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Re-designing Lignin for Industry and Agriculture

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Introduction

Research into the biology of lignification has received much attention in recent years, in part because of its huge economic importance. Lignin, as a major component of wood, is one of the most abundant and most renewable biopolymers on Earth. During pulp and paper manufacture, lignin has to be removed from cellulose, often using noxious chemicals, with associated financial and environmental costs. The polymer also has commercial relevance for agricultural crops, as it has a negative impact on digestibility and on the post-harvest deterioration of some vegetables, but may be beneficial for lodging resistance in crops such as maize. It is also extremely important for normal plant health, development, and disease resistance. Most of the genes on the lignin pathway have been cloned, and the function of individual genes has been investigated by reverse genetics. This work has also highlighted the possibilities of manipulating lignin, via genetic engineering, in order to adapt plants for specific agricultural or industrial processes. Although much of this research has been performed in model species, such as tobacco and *Arabidopsis*, results on lignin manipulation in more commercially relevant species, such as alfalfa and trees, is beginning to accumulate. Indeed, in some cases, field trials to underpin commercial exploitation have already taken place. This review presents the current status of research into the biosynthesis and manipulation of lignin, focusing particularly on the impact these have in controlling lignin quality for industrial applications such as wood pulping.

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Abbreviations: CAD, cinnamyl alcohol dehydrogenase; CCoAOMT, caffeoyl-CoA *O*-methyltransferase; CCR, cinnamoyl-CoA reductase; C3H, cinnamate 3-hydroxylase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; COMT, caffeate *O*-methyltransferase; F5H, ferulate 5-hydroxylase; G, guaiacyl; H, *p*-hydroxyphenyl; HCT, hydroxycinnamoyl-CoA:shikimate/quinate hydroxycinnamoyltransferase; PAL, phenylalanine ammonia lyase; S, syringyl.

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Lignin structure

The basic units of the lignin polymer are the three 'monolignols', *para*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol, which differ only in their degree of methoxylation. Lignin derived from these three different units is known as *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) lignin, respectively, although most lignins contain a combination of the units. Lignin content, and the monolignol composition of the polymer, vary between plant taxa, species, cell types, and even from one part of the cell wall to another. Lignin in gymnosperms, for example, is composed almost entirely of G units, with small amounts of *p*-hydroxyphenyl units. Lignin in most dicot angiosperms combines G and S monomers, but lignin of the monocot grasses can additionally contain a significant proportion of H units. Lignin is similarly structurally heterogeneous, with a variety of different chemical linkages, including non-condensed β–*O*–4 ether bonds and condensed carbon–carbon bonds (β–β, β–1, β–5, and 5–5) connecting monomers (*Figure 10.1*). Both the quantity of lignin, and the compositional and structural attributes of the polymer in different plant species/varieties, are relevant for agricultural and industrial applications. Conifer softwoods, for example, are harder to pulp using chemicals than angiosperm hardwoods. This is because softwoods are rich in G units, which have a free aromatic C_5 available to make carbon–carbon linkages that are resistant to chemical degradation. By comparison, woods rich in S units have relatively more non-condensed ether β–*O*–4-linkages in lignin that are amenable to chemical delignification methods such as Kraft pulping.

The lignin biosynthetic pathway

Only a brief description of the pathway, and recent revisions to it, will be given here, as comprehensive descriptions can be found in other recent reviews (e.g. Anterola and Lewis, 2002; Humphreys and Chapple, 2002; Baucher *et al.*, 2003; Boerjan *et al.*, 2003). Early reactions on the lignin pathway are common to other branches of phenylpropanoid metabolism, and are catalysed by phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), and 4-coumarate:CoA ligase (4CL) (*Figure 10.2*). Subsequent hydroxylation and methylation reactions add one or two methoxyl groups to the phenyl ring, and involve the enzymes cinnamate 3-hydroxylase (C3H), caffeoyl-CoA *O*-methyltransferase (CCoAOMT), ferulate 5-hydroxylase (F5H), and caffeate *O*-methyltransferase (COMT). Recent discoveries, principally the cloning of C3H and identification of C3H-defective mutants (Schoch *et al.*, 2001; Franke *et al.*, 2002a,b), have suggested that *p*-coumaroyl-shikimate and *p*-coumaroyl-quinate are the preferred substrates for this enzyme, and therefore important intermediates in lignin biosynthesis. From these data, the involvement in lignin biosynthesis of a novel hydroxycinnamoyl-CoA:shikimate/quinate hydroxycinnamoyltransferase (HCT), that could conjugate the shikimate and quinate groups onto *p*-coumarate, can be inferred. Indeed, a HCT gene has been cloned recently (Hoffmann *et al.*, 2003), opening up the possibility of verifying its proposed role in lignin biosynthesis using reverse genetics. The position of the second set of hydroxylation/methylation reactions on the pathway, whereby guaiacyl precursors can be converted into syringyl precursors via the action of F5H and COMT,

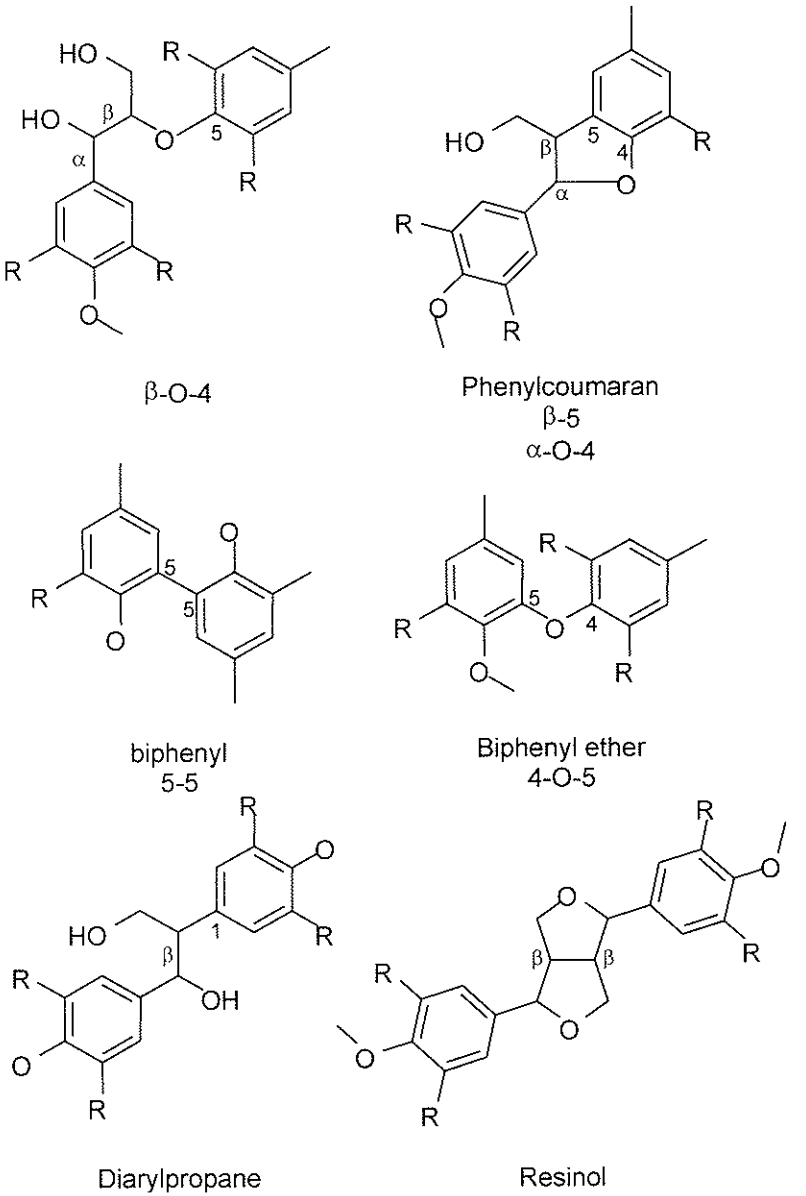

Figure 10.1. Common linkages within the lignin polymer.

has been revised recently. Traditionally, these reactions were thought to occur at the level of ferulic acid, converting it to sinapic acid, but it is now believed that these reactions really occur at the position of the aldehydes, and possibly the alcohols (Chen *et al.*, 1999; Humphreys *et al.*, 1999; Osakabe *et al.*, 1999; Li *et al.*, 2000). The final steps in monolignol production involve two successive reduction reactions catalysed by cinnamoyl-CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD). A novel gene encoding a sinapyl alcohol dehydrogenase (SAD) has

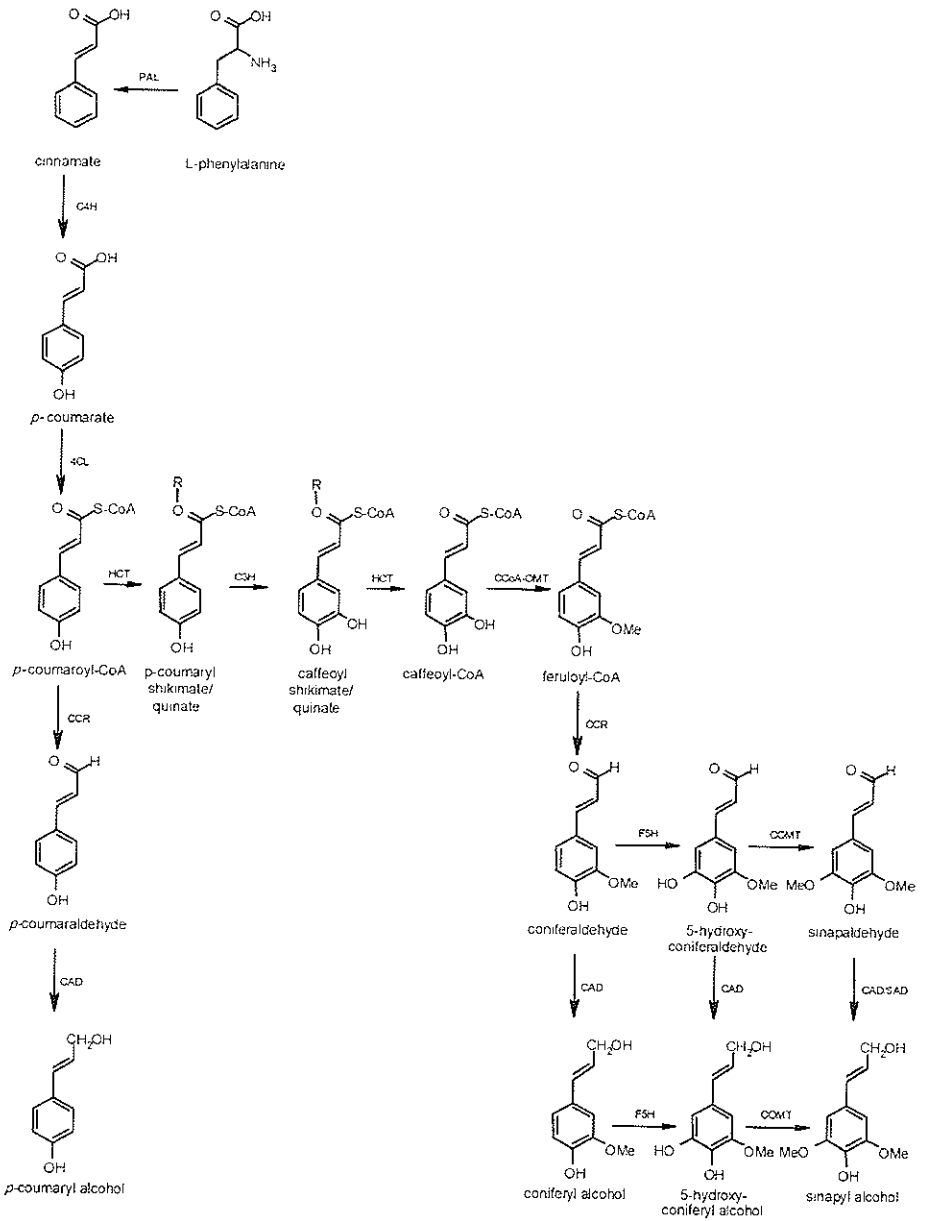

Figure 10.2. Current model of the major reactions on the lignin biosynthetic pathway. Other minor reactions are possible, but are not shown. COMT, caffeate *O*-methyltransferase; CCoAOMT, caffeoyl-CoA *O*-methyltransferase; C3H, cinnamate 3-hydroxylase; C4H, cinnamate 4-hydroxylase; CAD, cinnamyl alcohol dehydrogenase; CCR, cinnamoyl-CoA reductase; 4CL, 4-coumarate: CoA ligase; F5H, ferulate 5-hydroxylase; HCT, hydroxycinnamoyl-CoA:shikimate/quinate hydroxycinnamoyltransferase; PAL, phenylalanine ammonia lyase; SAD, sinapyl alcohol dehydrogenase.

been discovered recently in poplar (Li *et al.*, 2001), which preferentially converts sinapaldehyde into sinapyl alcohol. The discoverers propose a new pathway for lignin biosynthesis where CAD exclusively catalyses the conversion of coniferaldehyde to coniferyl alcohol, and SAD converts sinapaldehyde to sinapyl alcohol (Li *et al.*, 2001). However, as in the case of HCT, further work, particularly the production of *SAD* mutants or SAD-suppressed transgenics, is needed to confirm a role for this enzyme in lignin biosynthesis *in vivo*.

Most of the genes for enzymes involved in monolignol production have been cloned in a variety of species. It is thought that a common set of genes for woodiness exists in all seed plants, so that the whole genome sequencing of *Arabidopsis* can be used in comparative genomics experiments to illuminate common processes in other angiosperms, and even gymnosperms (Kirst *et al.*, 2003). Complete inventories of the genes involved in the monolignol biosynthetic pathway are already available for *Arabidopsis* (Costa *et al.*, 2003; Goujon *et al.*, 2003b; Raes *et al.*, 2003). This work indicates that, in *Arabidopsis*, most genes on the pathway are encoded by multigene families, although it is probable that only one or a few genes in each family have a real involvement in lignification. A couple of genes (C4H, HCT) are apparently unique in *Arabidopsis*, or have only one homologue (F5H) (Raes *et al.*, 2003).

Monolignol polymerization

The various different possible mechanisms whereby monolignols might be polymerized within the cell wall have been reviewed recently (Lewis, 1999; Boerjan *et al.*, 2003). A variety of cell wall oxygenases (peroxidases, laccases, and other phenoloxidases) have been proposed to be involved in converting the monolignols into phenoxy radicals (Savidge and Udagama-Randeniya, 1992; Dean and Eriksson, 1994; McDougall *et al.*, 1994; Richardson *et al.*, 1997). However, the exact identity of the oxygenases involved is still controversial, as is the mechanism of polymerization, be it a random, spontaneous phenomenon (Hatfield and Vermerris, 2001), or a highly ordered process (Gang *et al.*, 1999). In support of the ordered polymerization hypothesis, a protein ('dirigent' protein) capable of catalysing the stereoselective coupling of two coniferyl alcohol radicals into pinoresinol, has been isolated from *Forsythia* (Davin *et al.*, 1997). However, the lack of good evidence from transgenic or mutant plants supporting the roles of particular cell wall enzymes in lignin polymerization is still lacking, so this is likely to be an area of continuing heated debate.

Mutant or transgenic plants with altered lignin biosynthesis

The exact roles of different genes in lignin biosynthesis have been addressed by modifying their expression in transgenic plants using reverse genetic approaches to either suppress gene activity with antisense RNA/co-suppressing transgenes, or to over-express genes. To date, most of this work has been done in easy-to-transform species, such as tobacco and poplar. Mutants defective in particular lignin biosynthetic enzymes have also been identified in maize, sorghum, loblolly pine, and *Arabidopsis*. The significant body of work in these areas has been instrumental

in bringing us to our current understanding of lignin biosynthesis and how to manipulate it. In some cases, altered lignin plants have even proved superior for specific industrial or agricultural applications. That work is summarized below for each enzyme/gene.

PHENYLALANINE AMMONIA-LYASE (PAL)

Phenylalanine ammonia lyase catalyses the first step of the general phenylpropanoid pathway, a step that is common to the production of many metabolites, including flavonoids, coumarins and phytoalexins, and lignin. In tobacco, significant suppression of PAL expression leads to a range of severe phenotypes, including reduced growth, altered leaf shape, reduced pollen viability, and increased disease susceptibility (Elkind *et al.*, 1990; Maher *et al.*, 1994). PAL suppressed plants have reduced lignin content with an increased S/G ratio (Sewalt *et al.*, 1997a; Korth *et al.*, 2001). On the other hand, PAL over-expression causes a small increase in Klason lignin and reduces S/G (Korth *et al.*, 2001).

CINNAMIC ACID 4-HYDROXYLASE (C4H)

C4H suppression in transgenic tobacco significantly reduces lignin content but, in contrast to PAL, the incorporation of S units is particularly affected, and S/G ratio is therefore decreased (Sewalt *et al.*, 1997a). These opposing changes to lignin monomeric composition in C4H- and PAL-deficient plants is difficult to reconcile with the supposed sequential positioning of the two enzymes on the currently accepted scheme of the monolignol biosynthetic pathway. It has been suggested that C4H might have a different role and position on the pathway than that currently assumed, or that metabolic channelling, mediated by protein complexes incorporating C4H, may operate on the lignin pathway to direct precursors towards S lignin biosynthesis (Dixon *et al.*, 2001). A single C4H gene exists in *Arabidopsis*, and *ref3* plants (for '*reduced epidermal fluorescence*', Ruegger *et al.*, 1999) have a mutation in this gene (Franke *et al.*, 2002b). Similarly to reduced-C4H tobacco, *ref3* plants have decreased lignin content and are particularly deficient in lignin S units. The mutant has developmental abnormalities, being stunted and male sterile, with increased branching.

4-COUMARATE:COA LIGASE (4CL)

Plants with reduced 4CL activity have been produced in transgenic tobacco (Kajita *et al.*, 1996, 1997), *Arabidopsis* (Lee *et al.*, 1997), and aspen (Hu *et al.*, 1999; Li *et al.*, 2003). Plants with significant deficiencies in 4CL had lignin content reduced by 25–50% in all three species, but changes to lignin composition were less consistent. In tobacco, S units were apparently predominantly reduced, whereas only G units were reduced in *Arabidopsis*, and there was no change to the relative proportions of S and G units in aspen (Kajita *et al.*, 1996, 1997; Lee *et al.*, 1997; Hu *et al.*, 1999). It is possible that different 4CL isoforms have been suppressed in these distinct experiments, or that the certain 4CL isoforms have different substrate specificities in the species involved. However, it is at least equally likely that these discrepancies

reveal the considerable difficulty and caution that has to exist in directly comparing results obtained from different plants analysed using different lignin analytical techniques. For a criticism of existing lignin analytical techniques and discussion of their general inadequacies and limitations, see Anterola and Lewis (2002).

In tobacco, 4CL-deficient plants with the greatest lignin reductions were stunted and had collapsed xylem vessels (Kajita *et al.*, 1997). Conversely, in aspen, 4CL suppression has been reported to enhance growth (Hu *et al.*, 1999), although these effects were not reproduced when the transgene was expressed from a tissue-specific promoter (Li *et al.*, 2003). Surprisingly, both studies indicated increased cellulose contents for 4CL-suppressed aspen, although the apparent cellulose increase may partly be accounted for by a proportional increase in the cellulose component of whole tissues when the lignin component is reduced (see Anterola and Lewis, 2002 for discussion).

P-COUMARATE 3-HYDROXYLASE (C3H)

The *ref*8 mutant of *Arabidopsis* is defective in the single C3H gene in this species, CYP98A3 (Franke *et al.*, 2002a,b). Lignin content is drastically reduced in the mutant, and is almost entirely composed of *p*-coumaryl alcohol (H) units and esterified *p*-coumaric acid. The plants are severely dwarfed, with collapsed xylem vessels, increased cell wall degradability, and a higher susceptibility to fungal colonization (Franke *et al.*, 2002a).

CAFFEOYL COA *O*-METHYLTRANSFERASE (CCOAOMT)

Suppression of CCoAOMT reduces lignin content by 12–50% in transgenic tobacco (Zhong *et al.*, 1998, Pinçon *et al.*, 2001a), alfalfa (Guo *et al.*, 2001a; Marita *et al.*, 2003a), and poplar (Meyermans *et al.*, 2000; Zhong *et al.*, 2000). In all studies, the amount of G units was reduced, consistent with the proposed role for CCoAOMT in G lignin synthesis. In some studies, S units were also reduced, although small increases in the S/G ratio reflected a predominant influence on G units (Zhong *et al.*, 1998; Meyermans *et al.*, 2000). In alfalfa, and in one tobacco study, the amount of S units was not reduced (Guo *et al.*, 2001a, Pinçon *et al.*, 2001a). In poplar, the lignin produced by CCoAOMT-suppressed plants has been shown to be less cross-linked than normal (Zhong *et al.*, 2000). Vessel cell walls also showed enhanced fluorescence, possibly due to the increased levels of free and bound *p*-hydroxybenzoic acid that were detected. Similarly, increased amounts of methanol-extractable phenolics including the *O*-β-D-glucosides of caffeic acid, sinapic acid, and vanillic acid, were detected in the wood of the transgenic poplars (Meyermans *et al.*, 2000), while soluble caffeoyl glucoside accumulated in stem extracts of transgenic alfalfa (Guo *et al.*, 2001a). These glucosides may result from a detoxification of accumulating hydroxycinnamic acids, as indicated by feeding experiments with caffeic and sinapic acids (Meyermans *et al.*, 2000). The accumulation of *O*-β-D-glucopyranosyl-sinapic acid in plants with reduced S lignin supports the hypothesis that sinapic acid is not the main precursor for S units *in vivo*. Of all the CCoAOMT plants produced, only tobacco plants from one experiment showed an obvious phenotype with

altered growth and flower development (Pinçon *et al.*, 2001a). *Arabidopsis* mutants in CCoAOMT genes have not yet been identified.

CAFFEIC ACID *O*-METHYLTRANSFERASE (COMT)

COMT has been suppressed in tobacco, poplar, alfalfa, and maize transgenics (Dwivedi *et al.*, 1994; Ni *et al.*, 1994; Atanassova *et al.*, 1995; van Doorsselaere *et al.*, 1995; Tsai *et al.*, 1998; Jouanin *et al.*, 2000; Guo *et al.*, 2001a), and activity is reduced in maize, sorghum, and *Arabidopsis* mutants (Vignols *et al.*, 1995; Suzuki *et al.*, 1997; Bout and Vermerris, 2003; Goujon *et al.*, 2003c). These various plants showed no obvious external phenotype, although the wood of COMT-suppressed poplars had a rose (van Doorsselaere *et al.*, 1995) or red-brown colour (Tsai *et al.*, 1998; Jouanin *et al.*, 2000) that has been ascribed to an increased amount of coniferaldehyde (Tsai *et al.*, 1998). In all cases, the consistent effect of COMT suppression was a drastic reduction in the amounts of S lignin units and consequent decrease in S/G. Unusual monomers, the 5-hydroxyguaiacyl units (5OHG), have been detected in the polymer in both transgenic (Atanassova *et al.*, 1995; van Doorsselaere *et al.*, 1995; Tsai *et al.*, 1998; Lapierre *et al.*, 1999; Jouanin *et al.*, 2000; Guo *et al.*, 2001a; Marita *et al.*, 2003a) and mutant plants (Chabbert *et al.*, 1994; Suzuki *et al.*, 1997; Goujon *et al.*, 2003c), in some cases, even exceeding the residual amount of S units (Jouanin *et al.* 2000). In the *Arabidopsis* COMT knock-out mutant, no S units were detected.

Data concerning lignin contents in these various COMT-deficient plants is less consistent. Apparently similar transgenic tobacco have been reported to have normal amounts of lignin (Dwivedi *et al.*, 1994; Atanassova *et al.*, 1995; van Doorsselaere *et al.*, 1995) or reduced lignin content (Ni *et al.*, 1994; Jouanin *et al.*, 2000). In some cases, apparent reductions in lignin amount were dependent on the technique used to analyse the polymer (Guo *et al.*, 2001a; Marita *et al.*, 2003a), again highlighting the problems of comparing the work of different laboratories using different analytical and growth techniques (Baucher *et al.*, 2003). Lignin structure is also greatly altered in COMT-deficient plants. In poplar, the proportion of β–*O*–4 linkages was reduced, while condensed C–C linkages were increased and biphenyl (5–5) and phenylcoumaran (β–5) linkages were particularly more abundant (Lapierre *et al.*, 1999; Jouanin *et al.*, 2000; Guo *et al.*, 2001a), making the lignin more similar to softwood lignin. It is probably not surprising, therefore, that these poplar plants were found to be more difficult to pulp (Pilate *et al.*, 2002).

FERULATE 5-HYDROXYLASE (F5H)

An *Arabidopsis* F5H mutant, *fah1* (Chapple *et al.*, 1992; Meyer *et al.*, 1996) was identified over a decade ago and, more recently, F5H expression has been manipulated in transgenic tobacco, *Arabidopsis*, and poplar/aspen (Meyer *et al.*, 1998; Franke *et al.*, 2000; Sibout *et al.*, 2002; Li *et al.*, 2003). The *fah1* mutant produces a lignin deficient in S units with enhanced proportions of phenylcoumaran (β–5) and dibenzodioxocin (biphenyl; 5–5) linkages (Marita *et al.*, 1999), whereas over-expressing the gene in *Arabidopsis*, tobacco, or poplar results in lignin almost entirely composed of S units (Meyer *et al.*, 1998; Marita *et al.*, 1999; Franke *et al.*,

2000). These results confirm that F5H plays a crucial role in determining lignin monomer composition by controlling the production of S lignin units.

CINNAMOYL-COA REDUCTASE (CCR)

Transgenic plants deficient in CCR-activity have been described in tobacco (Piquemal *et al.*, 1998; Ralph *et al.*, 1998; O'Connell *et al.*, 2002) and *Arabidopsis* (Goujon *et al.*, 2003a). In both species, the transgenics have approximately 50% less Klason lignin than wild-type plants, as has the *Arabidopsis ccr* mutant, irregular xylem (*irx4*) (Jones *et al.*, 2001). Aberrant phenotypes are obvious for many of these CCR-deficient plants, including stunted growth, altered leaf morphology, and collapsed or irregular xylem vessels. The secondary walls of fibres and vessels appear to be disorganized and loosened, and particularly deficient in non-condensed lignin units (Chabannes *et al.*, 2001a,b; Pinçon *et al.*, 2001b; Goujon *et al.*, 2003a). The non-condensed fraction of lignin, particularly the β–*O*–4-linked guaiacyl units, is reduced (O'Connell *et al.*, 2002; Goujon *et al.*, 2003a) and S/G ratio is increased (Piquemal *et al.*, 1998; O'Connell *et al.*, 2002). Similar increases in S/G ratios were seen in some CCR-deficient *Arabidopsis* lines, depending on the growth conditions (Goujon *et al.*, 2003a). Increased amounts of cinnamic acids were present in cell walls of CCR-deficient plants (Piquemal *et al.*, 1998; O'Connell *et al.*, 2002; Goujon *et al.*, 2003a), and feruloyl-tyramine was detected in tobacco (Ralph *et al.*, 1998), possibly representing an alternative sink for feruloyl-CoA. Lignin in CCR-suppressed tobacco was also more easily extracted by alkali, and there was an increased proportion of free phenolic groups in the non-condensed fraction of lignin (O'Connell *et al.*, 2002).

CINNAMYL ALCOHOL DEHYDROGENASE (CAD)

Transgenic plants with reduced CAD activity exist 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), and *cad* mutants exist in pine (MacKay *et al.*, 1997), maize (Halpin *et al.*, 1998), *Arabidopsis* (Sibout *et al.*, 2003), and probably sorghum (Pillonel *et al.*, 1991). Lignin content is not significantly altered in most CAD-deficient plants, possibly because other phenolics, such as the aldehyde substrates of CAD, get incorporated into the polymer. An exception is the CAD-deficient *bm1* mutant of maize, which has significant reductions to lignin in certain genetic backgrounds (Kuc and Nelson, 1964; Colenbrander *et al.*, 1973) but not in others (Marita *et al.*, 2003b). In CAD-antisense poplar and in maize *bm1*, the S/G ratio was not altered (Halpin *et al.*, 1998; Lapierre *et al.*, 1999), whereas in CAD-antisense tobacco and alfalfa, the S/G ratio was reduced (Halpin *et al.*, 1994; Baucher *et al.*, 1999). Increased levels of cinnamaldehydes were detected in the lignin of CAD-antisense tobacco (Halpin *et al.*, 1994; Ralph *et al.*, 1998, 2001), and poplar (Kim *et al.*, 2002), and in the pine (Ralph *et al.*, 1997), *Arabidopsis* (Sibout *et al.*, 2003), and maize (Marita *et al.*, 2003b) *cad* mutants. This particular change to lignin composition/structure has been proposed to be connected to the striking red or brownish colour of xylem tissues in these plants (Higuchi *et al.*, 1994). Altered polymer structure is also indicated by its increased extractability in alkali (Halpin *et*

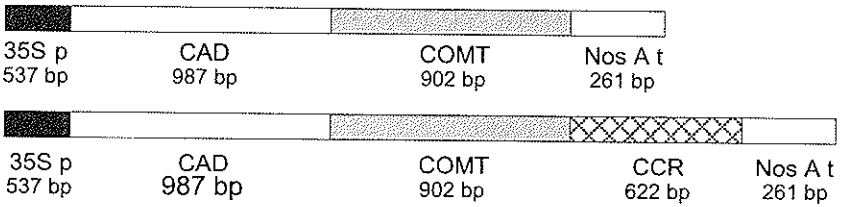

Figure 10.3. Chimeric constructs designed to suppress two or three lignin genes co-ordinately. 35S p, 35S promoter from cauliflower mosaic virus; CAD, cinnamyl alcohol dehydrogenase; COMT, caffeate *O*-methyltransferase; CCR, cinnamoyl CoA reductase; Nos A t, terminator from the nopaline synthase A gene; bp, base pairs.

al., 1994; Baucher *et al.*, 1996; Bernard-Vailhé *et al.*, 1996; Yahiaoui *et al.*, 1998; MacKay *et al.*, 1999) and by enrichment in free phenolic groups in both S and G units (Lapierre *et al.*, 1999; O'Connell *et al.*, 2002). This increase in free phenolic groups may be important in altering the solubility of lignin, which in turn has implications for the ease with which wood from these plants can be pulped (Lapierre *et al.*, 1999; O'Connell *et al.*, 2002).

MANIPULATION OF MULTIPLE GENES

Work is only beginning to attempt to combine different modified lignin characteristics by co-ordinate manipulation of multiple genes, but a few promising reports already exist in the literature (Zhong *et al.*, 1998; Chabannes *et al.*, 2001a; Guo *et al.*, 2001a; Pinçon *et al.*, 2001a,b; Abbott *et al.*, 2002; Li *et al.*, 2003). Stacking transgenes or traits in this way could provide great opportunities for crop and tree improvement in the future, but is still technically difficult to achieve (for discussion see Halpin *et al.*, 2001; Halpin and Boerjan, 2003). Chimeric constructs, incorporating sequences of two or even three different genes (*Figure 10.3*), appear to produce better effects in suppressing multiple genes than the more widely used method of crossing different single-transgene transgenics (Abbott *et al.*, 2002). Progress has also been made in suppressing one lignin biosynthetic gene (4CL) while overexpressing another (F5H) to concurrently improve several valuable wood quality traits (Li *et al.*, 2003).

Modified-lignin plants with improved digestibility

The 'brown-midrib' mutants of maize and sorghum have been shown to have reduced lignin content and increased digestibility (see Cherney *et al.*, 1991 for review) and some, at least, are deficient in enzymes of monolignol biosynthesis, notably COMT and CAD (Vignols *et al.*, 1995; Halpin *et al.*, 1998), suggesting a route to digestibility improvement via genetic engineering. Indeed, CAD- or COMT-deficient transgenic tobacco and alfalfa have been demonstrated to have slightly improved digestibility, assessed by a variety of methods (Bernard-Vailhé *et al.*, 1996, 1998; Sewalt *et al.*, 1997b; Baucher *et al.*, 1999; Guo *et al.*, 2001b), as has COMT-deficient *Stylosanthes* (Rae *et al.*, 2001). The *Arabidopsis* C3H mutant (*ref8*) has increased susceptibility to polysaccharide hydrolases (Franke *et al.*, 2002a), while enzymatic digestibility of PAL-suppressed transgenic tobacco or

CCR-deficient transgenic *Arabidopsis* indicated similar digestibility improvements (Sewalt *et al.*, 1997b; Goujon *et al.*, 2003a). Obviously, to be useful, genetic manipulation of lignin content must be accomplished without adverse effects on plant phenotype, disease resistance, or agronomic characters, such as lodging resistance. In practice, these restrictions are likely to limit the choice of genes for manipulation to those later on the monolignol branch pathway. For a more complete analysis of the opportunities for forage digestibility improvement by genetic engineering, see Barriere *et al.* (2003).

Modified-lignin plants improved for pulping

It is generally assumed that reducing the lignin content of wood or improving lignin extractability could make pulping easier and more environmentally benign. Transgenic tobacco and poplar with down-regulated CAD, 4CL, or CCR activity have been subjected to chemical pulping analyses (Baucher *et al.*, 1996; Lapierre *et al.*, 1999; Jouanin *et al.*, 2000; Kajita *et al.*, 2002; O'Connell *et al.*, 2002), and results indicate that the lignin modifications make Kraft pulping easier, while maintaining pulp yield and quality. In the only field trial of its kind yet reported on, the transgenic poplars were grown for 4 years in the field, and pulping improvements were maintained (Pilate *et al.*, 2002). By comparison, no improvements to Kraft pulping were evident, although soda pulping was improved, when wood from the pine *cad* mutant was tested (MacKay *et al.*, 1999), probably reflecting the differences in lignin structure between angiosperms and gymnosperms. Poplars over-expressing F5H have also been tested, and show particularly significant Kraft pulping improvements, potentially increasing pulp throughput by 60%, while concomitantly decreasing the consumption of pulping chemicals (Huntley *et al.*, 2003).

Conclusions and perspectives

A decade of research into the lignin biosynthetic pathway has yielded a clear picture of the roles of most of the genes involved in the process. Manipulation of gene activity using reverse genetic techniques has been a particularly valuable strategy in this work, and has also suggested potential routes to crop and tree improvement for particular applications. Current data suggest that manipulation of a variety of genes may improve digestibility or pulping performance to greater or lesser degrees. For pulping improvements, suppression of CAD or over-expression of F5H perhaps currently offer the most promise, with demonstrated pulping benefits and no adverse phenotypes.

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