulps represent the largest part of the world pulp market and are used to produce nearly all paper and board grades (Food and Agriculture Organization, 2001a). The aim of chemical pulping is to dissolve and remove the lignin from the fiber wall and to separate the fibers at the middle lamella without mechanical damage. Because of the insoluble and cross-linked nature of lignin, delignification requires harsh pulping conditions, including high temperatures, a combination of chemicals, and a long chemical treatment time. The different chemical reactions that occur in the production of chemical pulps have been described by Gierer (1985).

1. Alkaline Chemical Pulping Processes

a. Sulfate or Kraft Process

The most widely used pulping process for many lignocellulosic raw materials is the Kraft process. It is based on the use of NaOH and sodium sulfide (Na_2S), which act as delignifying agents. During cooking, Na_2S is hydrolyzed into NaOH, NaHS, and H_2S. The different sulfur compounds react with lignin, forming thiolignins that are more soluble. The liquor applied to the chips is called white liquor and the liquor extracted from the reactor and containing the removed compounds is called black liquor, a complex mixture of inorganic and organic compounds.

For the white liquor, an active alkali* value between 18 and 22% (on oven-dry weight of wood) and a sulfidity* value between 20 and 35%, are used. This chemical treatment cleaves the β-O-4 linkages and the methoxy groups, the latter being released as mercaptans. A high sulfidity increases the rate of delignification and protects the pulp against carbohydrate degradation in alkaline medium by buffering the cooking solution, although decreased cooking times also result in less carbohydrate degradation. When the sulfidity of the cooking solution is too low, the pulp may have a higher lignin content and significant carbohydrate degradation. When the sulfidity is excessive, the emission of reduced sulfur compounds may be too high.

In the Kraft process, most of the cooking chemicals from the black liquor are recovered. Addition of sodium sulfate (Na_2SO_4) to the black liquor allows Na_2S and CO_2 to be recovered. A reaction between CO_2 and NaOH produces sodium carbonate (Na_2CO_3). Subsequently, these compounds are dissolved in water to produce the green liquor. When $Ca(OH)_2$, formed from lime (CaO), and Na_2CO_3 are supplemented to the green liquor, NaOH and $CaCO_3$ are produced. Finally, the white liquor is regenerated by sedimentation of $CaCO_3$ and filtering for additional clarification. In addition to the regeneration of the white liquor, the combustion of organic materials generates steam that is used to produce electrical energy. Therefore, a Kraft pulp mill has a perfect electrical autonomy.

The low yield of Kraft pulp (45–55% of the initial biomass) is due to the removal of lignin and some of the hemicelluloses. The kappa number* (determined by oxidation of the pulp by a solution of potassium permanganate) is directly related to the residual lignin content and, before bleaching, is typically between 28 and 32 for softwoods and 18 to 22 for hardwoods.

The pulp quality of chemical pulps is clearly higher than that of mechanical pulps, mainly with respect to paper strength (Table 5). However, because of the perfect fiber separation, the fines are less numerous and, hence, the chemical pulps have a lower opacity.

b. Other Alkaline Processes for the Production of Chemical Pulps

In soda pulping, NaOH is used as a delignifying agent in the pulping liquor. In the soda-anthraquinone and Kraft-anthraquinone processes, catalysts are used, such as quinonic compounds (for instance, anthraquinone), that increase delignification and decrease carbohydrate degradation. Therefore, the cooking time can be reduced and the pulp yield increased (by 1–3%). Another process to produce pulps with a lower kappa number and reduce the charge of bleaching agents consists of prolonging the alkaline cooking step.

2. Acidic or Bisulfite Chemical Pulping Processes

The acidic or bisulfite process is mainly used for softwoods and is based on the reaction of the wood chips with calcium [$Ca(HSO_3)_2$], sodium [$NaHSO_3$], ammonium [NH_4HSO_3], or magnesium [$Mg(HSO_3)_2$] hydrogenosulfite, or bisulfite, in combination with free SO_2.

The chemical reactions taking place during the cooking induce the dissolution of lignin and hemicelluloses. Cellulose is also hydrolyzed when the SO_2 charge* is higher. The lignin is removed by sulfonation and hydrolysis, resulting in lignosulfonic acids. The pulp quality is higher than that of mechanical pulps but always lower than that obtained by Kraft pulping (Table 5). However, the high brightness of the pulp, which is due to the fact that these pulps can be bleached more easily than Kraft pulps, allows its utilization for some paper grades.

3. Bleaching of Chemical Pulps

The unbleached chemical pulps, containing residual lignin, can be used for some paper and board grades, such as wrapping papers and boxes; however, for the production of high-quality papers, such as printing-writing papers, the chemical pulps need to be bleached. Bleaching of chemical pulp aims at the total removal of the residual lignin without extensive polysaccharide degradation. Generally, a bleaching sequence consists of alternative steps of lignin oxidation and alkaline extraction steps. Each bleaching step is followed by a washing step to remove the dissolved compounds that would otherwise consume bleaching chemicals in the later stages.

Until the 1980's, the bleaching sequences were as follows: C-E-D-E-D [with C, chlorine (Cl_2); E, alkaline extraction; and D, chlorine dioxide (ClO_2)]. ClO_2 was used at the end of the bleaching sequence for its bleaching effect associated with its specific delignifying action, although ClO_2 is 10- to 15-fold more expensive than Cl_2. At the end of the 1980's, $ClO_2$was used instead of Cl_2 for environmental reasons: the use of high Cl_2 charges (3 to 6% on pulp weight) can induce the formation of organic compounds containing as much as five molecules of chlorine, such as dioxin or other chlorinated organic chemicals that generate toxicity problems in effluents. At the beginning of the 1990's, new bleaching sequences were developed, such as elemental chlorine-free (ECF) sequences that avoid the use of Cl_2, and totally chlorine-free (TCF) sequences that do not use chlorinated chemicals at all.

a. ECF Bleaching Sequences

Two ECF bleaching sequence categories exist: (i) the sequences based on ClO_2, such as

TABLE 6
Conditions Used in the Different Potential Stages of ECF Bleaching Sequences (Petit-Conil, 1995)

Conditions	D	O	E	P	Z	Q
Temperature (°C)	50–80	90–110	60–80	70–110	30–60	50–80
Duration	45 min-3 h	30–60 min	45–60 min	1–4 h	Few min	30–60 min
pH	Acidic	Alkaline	Alkaline	Alkaline	Acidic	Neutral
Pulp consistency (%)	3–12	10–12	10–12	10–20	10–35	3–12
Chemicals	ClO_2	O_2	NaOH	H_2O_2	O_3	EDTA
% on o.d. pulp	0.5–4%	3–5 bar NaOH 1–3%	1–3%	0.5–5% NaOH 1–3%	0.1–0.7%	<0.5%

D, O, E, P, Z, and Q, see text for explanation; o.d., oven dry.

D-E-D-E-D or D-E-D-D, and (ii) the sequences combining ClO_2 and oxygenated chemicals, such as oxygen (O_2) (O stage), hydrogen peroxide (H_2O_2) (P stage), or ozone (O_3) (Z stage). The efficiency of bleaching with O_2, H_2O_2, or O_3 can be negatively affected by the presence of metal ions (Fe, Cu, or Mn) in the pulp. Therefore, it is necessary to apply a chelation stage (Q) to complex the ions and to remove them from the pulp. The bleaching conditions of ECF sequences are given in Table 6. Frequently used sequences are: O-D-E-D-D, O-Q-P-D-E-D, or O-Z-E-D-D.

b. TCF Bleaching Sequences

In the TCF bleaching sequences, O_2, O_3, H_2O_2, and peracids (Pa), such as peracetic acid (CH_3CO_3H and H_2O_2) or permonosulfuric acid (H_2SO_5), are used as bleaching chemicals. Frequently used sequences are O-Q-P-P, O-Q-P-Z-Q-P, or O-Q-P-Pa-P.

D. Biological Pulps

Biopulping is defined as the treatment of wood chips with lignin-degrading fungi prior to pulping. Such a pretreatment reduces energy requirements and improves the paper strength. In addition to the economical benefits, the biopulping process is not harmful to the environment, because only benign materials are used and no additional waste streams are generated (Reid, 1991; Akhtar *et al*., 1998a; Highley & Dashek, 1998). Although the brightness of the pulp is significantly reduced after fungal pretreatment, pulps can easily be bleached with either alkaline hydrogen peroxide or sodium hydrosulfite. Biomechanical pulping is on the way toward being commercialized (Akhtar *et al*., 1998b; Breen & Singleton, 1999).

A fungal pretreatment in chemical pulping has also been shown to be beneficial for subsequent pulping processes because part of the lignin is removed or modified (Akhtar *et al*., 1998a). Although the results are too preliminary for the process to be carried out on an industrial scale, biochemical pulping could lead to pulps with lower kappa number, which are more easily bleachable, consume fewer chemicals, and need less waste effluent loading.

Biobleaching has been shown to be an effective treatment in which hemicellulases (such as xylanases and mannanases) and lignin-degrading fungi or their enzymes (such as laccases and peroxidases) are utilized to delignify and brighten the pulp, thus reducing the use of chemicals (reviewed by Onysko, 1993; Reid & Paice, 1994; Viikari *et al*., 1994). Several procedures, such as preatreatment with xylanase prior to bleaching, have been scaled up to full industrial scale (Viikari *et al*., 1994, 1998).

V. EFFECT OF LIGNIN MODIFICATIONS ON PULPING

Despite the fact that much of the research on genetic modification of lignin biosynthesis was motivated by a desire to improve wood for more efficient and environmentally benign papermaking, very few lignin-modified plants (either transgenic or mutant) have yet been tested in pulping and bleaching processes. The reasons for this regrettable situation are unclear, but many factors are likely to be involved. Most molecular biology laboratories where transgenic plants have been produced are unlikely to have easy access to expertise in pulping technology. It is

no accident that most of the instances where pulping characteristics of transgenics have been tested come from EU-funded consortia projects where the kind of interdisciplinary contacts that are necessary for such work are actively fostered. Obviously, pulping data that best approximate real industrial situations will come from reasonably large amounts of plant material grown in the field, which again poses problems for small laboratories that may not have access to field trial facilities. Similarly, navigating the regulatory process governing the controlled release of genetically modified (GM) plants is something many researchers would find daunting. Designing trials to the rigorous standards necessary to gain regulatory approval requires significant effort both in terms of the paperwork involved and in the implementation, maintenance, and eventual clearance of the trials themselves. The strongly negative attention such trials can attract from anti-GM activists is another factor that inhibits efforts in this area. The real prospect that such trials might be attacked and vandalized (as in the case of a poplar trial in the UK in 1999 and many recent trials in the US) or that laboratories perceived to be involved in such work might be firebombed (as happened at the University of Washington in 2001; Luce, 2002), not surprisingly puts many people off investing their research grants and efforts in this area. The end result is that some elements of the anti-GM lobby deliberately prevent the collection of the very data on the environmental impact of GM plants that more rational commentators agree is needed as part of the wider exploration of potential GM solutions to currently insoluble environmental problems. It is the responsibility of society as a whole, not scientists themselves, to assess this type of criminal activity aimed at field trial and laboratory destruction, which wastes significant amounts of public money and inhibits scientific advance. Unless a serious effort is made to tackle this problem, progress is bound to be slow.

The above comments notwithstanding, several pulping studies have been performed on lignin-modified GM plants including one involving trees grown for 4 years in the field (Pilate *et al*., 2002). The results of this field experiment will be discussed in some detail because they are currently the only data that can be drawn upon to predict the potential performance of GM lignin-modified trees in the field, in terms of both stability of transgene expression and persistence of lignin changes and pulping benefits. Pulping analyses have also been performed on the same poplar transgenics when grown in the greenhouse (Baucher *et al*., 1996; Lapierre *et al*., 1999, 2000; Petit-Conil *et al*., 1999, 2000; Jouanin *et al*., 2000), on the pine *cad* mutant (MacKay *et al*., 1999), on greenhouse-grown transgenic tobacco down-regulated for CAD or CCR (O'Connell *et al*., 2002), and on transgenic poplars overexpressing F5H (Huntley *et al*., 2003). The poplar and tobacco plants were analyzed by simulated Kraft pulping and/or mechanical pulping and the pine mutant both by Kraft and soda pulping.

A. Chemical Pulping of Wood from Transgenic Poplars Down-Regulated for COMT and CAD

Laboratory-scale Kraft pulping has been performed on greenhouse-grown transgenic poplars for two lines down-regulated for COMT (ASB2B and ASB10B) and two lines down-regulated for CAD (ASCAD21 and ASCAD52) (Baucher *et al*., 1996; Lapierre *et al*., 1999, 2000). Subsequently, field trials of the same tree lines were established at two sites, one in France and one in the United Kingdom (Petit-Conil *et al*., 2000; Pilate *et al*., 2002). Wood from the French trial was harvested after 2 and 4 years (i.e. after 2 years regrowth of the previously harvested trunks), while trees of the UK trial were harvested after 4 years of growth only. This harvesting strategy allowed an assessment of whether the Kraft pulping characteristics varied with age and enabled evaluation of the stability of transgene expression and lignin modification over several years of growth. Table 7 (COMT lines) and Table 8 (CAD lines) pull the data from publications on greenhouse- and field-grown trees together for ease of comparison.

For COMT-suppressed lines, enzyme activity was higher in field-grown trees than in younger greenhouse-grown plants (Table 7), whereas CAD-suppressed lines showed similar or lower enzyme activity in the field compared to the greenhouse (Table 8). Only ASCAD21 had altered lignin content[e] (a slight reduction) in greenhouse and field. Antisense COMT lines had a lower frequency of lignin units only involved in β-O-4 bonds[i], greatly reduced S/G[i], and significant amounts of 5OHG[i] units in lignin (Table 7). The lignin S/G ratio was unmodified in ASCAD lines, but altered lignin structure was indicated by the increased frequency of free phenolic groups in β-O-4 linked S or G units[i] (Table 8). These data demonstrate that trees modified in either COMT or CAD showed similar

TABLE 7
Lignin Characteristics and Properties of ECF-Bleached Kraft Pulps for COMT-Down-Regulated Poplar

	Control					ASB2B					ASB10B				
Characteristics	12 m G	24 m G	24 m F, Fr	48 m F, Fr	48 m F, UK	12 m G	24 m G	24 m F, Fr	48 m F, Fr	48 m F, UK	12 m G	24 m G	24 m F, Fr	48 m F, Fr	48 m F, UK
COMT enzymatic activity (%)	100[a]	—	—	100[d]	—	5[a]	—	—	44[d]	—	5[a]	—	—	32[d]	—
Lignin characteristics															
Klason lignin content (% of control)	100[c]	100[b]	100[b]	100[d]	100[d]	101[c]	109[b]	103[b]	100[d]	100[d]	107[c]	105[b]	97[b]	100[d]	100[d]
Yield in S + G + 5OHG (μmol/g lignin)	—	—	2580[a]	—	—	—	—	1570[a]	—	—	—	—	1610[a]	—	—
S/G	—	—	1.78[a]	2.00[d]	2.10[d]	—	—	0.77[a]	0.70[d]	1.50[d]	—	—	0.48[a]	0.80[d]	1.00[d]
Relative proportion of G/S/5OHG	—	—	36/64/tr[a]	—	—	—	—	53/41/6[a]	—	—	—	62/30/8[a]	—	—	—
Units involved only in β-O-4 (%)	—	—	60[d]	70[d]	60[d]	—	—	45[d]	50[d]	57[d]	—	—	42[d]	50[d]	45[d]
Free OH in β-O-4-linked G units	—	—	26.4[a]	—	—	—	—	—	—	—	—	—	21.5[a]	—	—
Free OH in β-O-4-linked S units	—	—	3.0[a]	—	—	—	—	—	—	—	—	—	2.6[a]	—	—
Pulp properties															
Kappa number	27.4[bc]	11.9[a]	17.7[b]	20.4[d]	19.7[d]	36.8[b,c]	14.3[a]	27.4[b]	—	—	37.7[bc]	14.8[a]	31.5[b]	32.7[d]	27.8[d]
Cellulose DP	1880[bc]	—	1440[b]	2090[d]	2190[d]	1940[bc]	—	1730[b]	—	—	—	—	1710[b]	2030[d]	2100[d]
Pulp yield (% wt)	—	46.9[a]	51.3[b]	51.9[d]	54.8[d]	—	47.2[a]	49.6[b]	—	—	—	47.6[a]	—	50.6[d]	52.0[d]
Pulp properties after ECF bleaching															
Brightness (%ISO)	88.1[c]	—	87.9[b]	89.8[d]	89.3[d]	80.1[c]	—	86.5[b]	—	—	83.2[c]	—	84.5[b]	89.4[d]	88.5[d]
Cellulose DP	1560[c]	—	1520[b]	—	—	1620[c]	—	1540[b]	—	—	1580[c]	—	1520[b]	—	—
Drainage index (°SR)	51[c]	—	—	—	—	50[c]	—	—	—	—	51[c]	—	—	—	—
Bulk* (cm^3/g)	1.10[c]	—	—	—	—	1.07[c]	—	—	—	—	1.07[c]	—	—	—	—
Tensile index (Nm/g)	82.3[c]	—	—	—	—	92.6[c]	—	—	—	—	82.6[c]	—	—	—	—
Burst index ($kPam^2$/g)	5.4[c]	—	—	6.1[e]	6.3[e]	5.8[c]	—	—	—	—	5.5[c]	—	—	6.0[e]	5.8[e]
Tear index (mNm^2/g)	4.5[c]	—	—	6.4[d]	4.3[d]	4.7[c]	—	—	—	—	5.1[c]	—	—	6.2[d]	6.4[d]
Fiber length (mm)	—	—	0.93[b]	—	—	—	—	0.91[b]	—	—	—	—	0.90[b]	—	—
Breaking length (km)	—	—	—	8.6[d,e]	9.2[d,e]	—	—	—	9.1[e]	8.9[e]	—	—	—	9.0[d,e]	9.3[d,e]

12 m, 24 m, 48 m, months of age; F, grown in the field; Fr, France; G, grown in the greenhouse; UK, United Kingdom;—not determined; tr, traces. [a,b,c,d] and [e]: data from Lapierre *et al.* (1999), Lapierre *et al.* (2000), Petit-Conil *et al.* (1999), Pilate *et al.* (2002), and Petit-Conil *et al.* (2000), respectively. Kraft pulps were produced in small-pressurized reactors placed in a rotating oil-thermoregulated bath. The conditions used for the pulping were 20%[a,b,c,] or 18%[d] active alkali, 25% sulfidity, liquor to wood ratio = 4, temperature raised to 170°C over 90 min and maintained for 90 min. The pulps were then washed and screened on a 150-μm sieve to retain uncooked particles. Kappa number and cellulose degree of polymerization (DP) were determined according to international standards. The pulps were subsequently bleached with an ECF sequence, O-D-E-D at 10% pulp consistency.

TABLE 8
Lignin Characteristics and Properties of ECF-Bleached Kraft Pulps for CAD-Down-Regulated Poplar

	Control					ASCAD21					ASCAD52				
Characteristics	3 m G	24 m G	24 m F, Fr	48 m F, Fr	48 m F, UK	3 m G	24 m G	24 m F	48 m F, Fr	48 m F, UK	3 m G	24 m G	24 m F	48 m F, Fr	48 m F, UK
CAD enzymatic activity (%)	100[a]	—	—	100[e]	—	30[a]	—	—	15[e]	—	30[a]	—	—	47[e]	—
Lignin characteristics															
Klason lignin content (% of control)	100[a]	100[b]	100[b]	—	—	100[a]	96[b]	96[b]	—	—	106[a]	100[b]	99[b]	—	—
Yield in S + G (μmol/g lignin or μmol/g CWR$*$)	414[a]*	—	2580[b]	—	—	311[a]*	—	2235[b]	—	—	323[a]*	—	2360[b]	—	—
S/G	1.71[a]	—	1.78[b]	—	—	1.32[a]	—	1.86[b]	—	—	1.63[a]	—	1.94[b]	—	—
Relative proportion of G/S/5OHG	37/63/tr[a]	—	36/64/tr[b]	—	—	43/57/tr[a]	—	35/65/tr[b]	—	—	38/62/tr[a]	—	34/66/tr[b]	—	—
Free OH in β-O-4-linked G units	—	—	26.4[b]	26.4[e]	—	—	—	33.6[b]	29.2[e]	—	—	—	30.5[b]	—	—
Free OH in β-O-4-linked S units	—	—	3.0[b]	3.0[e]	—	—	—	4.5[b]	4.5[e]	—	—	—	3.5[b]	—	—
Pulp properties															
Kappa number	20.8[a1]	18.7[c2]	17.6[d2]	20.4[e3]	19.7[e3]	19.2[a1]	15.6[c2]	16.3[d2]	18.6[e3]	13.9[e3]	18.8[a1]	15.7[c2]	—	—	—
Cellulose DP	1010[a1]	1900[c2]	1440[d2]	2090[e3]	2190[e3]	1140[a1]	1910[c2]	1670[d2]	2140[e3]	2270[e3]	990[a1]	1800[c2]	—	—	—
Pulp yield (% wt)	47.9[a1]	45.9[c2]	51.3[d2]	51.9[e3]	54.8[e3]	47.5[a1]	47.4[c2]	50.9[d2]	54.4[e3]	56.9[e3]	43.4[a1]	—	—	—	—
Pulp properties after ECF bleaching															
Brightness (%ISO)	—	90.6[c]	87.9[cd]	89.8[e]	89.3[e]	—	91.0[c]	89.6[c,d]	90.1[e]	90.7[e]	—	91.0[c]	89.2[c]	—	—
Cellulose DP	—	1510[c]	—	—	—	—	1500[c]	—	—	—	—	1470[c]	—	—	—
Drainage index (°SR)	—	54[c]	—	—	—	—	53[c]	—	—	—	—	56[c]	—	—	—
Bulk (cm^3/g)	—	1.12[c]	—	—	—	—	1.14[c]	—	—	—	—	1.10[c]	—	—	—
Tensile index (Nm/g)	—	91.8[c]	—	—	—	—	84.6[c]	—	—	—	—	96.7[c]	—	—	—
Burst index ($kPam^2$/g)	—	5.8[c]	—	6.1[f]	6.3[f]	—	5.5[c]	—	6.1[f]	6.1[f]	—	5.6[c]	—	5.9[f]	5.9[f]
Tear index (mNm^2/g)	—	5.7[c]	—	6.4[e,f]	4.3[e,f]	—	5.4[c]	—	5.9[e,f]	5.1[e,f]	—	5.6[c]	—	6.3[f]	5.9[f]
Kappa after O	—	12.5[c]	—	—	—	—	8.1[c]	—	—	—	—	8.6[c]	—	—	—
Cellulose DP after O	—	1600[c]	—	—	—	—	1560[c]	—	—	—	—	1530[c]	—	—	—
Fiber length (mm)	0.42[a]	—	0.93[d]	—	—	0.43[a]	—	0.92[d]	—	—	—	—	—	—	—
Breaking length (km)	—	—	—	8.6[e,f]	9.2[e,f]	—	—	—	9.0[e,f]	9.9[e,f]	—	—	—	9.2[f]	9.3[f]

3 m, 24 m, 48 m, months of age; [a], [b], [c], [d], [e] and [f]: data from Baucher *et al*. (1996), Lapierre *et al*. (1999), Petit-Conil *et al.* (1999), Lapierre *et al*. (2000), Pilate *et al*. (2002), and Petit-Conil *et al*. (2000), respectively. Conditions of Kraft pulping and bleaching were as described in Table 7. [1], 22% active alkali; [2], 20% active alkali; [3], 18% active alkali; —, not determined; tr, traces. CWR, cell wall residue; F, grown in the field; Fr, France; G, grown in the greenhouse; UK, United Kingdom.

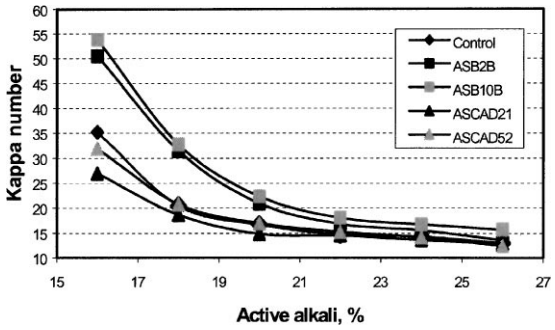

FIGURE 6. Evolution of kappa number with active alkali charge for Kraft pulping of 4-year-old poplar trees from the French field trial.

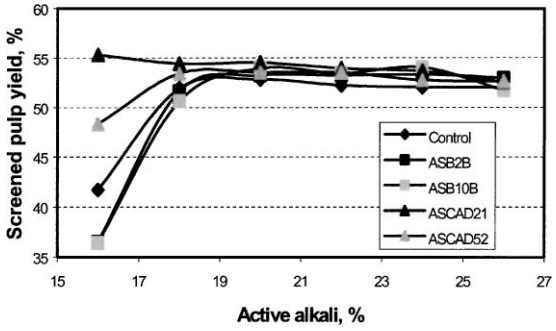

FIGURE 8. Evolution of screened pulp yield with active alkali charge for Kraft pulping of 4-year-old poplar trees from the French field trial.

changes to lignin whether grown in the greenhouse or in two different field environments.

Chemical pulping of the COMT antisense poplars demonstrated that their wood was more resistant to Kraft delignification than wood from control trees, as indicated by the higher kappa number of pulp from the transgenic plants (Table 7). Pulp brightness, after bleaching with an ECF sequence, was lower than that of the control (Table 7), although paper strength was similar according to its degree of depolymerization* (DP) of cellulose and its mechanical properties. Pulp from CAD antisense plants, however, was more easily delignified and had a lower kappa number than control pulp, a difference that was maintained after the oxygen delignification stage (O), the first step in the bleaching sequence (Table 8). Cellulose DP, pulp brightness, and paper strength were similar in control and CAD-suppressed lines (Table 8).

In parallel to the pulping experiments described above, wood from the French trial was Kraft pulped under a range of active alkali charges from 16 to 26% (Figure 6). Increasing the active alkali removes more lignin and yields lower kappa numbers. Compared with the control, less chemical was needed to reach a given kappa number for the pulp made from CAD antisense lines and more chemical was needed for COMT antisense poplars. The extent of cellulose degradation, for a given alkali charge, was the same for all lines (Figure 7), suggesting that down-regulation of COMT or CAD does not affect cellulose biosynthesis. Another important characteristic of the Kraft process is the evolution of the screened pulp yield with active alkali charge. Whereas a decrease in the screened pulp yield was observed at lower alkali charges for the control and COMT down-regulated poplars, the pulp yield remained high for CAD down-regulated poplars (Figure 8). The selectivity curve (Figure 9) shows the beneficial effect of CAD down-regulated lines on Kraft pulping in terms of cellulose preservation and delignification: for a given kappa number, the cellulose was less degraded for the CAD lines, particularly for ASCAD21. In contrast, when wood of COMT-down-regulated poplars was similarly pulped, the cellulose DP was lower than that of control wood.

The conclusions from these experiments are that suppression of CAD activity leads to the

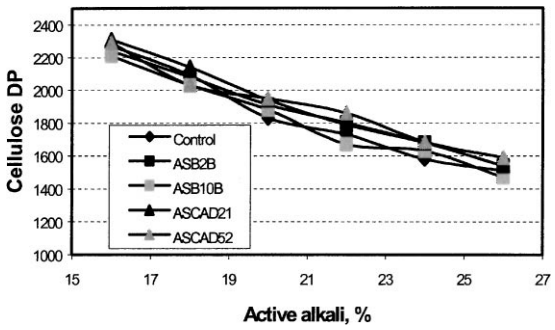

FIGURE 7. Evolution of cellulose DP with active alkali charge for Kraft pulping of 4-year-old poplar trees from the French field trial.

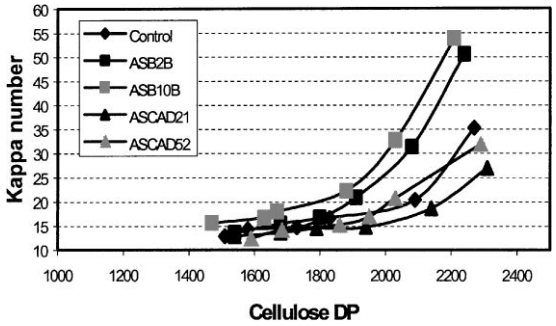

FIGURE 9. Selectivity curve: kappa number versus cellulose DP for Kraft pulping of 4-year-old poplar trees from the French field trial.

formation of wood with better Kraft pulping properties, probably because of the slightly decreased lignin content and modified lignin composition. Interestingly, ASCAD21, the line with the best pulping characteristics, had an increased proportion of free phenolic groups in both G and S lignin units, possibly influencing the solubility of the polymer during Kraft pulping. Savings in chemical use and increase in pulp yield for this line have been estimated at 6% and 2–3%, respectively, which is commercially valuable (Pilate *et al*., 2002). Most importantly, this line performed as well as wild-type trees in the field and showed no adverse effects of the genetic modification. In contrast, down-regulation of COMT has a negative effect on Kraft pulp properties, supporting the hypothesis that lignin rich in G units is difficult to extract by the Kraft process because of the more condensed lignin. The lower proportion of free phenolic groups in β-O-4-linked G units in COMT-suppressed poplars may also contribute to the higher kappa numbers (see section III.D). Although incorporation of 5OHG (with two hydroxyl functions) into lignin in COMT-suppressed plants might be expected to be beneficial for pulping because of an increase in solubility, this apparently did not counterbalance the detrimental effects of a more condensed lignin in these lines. The results obtained from both field trials demonstrated that, contrary to original expectations, depleting COMT activity reduces wood quality for Kraft pulping.

B. Chemical Pulping of Wood from Transgenic Poplar Overexpressing F5H

Lignin of transgenic poplars overexpressing F5H is significantly enriched in S units (Franke *et al*., 2000). Kraft pulping experiments on wood of 2-year-old greenhouse-grown poplars resulted in a pulp with lower kappa number and an increase in brightness (Huntley *et al*., 2003). This genetic improvement may increase pulp throughput by 60%, concomitantly with a decreased consumption of pulping chemicals (Huntley *et al*., 2003).

C. Chemical Pulping of Wood from Transgenic Tobacco Down-Regulated for CCR and CAD

Although tobacco is not normally used for pulping, its value as a model for species that are pulped has been investigated in laboratory-scale simulated Kraft pulping experiments. Stems of four transgenic tobacco lines, two down-regulated in CCR and two down-regulated in CAD were pulped. For each gene, enzyme activity was suppressed moderately in one line and severely in the other. Thus, lines CCR34 and CCR86 had 48% and 1% residual CCR activity, whereas lines CADJ40 and CADJ50 had 20% and 7% residual CAD activity, respectively. As shown in Table 9, the pulps of both types of transgenic plants had a lower kappa number than those of the control plants (O'Connell *et al*., 2002). The greatest reduction in kappa number was obtained for the lines with the lower CCR or CAD activity. The degree of cellulose DP was similar for the pulp of all lines tested. Micrographs of fibers from the unbleached pulps showed a higher level of cell separation for the transgenic than for the control lines. Whereas the CADJ40 pulp was bleached to a higher brightness than the control, the pulp from CCR86 was less bright than the control. This lower brightness was apparently due to a higher content of unextracted chlorophyll in CCR-suppressed stems (O'Connell *et al*., 2002). Because the pulping data obtained with CAD-down-regulated tobacco are comparable to those from CAD-suppressed poplar (both species had reduced kappa number and better bleaching with no modification of other pulp properties), these data indicate that tobacco may be an appropriate model for rapid evaluation of the impact of specific genetic modifications on pulping. In this respect, it will be interesting to see whether or not the results of pulping experiments on CCR-down-regulated trees, when these become available, are comparable with those already obtained in CCR-suppressed tobacco.

D. Chemical Pulping of Wood from a Pine *cad* Mutant

Different pulping experiments have been performed with wood from a 12-year-old pine *cad* mutant (MacKay *et al*., 1999). As shown in Table 10, soda pulping (26–28% active alkali) resulted in pulp with a lower kappa number than that of the wild type. These results are in agreement with the increased lignin reactivity observed with mild alkali treatment (MacKay *et al*., 1999). In contrast to the results obtained with the transgenic CAD-suppressed poplars, no improved delignification was obtained using either the Kraft-Pressure bomb* (20–24% active alkali) or the Kraft-micro autoclave* (17.5–25% active alkali), possibly because of the abundant presence of dihydroconiferyl alcohol in the lignin of

TABLE 9
Lignin Characteristics and Properties of Bleached Kraft Pulps for CCR- and CAD-Down-Regulated Tobacco

Characteristics	Control	CCR34	Control	CCR86	Control	CADJ40	Control	CADJ50
Enzymatic Activity (%)	100[a]	48[a]	100[a]	1[a]	100*[b]	20*[b]	100*[b]	7*[b]
Lignin Characteristics								
Klason lignin content (% of control)	100	100	100	58	100	100	100	100
Yield in S + G (μmol/g Klason lignin)	2106[c]	1695[c]	1295[c] (1711[d])	766[e] (726[d])	—	—	—	—
S/G	0.72	0.78	0.72	1.61	—	—	—	—
Free OH in linked β-*O*-4 G units	—	—	20.2	20.9	—	—	—	—
Pulp Properties								
Kappa number	21.6	20.6	20.3	17.7	20.0	18.0	27.0	24.0
Cellulose DP	1430	1430	1330	1220	1575	1600	—	—
Cellulose yield (% wt)	35.9	36.6	34.0	37.0	34.3	36.6	33.0	32.4
Pulp Properties After Bleaching								
Brightness (% ISO)	—	—	82.8[e]	77.5[e]	88.9[f]	88.9[f]	—	—
Breaking length (m)	—	—	5960[e]	8210[e]	9730[f]	9270[f]	—	—
Tear index (mNm^2/g)	—	—	4.9[e]	3.4[e]	5.3[f]	6.2[f]	—	—
Fiber length (mm)	—	—	0.93[e]	0.98[e]	0.77[f]	0.72[f]	—	—

All data are from O'Connell *et al*. (2002) except * which are from Halpin *et al*. (1994). All data were obtained by using mature stems as plant material except [a,b], and [c]; [a]determined on 34- to 45-day-old plantlets regenerated *in vitro*; [b]determined on 60- to 70-day-old greenhouse-grown plants; [c]determined on 7-week-old greenhouse-grown plants; [d]determined on 21-week-old greenhouse-grown plants. Conditions of Kraft pulping were 28% active alkali, 25% sulfidity. [e]Bleaching was performed using an O-D-E/O-D-E-C-F sequence; [f] Bleaching performed using an C-E-D-E-D sequence; —, not determined. CCR34 and CCR86 are suppressed CCR plants whereas CADJ40 and CADJ50 are suppressed CAD plants.

the pine mutant, which is involved in C-C linkages (MacKay *et al*., 1999). The latter compound has not been detected in the transgenic poplars, possibly reflecting the differences in the lignin biosynthetic pathway between gymnosperms and angiosperms. Interestingly, the reduced kappa number in the mutant after soda pulping opens up the possibility of lowering consumption of sulfide during pulping, thereby reducing the production of volatile organic sulfur compounds.

Generally, the pulp yield obtained for the wood of the mutant tree was lower than that of the wild type. MacKay *et al*. (1999) suggest this could be due to the difference in kappa number after soda pulping or excessive peeling of polysaccharides because of overpulping. Brightness of the soda pulp

TABLE 10
Properties of Soda Pulping for a 12-Year-Old *cad* Pine Mutant

Characteristics	Control	*cad* Mutant
26% active alkali		
Kappa number	53.1	41.6
Pulp yield (%)	31.1	26.9
Brightness (% ISO)	31.3	28.7
28% active alkali		
Kappa number	46.3	26.0
Pulp yield (%)	33.7	30.0
Brightness (% ISO)	33.0	34.0

Data are from MacKay *et al*. (1999).

of the pine *cad* mutant was similar to that of the wild type (Table 10). In contrast, brightness of the Kraft pulp of the mutant was slightly lower than that of the wild type, although kappa values were comparable (MacKay *et al*., 1999). Taken together, the data currently available concerning the potential of gene engineering for improving pulping suggest that CAD and CCR suppression affect lignin structure in ways that improve chemical pulping efficiency by reducing the amount of required chemicals, and thereby reducing the environmental impact of pulp production.

E. Mechanical Pulping of Wood from Transgenic Poplars Down-Regulated for COMT and CAD

Preliminary refiner mechanical pulp (RMP) experiments, carried out at a pilot scale, have been performed with wood of poplar trees down-regulated for COMT and CAD grown in both the French and UK field trials mentioned above (Petit-Conil *et al*., 1999, 2000). The mechanical properties of the RMP pulp were not modified, except for the ASCOMT line ASB10B which presented higher tensile and tear strength. For the wood of the ASCAD line ASCAD21, the brightness of the unbleached pulps and the pulp bleachability were lower than those of the control. These results are possibly caused by a modification in the chromophore nature caused by the alteration of CAD expression (Petit-Conil *et al*., 2000).

VI. CONCLUSIONS AND PERSPECTIVES

Our understanding of the lignin biosynthetic pathway is constantly and rapidly changing. The combination of classical biochemical approaches, allied with the use of transgenic plants to investigate the pathway *in vivo*, and the interest in improving wood and plant fibers for industrial applications, have contributed to recent progress in this research area. A new avenue for studying the molecular biology of plant cell walls is to characterize developmental processes, such as wood formation, at the level of the transcriptome and the metabolome (Hertzberg *et al*., 2001; Tretheway *et al*., 1999), and compare these processes in wild-type and transgenic plants modified in cell wall biosynthesis. Such analyses should provide a comprehensive and holistic view on cell wall assembly at a level that was not possible just a few years ago. In addition to the identification of novel genes involved in wood formation, whose function can be further studied by reverse genetics, applications of these techniques will shed light onto the interrelations between the biochemical pathways leading to the biosynthesis of the different cell wall macromolecules and onto their relation with plant growth and development.

Despite our currently poor understanding of the detailed biochemistry of plant cell walls, it is clear that we can modify this biochemistry for useful purposes. We already have evidence that genetic engineering of lignin biosynthesis has potential for improving both the efficiency and the environmental impact of pulp and papermaking. A major objective for the future will be to learn how to optimize lignin profiles for ease of pulping. Similarly, lignin-modified plants will need to be grown, experimentally at least, on a scale useful for assessing their impact for industrial pulping. Results obtained to date concern mainly model plants, such as tobacco and poplar. In the next step, the technology needs to be transferred to other species and to elite clones suitable to clonal forestry plantations. Transformation protocols for most of the commercially important tree species, including gymnosperms, have been established over the past years.

It is important to appreciate that tree breeding is only in the third or fourth generation of selection and, therefore, significant genetic improvements can be expected through conventional breeding alone. However, the genetic improvement by classical breeding is inevitably slow because of the long generation times typical of trees, and the fact that many traits can only be scored at rotation age. Because the current level of wood consumption is higher than the amount of wood that natural forests can sustainably produce, more wood will need to be grown in high-yielding plantations (Food and Agriculture Organization, 2001b). Molecular biology and genetic engineering can be of great value in advancing progress in this sector, for example, by allowing genes of interest to be identified and introduced into elite genotypes or by altering the expression of native genes. It is conceivable that future agro-forestry will utilize genetically engineered trees that produce wood of better quality and with higher yields, either for pulping or for timber use. In that context, plantation of transgenic trees could increase wood production and help the efforts to reduce the pressure on native forests

(Fenning and Gershenzon, 2002). Several forestry companies and Universities currently manage field trials of transgenic trees with potentially improved traits (for a list, see Information Systems for Biotechnology http://www.isb.vt.edu/) and these will hopefully pave the way to commercialization. Alternatively, when the function of agronomically important genes has been demonstrated in transgenic plants, a more efficient exploitation of the existing genetic diversity within a given species will be possible by combining favorable alleles for these genes by marker-assisted breeding, again accelerating the domestication of trees.

Potential risks of large-scale production of engineered trees have obviously to be taken into account, but these must be balanced with the clear potential benefits of cultivating genetically modified clones (Strauss, 2003). At present, the impact of transgenes on the environment is evaluated in controlled and environmentally safe field trials (Hancock & Hokanson, 2001; Dale *et al*., 2002). However, a variety of research strategies aimed at minimizing the possible escape of introduced genes are being investigated, such as the modification of flowering and sterility (Weigel & Nilsson, 1995; Rottmann *et al*., 2000; Peña & Séguin, 2001; Daniell, 2002), and the use of transformation methods to produce plants harboring only the target gene, devoid of selectable marker genes conferring antibiotic resistance (Ebinuma *et al*., 1997; Hare & Chua, 2002; Erikson *et al*., 2003). Because of the importance of high yielding plantation forests to sustainably supply wood and reduce destruction of natural forests, regulatory costs and hurdles for field trials involving certain classes of genetically modified trees, such as those that are transformed with species-own genes, should be reduced and field-trialing promoted (Fenning and Gershenzon, 2002; Strauss, 2003).

A critical point to consider is whether the gene engineering approach to tree improvement is economically attractive in comparison with the cost of traditional breeding methods. The traits introduced should have major economic value, as it is the case for lignin content or quality. In addition, the genetic engineering should confer improvement not easily obtainable by other means and should not adversely affect growth or survival of the plants. Important in this respect is the fact that current genetic engineering technology allows the stacking of several transgenes simultaneously in elite genotypes by co-transformation (Halpin & Boerjan, 2003; Li *et al*., 2003), further speeding-up the domestication of trees, and improving the cost effectivity of the transgenic approach. In conclusion, biotechnology and genetic engineering hold promise for the genetic improvement of trees and are important strategies to be considered to accelerate current breeding programs.

ACKNOWLEDGMENTS

The authors thank Martine De Cock for help in preparing the manuscript. This work was carried out in the framework of the European Union Research Programs AGRE-0021-C, FAIR-CT95-0424, and AIR2-CT93-1661 and by funding to CH from the UK Biotechnology and Biological Sciences Research Council (BBSRC). M.B. is a Research Associate of the Belgian "Fonds National de la Recherche Scientifique" (FNRS).

GLOSSARY

[a]Acetyl bromide extraction—Method to quantify lignin in which lignin is extracted by acetyl bromide and then quantified spectrophotometrically by measuring the absorbance at 280 nm (Iiyama & Wallis, 1988).

Active alkali—Percentage of active ingredients in the pulping liquor (NaOH + Na_2S in the Kraft pulping process; NaOH in the soda pulping process).

[b]Alkaline nitrobenzene oxidation—Method to determine lignin composition. Lignin is oxidized with nitrobenzene in alkali and the degradation products are measured after HPLC separation or gas chromatography. This method cleaves the β-*O*-4 and the C_α-C_β linkages in the polymer. The compounds recovered do not represent an absolute value for phenylpropanoid units in lignin and may also include esterified cell wall phenolics. Nitrobenzene oxidation yields vanillin (degradation product of coniferyl alcohol and ferulic acid [Van]), syringaldehyde (degradation product of sinapyl alcohol and sinapic acid [Syr]) and *p*-hydroxybenzaldehyde (degradation product of *p*-coumaryl alcohol and *p*-coumaric acid). The monomeric composition of lignin is generally expressed by the Syr/Van ratio.

Basis weight (g/m^2) or grammage—Weight of paper per ream (specified area of paper or paperboard). The TAPPI Standard ream is 1 m^2 of paper or board.

Breaking length (km)—Measure of tensile strength obtained by calculating the length at which a strip of paper would break under its own weight

when suspended by one end (NF EN ISO 1924-2). The breaking length of most papers varies from 2.5 to 12 km.

Brightness—Measure of the whiteness of the pulp or paper. It is the percentage of diffuse reflected blue light (457 nm) from the paper, relative to an MgO standard (NF Q 03039).

Bulk (cm^3/g)—Volume per unit weight (g) of a paper sheet (NF EN ISO 536-NF EN 20534). Bulk is the reciprocal of density (g/cm^3).

Burst index ($kPam^2/g$)—Burst strength is the resistance of a paper sheet to rupture by a stretching force perpendicular to the sheet (NF Q 03053). The burst strength measures the hydrostatic pressure required to rupture a piece of paper.

Canadian Standard Freeness (CSF)—Measure of pulp particle size and ability of a pulp to drain water. Water drainage is a crucial parameter in paper manufacture because it determines the consistency of paper products. A high drainage rate means a high freeness. The CSF test measures the drainage of 1 liter of pulp slurry at 0.3% pulp consistency through a calibrated screen.

Cellulose degree of polymerization (DP)—Number of monomers in the chain of the cellulose polymer. Cellulose DP is indicated by the measure of cellulose viscosity, which is determined after dissolving the pulp in a solvent, such as a cupriethylene-diamine solution. A higher viscosity indicates a higher cellulose DP, and is generally correlated with better pulp and paper properties.

[c]Derivatization followed by reductive cleavage (DFRC)—Method to determine the composition of lignin. The α- and β-aryl ether linkages in lignin are cleaved by acetyl bromide and the released monomers (cinnamyl acetates) are quantified by gas chromatography (Lu & Ralph, 1997). The results obtained are similar to those obtained by thioacidolysis. However, yields of released monomers are lower than with thioacidolysis.

Drainage index—Degree Schopper Reigler (°SR) or Canadian Standard Freeness* (ml CSF), both based on a similar concept. Drainage reflects the ease of removing water from the pulp by gravity or by mechanical means.

[d]Diffuse reflectance infrared Fourier transform (DRIFT) spectroscopy—See Fourier transform infrared (FTIR) spectroscopy

Fiber coarseness (mg/m)—Mass per chain of fibers 1 m long. Fiber coarseness reflects the relative thickness of cell walls.

Fibrillation—Loosening of the cross-linking within a fiber wall, allowing partial separation of constituent fibrils at the fiber surface.

[d]Fourier transform infrared (FTIR) and diffuse reflectance infrared Fourier transform (DRIFT) spectroscopy—Techniques based on interferometry, allowing the analysis of nanograms of material. FTIR and DRIFT spectroscopy rely on the absorption of energy from an illuminating laser. An FTIR spectrum is generated from radiation absorbed by molecules with polarized bonds or dipole bonds after excitation with infrared light. The ratio of absorbance intensities at different wavelengths is related to the concentration of different molecules in a cell wall sample. The DRIFT technique provides absorption spectra generated with light reflected from the surface of opaque materials. DRIFT of cell wall materials is non-destructive.

Kappa number—Measure of the residual lignin in the pulp, obtained by measuring the consumption of permanganate ions that react with lignin in the pulp. A high kappa number is indicative of a high lignin content. As a rough estimate, Klason[e] lignin content (%) = 0.15 × kappa number.

[e]Klason lignin determination—Method that quantifies lignin and consists of hydrolyzing the cell wall polysaccharides with sulfuric acid, leaving lignin as an insoluble material, which is dried and quantified gravimetrically (Effland, 1977).

Kraft-Pressure bomb and Kraft-micro autoclave—Small reactors designed for laboratory-scale simulation of chemical pulping processes.

Liquor—Aqueous solution of chemicals used to delignify wood. The white liquor is the fresh pulping liquor for the Kraft process and consists mainly of NaOH, Na_2S, a small quantity of Na_2CO_3, and several impurities coming from wood, when the white liquor is recycled. Black liquor is the waste liquor of the Kraft pulping process after completion of pulping. It includes most of the original cooking chemicals and the dissolved wood substances.

[f]NMR spectroscopy—Technique that provides a compositional and structural fingerprint of isolated lignin fractions and allows the characterization of the major lignin units (H, G, and S units), the determination of functional groups (such as methoxy and hydroxyl), and the main interunit bonds. Limitations are overlapping signals, the complexity of assigning signals, and the low sensitivity.

Opacity (%)—Ability of paper to hide or mask a color or object beyond the sheet. The higher the opacity, the better the hiding power (opaque papers have an opacity of 100%).

Pulp consistency—Ratio of dry to humid pulp weight.

Pulp viscosity—Measure of the average chain length (degree of polymerization, DP) of cellulose. A high viscosity is indicative of a high cellulose DP and is a sign of a strong pulp or paper. This parameter has little importance in mechanical pulping because the cellulose chains are not significantly degraded by mechanical pulping.

Pulp yield (% weight)—Ratio between the oven-dry weight of the pulp and the initial oven-dry wood mass used for pulping.

[g]Pyrolysis gas chromatography mass spectrometry (pyrolysis GC-MS)—Direct depolymerization of lignin by rapid heating followed by mass spectrometry of the breakdown products. The limitation is the presence of overlapping peaks originating from other compounds and the structural changes that may occur during pyrolysis. This technique allows evaluation of lignin content and composition but cannot provide information on interunit linkages.

Refining—Mechanical treatment of pulp fibers, performed in a beater or a refiner, to develop their optimal papermaking properties, such as strength properties. The effects of refining are increased flexibility of the fibers, fibrillation* of cellulose, and delamination of the cell wall. Refined pulp has a low freeness. Refining is monitored by the drainage rate of water through the pulp, related to the freeness.

S or scattering coefficient (m^2/kg)—Describes the ability of a paper sheet to scattering of light. Defined as S = the limiting fraction of light energy scattered backward per unit thickness as the thickness of the layer approaches zero. Alternatively, when the equations are written down in terms of basis weight: S = the fractional scattering loss of light energy per unit basis weight as the weight of the layer approaches zero.

Screening of the pulp—Process that consists of removing particles, such as rigid and long fibers, shives, and fines fragments, by passing the diluted pulp suspension through screens under pressure.

SO_2 charge (%)—For acidic cooking, the terms total SO_2, free SO_2, or combined SO_2 are used. The total SO_2 (free SO_2 + combined SO_2) charge is the quantity of SO_2 determined by iodometry. The combined SO_2 charge represents the weight of SO_2 equivalent to the CaO charge contained in bisulfite. The free SO_2 charge is the difference between the percentages of total SO_2 and combined SO_2.

Sulfidity—Ratio of Na_2S to the active alkali ($Na_2S/(NaOH + Na_2S) \times 100\%$). Typically, a mill runs at 24–28% sulfidity depending on the wood to be pulped. Sulfidity increases the rate of delignification by cleavage of the β-O-4 linkages and the methoxy groups. Because lower cooking times are required at high sulfidity, carbohydrates are less degraded.

Tear index—Ratio of the tear strength* by the basis weight* of the paper sheet and expressed as mNm^2/g.

Tear strength—Measure of the energy required to propagate an initial tear through several sheets of paper over a fixed distance (NF EN 21974).

Tensile index—Ratio of tensile strength* to the basis weight* of the paper sheet (NF EN ISO 1924-2) and expressed as mN/g.

Tensile strength–Resistance of a paper sheet to rupture by a stretching force parallel to the sheet and measured on paper strips using a constant rate of elongation according to TAPPI standard T 494.

[h]Thioglycolic acid extraction—Method to quantify lignin. After its extraction by thioglycolic acid and alkali, lignin is measured spectrophotometrically by measuring the absorbance at 280 nm (Campbell & Ellis, 1992).

[i]Thioacidolysis—Method to determine the composition of lignin. Thioacidolysis and subsequent gas chromatography analysis identifies monomers that are released by selectively breaking of the intermonomeric β-O-4 linkages, the major linkages in lignin (Lapierre, 1993). This method is specific for phenylpropanoids and is very sensitive. The yield of degradation products is 40–50% for softwoods and approximately 70% for hardwoods (Boudet *et al.*, 1995). The yield of the thioacidolysis products (S+G) reflects the frequency of β-O-4 linkages, which are referred to as "non-condensed" linkages, whereas C-C linkages are designated "condensed" linkages. An additional analysis of the released dimeric structures allows the determination of the different types of carbon-carbon and diphenyl ether bonds between monomers (Lapierre *et al.*, 1999).

REFERENCES

Abbott, J.C., Barakate, A., Pinçon, G., Legrand, M., Lapierre, C., Mila, I., Schuch, W., and Halpin, C. 2002. Simultaneous suppression of multiple genes by single transgenes. Down-regulation of three unrelated lignin biosynthetic genes in tobacco. *Plant Physiol* **128:**844–853.

Aitken, Y., Cadel, F., and Voillot, C. 1988. *Constituants fibreux des pâtes, papiers et cartons. Pratique de l'analyse*. Centre Technique de l'Industrie des Papiers, Cartons et Celluloses et Ecole française de Papeterie et des Industries Graphiques, Grenoble, France.

Akhtar, M., Lentz, M.J., Swaney, R.E., Scott, G.M., Horn, E., and Kirk, T.K. 1998a. Biopulping: technology learned from nature that gives back to nature. In *Competing and Cooperating for Stakeholder Value*, (Proceedings of the 23rd Annual Summit). pp. 149–157. Technology Transfer Society, Ed., T2S, Chicago, IL.

Akhtar, M., Scott, G.M., Lentz, M.J., Horn, E., Scaney, R.E., and Kirk, T.K. 1998b. Commercialization of biopulping for mechanical pulping. In *Biotechnology in the Pulp and Paper Industry, Vol. A: Oral Presentations*. pp. A55–A58. M. Paice and J. Saddler, Eds., Canadian Pulp and Paper Association, Montreal.

Allina, S.M., Pri-Hadash, A., Theilmann, D.A., Ellis, B.E., and Douglas, C.J. 1998. 4-Coumarate: coenzyme A ligase in hybrid poplar. *Plant Physiol* **116:**743–754.

Anterola, A.M. and Lewis, N.G. 2002. Trends in lignin modification: a comprehensive analysis of the effects of genetic manipulations/mutations on lignification and vascular integrity. *Phytochemistry* **61:**221–294.

Anterola, A.M., Jeon, J.-H., Davin, L.B., and Lewis, N.G. 2002. Transcriptional control of monolignol biosynthesis in *Pinus taeda*. Factors affecting monolignol ratios and carbon allocation in phenylpropanoid metabolism. *J Biol Chem* **277:**18272–18280.

Atanassova, R., Favet, N., Martz, F., Chabbert, B., Tollier, M.T., Monties, B., Fritig, B., and Legrand, M. 1995. Altered lignin composition in transgenic tobacco expressing *O*-methyltransferase sequences in sense and antisense orientation. *Plant J* **8:**465–477.

Bate, N.J., Orr, J., Ni, W., Meromi, A., Nadler-Hassar, T., Doerner, P.W., Dixon, R.A., Lamb, C.J., and Elkind, Y. 1994. Quantitative relationship between phenylalanine ammonia-lyase levels and phenylpropanoid accumulation in transgenic tobacco identifies a rate-determining step in natural product synthesis. *Proc Natl Acad Sci USA* **91:**7608–7612.

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

Baucher, M., Monties, B., Van Montagu, M., and Boerjan, W. 1998. Biosynthesis and genetic engineering of lignin. *Crit Rev Plant Sci* **17:**125–197.

Baucher, M., Bernard-Vailhé, M.A., Chabbert, B., Besle, J.-M., Opsomer, C., Van Montagu, M., and Botterman, J. 1999. Down-regulation of cinnamyl alcohol dehydrogenase in transgenic alfalfa (*Medicago sativa* L.) and the impact on lignin composition and digestibility. *Plant Mol Biol* **39:**437–447.

Bernard-Vailhé, M.A., Besle, J.M., Cornu, A., and Maillot, M.P. 1995. Composition and biological degradability of lignin modified transgenic plants. *Ann Zootech*:Supp. 44, 68.

Bernard-Vailhé, M.-A., Cornu, A., Robert, D., Maillot, M.-P., and Besle, J.-M. 1996. Cell wall degradability of transgenic tobacco stems in relation to their chemical extraction and lignin quality. *J Agric Food Chem* **44:**1164–1169.

Biermann, C.J. 1996. *Handbook of Pulping and Papermaking*, 2nd. Ed. Academic Press, San Diego, CA.

Blee, K., Choi, J.W., O'Connell, A.P., Jupe, S.C., Schuch, W., Lewis, N.G., and Bolwell, G.P. 2001. Antisense and sense expression of cDNA coding for CYP73A15, a class II cinnamate 4-hydroxylase, leads to a delayed and reduced production of lignin in tobacco. *Phytochemistry* **57:**1159–1166.

Blount, J.W., Korth, K.L., Masoud, S.A., Rasmussen, S., Lamb, C., and Dixon, R.A. 2000. Altering expression of cinnamic acid 4-hydroxylase in transgenic plants provides evidence for a feedback loop at the entry point into the phenylpropanoid pathway. *Plant Physiol* **122:**107–116.

Boerjan, W., Ralph, J., and Baucher, M. 2003. Lignin biosynthesis. *Annu Rev Plant Biol* **54:**519–546.

Boudet, A.-M. 2000. Lignins and lignification: selected issues. *Plant Physiol Biochem* **38:**81–96.

Boudet, A.M., Lapierre, C., and Grima-Pettenati, J. 1995. Biochemistry and molecular biology of lignification. *New Phytol* **129:**203–236.

Bout, S. and Vermerris, W. 2003. A candidate-approach to clone the sorghum *Brown midrib* gene encoding caffeic acid *O*-methyltransferase. *Mol Gen Genomics* **269:**205–214.

Breen, A. and Singleton, F.L. 1999. Fungi in lignocellulose breakdown and biopulping. *Curr Opin Biotech* **10:**252–258.

Campbell, M.M. and Ellis, B.E. 1992. Fungal elicitor-mediated responses in pine cell cultures. III. Purification and characterization of phenylalanine ammonia-lyase. *Plant Physiol* **98:**62–70.

Campbell, M.M. and Sederoff, R.R. 1996. Variation in lignin content and composition. Mechanisms of control and implications for the genetic improvement of plants. *Plant Physiol* **110:**3–13.

Chabannes, M., Ruel, K., Yoshinaga, A., Chabbert, B., Jauneau, A., Joseleau, J.-P., and Boudet, A.-M. 2001a. *In situ* analysis of lignins in transgenic tobacco reveals a differential impact of individual transformations on the spatial patterns of lignin deposition at the cellular and subcellular levels. *Plant J* **28:**271–282.

Chabannes, M., Barakate, A., Lapierre, C., Marita, J.M., Ralph, J., Pean, M., Danoun, S., Halpin, C., Grima-Pettenati, J., and Boudet, A.M. 2001b. Strong decrease in lignin content without significant alteration of plant development is induced by simultaneous down-regulation of cinnamoyl CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) in tobacco plants. *Plant J* **28:**257–270.

Chabbert, B., Monties, B., Liu, Y.T., and Lagrimini, M. 1992. Lignin content and composition in transgenic tobacco plants with altered peroxidase activity. In *Proceedings of the 5th International Conference on Biotechnology in the Pulp and Paper Industry*. pp. 481–485. M. Kuwahara and M. Shimada, Eds., Uni Publishers Co., Tokyo.

Chabbert, B., Tollier, M.T., and Monties, B. 1993. Lignin variability among different brown midrib sorghum lines. In *Proceedings of the 7th International Symposium on Wood and Pulping Chemistry*, Vol. 1, pp. 462–468. ISWPC, Ed., CTAPI publications, Beijing.

Chabbert, B., Tollier, M.T., Monties, B., Barrière, Y., and Argillier, O. 1994a. Biological variability in lignification of maize: expression of the brown midrib bm2 mutation. *J Sci Food Agric* **64:**455–460.

Chabbert, B., Tollier, M.T., Monties, B., Barrière, Y., and Argillier, O. 1994b. Biological variability in lignification of maize: expression of the brown midrib bm3 mutation in three maize cultivars. *J Sci Food Agric* **64:**349–355.

Chapple, C. 1998. Molecular-genetic analysis of plant cytochrome P450-dependent monooxygenases. *Annu Rev Plant Physiol Plant Mol Biol* **49:**311–343.

Chapple, C.C.S., Vogt, T., Ellis, B.E., and Somerville, C.R. 1992. An Arabidopsis mutant defective in the general phenylpropanoid pathway. *Plant Cell* **4:**1413–1424.

Chen, C., Meyermans, H., Burggraeve, B., De Rycke, R.M., Inoue, K., De Vleesschauwer, V., Steenackers, M., Van Montagu, M.C., Engler, G.J., and Boerjan, W.A. 2000. Cell-specific and conditional expression of caffeoyl-CoA *O*-methyltransferase in poplar. *Plant Physiol* **123:**853–867.

Chen, F., Yasuda, S., and Fukushima, K. 1999. Evidence for a novel biosynthetic pathway that regulates the ratio of syringyl to guaiacyl residues in lignin in the differentiating xylem of *Magnolia kobus* DC. *Planta* **207:**597–603.

Chiang, V.L. and Funaoka, M. 1990. The difference between guaiacyl and guaiacyl-syringyl lignins in their responses to kraft delignification. *Holzforschung* **44:**309–313.

Chiang, V.L., Puumala, R.J., Takeuchi, H., and Eckert, R.E. 1988. Comparison of softwood and hardwood kraft pulping. *Tappi J* **71:**173–176.

Christensen, J.C., Baucher, M., O'Connell, A.P., Van Montagu, M., and Boerjan, W. 2000. Control of lignin biosynthesis. In *Molecular Biology of Woody Plants, Volume 1*, (Forestry Sciences, Vol. 64). pp. 227–267. S.M. Jain and S.C. Minocha, Eds., Kluwer Academic Publishers, Dordrecht.

Christensen, J.H., Van Montagu, M., Bauw, G., and Boerjan, W. 2001a. Xylem peroxidases: purification and altered expression. In *Molecular Breeding of Woody Plants*, (Progress in Biotechnology Series, Vol. 18), pp. 171–176. N. Morohoshi and A. Komamine, Eds., Elsevier Science, Amsterdam.

Christensen, J.H., Overney, S., Rohde, A., Ardiles Diaz, W., Bauw, G., Simon, P., Van Montagu, M., and Boerjan, W. 2001b. The syringaldazine-oxidizing peroxidase PXP 3-4 from poplar xylem: cDNA isolation, characterization and expression. *Plant Mol Biol* **47:**581–593.

Dale, P.J., Clarke, B., and Fontes, E.M.G. 2002. Potential for the environmental impact of transgenic crops. *Nature Biotechnol* **20:**567–574.

Daniell, H. 2002. Molecular strategies for gene containment in transgenic crops. *Nature Biotechnol* **20:**581–586.

Daubresse, N., Rolando, C., Chupeau, Y., Pollet, B., and Lapierre, C. 1995. New insights into the biosynthesis of lignin precursors. In *Proceedings of the 8th International Symposium on Wood and Pulping Chemistry*, Vol. 2. pp. 29–34. ISWPC, Ed., Grafia Kirjapaino, Jyräskylä.

Davin, L.B., Wang, H.-B., Crowell, A.L., Bedgar, D.L., Martin, D.M., Sarkanen, S., and Lewis, N.G. 1997. Stereoselective bimolecular phenoxy radical

coupling by an auxiliary (dirigent) protein without an active center. *Science* **275:**362–366.

Dean, J.F.D. and Eriksson, K.-E. 1994. Laccase and the deposition of lignin in vascular plants. *Holzforschung* **48:**21–33.

Dean, J.F.D. 1997. Lignin analysis. In *Methods in Plant Biochemistry and Molecular Biology*. pp. 199–215. W.V. Dashek, Ed., CRC Press, Boca Raton, FL.

Dean, J.F.D., LaFayette, P.R., Rugh, C., Tristram, A.H., Hoopes, J.T., Eriksson, K.-E.L., and Merckle, S.A. 1998. Laccases associate with lignifying vascular tissues. In *Lignin and Lignan Biosynthesis* (ACS Symposium Series, Vol. 697). pp. 96–108. N.G. Lewis, and S. Sarkanen, Eds., American Chemical Society, Washington, DC.

Delmer, D.P. and Amor, Y. 1995. Cellulose biosynthesis. *Plant Cell* **7:**987–1000.

Dixon, R.A., Chen, F., Guo, D., and Parvathi, K. 2001. The biosynthesis of monolignols: a "metabolic grid," or independent pathways to guaiacyl and syringyl units? *Phytochemistry* **57:**1069–1084.

Douglas, C. 1996. Phenylpropanoid metabolism and lignin biosynthesis: from weeds to trees. *Trends Plant Sci* **1:**171–178.

Dwivedi, U.N., Campbell, W.H, Yu, J., Datla, R.S.S., Bugos, R.C., Chiang, V.L., and Podila, G.K. 1994. Modification of lignin biosynthesis in transgenic *Nicotiana* through expression of an antisense *O*-methyltransferase gene from *Populus*. *Plant Mol Biol* **26:**61–71.

Ebinuma, H., Sugita, K., Matsunaga, E., and Yamakado, M. 1997. Selection of marker-free transgenic plants using the isopentenyl transferase gene. *Proc Natl Acad Sci USA* **94:**2117–2121.

Effland, M.J. 1977. Modified procedure to determine acid-insoluble lignin in wood and pulp. *TAPPI* **60:**143–144.

Ehlting, J., Büttner, D., Wang, Q., Douglas, C.J., Somssich, I.E., and Kombrink, E. 1999. Three 4-coumarate:coenzyme A ligases in *Arabidopsis thaliana* represent two evolutionarily divergent classes in angiosperms. *Plant J* **19:**9–20.

Elfstrand, M., Sitbon, F., Lapierre, C., Bottin, A., and von Arnold, S. 2002. Altered lignin structure and resistance to pathogens in *spi 2*-expressing tobacco plants. *Planta* **214:**708–716.

Elkind, Y., Edwards, R., Mavandad, M., Hedrick, S.A., Ribak, O., Dixon, R.A., and Lamb, C.J. 1990. Abnormal plant development and down-regulation of phenylpropanoid biosynthesis in transgenic tobacco containing a heterologous phenylalanine ammonia-lyase gene. *Proc Natl Acad Sci USA* **87:**9057–9061.

El Mansouri, I., Mercado, J.A., Santiago-Domenech, N., Pliego-Alfaro, F., Valpuesta, V., and Quesada, M.A. 1999. Biochemical and phenotypical characterization of transgenic tomato plants overexpressing a basic peroxidase. *Physiol Plant* **106:**355–362.

Erikson, O., Clapham, D., Hertzberg, M., and Näsholm, T. 2003. D-amino acid selectable marker genes. Abstract presented at the Tree Biotechnology Meeting, June 7–12, Umeå, Sweden.

Fengel, D. and Wegener, G. 1984. Lignin. In *Wood: Chemistry, Ultrastructure, Reactions*. pp. 132–181. D. Fengel and G. Wegener, Eds., De Gruyter, Berlin.

Fenning, T.M. and Gershenzon, J. 2002. Where will the wood come from? Plantation forests and the role of biotechnology. *Trends Biotechnol* **20:**291–296

Food and Agriculture Organization of the United Nations. 2001a. *Pulp and Paper Capacities—Survey 2000–2005*. FAO, Rome.

Food and Agriculture Organization of the United Nations. 2001b. *State of the World's Forests* 2001 [www.fao.org/forestry/FO/SOFO/SOFO2001/publi-e.stm].

Fergus, B.J., Procter, A.R., Scott, J.A.N., and Goring, D.A.I. 1969. The distribution of lignin in sprucewood as determined by ultraviolet microscopy. *Wood Sci Technol* **3:**117–138.

Franke, R., McMichael, C.M., Meyer, K., Shirley, A.M., Cusumano, J.C., and Chapple, C. 2000. Modified lignin in tobacco and poplar plants overexpressing the *Arabidopsis* gene encoding ferulate 5-hydroxylase. *Plant J* **22:**223–234.

Franke, R., Humphreys, J.M., Hemm, M.R., Denault, J.W., Ruegger, M.O., Cusumano, J.C., and Chapple, C. 2002a. The Arabidopsis *REF8* gene encodes the 3-hydroxylase of phenylpropanoid metabolism. *Plant J* **30:**33–45.

Franke, R., Hemm, M.R., Denault, J.W., Ruegger, M.O., Humphreys, J.M., and Chapple, C. 2002b. Changes in secondary metabolism and deposition of an unusual lignin in the *ref8* mutant of Arabidopsis. *Plant J* **30:**47–59.

Gang, D.R., Costa, M.A., Fujita, M., Dinkova-Kostova, A.T., Wang, H.-B., Burlat, V., Martin, W., Sarkanen, S., Davin, L.B., and Lewis, N.G. 1999. Regiochemical control of monolignol radical coupling: a new paradigm for lignin and lignan biosynthesis. *Chem Biol* **6:**143–151.

Gierer, J. 1985. Chemistry of delignification. Part 1: General concept and reaction during pulping. *Wood Sci Technol* **19:**289–312.

Goujon, T., Sibout, R., Pollet, B., Maba, B., Nussaume, L., Bechtold, N., Lu, F., Ralph, J., Mila, I., Barrière, Y., Lapierre, C., and Jouanin, L. 2003a. A new *Arabidopsis thaliana* mutant deficient in the expression of *O*-methyltransferase impacts lignins and sinapoyl esters. *Plant Mol Biol* **51:**973–989.

Goujon, T., Ferret, V., Mila, I., Pollet, B., Ruel, K., Burlat, V., Joseleau, J.-P., Barrière, Y., Lapierre, C., and Jouanin, L. 2003b. Down-regulation of the *AtCCR1* gene in *Arabidopsis thaliana*: effects on phenotype, lignins and cell wall degradability. *Planta* **217:**218–228.

Grand, C., Boudet, A., and Boudet, A.M. 1983. Isoenzymes of hydroxycinnamate:CoA ligase from poplar stems: properties and tissue distribution. *Planta* **158:**225–229

Grand, C., Parmentier, P., Boudet, A., and Boudet, A.M. 1985. Comparison of lignins and of enzymes involved in lignification in normal and brown midrib (bm_3) mutant corn seedlings. *Physiol Vég* **23:**905–911.

Grima-Pettenati, J. and Goffner, D. 1999. Lignin genetic engineering revisited. *Plant Sci* **145:**51–65.

Guo, D., Chen, F., Inoue, K., Blount, J.W., and Dixon, R.A. 2001. Downregulation of caffeic acid 3-*O*-methyltransferase and caffeoyl CoA 3-*O*-methyltransferase in transgenic alfalfa: impacts on lignin structure and implications for the biosynthesis of G and S lignin. *Plant Cell* **13:**73–88.

Guo, D., Chen, F., and Dixon, R.A. 2002. Monolignol biosynthesis in microsomal preparations from lignifying stems of alfalfa (*Medicago sativa* L.). *Phytochemistry* **61:**657–667.

Halpin, C. and Boerjan, W. 2003. Stacking transgenes in forest trees. *Trends Plant Sci* **8:**363–365.

Halpin, C., Knight, M.E., Foxon, G.A., Campbell, M.M., Boudet, A.M., Boon, J.J., Chabbert, B., Tollier, M.-T., and Schuch, W. 1994. Manipulation of lignin quality by downregulation of cinnamyl alcohol dehydrogenase. *Plant J* **6:**339–350.

Halpin, C., Holt, K., Chojecki, J., Oliver, D., Chabbert, B., Monties, B., Edwards, K., Barakate, A., and Foxon, G.A. 1998. *Brown-midrib* maize (*bm1*)—a mutation affecting the cinnamyl alcohol dehydrogenase gene. *Plant J* **14:**545–553.

Hancock, J. and Hokanson, K. 2001. Invasiveness of transgenic vs. exotic plant species: how useful is the analogy? In *Proceedings of the First International Symposium on Ecological and Societal Aspects of Transgenic Plantations*. pp. 187–192. S.H. Strauss and H.D. Bradshaw, Eds., Oregon State University, Corvallis, [www.fsl.orst.edu/tgerc/iufro2001/eprocd.pdf].

Harding, S.A., Leshkevich, J., Chiang, V.L., and Tsai, C.-J. 2002. Differential substrate inhibition couples kinetically distinct 4-coumarate: coenzyme A ligases with spatially distinct metabolic roles in quaking aspen. *Plant Physiol* **128:**428–438.

Hare, P.D. and Chua, N.-H. 2002. Excision of selectable marker genes from transgenic plants. *Nature Biotechnol* **20:**575–580.

Hatfield, R. and Vermerris, W. 2001. Lignin formation in plants. The dilemma of linkage specificity. *Plant Physiol* **126:**1351–1357.

Hertzberg, M., Aspeborg, H., Schrader, J., Andersson, A., Erlandsson, R., Blomqvist, K., Bhalerao, R., Uhlén, M., Teeri, T.T., Lundeberg, J., Sundberg, B., Nilsson, P., and Sandberg, G. 2001. A transcriptional roadmap to wood formation. *Proc Natl Acad Sci USA* **98:**14732–14737.

Hibino, T., Takabe, K., Kawazu, T., Shibata, D., and Higuchi, T. 1995. Increase of cinnamaldehyde groups in lignin of transgenic tobacco plants carrying an antisense gene for cinnamyl alcohol dehydrogenase. *Biosci Biotechnol Biochem* **59:**929–931.

Highley, T.L. and Dashek, W.V. 1998. Biotechnology in the study of brown- and white-rot decay. In *Forest Products Biotechnology*. pp. 15–36. A.M. Bruce, and J.W. Palfreyman, Eds., Taylor & Francis, London.

Higuchi, T. 1985. Biosynthesis of lignin. In *Biosynthesis and Biodegradation of Wood Components*. pp. 141–160. T. Higuchi, Ed., Academic Press, Orlando.

Higuchi, T. 1990. Lignin biochemistry: biosynthesis and biodegradation. *Wood Sci Technol* **24:**23–63.

Higuchi, T. 1997. *Biochemistry and Molecular Biology of Wood* (Springer Series in Wood Science). Springer, Berlin.

Higuchi, T., Ito, T., Umezawa, T., Hibino, T., and Shibata, D. 1994. Red-brown color of lignified tissues of transgenic plants with antisense CAD gene: wine-red lignin from coniferyl aldehyde. *J Biotechnol* **37:**151–158.

Holder, D.A. and Murray, W.A. 1970. *Bibliography of the Mechanical Pulping of Hardwood and Resinous Species*. Canadian Pulp and Paper Association: Technical Section, Montreal.

Hoffmann, L., Maury, S., Martz, F., Geoffroy, P., and Legrand, M. 2003. Purification, cloning and properties of an acyltransferase controlling shikimate and quinate ester intermediates in phenylpropanoid metabolism. *J Biol Chem* **278:**95–103.

Howles, P.A., Sewalt, V.J.H., Paiva, N.L., Elkind, Y., Bate, N.J., Lamb, C., and Dixon, R.A. 1996. Overexpression of L-phenylalanine ammonia-lyase in transgenic tobacco plants reveals control points for flux into phenylpropanoid biosynthesis. *Plant Physiol* **112:**1617–1624.

Hu, W.-J., Kawaoka, A., Tsai, C.-J., Lung, J., Osakabe, K., Ebinuma, H., and Chiang, V.L. 1998. Compartmentalized expression of two structurally and functionally distinct 4-coumarate: CoA ligase genes in aspen (*Populus tremuloides*). *Proc Natl Acad Sci USA* **95:**5407–5412.

Hu, W.-J., Harding, S.A., Lung, J., Popko, J.L., Ralph, J., Stokke, D.D., Tsai, C.-J., and Chiang, V.L. 1999. Repression of lignin biosynthesis promotes cellulose accumulation and growth in transgenic trees. *Nature Biotechnol* **17:**808–812.

Humphreys, J.M. and Chapple, C. 2002. Rewriting the lignin roadmap. *Curr Opin Plant Biol* **5:**224–229.

Humphreys, J.M., Hemm, M.R., and Chapple, C. 1999. New routes for lignin biosynthesis defined by biochemical characterization of recombinant ferulate 5-hydroxylase, a multifunctional cytochrome P450-dependent monooxygenase. *Proc Natl Acad Sci USA* **96:**10045–10050.

Huntley, S.K., Ellis, D., Gilbert, M., Chapple, C., and Mansfield, S.D. 2003. Significant increases in pulping efficiency in C4H-F5H transformed poplar. Abstract presented at the Tree Biotechnology Symposium, Umeå (Sweden), June 7–12, 2003 (S10.5).

Hwang, B.H. and Sakakihara, A. 1981. Hydrogenolysis of protolignin. XVIII. Isolation of a new dimeric compound with a heterocycle involving α-,β-diether. *Holzforschung* **35:**297–300.

Iiyama, K. and Wallis, A.F.A. 1988. An improved acetyl bromide procedure for determining lignin in woods and wood pulps. *Wood Sci Technol* **22:**271–280.

Inoue, K., Sewalt, V.J.H., Ballance, G.M., Ni, W., Stürzer, C., and Dixon, R.A. 1998. Developmental expression and substrate specificities of alfalfa caffeic acid 3-*O*-methyltransferase and caffeoyl coenzyme A 3-*O*-methyltransferase in relation to lignification. *Plant Physiol* **117:**761–770.

Jones, L., Ennos, A.R., and Turner, S.R. 2001. Cloning and characterization of *irregular xylem4* (*irx4*): a severely lignin-deficient mutant of *Arabidopsis*. *Plant J* **26:**205–216.

Joseleau, J.-P. and Ruel, K. 1997. Study of lignification by noninvasive techniques in growing maize internodes. An investigation by Fourier transform infrared cross-polarization-magic angle spinning ^{13}C-nuclear magnetic resonance spectroscopy and immunocytochemical transmission electron microscopy. *Plant Physiol* **114:**1123–1133.

Jouanin, L., Goujon, T., de Nadaï, V., Martin, M.-T., Mila, I., Vallet, C., Pollet, B., Yoshinaga, A., Chabbert, B., Petit-Conil, M., and Lapierre, C. 2000. Lignification in transgenic poplars with extremely reduced caffeic acid *O*-methyltransferase activity. *Plant Physiol* **123:**1363–1373.

Kajita, S., Katayama, Y., and Omori, S. 1996. Alterations in the biosynthesis of lignin in transgenic plants with chimeric genes for 4-coumarate: coenzyme A ligase. *Plant Cell Physiol* **37:**957–965.

Kajita, S., Hishiyama, S., Tomimura, Y., Katayama, Y., and Omori, S. 1997. Structural characterization of modified lignin in transgenic tobacco plants in which the activity of 4-coumarate: coenzyme A ligase is depressed. *Plant Physiol* **114:**871–879.

Kim, H., Ralph, J., Yahiaoui, N., Pean, M., and Boudet, A.-M. 2000. Cross-coupling of hydroxycinnamyl aldehydes into lignins. *Org Lett* **2:**2197–2200.

Kim, H., Ralph, J., Lu, F., Pilate, G., Leplé, J.-C., Pollet, B., and Lapierre, C. 2002. Identification of the structure and origin of thioacidolysis marker compounds for cinnamyl alcohol dehydrogenase deficiency in angiosperms. *J Biol Chem* **277:**47412–47419.

Kirk, T.K. and Akhtar, M. 2000. Biopulping is nearing commercialization. In *International Symposium on Environmentally Friendly and Emerging Technologies for a Sustainable Pulp and Paper Industry* (TFRI Extension Series No. 115). pp. 294–299. Y.-C. Su and E.I.C. Wang, Eds., Taiwan Forestry Research Institute, Taipei.

Korth, K. L., Blount, J.W., Chen, F., Rasmussen, S., Lamb, C., and Dixon, R.A. 2001. Changes in phenylpropanoid metabolites associated with homology-dependent silencing of phenylalanine ammonia-lyase and its somatic reversion in tobacco. *Physiol Plant* **111:**137–143.

Kuč, J., Nelson, O.E., and Flanagan, P. 1968. Degradation of abnormal lignins in the brown-midrib mutants and double mutants of maize. *Phytochemistry* **7:**1435–1436.

Lacombe, E., Hawkins, S., Van Doorsselaere, J., Piquemal, J., Goffner, D., Poeydomenge, O., Boudet, A.-M., and Grima-Pettenati, J. 1997. Cinnamoyl CoA reductase, the first committed enzyme

of the lignin branch biosynthetic pathway: cloning, expression and phylogenetic relationships. *Plant J* **11:**429–441.

Lagrimini, L.M. 1991. Wound-induced deposition of polyphenols in transgenic plants overexpressing peroxidase. *Plant Physiol* **96:**577–583.

Lagrimini, L.M., Burkhart, W., Moyer, M., and Rothstein, S. 1987. Molecular cloning of complementary DNA encoding the lignin-forming peroxidase from tobacco: molecular analysis and tissue-specific expression. *Proc Natl Acad Sci USA* **84:**7542–7546.

Lagrimini, L.M., Bradford, S., and Rothstein, S. 1990. Peroxidase-induced wilting in transgenic tobacco plants. *Plant Cell* **2:**7–18.

Lagrimini, L.M., Vaughn, J., Erb, W.A., and Miller, S.A. 1993. Peroxidase overproduction in tomato: wound-induced polyphenol deposition and disease resistance. *HortScience* **28:**218–221.

Lagrimini, L.M., Gingas, V., Finger, F., Rothstein, S., and Liu, T.-T.Y. 1997a. Characterization of antisense transformed plants deficient in the tobacco anionic peroxidase. *Plant Physiol* **114:**1187–1196.

Lagrimini, L.M., Joly, R.J., Dunlap, J.R., and Liu, T.-T.Y. 1997b. The consequence of peroxidase overexpression in transgenic plants on root growth and development. *Plant Mol Biol* **33:**887–895.

Lapierre, C. 1993. Application of new methods for the investigation of lignin structure. In *Forage Cell Wall Structure and Digestibility*. pp. 133–166. H.G. Jung, D.R. Buxton, R.D. Hatfield, and J. Ralph, Eds., American Society of Agronomy, Madison, WI.

Lapierre, C., Tollier, M.-T., and Monties, B. 1988. Mise en évidence d'un nouveau type d'unité constitutive dans les lignines d'un mutant de Maïs bm3. *C R Acad Sci Paris, Série III* **307:**723–728.

Lapierre, C., Pollet, B., Petit-Conil, M., Toval, G., Romero, J., Pilate, G., Leplé, J.-C., Boerjan, W., Ferret, V., De Nadai, V., and Jouanin, L. 1999. Structural alterations of lignins in transgenic poplars with depressed cinnamyl alcohol dehydrogenase or caffeic acid *O*-methyltransferase activity have opposite impact on the efficiency of industrial Kraft pulping. *Plant Physiol* **119:**153–163.

Lapierre, C., Pollet, B., Petit-Conil, M., Pilate, G., Leplé, C., Boerjan, W., and Jouanin, L. 2000. Genetic engineering of poplar lignins: impact of lignin alterations on Kraft pulping performances. In *Lignin: Historical, Biological, and Materials Perspectives*, (ACS Symposium Series, Vol. 742). pp. 145–160. W.G. Glasser, R.A. Northey, and T.P. Schultz, Eds., American Chemical Society, Washington, DC.

Leask, R.A., Kocurek, M.J., and Stevens, F. 1987. *Pulp and Paper Manufacture, Vol. 2: Mechanical Pulping*, 3rd ed. Tappi and Canadian Pulp and Paper Association Joint Textbook Committee of the Paper Industry, Montreal.

Lee, D., Meyer, K., Chapple, C., and Douglas, C.J. 1997. Antisense suppression of 4-coumarate: coenzyme A ligase activity in Arabidopsis leads to altered lignin subunit composition. *Plant Cell* **9:**1985–1998.

Lewis, N.G. 1999. A 20th century roller coaster ride: a short account of lignification. *Curr Opin Plant Biol* **2:**153–162.

Li, L., Popko, J.L., Zhang, X.-H., Osakabe, K., Tsai, C.-J., Joshi, C.P., and Chiang, V.L. 1997. A novel multifunctional *O*-methyltransferase implicated in a dual methylation pathway associated with lignin biosynthesis in loblolly pine. *Proc Natl Acad Sci USA* **94:**5461–5466.

Li, L., Popko, J.L., Umezawa, T., and Chiang, V.L. 2000. 5-Hydroxyconiferyl aldehyde modulates enzymatic methylation for syringyl monolignol formation, a new view of monolignol biosynthesis in angiosperms. *J Biol Chem* **275:**6537–6545.

Li, L., Cheng, X.F., Leshkevich, J., Umezawa, T., Harding, S.A., and Chiang, V.L. 2001. The last step of syringyl monolignol biosynthesis in angiosperms is regulated by a novel gene encoding sinapyl alcohol dehydrogenase. *Plant Cell* **13:**1567–1585.

Li, L., Zhou, Y., Cheng, X., Sun, J., Marita, J.M., Ralph, J., and Chiang, V.L. 2003. Combinatorial modification of multiple lignin traits in trees through multigene cotransformation. *Proc Natl Acad Sci USA* **100:**4939–4944.

Lindermayr, C., Möllers, B., Fliegmann, J., Uhlmann, A., Lottspeich, F., Meimberg, H., and Ebel, J. 2002a. Divergent members of a soybean (*Glycine max* L.) 4-coumarate:coenzyme A ligase gene family. Primary structures, catalytic properties, and differential expression. *Eur J Biochem* **269:**1304–1315.

Lindermayr, C., Fliegmann, J., and Ebel, J. 2002b. Deletion of a single amino acid residue from different 4-coumarate-CoA ligases from soybean results in the generation of new substrate specificities. *J Biol Chem* **278:**2781–2786.

Lu, F. and Ralph, J. 1997. Derivatization followed by reductive cleavage (DFRC method), a new method for lignin analysis: protocol for analysis of DFRC monomers. *J Agric Food Chem* **45:**2590–2592.

Luce, B. 2002. http://www.washington.edu/alumni/columns/dec02/groundup01.html.

MacKay, J.J., O'Malley, D.M., Presnell, T., Booker, F.L., Campbell, M.M., Whetten, R.W., and Sederoff, R.R. 1997. Inheritance, gene expression, and lignin characterization in a mutant pine deficient in cinnamyl alcohol dehydrogenase. *Proc Natl Acad Sci USA* **94:**8255–8260.

MacKay, J., Presnell, T., Jameel, H., Taneda, H., O'Malley, D., and Sederoff, R. 1999. Modified lignin and delignification with a CAD-deficient loblolly pine. *Holzforschung* **53:**403–410.

Mäder, M. and Füssl, R. 1982. Role of peroxidase in lignification of tobacco cells. II. Regulation by phenolic compounds. *Plant Physiol* **70:**1132–1134.

Maher, E.A., Bate, N.J., Ni, W., Elkind, Y., Dixon, R.A., and Lamb, C.J. 1994. Increased disease susceptibility of transgenic tobacco plants with suppressed levels of preformed phenylpropanoid products. *Proc Natl Acad Sci USA* **91:**7802–7806.

Marita, J.M., Ralph, J., Hatfield, R.D., and Chapple, C. 1999. NMR characterization of lignins in *Arabidopsis* altered in the activity of ferulate 5-hydroxylase. *Proc Natl Acad Sci USA* **96:**12328–12332.

Marita, J.M., Ralph, J., Hatfield, R.D., Guo, D., Chen, F., and Dixon, R.A. 2003. Structural and compositional modifications in lignin of transgenic alfalfa down-regulated in caffeic acid 3-*O*-methyltransferase and caffeoyl coenzyme A 3-*O*-methyltransferase. *Phytochemistry* **62:**53–65.

Martz, F., Maury, S., Pinçon, G., and Legrand, M. 1998. cDNA cloning, substrate specificity and expression study of tobacco caffeoyl-CoA 3-*O*-methyltransferase, a lignin biosynthetic enzyme. *Plant Mol Biol* **36:**427–437.

Matsui, N., Fukushima, K., Yasuda, S., and Terashima, N. 1994. On the behavior of monolignol glucosides in lignin biosynthesis. II. Synthesis of monolignol glucosides labeled with ^{3}H at the hydroxymethyl group of side chain, and incorporation of the label into magnolia and ginkgo lignin. *Holzforschung* **48:**375–380.

Matsui, N., Chen, F., Yasuda, S., and Fukushima, K. 2000. Conversion of guaiacyl to syringyl moieties on the cinnamyl alcohol pathway during the biosynthesis of lignin in angiosperms. *Planta* **210:**831–835.

Maury, S., Geoffroy, P., and Legrand, M. 1999. Tobacco *O*-methyltransferases involved in phenylpropanoid metabolism. The different caffeoyl-coenzyme A/5-hydroxyferuloyl-coenzyme A 3/5-*O*-methyltransferase and caffeic acid/5-hydroxyferulic acid 3/5-*O*-methyltransferase classes have distinct substrate specificities and expression patterns. *Plant Physiol* **121:**215–223.

McDougall, G.J., Stewart, D., and Morrison, I.M. 1994. Cell-wall-bound oxidases from tobacco (*Nicotiana tabacum*) xylem participate in lignin formation. *Planta* **194:**9–14.

Mellerowicz, E.J., Baucher, M., Sundberg, B., and Boerjan, W. 2001. Unravelling cell wall formation in the woody dicot stem. *Plant Mol Biol* **47:**239–247.

Meng, H. and Campbell, W.H. 1998. Substrate profiles and expression of caffeoyl coenzyme A and caffeic acid *O*-methyltransferases in secondary xylem of aspen during seasonal development. *Plant Mol Biol* **38:**513–520.

Messner, K. and Srebotnik, E. 1994. Biopulping: an overview of developments in an environmentally safe paper-making technology. *FEMS Microbiol Rev* **13:**351–364.

Meyer, K., Shirley, A.M., Cusumano, J.C., Bell-Lelong, D.A., and Chapple, C. 1998. Lignin monomer composition is determined by the expression of a cytochrome P450-dependent monooxygenase in *Arabidopsis*. *Proc Natl Acad Sci USA* **95:**6619–6623.

Meyermans, H., Morreel, K., Lapierre, C., Pollet, B., De Bruyn, A., Busson, R., Herdewijn, P., Devreese, B., Van Beeumen, J., Marita, J.M., Ralph, J., Chen, C., Burggraeve, B., Van Montagu, M., Messens, E., and Boerjan, W. 2000. Modification in lignin and accumulation of phenolic glucosides in poplar xylem upon down-regulation of caffeoyl-coenzyme A *O*-methyltransferase, an enzyme involved in lignin biosynthesis. *J Biol Chem* **275:**36899–36909.

Monties, B. 1989. Lignins. In *Methods in Plant Biochemistry: Plant Phenolics*, Vol. 1, pp. 113–157. P.M. Dey and J.B. Harborne, Eds., Academic Press, New York.

Monties, B. 1998. Novel structures and properties of lignins in relation to their natural and induced variability in ecotypes, mutants and transgenic plants. *Polymer Degrad Stabil* **59:**53–64.

Nair, R.B., Xia, Q., Kartha, C.J., Kurylo, E., Hirji, R.N., Datla, R., and Selvaraj, G. 2002. Arabidopsis CYP98A3 mediating aromatic 3-hydroxylation. Developmental regulation of the gene, and expression in yeast. *Plant Physiol* **130:**210–220.

Ni, W., Paiva, N.L., and Dixon, R.A. 1994. Reduced lignin in transgenic plants containing a caffeic acid *O*-methyltransferase antisense gene. *Transgenic Res* **3:**120–126.

O'Connell, A., Holt, K., Piquemal, J., Grima-Pettenati, J., Boudet, A., Pollet, B., Lapierre, C., Petit-Conil,

M., Schuch, W., and Halpin, C. 2002. Improved paper pulp from plants with suppressed cinnamoyl-CoA reductase or cinnamyl alcohol dehydrogenase. *Transgenic Res* **11:**495–503.

Odendahl, S. 1994. Environmental protection and consumer demands: a review of trends and impacts. *Pulp Pap-Can* **95:**144–148.

Ona, T., Sonoda, T., Ito, K., Shibata, M., Tamai, Y., and Kojimo, Y. 1996. Use of the radially divided increment core method to assess pulpwood quality for eucalypt breeding in *E. camaldulensis* and *E. globulus*. *Appita J* **49:**325–331.

Önnerud, H., Zhang, L., Gellerstedt, G., and Henriksson, G. 2002. Polymerization of monolignols by redox shuttle-mediated enzymatic oxidation: a new model in lignin biosynthesis I. *Plant Cell* **14:**1953–1962.

Onysko, K.A. 1993. Biological bleaching of chemical pulps: a review. *Biotechnol Adv* **11:**179–198.

Osakabe, K., Tsao, C.C., Li, L., Popko, J.L., Umezawa, T., Carraway, D.T., Smeltzer, R.H., Joshi, C.P., and Chiang, V.L. 1999. Coniferyl aldehyde 5-hydroxylation and methylation direct syringyl lignin biosynthesis in angiosperms. *Proc Natl Acad Sci USA* **96:**8955–8960.

Paice, M.G., Bourbonnais, R., Reid, I.D., Archibald, F.S., and Jurasek, L. 1995. Oxidative bleaching enzymes—a review. *J Pulp Pap Sci* **21:**J280–J284.

Pakusch, A.-E., Matern, U., and Schiltz, E. 1991. Elicitor-inducible caffeoyl-coenzyme A 3-*O*-methyltransferase from *Petroselinum crispum* cell suspensions. Purification, partial sequence, and antigenicity. *Plant Physiol* **95:**137–143.

Parham, R.A. 1987. Structure, chemistry and physical properties of woody raw materials. In *Pulp and Paper Manufacture, Vol. 1: Properties of Fibrous Raw Materials and Their Preparation for Pulping*, 3rd ed. M.J. Kocurek, and F. Stevens, Eds., Tappi and Canadian Pulp and Paper Association Joint Textbook Committee of the Paper Industry, Montreal.

Parvathi, K., Chen, F., Guo, D., Blount, J.W., and Dixon, R.A. 2001. Substrate preferences of *O*-methyltransferases in alfalfa suggest new pathways for 3-*O*-methylation of monolignols. *Plant J* **25:**193–202.

Peña, L. and Séguin, A. 2001. Recent advances in the genetic transformation of trees. *Trends Biotechnol* **19:**500–506.

Petit-Conil, M. 1995. Principes de préparation de pâtes chimicothermomécaniques de bois résineux et feuillus. Ph. D. thesis, Institut National Polytechnique, Grenoble, France.

Petit-Conil, M., Lapierre, C., Pollet, B., Mila, I., Meyermans, H., Pilate, G., Leplé J.-C., Jouanin, L., Boerjan, W., and de Choudens, C. 1999. Impact of lignin engineering on pulping processes of poplar trees. In *Proceedings of the 27th EUCEPA Conference*, pp. 1–10. Association Technique de l'Industrie Papetière, Ed., ATIP Press, Paris.

Petit-Conil, M., Chirat, C., Lapierre, C., Pollet, B., Mila, I., Jouanin, L., Guiney, E., Schuch, W., Boerjan, W., Leplé, J.C., and Pilate, G. 2000. Designing lignin structure for modifying pulping performances: application to poplar tree. In *Proceedings of the 2000 TAPPI Engineering Conference on Process and Product Quality*. PULP0036. Technical Association for Paper and Pulp Industry, Ed., TAPPI Press, Atlanta, GA [CD-ROM].

Pilate, G., Guiney, E., Holt, K., Petit-Conil, M., Lapierre, C., Leplé, J.-C., Pollet, B., Mila, I., Webster, E.A., Marstorp, H.G., Hopkins, D.W., Jouanin, L., Boerjan, W., Schuch, W., Cornu, D., and Halpin, C. 2002. Field and pulping performances of transgenic trees with altered lignification. *Nature Biotechnol* **20:**607–612.

Pillonel, C., Mulder, M.M., Boon, J.J., Forster, B., and Binder, A. 1991. Involvement of cinnamyl-alcohol dehydrogenase in the control of lignin formation in *Sorghum bicolor* L. Moench. *Planta* **185:**538–544.

Pinçon, G., Maury, S., Hoffmann, L., Geoffroy, P., Lapierre, C., Pollet, B., and Legrand, M. 2001a. Repression of *O*-methyltransferase genes in transgenic tobacco affects lignin synthesis and plant growth. *Phytochemistry* **57:**1167–1176.

Pinçon, G., Chabannes, M., Lapierre, C., Pollet, B., Ruel, K., Joseleau, J.-P., Boudet, A.M., and Legrand, M. 2001b. Simultaneous down-regulation of caffeic/5-hydroxy ferulic acid-*O*-methyltransferase I and cinnamoyl-coenzyme A reductase in the progeny from a cross between tobacco lines homozygous for each transgene. Consequences for plant development and lignin synthesis. *Plant Physiol* **126:**145–155.

Piquemal, J., Lapierre, C., Myton, K., O'Connell, A., Schuch, W., Grima-Pettenati, J., and Boudet, A.-M. 1998. Down-regulation in cinnamoyl-CoA reductase induces significant changes of lignin profiles in transgenic tobacco plants. *Plant J* **13:**71–83.

Raes, J., Rohde, A., Christensen, J.H., Van de Peer, Y., and Boerjan, W. 2003. Genome-wide characterization of the lignification toolbox in *Arabidopsis thaliana*. *Plant Physiol*: in press.

Ralph, J. 1996. An unusual lignin from kenaf. *J Nat Prod* **59:**341–342.

Ralph, J., MacKay, J.J., Hatfield, R.D., O'Malley, D.M., Whetten, R.W., and Sederoff, R.R. 1997. Abnormal lignin in a loblolly pine mutant. *Science* **277:**235–239.

Ralph, J., Hatfield, R.D., Piquemal, J., Yahiaoui, N., Pean, M., Lapierre, C., and Boudet, A.M. 1998. NMR characterization of altered lignins extracted from tobacco plants down-regulated for lignification enzymes cinnamyl-alcohol dehydrogenase and cinnamoyl-CoA reductase. *Proc Natl Acad Sci USA* **95:**12803–12808.

Ralph, J., Marita, J.M., Ralph, S.A., Hatfield, R.D., Lu, F., Ede, R.M., Peng, J., Quideau, S., Helm, R.F., Grabber, J.H., Kim, H., Jimenez-Monteon, G., Zhang, Y., Jung, H.-J.G., Landucci, L.L., MacKay, J.J., Sederoff, R.R., Chapple, C., and Boudet, A.M. 1999a. Solution-state NMR of lignins. In *Advances in Lignocellulosic Characterization*. pp. 55–108. D.S. Argyropoulos, Ed., TAPPI Press, Atlanta, GA.

Ralph, J., Kim, H., Peng, J., and Lu, F. 1999b. Arylpropane-1,3-diols in lignins from normal and CAD-deficient pines. *Org Lett* **1:**323–326.

Ralph, J., Lapierre, C., Marita, J.M., Kim, H., Lu, F., Hatfield, R.D., Ralph, S., Chapple, C., Franke, R., Hemm, M.R., Van Doorsselaere, J., Sederoff, R.R., O'Malley, D.M., Scott, J.T., Mackay, J.J., Yahiaoui, N., Boudet, A.-M., Pean, M., Pilate, G., Jouanin, L., and Boerjan, W. 2001a. Elucidation of new structures in lignins of CAD- and COMT-deficient plants by NMR. *Phytochemistry* **57:**993–1003.

Ralph, J., Lapierre, C., Lu, F., Marita, J.M., Pilate, G., Van Doorsselaere, J., Boerjan, W., and Jouanin, L. 2001b. NMR evidence for benzodioxane structures resulting from incorporation of 5-hydroxyconiferyl alcohol into lignins of *O*-methyltransferase-deficient plants. *J Agric Food Chem* **49:**86–91.

Ranocha, P., McDougall, G., Hawkins, S., Sterjiades, R., Borderies, G., Stewart, D., Cabanes-Macheteau, M., Boudet, A.-M., and Goffner, D. 1999. Biochemical characterization, molecular cloning and expression of laccases—a divergent gene family—in poplar. *Eur J Biochem* **259:**485–495.

Ranocha, P., Goffner, D., and Boudet, A.M. 2000. Plant laccases: are they involved in lignification. In *Cell and Molecular Biology of Wood Formation*. pp. 397–410. R. Savidge, J. Barnett, and R. Napier, Eds., BIOS Scientific Publications, Oxford.

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

Rasmussen, S., and Dixon, R.A. 1999. Transgene-mediated and elicitor-induced perturbation of metabolic channeling at the entry point into the phenylpropanoid pathway. *Plant Cell* **11:**1537–1551.

Reid, I.D. 1991. Biological pulping in paper manufacture. *Trends Biotechnol* **9:**262–265.

Reid, I.D. and Paice, M.G. 1994. Biological bleaching of kraft pulps by white-rot fungi and their enzymes. *FEMS Microbiol Rev* **13:**369–376.

Richardson, A., Stewart, D., and McDougall, G.J. 1997. Identification and partial characterization of a coniferyl alcohol oxidase from lignifying xylem of Sitka spruce (*Picea sitchensis*). *Planta* **203:**35–43.

Rottmann, W.H., Meilan, R., Sheppard, L.A., Brunner, A.M., Skinner, J.S., Ma, C., Cheng, S., Jouanin, L., Pilate, G., and Strauss, S.H. 2000. Diverse effects of overexpression of *LEAFY* and *PTLF*, a poplar (*Populus*) homolog of *LEAFY/FLORICAULA*, in transgenic poplar and *Arabidopsis*. *Plant J* **22:**235–245.

Savidge, R. and Udagama-Randeniya, P. 1992. Cell wall-bound coniferyl alcohol oxidase associated with lignification in conifers. *Phytochemistry* **31:**2959–2966.

Schneider, K., Hövel, K., Witzel, K., Hamberger, B., Schomburg, D., Kombrink, E., and Stuible, H.-P. 2003. The substrate specificity-determining amino acid code of 4-coumarate:CoA ligase. *Proc Natl Acad Sci USA* **100:**8601–8606.

Schoch, G., Goepfert, S., Morant, M., Hehn, A., Meyer, D., Ullmann, P., and Werck-Reichhart, D. 2001. CYP98A3 from *Arabidopsis thaliana* is a 3′-hydroxylase of phenolic esters, a missing link in the phenylpropanoid pathway. *J Biol Chem* **276:**36566–36574.

Sederoff, R.R., MacKay, J.J., Ralph, J., and Hatfield, R.D. 1999. Unexpected variation in lignin. *Curr Opin Plant Biol* **2:**145–152.

Sewalt, V.J.H., Ni, W., Blount, J.W., Jung, H.G., Masoud, S.A., Howles, P.A., Lamb, C., and Dixon, R.A. 1997. Reduced lignin content and altered lignin composition in transgenic tobacco down-regulated in expression of L-phenylalanine ammonia-lyase or cinnamate 4-hydroxylase. *Plant Physiol* **115:**41–50.

Sibout, R., Baucher, M., Van Doorsselaere, J., Gatineau, M., Pollet, B., Mila, I., Maba, B., Pilate, G.,

Lapierre, C., Boerjan, W., and Jouanin, L. 2002. Expression of a poplar ferulate-5-hydroxylase in *Arabidopsis thaliana*. *Plant Physiol Biochem* **40:**1087–1096.

Sibout, R., Eudes, A., Pollet, B., Goujon, T., Mila, I., Granier, F., Séguin, A., Lapierre, C., and Jouanin, L. 2003. Expression pattern of two paralogs encoding cinnamyl alcohol dehydrogenase in Arabidopsis. Isolation and characterization of the corresponding mutants. *Plant Physiol* **132:**848–860.

Sterjiades, R., Dean, J.F.D., Gamble, G., Himmelsbach, D.S., and Eriksson, K.-E.L. 1993. Extracellular laccases and peroxidases from sycamore maple (*Acer pseudoplatanus*) cell-suspension cultures. Reactions with monolignols and lignin model compounds. *Planta* **190:**75–87.

Stewart, D., Yahiaoui, N., McDougall, G.J., Myton, K., Marque, C., Boudet, A.M., and Haigh, J. 1997. Fourier-transform infrared and Raman spectroscopic evidence for the incorporation of cinnamaldehydes into the lignin of transgenic tobacco (*Nicotiana tabacum* L.) plants with reduced expression of cinnamyl alcohol dehydrogenase. *Planta* **201:**311–318.

Strauss, S. 2003. Genomics, genetic engineering, and domestication of crops. *Science* **300:**61–62.

Suzuki, S., Lam, T.B.T., and Iiyama, K. (1997). 5-Hydroxyguaiacyl nuclei as aromatic constituents of native lignin. *Phytochemistry* **46:**695–700.

Teutsch, H.G., Hasenfratz, M.P., Lesot, A., Stoltz, C., Garnier, J.-M., Jeltsch, J.-M., Durst, F., and Werck-Reichhart, D. 1993. Isolation and sequence of a cDNA encoding the Jerusalem artichoke cinnamate 4-hydroxylase, a major plant cytochrome P450 involved in the general phenylpropanoid pathway. *Proc Natl Acad Sci USA* **90:**4102–4106.

Trethewey, R.N., Krotzky, A.J., and Willmitzer, L. 1999. Metabolic profiling: a Rosetta Stone for genomics? *Curr Opin Plant Biol* **2:**83–85.

Tognolli, M., Penel, C., Greppin, H., and Simon, P. 2002. Analysis and expression of the class III peroxidase large gene family in *Arabidopsis thaliana*. *Gene* **288:**129–138.

Tsai, C.-J., Popko, J.L., Mielke, M.R., Hu, W.-J., Podila, G.K., and Chiang, V.L. 1998. Suppression of *O*-methyltransferase gene by homologous sense transgene in quaking aspen causes red-brown wood phenotypes. *Plant Physiol* **117:**101–112.

Vallette, P. and de Choudens, C. 1992. *Le bois, la pâte, le papier*, 3rd Ed. Centre Technique de l'Industrie des Papiers, Cartons et Celluloses, Grenoble, France.

Van Doorsselaere, J., Baucher, M., Chognot, E., Chabbert, B., Tollier, M.-T., Petit-Conil, M., Leplé, J.-C., Pilate, G., Cornu, D., Monties, B., Van Montagu, M., Inzé, D., Boerjan, W., and Jouanin, L. 1995. A novel lignin in poplar trees with a reduced caffeic acid/5-hydroxyferulic acid *O*-methyltransferase activity. *Plant J* **8:**855–864.

Vignols, F., Rigau, J., Torres, M.A., Capellades, M., and Puigdomènech, P. 1995. The *brown midrib3* (*bm3*) mutation in maize occurs in the gene encoding caffeic acid *O*-methyltransferase. *Plant Cell* **7:**407–416.

Viikari, L., Kantelinen, A., Sundquist, J., and Linko, M. 1994. Xylanases in bleaching: from an idea to the industry. *FEMS Microbiol Rev* **13:**335–350.

Viikari, L., Buchert, J., and Suurnäkki, A. 1998. Enzymes in pulp bleaching. In *Forest Products Biotechnology*. pp. 83–97. A.M. Bruce, and J.W. Palfreyman, Eds., Taylor & Francis, London.

Weigel, D. and Nilsson, O. 1995. A developmental switch sufficient for flower initiation in diverse plants. *Nature* **377:**495–500.

Weisshaar, B. and Jenkins, G.I. 1998. Phenylpropanoid biosynthesis and its regulation. *Curr Opin Plant Biol* **1:**251–257.

Whetten, R. and Sederoff, R. 1995. Lignin biosynthesis. *Plant Cell* **7:**1001–1013.

Whetten, R.W., MacKay, J.J., and Sederoff, R.R. 1998. Recent advances in understanding lignin biosynthesis. *Annu Rev Plant Physiol Plant Mol Biol* **49:**585–609.

Yahiaoui, N., Marque, C., Myton, K.E., Negrel, J., and Boudet, A.M. 1998. Impact of different levels of cinnamyl alcohol dehydrogenase down-regulation on lignins of transgenic tobacco plants. *Planta* **204:**8–15.

Yahong, L., Tsuji, Y., Nishikubo, N., Kajita, S., and Morohoshi, N. 2001. Analysis of transgenic poplar in which the expression of peroxidase gene is suppressed. In *Molecular Breeding of Woody Plants*, (Progress in Biotechnology Series, Vol. 18). pp. 195–204. N. Morohoshi, and A. Komamine, Eds., Elsevier Science, Amsterdam.

Yamauchi, K., Yasuda, S., Hamada, K., Tsutsumi, Y., and Fukushima, K. 2003. Multiform biosynthetic pathway of syringyl lignin in angiosperms. *Planta* **216:**496–501.

Ye, Z.-H. 1997. Association of caffeoyl coenzyme A 3-*O*-methyltransferase expression with lignifying tissues in several dicot plants. *Plant Physiol* **115:**1341–1350.

Ye, Z.-H. and Varner, J.E. 1995. Differential expression of two *O*-methyltransferases in lignin biosynthesis in *Zinnia elegans*. *Plant Physiol* **108:**459–467.

Ye, Z.-H., Kneusel, R.E., Matern, U., and Varner, J.E. 1994. An alternative methylation pathway in lignin biosynthesis in *Zinnia*. *Plant Cell* **6:**1427–1439.

Zhong, R., Morrison, W.H. III, Negrel, J., and Ye, Z.-H. 1998. Dual methylation pathways in lignin biosynthesis. *Plant Cell* **10:**2033–2046.

Zhong, R., Morrison III, W.H., Himmelsbach, D.S., Poole II, F.L., and Ye, Z.-H. 2000. Essential role of caffeoyl coenzyme A *O*-methyltransferase in lignin biosynthesis in woody poplar plants. *Plant Physiol* **124:**563–577.

Zubieta, C., Kota, P., Ferrer, J.-L., Dixon, R.A., and Noel, J.P. 2002. Structural basis for the modulation of lignin monomer methylation by caffeic acid/5-hydroxyferulic acid 3/5-*O*-methyltransferase. *Plant Cell* **14:**1265–1277.