Chapter 1

Monolignol Biosynthesis and its Genetic Manipulation: the Good, the Bad and the Ugly

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Abstract: Economic and environmental factors favor the adoption of lignocellulosic bioenergy crops for production of liquid transportation fuels. However, lignocellulosic biomass is recalcitrant to saccharification (sugar release from cell walls), and this is, at least in part, due to the presence of the phenylpropanoid-derived cell wall polymer lignin. A large body of evidence exists documenting the impacts of lignin modification in plants. This technology can lead to improved forage quality and enhanced processing properties for trees (paper pulping) and lignocellulosic energy crops. We here provide a comprehensive review of the literature on lignin modification in plants. The pathway has been targeted through down-regulating expression of the enzymes of the monolignol pathway, as well as through down-regulation or over-expression of transcription factors that control lignin biosynthesis and/or programs of secondary cell wall development. Targeting lignin modification at some steps in the monolignol pathway can result in impairment of plant growth and development, often associated with the triggering of endogenous host defense mechanisms. Recent studies suggest that it may be possible to decouple negative growth impacts from lignin reduction.

Keywords: monolignol biosynthesis, genetic modification, transcription factor, gene silencing, saccharification.

1.1 Introduction

Lignin is a major component of plant secondary cell walls, and the second most abundant plant polymer on the planet. Lignin constitutes about 15-35% of the dry mass of vascular plants (Adler, 1977). Considerable attention has been given over the past several years to the reduction of lignin content in model plant species, forages, trees, and dedicated bioenergy feedstocks. This is because forage digestibility, paper pulping and liquid fuel production from biomass through fermentation are all affected by recalcitrance of lignocellulose, primarily due to the presence of lignin, which blocks accessibility of the sugar-rich cell wall polysaccharides cellulose and hemicellulose to enzymes and microorganisms (Pilate *et al.*, 2002; Reddy *et al.*, 2005; Chen & Dixon, 2007).

Much is now known of the biosynthesis of lignin and its control at the transcriptional level. This information informs the targets that have been selected for genetic modification of lignin content and composition in transgenic plants. There is considerable variation in the effects on lignin content and composition depending on the gene that is targeted for down- or up-regulation. Equally important, lignin modification can have profound impacts on plant growth and development, ranging from good through bad to “downright ugly”, but these impacts are again strongly target dependent. Understanding the mechanisms that may impact plant growth, which equate to agronomic performance, in crop species “improved” through lignin modification is critical for economic advancement of the forage and biofuels industries. Although still poorly understood, these mechanisms may also throw light on basal plant developmental and defense processes.

1.2 Function and distribution of lignin in plants

Lignin is an aromatic heteropolymer derived primarily from three hydroxycinnamyl alcohols; 4-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol, which give rise, respectively, to the 4-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) subunits of lignin (Freudenberg and Neish, 1968; Ralph *et al.*, 2004). G units are mono-methoxylated, S units are di-methoxylated and H units are not methoxylated (Fig. 1.1). These monomers are linked through oxidative coupling catalyzed by both peroxidases and laccases (Boudet *et al.*, 1995). Unlike cellulose and other polymers that have labile linkages (e.g., glycosidic or peptide) between their building blocks, the units of lignin are linked by strong ether and carbon-carbon bonds (Sarkanen 1971). Lignin is present in

secondarily thickened cell walls of plants where it is critical for structural integrity of the cell wall and gives strength to stems (Chabannes *et al.*, 2001; Jones *et al.*, 2001). Lignin also imparts hydrophobicity to vascular elements for water transport. The lignin content of mature internodes of stems of alfalfa (*Medicago sativa*), the world's major forage legume and a target of much of the work to be described in this article, is about 17% of the dry weight (Guo *et al.*, 2001a).

Lignin composition varies among major phyla of vascular plants (Boerjan *et al.*, 2003). Dicotyledonous and monocotyledonous angiosperm lignins contain G and S units as the two major monomer species, with low levels of H units. Monocotyledonous lignins have more H units than dicotyledonous lignins (Baucher *et al.*, 1998), but care must be taken not to attribute other components to H-units as often happens (Boerjan, 2003). Fern and gymnosperm lignins have primarily G units and low levels of H units, but S units have been found in cuplet fern, yew plum pine, sandarac-cypress and a few genera in the Gnetophyta (Weng *et al.*, 2008b). Some lower plants like *Selaginella moellendorffii* (Weng *et al.*, 2008a,b) and *Marchantia polymorpha* have both G and S units in their lignins (Espineira *et al.*, 2011), despite predating hardwoods/dicots and even softwoods. The apparent presence of H, G and S units in the lignin from the seaweed *Calliarthron cheilosporioides* (Martone *et al.*, 2009) may indicate convergent evolution of lignin.

The presence of each methoxyl group on a monolignol units results in one less reactive site, and therefore fewer available potential coupling combinations during polymerization. Thus, S lignin is more linear and less crosslinked than G/S lignin, and provides a strong yet flexible polymer that is especially advantageous to herbaceous angiosperms (Bonavitz & Chapple 2010). A correlation has been shown between degradability of the cell walls in forages and the amount of G lignin, as lignin rich in G units is more highly condensed, making it less amenable to degradation (Jung & Deetz 1993). Thus, transgenic poplar plants with lignin rich in G units are, like softwoods, more difficult to pulp because of their more condensed lignin (Lapierre *et al.*, 1999).

Lignin content increases with progressive maturity of stems; these relationships have been studied in detail in alfalfa (Jung *et al.*, 1997; Chen *et al.*, 2006), ryegrass (Tu *et al.*, 2010), tall fescue (Buxton & Redfearn 1997; Chen *et al.*, 2002) and switchgrass (Mann *et al.*, 2009; Shen *et al.*, 2009). Decreasing the lignin content increases the digestibility of alfalfa for ruminant animals (Baucher *et al.*, 1999; Guo *et al.*, 2001a,b; Reddy *et al.*, 2005)

and improves processing efficiency for production of liquid biofuels through saccharification and fermentation (Chen *et al.*, 2007). Lignin composition has also been linked with reduced cell wall digestibility (Jung & Deetz 1993). However, the importance of lignin composition for digestibility has been questioned based on the results of studies with synthetic lignins which show lignin composition *per se* to have no effect (Grabber *et al.*, 1997).

Plants have primary and secondary cell walls that differ in function as well as composition. Primary walls allow cells to expand and divide while providing mechanical strength. Once cell growth stops, a much thicker secondary cell wall is deposited in some specialized cell types. These include vessels and fibers in the stem, sclereid cells, endodermal tissue of roots, some cells of anthers and pods important for dehiscence (Zhong & Ye, 2009), and seed coats (Marles *et al.*, 2008; Chen *et al.*, 2012). Generally, secondary cell walls consist of three layers named S1 (outer), S2 (middle) and S3 (inner). Lignin deposition starts at the cell corners in the region of the middle lamella and the primary wall when S1 formation has started. Most of the lignin is deposited in the S2 layer and impregnates the cellulose and hemicelluloses that are deposited there (Donaldson, 2001; Boerjan *et al.*, 2003). Based on UV microscopy, the density of lignin is higher in middle lamella and primary walls than in secondary walls of secondarily thickened cells, but the secondary walls have more lignin content as they constitute the largest proportion of the total cell wall (Fergus *et al.*, 1969). Usually H units are deposited first during cell wall formation, followed by G units and then S units (Terashima *et al.*, 1993, 1998; Donaldson, 2001). However, S units have been identified in lignin from corn coleoptiles, indicating that S lignin deposition may also start early in development (Musel *et al.*, 1997). H lignin is believed to determine the shape of the cells by acting as a matrix for deposition of G and S units (Terashima *et al.*, 1998). Vascular cells without H units may be free to expand and assume a round shape. In general, a higher amount of G units is present in vessels than in fibers, which are rich in S units (Saka & Goring, 1985).

There is considerable variation in lignin content and composition not only between different plant species but also in different tissues of the same plant, between various developmental stages of the plant, and in response to environmental conditions (Terashima *et al.*, 1993; Musel *et al.*, 1997; Vermerris & Boon, 2001; Donaldson, 2002; Chen *et al.*, 2006). For example, the S/G ratio increases to alter the cell wall mechanical properties in

poplar plants grown under simulated wind influence compared to plants grown in non-windy conditions (Koehler & Telewski, 2006).

Two examples, one for a bioenergy crop (switchgrass, *Panicum virgatum*), the other for a forage crop (alfalfa) are given to demonstrate the extent of variation in lignin encountered in wild-type, non-genetically modified plant biomass. Lignin content and composition of switchgrass cv. Alamo grown in the field, greenhouse and growth chambers was compared using different techniques (Mann *et al.*, 2009). Lignin content was not different in leaves from different parts of the tiller but stem tissues had increasing lignin content from the top to the bottom of the tiller. Younger stem tissue from the field had slightly higher lignin content compared to the greenhouse- and growth chamber- grown plants. The S/G ratio of leaf and stem tissues varied between different environments (Mann *et al.*, 2009). Similar observations have been made in tall fescue (Chen *et al.*, 2002) and perennial ryegrass (Tu *et al.*, 2010), where lignin content increases moderately during the stem elongation stage but a dramatic increase in lignin content occurs when plants progress from the elongation stage to the reproductive stage.

In a study with alfalfa, the lignin content of young internodes (internodes 1-2) was 93 mg/g CWR (cell wall residue), increasing towards a value of 250 mg/g CWR in the mature 8th internode (Chen *et al.*, 2006). This was accompanied by an increase in S/G ratio from 0.087 to 0.64. The lignin content (thioacidolysis yields) and lignin monomer composition of individual cell types (vascular elements, phloem fibers and vascular parenchyma) from the fifth internode were quite different (Nakashima *et al.*, 2008). Thioacidolysis yields are higher in vascular cells compared to parenchyma and fiber cells (430, 267 and 76 $\mu\text{mol/g}$ dry weight respectively), with fiber and parenchyma cells enriched in S lignin units with S/G ratio of 0.6 and 0.72 respectively, and vascular cells enriched in G lignin units with S/G ratio of 0.17 (Nakashima *et al.*, 2008). The H/total lignin ratios were 0.06, <0.01 and 0.03 respectively in vascular elements, fiber and parenchyma cells from the fifth internodes of greenhouse-grown alfalfa.

Coherent anti-stokes Raman scattering microscopy (CARS) has been used to determine the spatial distribution of lignin across secondary cell walls from the stems of alfalfa plants (Zeng *et al.*, 2010). At the tissue level, CARS intensity decreased in the order of fiber > xylem > epidermis > phloem > parenchyma. In general,

the CARS signal at the cellular level was highest in the cell corner compared to the compound middle lamella (middle lamella and primary walls from adjacent cells), and the signal in the middle lamella was higher than in the secondary walls (Zeng *et al.*, 2010).

1.3 Targets for modification of lignin biosynthesis

Fig. 1.1 shows a current view of the pathways for monolignol biosynthesis, and Fig. 1.2 outlines the transcriptional control mechanisms that regulate lignin deposition during secondary cell wall formation. Both biosynthetic enzymes and transcription factors have been targeted to reduce (or occasionally increase) lignin levels. In the following sections we briefly describe the gene targets, and then describe the effects of their down-regulation on lignin content and composition and plant phenotype in model systems, forages, and industrial pulp or bioenergy species.

1.3.1 Gene targets 1. Biosynthetic enzymes

The reader is referred to Fig. 1.1, which illustrates the positions of the various enzymes in the monolignol pathway.

1.3.1.1 L-Phenylalanine ammonia-lyase (PAL)

PAL has been characterized biochemically from many plant species since its discovery in 1961 (Koukol & Conn, 1961). *PAL* genes were first cloned from French bean (*Phaseolus vulgaris*) (Edwards *et al.*, 1985). The enzyme is tetrameric, and contains an unusual methylidene imidazolone residue at the active site that is formed post-translationally (Calabrese *et al.*, 2004). PAL is usually encoded by a multigene family, and it is possible that expression of different members can lead to formation of heterotetramers, the functional significance of which is not clear (Reichert *et al.*, 2009).

1.3.1.2 Cinnamate 4-hydroxylase (C4H)

C4H catalyzes 4-hydroxylation of cinnamic acid to 4-coumaric acid (Russell & Conn, 1967; Russell, 1971). C4H is the most abundant plant cytochrome P450 enzyme. The cloning of the *C4H* gene was described almost simultaneously from alfalfa (Fahrendorf & Dixon, 1993), artichoke (Teutsch *et al.*, 1993) and mung bean (Mizutani *et al.*, 1993).

1.3.1.3 4-Coumarate: Coenzyme-A ligase (4CL)

4CL has been extensively studied since the 1970s (Hahlbrock & Grisebach, 1970; Knobloch & Hahlbrock 1975, 1979). The enzyme converts hydroxycinnamic acids, preferably 4-coumaric acid, to the corresponding Coenzyme-A thioesters. 4CL exists as a multigene family in the species studied to date (Hu *et al.*, 1998; Ehltting *et al.*, 1999; Lindermayr *et al.*, 2002; Dixon & Reddy, 2003; Xu *et al.*, 2009). Only four of the eleven putative 4CLs in *Arabidopsis* appear to encode catalytically active 4CL enzymes, and the knockout mutant of At4CL5 does not show any changes in lignin content or monomer composition (Costa *et al.*, 2005), suggesting functional redundancy.

1.3.1.4 Enzymes of the coumaroyl shikimate shunt

Even though the biochemical formation of 4-coumaroyl shikimate and 4-coumaroyl quinate had been known for many years (Rhodes & Woollorton, 1976; Ulbrich & Zenk, 1980), it was not originally appreciated that these reactions may be involved in lignin biosynthesis. Discovery of the *Arabidopsis thaliana* cytochrome P450-dependent monooxygenase enzyme CYP98A3 (4-coumaroyl shikimate 3'-hydroxylase, C3'H), which hydroxylates the shikimate and quinate esters of 4-coumarate, prompted a revision of the monolignol pathway with suggestion of a new route for 3-hydroxylation of the 4-hydroxyphenyl moiety (Schoch *et al.*, 2001, Franke *et al.*, 2002a). Soon after, tobacco hydroxycinnamoyl-CoA: shikimate hydroxycinnamoyl transferase (HCT) was characterized (Hoffmann *et al.*, 2003). The monolignol pathway was then revised to involve HCT utilizing 4-coumaroyl-CoA as acyl donor and shikimate or quinate as acceptor, followed by subsequent 3-hydroxylation of the 4-coumaroyl moiety by C3'H leading to a caffeoyl ester and its subsequent conversion to caffeoyl-CoA by HCT acting in the reverse direction (Figure 1). Identification of a separate hydroxycinnamoyl-CoA: quinate

hydroxycinnamoyl transferase (HQT) involved in the synthesis of chlorogenic acid (caffeoyl quinate) (Niggeweg *et al.*, 2004) suggested that the 4-coumaroyl ester of shikimate was likely the preferred intermediate in lignin biosynthesis. In tomato, HQT down-regulation or over-expression does not change lignin content, but leads, respectively, to a decrease or increase in chlorogenic acid content (Niggeweg *et al.*, 2004).

There is good genetic evidence for the operation of the “shikimate shunt” in lignin biosynthesis in several plant species (Franke *et al.*, 2002a; Hoffmann *et al.*, 2004; Reddy *et al.*, 2005; Shadle *et al.*, 2007; Wagner *et al.*, 2007; Coleman *et al.*, 2008a). However, it is still not clear whether this pathway operates universally (e.g., in monocots). Other enzyme systems are known to exist for the conversion of a coumaroyl moiety to a caffeoyl moiety (e.g., Kneusel *et al.*, 1989), although most have yet to be analyzed beyond the level of protein biochemistry.

1.3.1.5 Caffeoyl-CoA 3-O-methyltransferase (CCoAOMT)

CCoAOMT is an S-adenosyl L-methionine and divalent cation dependent *O*-methyltransferase that preferentially converts caffeoyl-CoA to feruloyl-CoA (Kuhnl *et al.*, 1989; Ye *et al.*, 1994; Inoue *et al.*, 1998; Parvathi *et al.*, 2001). Demonstration of the involvement of CCoAOMT in lignin biosynthesis first came from studies on xylogenesis in *Zinnia* (Ye *et al.*, 1994), and this resulted in the first major revision of the monolignol pathway. Prior to that, caffeic acid 3-*O*-methyltransferase (COMT) was believed to be involved in methylation at both the C3 and C5 positions of monolignols (Finkle & Nelson, 1963; Davin & Lewis, 1992). The alfalfa CCoAOMT crystal structure has been obtained, and the enzyme forms a homodimer in solution, although the dimer is not necessary for substrate recognition and transmethylation as the substrate and cofactor interact with the monomer (Ferrer *et al.*, 2005).

1.3.1.6 Ferulate 5-hydroxylase (F5H)

F5H is the third cytochrome P450-dependent monooxygenase enzyme in the monolignol pathway. The *F5H* gene was cloned from the *fah1* mutant of *Arabidopsis* using a forward genetics approach (Chapple *et al.*, 1992). The F5H enzyme has a higher affinity for coniferaldehyde and coniferyl alcohol compared to ferulate and is

therefore more correctly referred to as coniferaldehyde 5-hydroxylase or Cald5H (Humphreys *et al.*, 1999; Osakabe *et al.*, 1999). This discovery led to a reappraisal of the monolignol pathway that no longer supported involvement of ferulate and sinapate in lignin biosynthesis.

1.3.1.7 Caffeic acid 3-*O*-methyltransferase (COMT)

Caffeic acid 3-*O*-methyltransferase (COMT) has been studied for many years (Finkle & Nelson, 1963). However, the common name of the enzyme appears to be a misnomer; caffeic acid may not be a substrate for COMT during monolignol biosynthesis as COMT from many species, including *Arabidopsis*, aspen and alfalfa, has a significantly higher affinity for 5-hydroxyconiferaldehyde than for caffeic acid (Li *et al.*, 2000; Parvathi *et al.*, 2001). In *Arabidopsis*, *O*-methylation of 5-hydroxyconiferyl alcohol is inhibited in the presence of 5-hydroxyconiferaldehyde such that, when both substrates are present, AtCOMT preferentially catalyzes *O*-methylation of 5-hydroxyconiferaldehyde (Nakatsubo *et al.*, 2008). Alfalfa COMT can efficiently methylate caffealdehyde and caffeyl alcohol (Parvathi *et al.*, 2001). COMT from tall fescue (Chen *et al.*, 2004) and wheat (Ma & Xu, 2008) efficiently utilizes both caffealdehyde and 5-hydroxyconiferaldehyde. Further studies are needed to determine unequivocally the preferred routes for monolignol *O*-methylation *in vivo*.

1.3.1.8 Cinnamoyl-CoA reductase (CCR)

CCRs are involved in the reduction of hydroxycinnamoyl-CoA thioesters to the corresponding aldehydes, and have been studied for many years (Gross *et al.*, 1973). There are two well-characterized CCRs in *Arabidopsis*; AtCCR1 is five times more efficient with feruloyl-CoA and sinapoyl-CoA than is AtCCR2, and is involved in developmentally-regulated lignification whereas AtCCR2 is expressed in response to pathogen infection and hence may be involved in disease resistance (Lauvergeat *et al.*, 2001). Feruloyl-CoA and caffeoyl-CoA are the most and least preferred substrates, respectively, for CCRs from *Arabidopsis* (Patten *et al.*, 2005). Feruloyl-CoA and sinapoyl-CoA are the preferred substrates for *M. truncatula* MtCCR1, whereas caffeoyl-CoA and 4-coumaroyl-CoA are the preferred substrates for MtCCR2 (Zhou *et al.*, 2010). MtCCR2 may be involved in a route to lignin biosynthesis whereby caffeoyl-CoA is converted to caffealdehyde which is then 3-*O*-methylated

to coniferaldehyde by COMT (Zhou *et al.*, 2010) (Fig. 1.1), a pathway previously suggested to occur in *Arabidopsis* (Do *et al.*, 2007).

1.3.1.9 Cinnamyl alcohol dehydrogenase (CAD)

CAD is a zinc-dependent enzyme that catalyzes the reduction of hydroxy-cinnamaldehydes to their corresponding alcohols (Mansell *et al.*, 1974). Coniferaldehyde and sinapaldehyde are preferred substrates for CAD in tall fescue (Chen *et al.*, 2003).

1.3.2 Gene targets 2. Transcription factors

The genes involved in cellulose, xylan, and lignin synthesis are coordinately expressed during secondary wall biosynthesis and, in the past decade, many transcription factors (TFs) involved in the biosynthesis of lignin and the other secondary cell wall polymers have been identified and characterized (Fig. 1.2). In *Arabidopsis*, several closely related NAC TFs regulate secondary wall biosynthesis; these are NST1 (NAC SECONDARY WALL THICKENING PROMOTING FACTOR1), NST2 /NST3/SND1 (SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN1), VASCULAR-RELATED NAC-DOMAIN6 (VND6) and VND7 (Mitsuda *et al.*, 2005, 2007; Zhong *et al.*, 2006; Yamaguchi *et al.*, 2008). Although two *NST* genes act redundantly to control lignification in the interfascicular tissues in the stem of *Arabidopsis* (Mitsuda *et al.*, 2005, 2007), a single *NST* gene was cloned from the model legume *Medicago truncatula* by forward genetic screening, and shown to function as a master switch for secondary wall synthesis in various tissues (Zhao *et al.*, 2010a). MYB family TFs are also important for biosynthesis of other secondary wall components, and act downstream of the *NST* genes (Zhong *et al.*, 2007; Ko *et al.*, 2009; McCarthy *et al.*, 2009; Zhou *et al.*, 2009) (Fig. 1.2). Among these, MYB46 is a direct target of NST3/SND1 *in vitro* and *in vivo*, and may be the master switch that turns on expression of genes for the biosynthesis of cellulose, xylan and lignin (Zhong *et al.*, 2007; Ko *et al.*, 2009). Detailed promoter and electrophoretic mobility shift assay analyses have revealed that AC-rich elements corresponding to MYB TF binding motifs in the promoters of many monolignol pathway genes are necessary for the coordinated activation of these genes (Lacombe *et al.*, 2000; Patzlaff *et al.*, 2003; Zhou *et al.*, 2009). Interestingly, F5H is not regulated

by a “lignin MYB” gene, but directly by the upstream NAC TF (Zhao *et al.*, 2010b). Finally, MYB and WRKY TFs can act as transcriptional repressors of lignification (Wang *et al.*, 2010; Shen *et al.*, 2012). Further details of the TFs that regulate secondary wall formation can be found in the following review articles (Demura & Fukuda, 2007; Zhong & Ye, 2007; Demura & Ye, 2010; Zhong *et al.*, 2010).

1.4 Impacts of lignin modification by targeting the monolignol biosynthetic pathway

In this section, we review both the biochemical and broader plant growth phenotypes following down-regulation of monolignol biosynthesis at each of the enzymatic steps in the pathway. A similar analysis of impacts of targeting TFs affecting the monolignol pathway is given in the following section. Clearly, reducing lignin levels in transgenic plants is facile. However, depending on the step targeted, the results can be good, bad or ugly!

1.4.1 L-Phenylalanine ammonia-lyase

The lignin content of PAL down-regulated alfalfa total stem tissue (internodes 1-8) was 97 mg/g of total cell wall residue (CWR) compared to ~150 mg/g CWR for control alfalfa, and varied from 72 mg/g CWR in internodes 1-2 to 127 mg/g CWR in the eighth internode, indicating that the lignin content was reduced in all developmental stages of the stem (Chen *et al.*, 2006). Analysis of cell-specific effects of PAL down-regulation in the fifth internode, using laser-capture microdissection to collect specific cell types, showed an increase in S/G ratio in vascular elements and fibers but not in parenchyma (Nakashima *et al.*, 2008). Cell wall autofluorescence almost completely disappeared in the stem sections of these plants, except in a few vascular cells.

Down-regulation of PAL in transgenic tobacco leads to a decrease in lignin content (Elkind *et al.*, 1990; Bate *et al.*, 1994; Sewalt *et al.*, 1997a; Korth *et al.*, 2001), altered leaf shape and texture, stunted growth, reduced pollen viability, and altered flower morphology and pigmentation. The leaves have 10-fold reduced levels of the hydroxycinnamate ester chlorogenic acid and 70% reduced salicylic acid content compared to controls (Pallas *et*

al., 1996). The plants are more susceptible to the fungal pathogen *Cercospora nicotianae* (Maher *et al.*, 1994), and to viral pathogens (Felton *et al.*, 1999).

Arabidopsis thaliana has four PAL genes. Single mutants in *pal1* or *pal2*, *pal1 pal2* double mutants and *pal1 pal2 pal3* triple mutants show normal morphology with slight to significant reduction in lignin content (Rohde *et al.*, 2004; Huang *et al.*, 2010). In contrast, *pal1 pal2 pal4* triple mutants and *pal1 pal2 pal3 pal4* quadruple mutants have a dwarf phenotype and are sterile (Huang *et al.*, 2010). The *pal1 pal2* double mutants have lower levels of feruloyl malate esters, increased phenylalanine content, lack three kaempferol glycosides and are sensitive to UV light but are more drought resistant than wild-type (Rohde *et al.*, 2004; Huang *et al.*, 2010). The quadruple mutant has lower salicylic acid levels and is susceptible to the bacterial pathogen *Pseudomonas syringae*.

In *Salvia miltiorrhiza*, PAL down-regulation leads to reduction in the contents of lignin and the water soluble phenolics rosmarinic acid and salvianolic acid B. This is accompanied by stunted growth, altered leaf morphology, and delayed root formation (Song & Wang, 2011).

Because of the extensive negative pleiotropic effects of PAL down-regulation on phenylpropanoid metabolism and growth, this enzyme is probably not a suitable target for reducing recalcitrance of cell wall material in forage or bioenergy crops.

1.4.2 Cinnamate 4-hydroxylase

C4H down-regulation in alfalfa leads to reduced lignin content in stem tissue, accompanied by a decreased S/G ratio, but with less effect on soluble phenolic compounds than observed in PAL down-regulated plants (Reddy *et al.*, 2005, Chen *et al.*, 2006). Based on laser capture microdissection analysis, S/G ratio is decreased in vascular and fiber cells but is similar to control values in parenchyma cells (Nakashima *et al.*, 2008). Stem sections of these plants show a few distorted vascular cells, and the sections have very low lignin autofluorescence, similar to PAL down-regulated alfalfa. Vascular cells are smaller than in wild-type, and less in number (Fig. 1.3A). The saccharification efficiency of C4H down-regulated alfalfa is higher than that of wild-type plants, but lower than

that of HCT down-regulated alfalfa (Chen & Dixon, 2007). C4H down-regulated alfalfa forage is approximately 15% more digestible in the rumens of steers than forage from wild-type plants (Reddy *et al.*, 2005).

Down-regulation of C4H activity in tobacco results in reduced lignin content with decreased S/G ratio, and decreased levels of chlorogenic acid and soluble esters of caffeic acid in leaf tissue (Sewalt *et al.*, 1997a; Blount *et al.*, 2000; Blee *et al.*, 2001). C4H mis-sense mutants of Arabidopsis show decreased lignin content and decreased levels of several phenolic compounds as well as pleiotropic effects including male sterility (Schilmeller *et al.*, 2009). Sense-suppression of C4H in transgenic tomato results in reduced lignin content with increased S/G ratio (Millar *et al.*, 2007), in contrast to the decreased S/G ratio in tobacco and alfalfa. Down-regulation of C4H in all these species results in a dwarf phenotype.

1.4.3 4-Coumarate: Coenzyme-A ligase

Down-regulation of 4CL activity results in decreased lignin content in alfalfa, Arabidopsis, tobacco, aspen (*Populus tremuloides*) and hybrid white poplar (*Populus tremula* X *Populus alba*). This is accompanied by a slight increase in the S/G ratio and increased salicylic acid levels in alfalfa, a greater increase in S/G ratio in Arabidopsis, an unchanged S/G ratio in aspen, and decreased S/G ratio in hybrid white poplar.

Autofluorescence of stem sections of 4CL down-regulated alfalfa confirms overall reduction in lignin content, while Mäule staining shows lower amounts of S lignin in vascular cells and pith rays (Figure 3A). The lignin thioacidolysis yield is most noticeably decreased in vascular and fiber cells (Nakashima *et al.*, 2008). These lines also have reduced mean areas of individual vascular cells and decreased numbers of vascular cells compared to wild-type. The biomass yield of 4CL down-regulated alfalfa at early bud stage is decreased by around 37% (Nakashima *et al.*, 2008).

Down-regulation of 4CL in tobacco decreases the lignin content and leads to plants with a dwarf phenotype and/or brown stems. In these plants, the amount of 4-hydroxybenzaldehyde increases, levels of vanillin and syringaldehyde are reduced, and there are also changes in several other phenolic compounds (Kajita *et al.*, 1996, 1997).

The reduction in lignin level in Arabidopsis following down-regulation of 4CL is achieved by a decrease in G units but not S units, leading to higher S/G ratio, and the plants appear phenotypically normal (Lee *et al.*, 1997).

Transgenic aspen trees with suppressed Pt4CL1 expression have up to a 45% reduction in lignin content with no significant change in S/G ratio. The reduction in lignin content was proposed to be compensated for by an up to 15% increase in cellulose content, accompanied by increased or unchanged biomass (Hu *et al.*, 1999; Li *et al.*, 2003). It is debatable as to whether the plants are synthesizing additional cellulose, or whether the effect is simply one of compensation. Aspen trees with 4CL1 down-regulation and F5H over-expression show an additive effect, with up to 52% reduction in lignin, 64% higher S/G ratio and 30% more cellulose (Li *et al.*, 2003).

Transgenic field-grown hybrid white poplar trees down-regulated in 4CL1 have decreased lignin content with decreased S/G ratio (Voelker *et al.*, 2010). However, unlike aspen (Hu *et al.*, 1999; Li *et al.*, 2003), the white poplar with greatest suppression of 4CL shows reduced biomass with brown stems similar to those seen in tobacco (Kajita *et al.*, 1996) and alfalfa (Nakashima *et al.*, 2008). The brown wood is enriched in phenolics such as naringenin, dihydrokaempferol, and their corresponding glucosides. However, the saccharification rates of these trees do not correlate well with reduced lignin content.

1.4.4 Hydroxycinnamoyl-CoA: shikimate hydroxycinnamoyl transferase

Silencing of HCT causes a strong reduction of lignin content and a large increase of H units in alfalfa, Arabidopsis, *Nicotiana benthamiana* and *Pinus radiata*. This is accompanied by a dwarf phenotype in alfalfa, Arabidopsis and *N. benthamiana*.

In alfalfa, silencing of HCT results in an approximately 50% reduction in lignin content, accompanied by a decrease in S and G units and a corresponding increase in H units from trace amounts to almost 50% of the total lignin as determined by thioacidolysis. The H unit-rich lignin is of low molecular weight and more extractable than the bulk lignin (Ziebell *et al.*, 2010). The thioacidolysis yield of HCT down-regulated alfalfa is more severely reduced in parenchyma and fiber cells compared to vascular cells. Stem sections show very low lignin autofluorescence in vascular cells, and these are very small (<50% of the size of the cells in the control), reduced in number and severely distorted (Fig. 1.3A). The pith rays show a characteristic pattern with S lignin localized

to the middle layers of the cell walls (Nakashima *et al.*, 2008). Levels of wall-bound vanillin and ferulic acid are decreased whereas levels of 4-hydroxybenzaldehyde and 4-coumaric acid are increased (Chen *et al.*, 2006).

Alfalfa HCT down-regulated transgenics exhibit up to a 166% increase in enzymatic saccharification efficiency (Chen & Dixon, 2007) and up to a 20% increase in *in vitro* dry matter digestibility using rumen fluid (Shadle *et al.*, 2007). However, the most strongly down-regulated lines are dwarf with a bushy phenotype (Fig. 1.4A), and exhibit reduced photosynthetic and transpiration rates, and delayed flowering in some lines (Chen *et al.*, 2006; Shadle *et al.*, 2007; Gallego-Giraldo *et al.*, 2011b). Surprisingly, these plants also showed increased drought and fungal tolerance (Gallego-Giraldo *et al.*, 2011b).

Transgenic suppression of HCT in *P. radiata* tracheary elements results in a 42% reduction in lignin content, an increased proportion of H units (from trace amounts to 31%), and a decrease in the proportion of G units from 99.6% in controls to 69% in transgenics (Wagner *et al.*, 2007). NMR analysis of lignin from HCT down-regulated *P. radiata* and alfalfa confirmed the increase in H units and some corresponding interunit linkage ratio changes in the polymer structure (Wagner *et al.*, 2007; Pu *et al.*, 2009).

Virus induced gene silencing of HCT in *N. benthamiana* resulted in dwarf plants with a 15% reduction in lignin content, increased H units from trace amounts to 8% of total lignin units, and a 9% decrease in S units without any change in G units (Hoffmann *et al.*, 2004). The corresponding increase in saccharification efficiency was similar to that seen in HCT down-regulated alfalfa.

In Arabidopsis, silencing of HCT resulted in dwarf plants with green/purple coloration, reduced lignin content with increased amount of H units (from trace to 85%), and increased flavonoid content (Hoffmann *et al.*, 2004; Besseau *et al.*, 2007). The HCT-deficient plants had small vascular cells, many of which were collapsed (Besseau *et al.*, 2007).

1.4.5 4-Coumaroyl shikimate 3'-hydroxylase

Silencing of C3'H expression in alfalfa (Fig. 1.4B), Arabidopsis and hybrid poplar (*Populus grandidentata* X *Populus alba*) resulted in all cases in dwarf plants with distorted xylem elements, reduced lignin content and a massive increase in the proportion of H lignin units. These plants also had increased flavonoid content.

Down-regulation of C3'H in alfalfa results in a greater than 30% reduction in lignin content, an increase in the H/(total lignin) ratio from 0.03 to 0.48, and a small increase in S/G ratio (Reddy *et al.*, 2005; Chen *et al.*, 2006). Stem sections of C3'H down-regulated alfalfa show very low lignin autofluorescence, except in a few vascular cells that have low amounts of S lignin, and some of the vessels are collapsed (Nakashima *et al.*, 2008) (Figure 3A). Patten *et al.*, (2007) reported that C3'H-silenced alfalfa plants produce more xylem than control lines, but Nakashima *et al.*, (2008) found fewer and smaller vessel elements compared to controls; however, this study was restricted to the fifth internode. Laser capture microdissection studies indicated that lignin thioacidolysis yield was considerably reduced in vascular, fiber and parenchyma cells (Nakashima *et al.*, 2008). NMR studies confirmed the increased proportion of H units in the lignin, and revealed a low molecular weight for the H-rich lignin, higher levels of phenylcoumarans and resinols, and doubling of the proportion of, and an increased variability in, dibenzodioxocin structures (Ralph *et al.*, 2006; Pu *et al.*, 2009; Ziebell *et al.*, 2010). There was an increase in the content of wall-bound 4-hydroxybenzaldehyde and 4-coumaric acid, and a decrease in vanillin and ferulic acid, in these plants (Chen *et al.*, 2006). Salicylic acid content was also increased (Lee *et al.*, 2011; Gallego-Giraldo *et al.*, 2011b), along with a corresponding increase in the expression of pathogenesis-related genes, which are inducible by salicylate.

The saccharification efficiency of C3'H down-regulated alfalfa is higher than wild-type but slightly lower than that of HCT down-regulated alfalfa (Chen & Dixon, 2007). *In situ* digestibility of C3'H silenced alfalfa in fistulated steers was increased by more than 18% (Reddy *et al.*, 2005); the separation in digestibility between C3'H silenced and control alfalfa was measurable within 12 h of incubation in the rumen, and that difference increased towards 72 h of incubation (Reddy *et al.*, 2005).

C3'H down-regulation in hybrid poplar leads to dwarf plants with >50% reduction in lignin content, with H units increased from trace amounts in controls to ~20% of the total, accompanied by a reduction in G units but little change in S units (Coleman *et al.*, 2008a,b). As with HCT down-regulated alfalfa, these plants have collapsed xylem, and reduced photosynthesis and stomatal conductance.

C3'H mutants of *Arabidopsis* are dwarf, and have more than 60% reduction in lignin content, with approximately 95% of the lignin being made of H units (Franke *et al.*, 2002a,b; Abdulrazzak *et al.*, 2006). The

plants have collapsed vessel elements with smaller cells similar to C3H down-regulated alfalfa, and flavonoid content is increased (Abdulrazzak *et al.*, 2006). There is high expression of genes related to response to biotic and abiotic stress, in particular those mediated by jasmonate and abscisic acid. Sinapoylated cyanidin glucosides accumulate in C3'H gene-silenced Arabidopsis, but very low levels of sinapate esters are seen in the true null mutants (Abdulrazzak *et al.*, 2006). Cell walls of C3'H mutants are approximately 80% more digestible by polysaccharide hydrolases than are wild-type cell walls (Franke *et al.*, 2002b).

1.4.6 Caffeoyl CoA 3-O-methyltransferase

CCoAOMT down-regulated alfalfa plants appear phenotypically normal, although knock-out mutants in *Medicago truncatula* have reduced biomass yield (Fig. 1.4D). The lignin has an increased proportion of β -5-linked dimers of G units (Guo *et al.*, 2001a, Chen *et al.*, 2006). The approximately 50% reduction in G units with little change in S units suggests that CCoAOMT may not be essential for S lignin synthesis in alfalfa, and recent studies in *M. truncatula* have led to the hypothesis that there may be a shunt around the CCoAOMT reaction involving the activity of cinnamoyl-CoA reductase with caffeoyl-CoA (Zhou *et al.*, 2010); this leads to formation of caffealdehyde, a preferred substrate for Medicago COMT (Parvathi *et al.*, 2001). This model is supported by mathematical modeling of flux partitioning into lignin in alfalfa lines down-regulated at different steps in the monolignol pathway (Lee *et al.*, 2011).

The large change in S/G ratio in CCoAOMT down-regulated alfalfa is primarily in the vascular elements (Fig. 1.3A), but the thioacidolysis yields are much lower in parenchyma and fiber cells compared to vascular elements (Nakashima *et al.*, 2008). S lignin deposition in pith rays shows a distinctive pattern with S lignin confined to the middle layer of the cell wall (Nakashima *et al.*, 2008). The mean area of the vascular cells is smaller than that of the wild-type (Nakashima *et al.*, 2008). CCoAOMT down-regulated alfalfa accumulates caffeic acid β -D-glucoside and salicylic acid (Guo *et al.*, 2001a; Chen *et al.*, 2006; Lee *et al.*, 2011). The CCoAOMT down-regulated alfalfa plants have increased saccharification efficiency (Chen & Dixon, 2007) and a 6% increase in forage digestibility (Guo *et al.*, 2001b; Reddy *et al.*, 2005) compared to wild-type.

CCoAOMT down-regulation in tobacco results in up to 15% reduction in lignin content with preferentially more reduction in G lignin, resulting in an increased S/G ratio (Zhong *et al.*, 1998; Pincon *et al.*, 2001a). Reduction in plant growth was reported in one of the two studies.

CCoAOMT down-regulation in poplar results in 12-40% reduction in lignin content, decreases in both G and S units but an overall 11% increase in S/G ratio, with no change in plant growth (Meyermans *et al.*, 2000; Zhong *et al.*, 2000). Levels of 4-hydroxybenzoate, and glucosides of caffeic acid, vanillic acid and sinapic acid are increased (Meyermans *et al.*, 2000). Cross-sections of CCoAOMT down-regulated poplar stems have red (Meyermans *et al.*, 2000) or orange (Zhong *et al.*, 2000) coloration, with a few slightly deformed vessels.

Flax lignin is unusual in having a high proportion of G units; down-regulation of CCoAOMT in flax reduces the lignin content by 8-18% with reduction in the proportion of G units. The plants are phenotypically normal, except that the thickness of the vascular cells is reduced (Day *et al.*, 2009).

Arabidopsis CCoAOMT knockout mutants exhibit an approximately 26% reduction in lignin content, accompanied by a 56% higher S/G ratio due to a 16% reduction in the level of G units. The stems are slightly shorter than wild-type under short-day growth conditions (Do *et al.*, 2007). FTIR microscopy showed that the reduction in lignin content is more severe in xylem fibers that have only G lignin than in fiber cells that have both S and G lignins. The fact that the mutant has a significant amount of lignin with G units despite the complete loss of CCoAOMT enzyme activity suggests the presence of an alternative pathway for 3-*O*-methylation. Arabidopsis double mutants generated by crossing *CCoAOMT* and *COMT* mutants (Goujon *et al.*, 2003a) are compromised in development and have very low lignin content, composed mostly of H units. The flavonoid isorhamnetin is not detectable in the mutants (consistent with roles for COMT and CCoAOMT in methylation of flavonoids) and sinapoylmalate is barely detectable (Do *et al.*, 2007). Simultaneous strong down-regulation of both CCoAOMT and COMT in alfalfa likewise results in plants that are compromised in development, with abberantly thickened vascular cell walls (Fig. 1.3B) (Nakashima *et al.*, 2008).

Down-regulation of CCoAOMT in tracheary element cultures of *Pinus radiata* results in 5-20% reduction of lignin content with reduction in G units leading to a 10-fold increase in H/G ratio. The lignin of these transgenic cultures has a unique catechyl (C) unit signature in addition to the H and G units that are normally present

(Wagner *et al.*, 2011). NMR analysis revealed small amounts of caffeyl alcohol as the new monolignol in these transgenic tracheary elements. The incorporation of caffeyl alcohol into the lignin polymer reflects the incorporation of 5-hydroxyconiferyl alcohol in COMT down-regulated alfalfa and other angiosperms (Marita *et al.*, 2003a). However, CCoAOMT down-regulation in alfalfa and other angiosperms does not result in the incorporation of C units into lignin. Recently, a lignin composed entirely of caffeyl alcohol units has been discovered in the seed coats of vanilla orchid and several species of cacti (Chen *et al.*, 2012).

1.4.7 Ferulate 5-hydroxylase

The *fah1* mutant of Arabidopsis is devoid of F5H activity, fails to accumulate sinapoyl malate, a major leaf metabolite in wild-type Arabidopsis, and contains lignin that is almost completely made up of G units as in gymnosperms (Chapple *et al.*, 1992; Meyer *et al.*, 1998; Marita *et al.*, 1999). The lignin of the mutant has enhanced levels of phenylcoumaran and dibenzodioxocin structures, and over-expression of the wild-type copy of F5H in the *fah1* background results in restoration of the wild-type lignin profile and the disappearance of these structures (Marita *et al.*, 1999).

Over-expression of F5H under control of the *C4H* promoter results in lignin composed of close to 100% S units, mostly linked through β -aryl ether linkages (Marita *et al.*, 1999). Benzodioxane structures are also detected in F5H over-expressing Arabidopsis, probably due to increased flux into 5-hydroxyconiferyl alcohol that is not sufficiently rapidly methylated by the Arabidopsis COMT (Ralph *et al.*, 2001b). Similarly, over-expression of F5H from *Selaginella moellendorffii* (SmF5H) in the *fah1* mutant results in lignin with up to 80% S lignin (Weng *et al.*, 2008b). Neither over-expression nor down-regulation of F5H alters the overall growth phenotype of Arabidopsis. F5H suppression does not affect saccharification efficiency or digestibility of Arabidopsis, but over-expression of F5H increases saccharification efficiency, most noticeably after treatment with hot water prior to enzyme hydrolysis (Li *et al.*, 2010a).

Down-regulation of F5H in alfalfa results in only a slight reduction in S units with no significant change in lignin content, and plant growth is unaffected (Reddy *et al.*, 2005; Chen *et al.*, 2006). Based on autofluorescence of stem sections, it was hard to detect effects of F5H down-regulation, but Mäule staining showed reduction in S

lignin content (Nakashima *et al.*, 2008) (Fig. 1.3A). The plants had smaller vascular cells than controls, but the number of vascular cells per cluster was normal. Thioacidolysis showed no reduction in S lignin content of vascular cells despite the fact that the *PAL* promoter driving the F5H transgene is most strongly expressed in this tissue type. However, there was a reduction in S units in fibers and vascular parenchyma cells (Nakashima *et al.*, 2008). The F5H-suppressed alfalfa showed neither improved digestibility nor increased fermentable sugar yield (Reddy *et al.*, 2005; Chen & Dixon, 2007).

Over-expression of sweetgum F5H under the control of the *Populus trichocarpa 4CLI* promoter in transgenic aspen results in accelerated lignification of stem xylem cells without a change in lignin content, and a 2.5-fold increase in S/G ratio compared to controls, with S units constituting approximately 80% of the lignin units (Li *et al.*, 2003). Similarly, over-expression of F5H in hybrid poplar results in lignin comprising up to 97.5% S units, leading to a more linear and less polymerized lignin (Stewart *et al.*, 2009). The benzodioxane structures detected in F5H over-expressing *Arabidopsis* (Ralph *et al.*, 2001b) are barely detectable in F5H over-expressing aspen (Li *et al.*, 2003) and hybrid poplar, suggesting that COMT is not limiting in these plants (Stewart *et al.*, 2009). In hybrid poplar, *C4H*-promoter-driven F5H over-expression results in lower molecular weight lignin, a two-fold decrease in 4-hydroxybenzoate incorporation, decreased levels of phenylcoumarans and increased levels of resinols and spirodienones (Stewart *et al.*, 2009). Pulping efficiency is increased in F5H over-expressing poplar (Huntley *et al.*, 2003), possibly as a result of the lower molecular weight of the lignin as well as the high syringyl (and hence, high β -ether) content (Stewart *et al.*, 2009).

1.4.8 Caffeic acid O-methyltransferase

COMT down-regulation has been analyzed in many plant species. In alfalfa (Fig. 1.3A), *Arabidopsis*, aspen (*Populus tremuloides*), corn, perennial ryegrass, poplar (*Populus tremula* x *alba*), silver birch, *Stylosanthes humilis*, switchgrass, tall fescue and tobacco, COMT down-regulation causes a large reduction in S units, leading to lower S/G ratio and highlighting the involvement of COMT in S lignin biosynthesis. COMT down-regulation is usually accompanied by the appearance of 5-hydroxyguaiacyl units in lignin due to incorporation of 5-hydroxy-coniferyl alcohol with unique benzodioxane structures (Jouanin *et al.*, 2000; Ralph *et al.*, 2001a, 2001b;

Marita *et al.*, 2003a; Morreel *et al.*, 2004; Lu *et al.*, 2010). An increase in biphenyl (5–5) and phenylcoumaran (β –5) lignin linkages has also been detected in COMT-deficient plants. Decreased lignin content was observed in most studies. Cell wall digestibility is increased in alfalfa (Guo *et al.*, 2001b; Reddy *et al.*, 2005), perennial ryegrass, *Stylosanthes humilis*, switchgrass, tall fescue and tobacco; saccharification efficiency was shown to be increased in alfalfa (Chen & Dixon, 2007) and sorghum (Sattler *et al.*, 2010), and pulping efficiency was decreased in poplar as a result of COMT down-regulation (Lapierre *et al.*, 1999). Analysis of early and late maturity COMT down-regulated alfalfa revealed a mean ethanol conversion efficiency increase of 8% compared to wild-type (Dien *et al.*, 2011).

In stem cross sections of COMT down-regulated alfalfa, lignin accumulates unevenly between the pith rays, with lignin autofluorescence being strong in some portions compared to others (Nakashima *et al.*, 2008). The portions with strong autofluorescence have larger vascular cells with a normal number of cells per cluster, whereas the portions of stem sections with less autofluorescence have normal sized cells with a lower number of cells per cluster. This imbalance across the stem sections may be the reason for the increased lodging compared to controls observed in the field (Nakashima *et al.*, 2008). There is greater reduction in thioacidolysis lignin content in fiber cells and to some extent in parenchyma cells but an increase in lignin content of vascular cells in the fifth internode of COMT down-regulated alfalfa compared to controls (Nakashima *et al.*, 2008). However, it must be remembered that thioacidolysis yields are deceptive here, as 5-hydroxyguaiacyl monomers are released from benzodioxane structures significantly less efficiently than are the normal S and G monomers from β -ether units.

In the forage legume *Stylosanthes humilis*, histochemical analysis revealed that COMT down-regulation leads to no apparent change in lignin content, but a drastic reduction in S lignin levels (Rae *et al.*, 2001). The *in vitro* digestibility in rumen fluid and acid pepsin is increased by 16% compared to controls.

The corn *bm3* mutant has a brown coloration of the leaf midrib in internodes at the flowering stage. The Klason lignin content is reduced by ~20%, with reduced S lignin content, appearance of 5-hydroxy guaiacyl units, and elevated levels of ferulic acid monomers and dimers (Marita *et al.*, 2003b). The *bm3* mutant and transgenic COMT down-regulated corn show similar (30–40%) decreases in wall esterified 4-coumaric acid

content (Piquemal *et al.*, 2002; Marita *et al.*, 2003b). The S/G ratio in *bm3* is decreased by more than 2-fold to 0.06, and NMR analysis reveals the presence of benzodioxane units in the isolated lignin. The *bm3* mutant has 15% higher cell wall degradability by cellulase treatment (Marita *et al.*, 2003b), whereas in COMT down-regulated corn, the digestibility of stems and leaves is increased by 7% and 2%, respectively (Piquemal *et al.*, 2002). Commercial *bm3* corn has been developed and comprises about 5% of the silage market in Canada (Zandbergen, 2006). It has lower yield compared to controls, but improves milk production (Oba & Allen, 1999).

Sorghum *bmr12* is a knockout mutant of COMT with 13% reduced acid detergent lignin content, decreased S lignin content and increased *in vitro* dry matter digestibility (Sattler *et al.*, 2010). Conversion of cellulose to ethanol in sorghum *bmr12* biomass after acid pretreatment is increased by 21% compared to controls (Dien *et al.*, 2009).

COMT down-regulation in switchgrass results in plants with normal growth and development (Fu *et al.*, 2011a); the same is observed in perennial ryegrass (Tu *et al.*, 2010) and tall fescue (Chen *et al.*, 2004). The digestibility of switchgrass, perennial ryegrass and tall fescue biomass increased by 9%, 8.6% and 10.8% respectively compared to their respective controls. In switchgrass, the overall ethanol yield after saccharification and fermentation is increased by 38% compared to controls (Fu *et al.*, 2011).

COMT-suppression results in the formation of a red-brown woody stem with no change in lignin content in aspen (Tsai *et al.*, 1998), and brownish coloration at the basal internode of the stem in switchgrass (Fu *et al.*, 2011). Suppression of COMT in poplar was shown to reduce lignin content, as determined by the Klason and acetyl bromide methods, in one study (Jouanin *et al.*, 2000), but no change was reported in another using the same analytical methods (Van Doorsselaere *et al.*, 1995). The pulping efficiency of COMT suppressed poplar plants with reduced S lignin content is reduced drastically (Lapierre *et al.*, 1999; Pilate *et al.*, 2002). This is presumably because, despite there being β -ethers, benzodioxanes are inefficiently cleaved under alkaline pulping conditions, unlike the syringyl counterparts they replace (Ralph *et al.*, 2004).

Field trials of COMT down-regulated transgenic poplar plants showed that growth indicators and interactions with insects were normal (Pilate *et al.*, 2002). The down-regulation of COMT in silver birch led to no change in

lignin content, and did not affect the feeding preference of insect herbivores or the interactions with the ectomycorrhizal fungus *Paxillus involutus* (Tiimonen *et al.*, 2005; Sutela *et al.*, 2009).

The *Atcomt1* mutant of Arabidopsis totally lacks S units and has a slight increase in lignin content (Goujon *et al.*, 2003a). COMT down-regulation in tobacco has been reported to either lower the lignin content (Ni *et al.*, 1994) or result in no change (Dwivedi *et al.*, 1994; Atanassova *et al.*, 1995, Zhong *et al.*, 1998). Cell wall digestibility of COMT down-regulated tobacco is increased by up to 48% (Sewalt *et al.*, 1997b).

1.4.9 Cinnamoyl-CoA Reductase

CCR suppressed alfalfa, Arabidopsis, *Medicago truncatula*, Norway spruce, poplar (*Populus tremula x alba*), perennial ryegrass, tobacco and tomato have been reported with various degrees of reduction in lignin content, and with severe down-regulation leading to a dwarf phenotype (Fig. 1.4C) with collapsed vessels.

In alfalfa, CCR down-regulation results in reduced biomass, 30% reduction in lignin content of mature stem internodes, and an increase in S/G ratio (Jackson *et al.*, 2008). Total lignin autofluorescence is low in stem sections, with significantly weaker S lignin staining than in controls and with the tangential walls staining unevenly, indicating the presence of pits between vascular elements (Nakashima *et al.*, 2008). The diameter of the vascular cells of these plants is significantly increased, and the vessels are rounder in shape than those of the wild-type (Fig. 1.3A). The thioacidolysis lignin yield is reduced drastically in vascular, fiber and parenchyma cells, with H lignin almost completely absent in vascular cells (Nakashima *et al.*, 2008). This may explain the unusual cell shape, as H lignin has been proposed to determine the shape of vascular cells by acting as a matrix for deposition of G and S units (Terashima *et al.*, 1998). *In vitro* dry matter digestibility increases by 5-10% in these plants and the saccharification efficiency is increased by up to 50% (Jackson *et al.*, 2008).

Several Arabidopsis *AtCCR1* knockout mutants have been characterized. An *irx4* mutant (Jones *et al.*, 2001), as well as *ccr1s* and *ccr1g* (Mir Derikvand *et al.*, 2008) exhibit about 25-50% reductions in lignin content, similar to transgenic *CCR1* down-regulated Arabidopsis (Goujon *et al.*, 2003b). *CCR1* mutants of Arabidopsis show delayed senescence compared to wild-type (Patten *et al.*, 2005; Laskar *et al.*, 2006; Mir Derikvand *et al.*, 2008) and dwarfness with altered architecture of the cell walls where the S2 sublayer is affected (Jones *et al.*,

2001; Goujon *et al.*, 2003, Ruel *et al.*, 2009). The S/G ratio is decreased in interfascicular fibers but increased in vascular bundles (Ruel *et al.*, 2009), and the overall S/G ratios are decreased at the 6 week-old stage but comparable to wild-type at the 9 week-old stage (Laskar *et al.*, 2006). Increased accumulation of ferulic acid is seen in the walls of *ccr1* mutants (Mir Derikvand *et al.*, 2008), and there are also changes in soluble phenolics including an increase in feruloyl malate. *CCR1* down-regulation has a positive impact on cell wall digestibility of *Arabidopsis* (Goujon *et al.*, 2003b). Simultaneous suppression of CCR and cinnamyl alcohol dehydrogenase (CAD) in *Arabidopsis* leads to a severe dwarf phenotype, red coloration of xylem, accumulation of soluble phenolics (sinapoyl malate, feruloyl malate and flavanol glucosides), and male sterility due to the absence of lignified thickening in the endothecium resulting in indehiscence of anthers (Thevenin *et al.*, 2011).

Transgenic tobacco plants with strong down-regulation of CCR activity leading to a 50% reduction in lignin exhibit a dwarf phenotype, abnormal leaf development, and orange-brown xylem probably due to the presence of phenolics like ferulate and sinapate (Piquemal *et al.*, 1998; Chabannes *et al.*, 2001a). The S/G ratio is increased, with lignin deposition affected in the inner S2 and S3 layers of the fibers and vessels (Piquemal *et al.*, 1998; Chabannes *et al.*, 2001a). The lignin of CCR down-regulated tobacco has increased content of tyramine ferulate, an unusual component of tobacco cell walls (Ralph *et al.*, 1998). Inclusion of ferulic acid, as a monomer, into lignin provides a new mechanism for producing branch points in the polymer (Ralph *et al.*, 2008). Transcriptomic and metabolomic analysis of CCR down-regulated tobacco suggest increased photo-oxidative stress and photorespiration (Dauwe *et al.*, 2007). Surprisingly, and in sharp contrast to the situation in *Arabidopsis* described above, crossing of CCR and CAD down-regulated tobacco resulted in progeny that looked normal, with increased S/G ratio and slightly elevated tyramine ferulate levels when compared to plants down-regulated in CAD or CCR alone (Chabannes *et al.*, 2001a).

CCR down-regulated tomato plants show a severe dwarf phenotype with 38% reduction in Klason lignin content, brown-orange coloration of the xylem, and slightly collapsed xylem vessels (van der Rest *et al.*, 2006). These plants have higher contents of chlorogenic acid and rutin, and accumulate N-caffeoyl putrescine and kaempferol rutinoside that are not seen in the wild-type.

CCR1 down-regulated perennial ryegrass shows up to 37% reduction in lignin content, little change in S/G ratio, and increased accumulation of ferulic, sinapic, and *O*-caffeoyl quinic acids and flavonol glucosides. Field-grown transgenics exhibited a normal growth phenotype and up to 13.6% improved digestibility (Tu *et al.*, 2010).

In poplar, down-regulation of CCR is associated with reduced growth, orange-brown coloration of the outer xylem, concentric sublayers in the S2 layer of fibers and S2 and S3 layers of vessels in the orange-brown area, reduced lignin and hemicellulose content, lower S/G ratio, and increased accumulation of ferulate esters (Leple *et al.*, 2007). Similarly to in CCoAOMT down-regulated poplar plants (Meyermans *et al.*, 2000), glucosides of vanillic and sinapic acids accumulate in CCR down-regulated poplar, but caffeic acid glucosides are absent. Down-regulation of CCR in Norway spruce results in plants with reduced stem diameter, 8% reduced lignin content and 34% reduced H lignin compared to the control (Wadenback *et al.*, 2008). CCR down-regulated tobacco, poplar and Norway spruce show improved pulping efficiency but the plants are compromised due to the growth defects (O'Connell *et al.*, 2002; Leple *et al.*, 2007; Wadenback *et al.*, 2008).

CCR1 down-regulated alfalfa lines have reduced lignin content and increased S/G ratio, but plants do not show a colored xylem phenotype (Jackson *et al.*, 2008). Some lines exhibit reduced biomass production. *M. truncatula* CCR1 completely, and CCR2 partially, complement the *irx4* CCR mutation in Arabidopsis (Zhou *et al.*, 2010). *M. truncatula* CCR1 knock-out mutants exhibit a severely dwarf phenotype (Fig. 1.4C) with highly reduced lignin content, whereas *MtCCR2* mutants have only a moderate reduction in lignin content and appear phenotypically normal (Zhou *et al.*, 2010).

1.4.10 Cinnamyl alcohol dehydrogenase

CAD down-regulation has been studied in transgenic alfalfa, tobacco, poplar (*Populus tremula* x *alba*), flax, tall fescue, and switchgrass, and CAD mutants of Arabidopsis, *Medicago truncatula*, pine, corn, rice and sorghum have been identified. In general, CAD-suppressed plants do not show a dwarf phenotype, have modest changes in lignin content, and have a red-brown coloration of the xylem in stems.

Alfalfa and *M. truncatula* plants with reduced CAD activity show decreased biomass yields in some lines (Fig. 1.4E), red pigmentation in vascular tissue due to accumulation of monolignol precursor hydroxycinnamaldehyde units, up to 10% reduction in lignin content, and a decreased S/G ratio (Baucher *et al.*, 1999; Jackson *et al.*, 2008). The mean area of individual vascular cells is reduced in comparison to the controls (Nakashima *et al.*, 2008) (Fig. 1.3A). The thioacidolysis yield from lignin is reduced in vascular, fiber and parenchyma cells by more than 70%. Sinapaldehyde and coniferaldehyde markers (Lapierre *et al.*, 2004) were detected in CAD down-regulated alfalfa using GC/MS during thioacidolysis, but could not be quantified due to the low amount (Jackson *et al.*, 2008) – incorporated aldehydes as their 8-O-4-ethers cleave more poorly than their β -O-4-ether counterparts in normal units. CAD down-regulation had little impact on saccharification efficiency, which was improved marginally only in a few lines, but *in vitro* dry matter digestibility of CAD down-regulated alfalfa was increased by up to 16% compared to controls (Baucher *et al.*, 1999; Jackson *et al.*, 2008).

The *gold hull and internode (gh)* mutants of rice exhibit reddish brown pigmentation in the hull and internode and turn golden yellow at maturation. Using map based cloning, the *gh2* mutation was identified to be in a *CAD* gene (Zhang *et al.*, 2006). The lignin content is reduced by 5-6% compared to wild-type, and H, G and S units are reduced in similar proportion (Zhang *et al.*, 2006). The *brown midrib6* mutant of sorghum is a null *CAD* allele (Saballos *et al.*, 2008, 2009; Sattler *et al.*, 2009) with a reduction in H, G and S units but greater reduction in S units leading to a reduced S/G ratio (Sattler *et al.*, 2009). Cows fed with *bmr6* silage exhibited a 10% increase in milk production (Oliver *et al.*, 2004).

The *bm1* mutation of corn has very low CAD activity (Halpin *et al.*, 1998) and increased levels of cinnamaldehydes (Halpin *et al.*, 1998; Marita *et al.*, 2003b). Lignin content is reduced by 20% with no change in S/G ratio. The *bm1* gene may encode a transcription factor that controls monolignol genes and suppresses CAD (Guillaumie *et al.*, 2007).

CAD down-regulation in tall fescue leads to ~15% decreased lignin content, with reduction in G and S units but not H units, and decreased S/G ratio (Chen *et al.*, 2003). The *in vitro* dry matter digestibility is increased by 7.2-9.5% compared to controls (Chen *et al.*, 2003). Similarly, CAD down-regulation in switchgrass results in a

14-22% reduction in lignin content and decreased S/G ratio, no changes in wall-bound phenolics, an increase in soluble chlorogenic acid content, and up to 20% increased digestibility and 15-35% increased saccharification efficiency (Fu *et al.*, 2011). Another study showed a more modest (2-11%) increase in saccharification efficiency in CAD down-regulated switchgrass (Saathoff *et al.*, 2011). CAD deficient sorghum also shows improved saccharification efficiency (Dien *et al.*, 2009).

The *Atcad-c* and *Atcad-d* mutants of Arabidopsis have reduced CAD activities, but only *Atcad-d* shows a (slight) reduction in lignin content, reduction of S units and incorporation of sinapaldehyde in lignin (Sibout *et al.*, 2003). According to Kim *et al.*, (2004), CAD mutants of Arabidopsis attain wild-type lignin levels and composition by maturity. The Arabidopsis *cad-c cad-d* double mutant has reduced lignin content, with incorporation of coniferaldehyde and sinapaldehyde into lignin, in both xylem vessels and fibers, leading to limp floral stems (Sibout *et al.*, 2005). The Arabidopsis *cad-c cad-d ccr1* triple mutant exhibits 50% reduction in lignin content accompanied by severely reduced growth, and male sterility due to failure of anther dehiscence (Thevenin *et al.*, 2011).

Down-regulation of CAD in poplar is associated with red xylem but no change in overall lignin amount or S/G ratio (Baucher *et al.*, 1996). Pulp efficiency is higher in these plants (Baucher *et al.*, 1996; Lapierre *et al.*, 1999; Pilate *et al.*, 2002). Levels of thioacidolysis-derived indene compounds that originate from incorporation of sinapaldehyde into lignins through 8–O–4-cross-coupling increase as a function of CAD suppression in poplar (Lapierre *et al.*, 2004). Interaction of insects with field-grown transgenic poplar trees with reduced CAD activity was normal, and no changes were detected in the microbial communities growing below these trees (Pilate *et al.*, 2002).

The CAD mutant of loblolly pine has reduced lignin content and slightly bent stems (MacKay *et al.*, 1997). In addition to mildly elevated vanillin and coniferaldehyde levels in the lignin, dihydroconiferyl alcohol is present in the lignin in high amounts (Ralph *et al.*, 1997); its derivation remains unclear.

Down-regulation of CAD in tobacco does not alter the lignin content (Stewart *et al.*, 1997), although it leads to higher levels of hydroxycinnamaldehyde and hydroxybenzaldehyde (mainly syringaldehyde) incorporation

(Kim *et al.*, 2003) and improved pulping efficiency (O'Connell *et al.*, 2002). Transcripts of genes related to light harvesting/signaling and cell wall expansion are elevated in CAD down-regulated tobacco (Dauwe *et al.*, 2007).

In flax, decreased CAD activity results in reduced lignin content and increased levels of ferulic, 4-coumaric, caffeic and chlorogenic acids (Wrobel-Kwatowska *et al.*, 2007). Many of the transformants show dramatic growth reduction, but some plants look phenotypically normal. These CAD down-regulated plants also show improved mechanical properties but reduced resistance to *Fusarium oxysporium* (Wrobel-Kwatowska *et al.*, 2007).

1.5 Impacts of lignin modification by targeting TFs

Lignin deposition during plant secondary cell wall formation is controlled by a network of tissue specific TFs (Umezawa, 2009; Zhao & Dixon, 2011). This network coordinates monolignol biosynthesis into the broader program of secondary cell wall biosynthesis, and therefore consists of both master switches, that control multiple features of secondary wall formation, and lignin-pathway specific downstream TFs (Fig. 1.2). There are clear distinctions and outcomes between down-regulating lignin biosynthesis through targeting biosynthetic enzymes or regulatory TFs. In the former case, the monolignol pathway may remain functional up to the step that has down-regulated, resulting in metabolic spillover. In the latter case, metabolic spillover may not occur as the pathway has not been activated at any stage. It might therefore be predicted that targeting TFs would have less deleterious effects on plant growth and development than targeting biosynthetic enzymes. This appears to be at least partly correct, as seen in the following examples.

1.5.1 NAC master switches

Secondary wall containing cell types in stems provide mechanical strength to support the plant's weight, and facilitate the transport of water and nutrients. In reproductive organs, secondary cell walls at the valve margin of the siliques and in the endothecium of the anthers are required for seed shedding and pollen release, respectively. In the anther endothecium of *Arabidopsis thaliana*, secondary wall thickening is controlled non-redundantly by two NAC transcription factors, NST1 and NST2, and knock-out of both is required to prevent anther dehiscence

(Mitsuda *et al.*, 2005). Likewise, double knock-out of *NST1* and another NAC TF, *NST3/SND1*, is necessary for the striking phenotype of loss of secondary cell wall development in interfascicular and vascular fibers of the stem. In contrast to the requirement for redundant NAC TFs in Arabidopsis, *M. truncatula* possesses a single fiber-specific secondary wall NAC TF, MtNST1, which orchestrates multiple features of cell wall development in different tissues (Zhao *et al.*, 2010a). *Tnt1* retrotransposon insertions in the *MtNST1* gene result not only in loss of lignification in the interfascicular region of the stem (Fig. 1.4F & Fig. 1.4G) and in prevention of anther dehiscence, but also in alterations in the cell walls of stomatal guard cells (loss of fluorescence likely due to the loss of wall-bound ferulate esters). Saccharification efficiency of *MtNST1* transposon insertion mutants is increased by up to 23% following acid pre-treatment, and by 18% with no pretreatment. Loss of function of *MtNST1* has a relatively minor impact on overall plant growth; stem length is reduced by around 25% but average leaf area is increased by around 14%. The reduction in stem length is mainly the result of reduction of internode length in the most mature internodes. Flowering time and stem diameter are not affected in the mutant lines, but lodging is increased (Fig. 1.4H) (Zhao *et al.*, 2010a).

1.5.2 MYB repressors of monolignol biosynthesis

MYB transcriptional repressors of phenylpropanoid biosynthesis were first described in *Antirrhinum majus* (Tamagnone *et al.*, 1998). A switchgrass ortholog, PvMYB4, binds to the AC-rich elements of lignin biosynthetic genes both *in vitro* and in a yeast transcription system (Shen *et al.*, 2012). Ectopic over-expression of PvMYB4 in transgenic switchgrass results in the down-regulation of most monolignol pathway genes, reduced lignin content and ester linked 4-coumarate to ferulate ratio, reduced plant stature but increased tillering, and an approximately three-fold increase in sugar release efficiency from cell wall residues without pre-treatment (Shen *et al.*, 2012).

1.5.3 WRKY repressors of lignification in pith

During a forward genetic screen for *Medicago truncatula* mutants with reduced lignin autofluorescence, some plants were observed to possess ectopic lignification throughout the central pith tissues of the stem (Fig. 1.4I &

Fig. 1.4J) (Wang *et al.*, 2011). This phenotype was caused by the presence of a *Tnt1* retrotransposon insertion in a gene encoding a WRKY family transcription factor. *T-DNA* insertional knock-out of the orthologous *WRKY-12* gene in *Arabidopsis* led to the same phenotype, and this could be complemented by the *Medicago* *WRKY* gene. In addition to increased lignification, *WRKY* knock-out plants possess highly thickened pith cell walls with increased levels of cellulose and hemicellulose, such that the overall biomass density of the stems is increased by up 50% (Wang *et al.*, 2011). Remarkably, this does not appear to negatively affect overall biomass in either *Arabidopsis* or *Medicago* (Fig. 1.4K).

1.6 Monolignol pathway modification and plant growth

Generally, down-regulation of lignin biosynthesis at the level of CCoAOMT, COMT, F5H and CAD does not result in severe negative effects on plant growth. This can be explained by the possibility of redundancy at the 3-*O*-methylation step (COMT/CCoAOMT double knock-down lines do have a dwarf phenotype), replacement of S units by 5-OH-G units in F5H knock-outs, or incorporation of aldehydes into the lignin of CAD deficient plants. Excluding PAL and C4H, deficiency of which has major pleiotropic effects, this leaves the question of why targeting 4CL, HCT, C3H and CCR leads to dwarf plants. Several hypotheses have been proposed, of two types. In the first type, metabolic spillover from monolignol biosynthesis into flavonoids (levels of which are clearly elevated in C3'H and HCT-down-regulated alfalfa and *Arabidopsis*) indirectly leads to growth inhibition through processes such as inhibition of auxin transport (Besseau *et al.*, 2007), or else growth is affected by other metabolites that might interfere with hormone action. In the second type, it is not the metabolic spillover itself that is the primary reason for impaired growth, but rather the “incorrectly” lignified cell walls which the plant senses (Li *et al.*, 2010b), possibly through the release of oligosaccharide signal molecules (Gallego-Giraldo *et al.*, 2011b). Another obvious question is whether the mechanisms of growth inhibition are the same for plants down-regulated in 4CL, C3'H, HCT or CCR.

These points are well illustrated by attempts to understand the growth phenotype of HCT down-regulated *Arabidopsis* and alfalfa. These plants are dwarf, and silencing *HCT* in *Arabidopsis* leads to accumulation of flavonoids and inhibition of auxin transport (Besseau *et al.*, 2007). Restoring auxin transport by reducing

flavonoid content was reported to overcome the dwarf phenotype while maintaining the reduced lignin phenotype (Besseau *et al.*, 2007), although these results could not be reproduced in a more recent study (Li *et al.*, 2010b). Furthermore, HCT down-regulated alfalfa plants also have increased levels of flavonoids, but auxin transport appears normal (Gallego-Giraldo *et al.*, 2011b). The dwarf phenotype of HCT-down-regulated *Arabidopsis* plants is overcome when lignin accumulation is restored by expression of a *Selaginella* enzyme that by-passes the HCT reactions (Li *et al.*, 2010b). This suggests that structural effects in partially lignified secondary cell walls are in some way linked to the growth defects. These could operate in two ways: either defective cell wall structure leads to physiological stress effects associated, for example, with leakiness of conducting vessels, or possibly incorrectly lignified cell walls release signal molecules that actively perturb growth.

Evidence for the latter model comes from studies with HCT down-regulated alfalfa. Microarray analysis indicated that these plants massively over-express a number of pathogenesis-related (PR) genes, as though they were under microbial attack (Gallego-Giraldo *et al.*, 2011b), and cold water treatment of cell walls from the transgenic plants, but not control plants, releases pectic polysaccharides that induce the same set of genes when added to cell suspension cultures (Gallego-Giraldo *et al.*, 2011b). Furthermore, the plants have elevated levels of the defense hormone salicylic acid, known to be required for induction of PR proteins (Gallego-Giraldo *et al.*, 2011b). At this time it is not possible to be sure whether the elevated salicylate levels occur as a result of the action of wall-released pectic elicitors, or whether they occur through spillover from the lignin pathway (salicylate is biosynthetically related to lignin via the shikimic acid pathway, and can also be formed from cinnamate). The interesting observation that levels of salicylate are inversely proportional to lignin levels and growth in a series of transgenic alfalfa plants in which lignin biosynthesis has been perturbed at different biosynthetic steps (Lee *et al.*, 2011; Gallego-Giraldo *et al.*, 2011a) is essentially consistent with either hypothesis.

Reduction of salicylate levels by genetically blocking its formation or causing its removal restores growth in HCT down-regulated *Arabidopsis*, although the plants maintain reduced lignin levels (Gallego-Giraldo *et al.*,

2011a). This important observation indicates that it is theoretically possible to engineer plants with highly reduced cell wall recalcitrance without negatively impacting growth.

HCT-down-regulated alfalfa and *Arabidopsis* lack responsiveness to gibberellic acid, and this responsiveness is restored upon genetic removal of salicylate in plants that still retain the low lignin phenotype (Gallego-Giraldo *et al.*, 2011a). These data place salicylate as a central component in growth signaling pathways that either sense flux into the monolignol pathway or respond to secondary cell wall integrity. However, salicylate is clearly not central to the orchestration of growth and genetic responses in all lignin down-regulated plants. For example, although salicylate levels are increased in CCR-down-regulated alfalfa, microarray analysis of CCR down-regulated tobacco showed activation of a set of stress-responsive genes that did not include the PR protein genes induced in alfalfa and *Arabidopsis*; rather, these genes were associated with oxidative stress, perhaps arising as a result of increased photorespiration (Dauwe *et al.*, 2007).

1.7 Conclusions - it isn't all that bad!

In summary, lignin modification is a well supported approach for reducing cell wall recalcitrance in higher plants. Some genes, such as CCoAOMT and COMT, can be targeted to give significant improvements in saccharification efficiency or forage digestibility without significant effects on agronomic properties. Targeting other genes such as HCT can lead to much better improvements in saccharification efficiency, but at the cost of significantly reduced biomass yield. The negative growth impacts can, however, be overcome by additional breeding or engineering, for example to remove excess salicylate. This latter manipulation is probably not viable for field-grown plants, as it may compromise disease resistance. The fact that low lignin and poor growth can be uncoupled, however, gives hope that metabolic engineering strategies can be developed that will allow for improved growth of very low lignin plants. An alternative approach is to re-wire the transcriptional control networks to target lignin modification to specific cell types, and to replace the reduced lignin with additional polysaccharide biosynthesis; this has recently been demonstrated in *Arabidopsis* (Yang *et al.*, 2013). Finally, but beyond the scope of the present article, there is increasing evidence that reducing lignin levels results, paradoxically, in enhanced resistance against microbes (Funnell-Harris *et al.*, 2010; Gallego-Giraldo *et al.*,

2011b). Clearly, the bad and the ugly outcomes of lignin modification may yet prove to have good and attractive sides.

1.8 References

- Abdulrazzak, N., Pollet, B., Ehlting, J., *et al.* (2006) A *coumaroyl-ester-3-hydroxylase* insertion mutant reveals the existence of nonredundant *meta*-hydroxylation pathways and essential roles for phenolic precursors in cell expansion and plant growth. *Plant Physiology*, **140**, 30-48.
- Adler, E. (1977) Lignin chemistry – Past, present and future. *Wood Science and Technology*, **11**, 169-218.
- Atanassova, R., Favet, N., Martz, F., *et al.* (1995) Altered lignin composition in transgenic tobacco expressing *O*-methyltransferase sequences in sense and antisense orientation. *Plant Journal*, **8**, 465-477.
- Bate, N.J., Orr, J., Ni, W., *et al.* (1994) Quantitative relationship between phenylalanine ammonia-lyase levels and phenylpropanoid accumulation in transgenic tobacco identifies a rate-determining step in natural product synthesis. *Proceedings of the National Academy of Sciences USA*, **91**, 7608-7612.
- Baucher, M., Bernard-Vailhé, M.A., Chabbert, B., *et al.* (1999) Down-regulation of cinnamyl alcohol dehydrogenase in transgenic alfalfa (*Medicago sativa* L.) and the effect on lignin composition and digestibility. *Plant Molecular Biology*, **39**, 437-447.
- Baucher, M., Chabbert, B., Pilate, G., *et al.* (1996) Red xylem and higher lignin extractability by down-regulating a cinnamyl alcohol dehydrogenase in poplar. *Plant Physiology*, **112**, 1479-1490.
- Baucher, M., Monties, B., Van Montagu, M. & Boerjan, W. (1998) Biosynthesis and genetic engineering of lignin. *Critical Reviews in Plant Science*, **17**, 125-197.
- Besseau, S., Hoffmann, L., Geoffroy, P., Lapierre, C., Pollet, B. & Legrand, M. (2007) Flavonoid accumulation in *Arabidopsis* repressed in lignin synthesis affects auxin transport and plant growth. *Plant Cell*, **19**, 148-162.
- Blee, K., Choi, J.W., O'Connell, A.P., *et al.* (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.J. & 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 Physiology*, **122**, 107-116.
- Boerjan, W., Ralph, J. & Baucher, M. (2003) Lignin biosynthesis. *Annual Review of Plant Biology*, **54**, 519-546.
- Bonavitz, N.D. & Chapple, C. (2010) The genetics of lignin biosynthesis: connecting genotype to phenotype. *Annual Review of Genetics*, **44**, 337-63.
- Boudet, A.M., Lapierre, C. & Grima-Pettenati, J. (1995) Biochemistry and molecular biology of lignification. *New Phytologist*, **129**, 203-236.
- Buxton, D.R., & Redfearn, D.D. (1997) Plant limitations to fiber digestion and utilization. *Journal of Nutrition*, **127**, 814S-818S.
- Calabrese, J.C., Jordan, D.B., Boodhoo, A., Sariaslani, S. & Vannelli, T. (2004) Crystal structure of phenylalanine ammonia-lyase: Multiple helix dipoles implicated in catalysis. *Biochemistry*, **43**, 11403-11416.
- Chabannes, M., Barakate, A., Lapierre, C., *et al.* (2001a) Strong decrease in lignin content without significant alteration of plant development is induced by simultaneous down-regulation of cinnamyl alcohol dehydrogenase (CAD) in tobacco plants (2001) *Plant Journal*, **28**, 257-270.
- Chabannes, M., Ruel, K., Yoshinaga, A., *et al.* (2001b) 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 Journal*, **26**, 271-282.
- Chapple, C.C., Vogt, T., Ellis, B.E. & Somerville, C.R. (1992) An Arabidopsis mutant defective in the general phenylpropanoid pathway. *Plant Cell*, **4**, 1413-1424.
- Chen, F. & Dixon, R.A. (2007) Lignin modification improves fermentable sugar yields for biofuel production. *Nature Biotechnology*, **25**, 759-761.
- Chen, F., Reddy, M.S.S., Temple, S., Jackson, L., Shadle, G. & Dixon, R.A. (2006) Multi-site genetic modulation of monolignol biosynthesis suggests new routes for formation of syringyl lignin and wall-bound ferulic acid in alfalfa (*Medicago sativa* L.) *Plant Journal*, **48**, 113-124.

- Chen, F., Tobimatsu, Y., Havkin-Frenkel, D., Dixon, R.A. & Ralph, J. (2012) A polymer of caffeyl alcohol in plant seeds. *Proceedings of the National Academy of Sciences USA*, **109**, 1772-1777.
- Chen, L., Auh, C.-K., Chen, F., *et al.* (2002) Lignin deposition and associated changes in anatomy, enzyme activity, gene expression, and ruminal degradability in stems of tall fescue at different developmental stages. *Journal of Agricultural and Food Chemistry*, **50**, 5558-5565.
- Chen, L., Auh, C.-K., Dowling, P., Bell, J., Lehman, D. & Wang, Z.-Y. (2004) Transgenic down-regulation of caffeic acid *O*-methyltransferase (COMT) led to improved digestibility in tall fescue (*Festuca arundinacea*). *Functional Plant Biology*, **31**, 235-245.
- Chen, L., Auh, C.K., Dowling, P., *et al.* (2003) Improved forage digestibility of tall fescue (*Festuca arundinacea*) by transgenic down-regulation of cinnamyl alcohol dehydrogenase. *Plant Biotechnology Journal*, **1**, 437-449.
- Coleman, H.D., Park, J.Y., Nair, R., Chapple, C. & Mansfield, S.D. (2008a) RNAi-mediated suppression of *p*-coumaroyl-CoA 3'-hydroxylase in hybrid poplar impacts lignin deposition and soluble secondary metabolism. *Proceedings of the National Academy of Sciences USA*, **105**, 4501-4506.
- Coleman, H.D., Samuels, A.L., Guy, R.D. & Mansfield, S.D. (2008b) Perturbed lignification impacts tree growth in hybrid poplar-A function of sink strength, vascular integrity, and photosynthetic assimilation. *Plant Physiology*, **148**, 1229-1237.
- Costa, M.A., Bedgar, D.L., Moinuddin, S.G., *et al.* (2005) Characterization *in vitro* and *in vivo* of the putative multigene 4-coumarate:CoA ligase network in Arabidopsis: syringyl lignin and sinapate/sinapyl alcohol derivative formation. *Phytochemistry*, **66**, 2072-2091.
- Dauwe, R., Morreel, K., Goeminne, G., *et al.* (2007) Molecular phenotyping of lignin-modified tobacco reveals associated changes in cell-wall metabolism, primary metabolism and photorespiration. *Plant Journal*, **52**, 263-285.
- Davin, L.B. & Lewis, N.G. (1992) Phenylpropanoid metabolism: biosynthesis of monolignols, lignans and neolignans, lignins and suberins. In: *Phenolic Metabolism in Plants*, (eds. H.A. Stafford & R.K. Ibrahim), pp. 325-375. Plenum Press, New York.

- Day, A., Neutelings, G., Nolin, F., *et al.* (2009) Caffeoyl coenzyme A *O*-methyltransferase down-regulation is associated with modifications in lignin and cell-wall architecture in flax secondary xylem. *Plant Physiology and Biochemistry*, **47**, 9-19.
- Demura, T. & Fukuda, H. (2007) Transcriptional regulation in wood formation. *Trends in Plant Science*, **12**, 64-70.
- Demura, T., & Ye, Z.H. (2010) Regulation of plant biomass production. *Current Opinion in Plant Biology*, **13**, 299-304.
- Dien, B.S., Miller, D.J., Hector, R.E., *et al.* (2011) Enhancing alfalfa conversion efficiencies for sugar recovery and ethanol production by altering lignin composition. *Bioresource Technology*, **102**, 6479-6486.
- Dien, B.S., Sarath, G., Pedersen, J.F., *et al.* (2009) Improved sugar conversion and ethanol yield for forage sorghum (*Sorghum bicolor* L. Moench) lines with reduced lignin contents. *BioEnergy Research*, **2**, 153-164.
- Dixon, R.A. & Reddy, M.S.S. (2003) Biosynthesis of monolignols. Genomic and reverse genetic approaches. *Phytochemical Reviews*, **2**, 289-306.
- Do, C.-T., Pollet, B., Thevenin, J., *et al.* (2007) Both caffeoyl coenzyme A 3-*O*-methyltransferase 1 and caffeic acid *O*-methyltransferase 1 are involved in redundant functions for lignin, flavonoids and sinapoyl malate biosynthesis in Arabidopsis. *Planta*, **226**, 1117-1129.
- Donaldson, L.A. (2001) Lignification and lignin topochemistry-an ultrastructural view. *Phytochemistry*, **57**, 859-873.
- Donaldson, L.A. (2002) Abnormal lignin distribution in wood from severely drought stressed *Pinus radiata* trees. *IAWA Journal*, **23**, 161-178.
- Dwivedi, U.N., Campbell, W.H., Yu, J., *et al.* (1994) Modification of lignin biosynthesis in transgenic *Nicotiana* through expression of an antisense *O*-methyltransferase gene from *Populus*. *Plant Molecular Biology*, **26**, 61-71.

- Edwards, K., Cramer, C.L., Bolwell, G.P., Dixon, R.A., Schuch, W. & Lamb, C.J. (1985) Rapid transient induction of phenylalanine ammonia-lyase mRNA in elicitor-treated bean cells. *Proceedings of the National Academy of Sciences USA*, **82**, 6731-6735.
- Ehlting, J., Buttner, D., Wang, Q., Douglas, C.J., Somssich, I.E. & Kombrink, E. (1999) Three 4-coumarate: Coenzyme A ligases in *Arabidopsis thaliana* represent two evolutionary classes in angiosperms. *Plant Journal*, **19**, 9-20.
- Elkind, Y., Edwards, R., Mavandad, M., *et al.* (1990) Abnormal plant development and down-regulation of phenylpropanoid biosynthesis in transgenic tobacco containing a heterologous phenylalanine ammonia-lyase gene. *Proceedings of the National Academy of Sciences USA*, **87**, 9057-9061.
- Espineira, J.M., Novo Uzal, E., Gomez Ros, L.V., *et al.* (2011) Distribution of lignin monomers and the evolution of lignification among lower plants. *Plant Biology*, **13**, 59-68.
- Fahrendorf, T. & Dixon, R.A. (1993) Stress responses in alfalfa (*Medicago sativa* L.). XVIII: Molecular cloning and expression of the elicitor-inducible cinnamic acid 4-hydroxylase cytochrome P450. *Archives of Biochemistry and Biophysics*, **305**, 509-515.
- Felton, G.W., Korth, K.L., Bi, J.L., *et al.* (1999) Inverse relationship between systemic resistance of plants to microorganisms and to insect herbivory. *Current Biology*, **9**, 317-320.
- Fergus, B.J., Procter, A.R., Scott, J.A.N. & Goring, D.A.I. (1969) The distribution of lignin in spruce-wood as determined by ultraviolet microscopy. *Wood Science and Technology*, **3**, 117-138.
- Ferrer, J.-L., Zubieta, C., Dixon, R.A. & Noel, J.P. (2005) Crystal structures of alfalfa caffeoyl Coenzyme A 3-O-methyltransferase. *Plant Physiology*, **137**, 1009-1017.
- Finkle, B.J. & Nelson, R.F. (1963) Enzyme reactions with phenolic compounds: a *meta*-O-methyltransferase in plants. *Biochimica et Biophysica Acta*, **78**, 747-749.
- Franke, R., Hemm, M.R., Denault, J.W., Ruegger, M.O., Humphreys, J.M. & Chapple, C. (2002b) Changes in secondary metabolism and deposition of an unusual lignin in the ref8 mutant of *Arabidopsis*. *Plant Journal*, **30**, 47-59.

- Franke, R., Humphreys, J.M., Hemm, M.R., *et al.* (2002a) The Arabidopsis REF8 gene encodes the 3-hydroxylase of phenylpropanoid metabolism. *Plant Journal*, **30**, 33-45.
- Freudenberg, K. & Neish, A.C. (1968) *Constitution and Biosynthesis of Lignin*, Springer-Verlag, Berlin-Heidelberg-New York.
- Fu, C., Mielenz, J.R., Xiao, X., *et al.* (2011a) Genetic manipulation of lignin reduces recalcitrance and improves ethanol production from switchgrass. *Proceedings of the National Academy of Sciences USA*, **108**, 3803-3808.
- Fu, C.-X., Xiao, X., Xi, Y., *et al.* (2011b) Downregulation of cinnamyl alcohol dehydrogenase (CAD) leads to improved saccharification efficiency in switchgrass. *BioEnergy Research*, **4**, 153-164.
- Funnell-Harris, D.L., Pedersen, J.F. & Sattler, S.E. (2010) Alteration in lignin biosynthesis restricts growth of *Fusarium* spp. in brown midrib sorghum. *Phytopathology*, **100**, 671-681.
- Gallego-Giraldo, L., Escamilla-Trevino, L., Jackson, L.A. & Dixon, R.A. (2011a) Salicylic acid mediates the reduced growth of lignin down-regulated plants. *Proceedings of the National Academy of Sciences USA*, **108**, 20814-20819.
- Gallego-Giraldo, L., Jikumaru, Y., Kamiya, Y., Tang, Y. & Dixon, R.A. (2011b) Selective lignin downregulation leads to constitutive defense response expression in alfalfa (*Medicago sativa* L.) *New Phytologist*, **190**, 627-639.
- Goujon, T., Ferret, V., Mila, I., *et al.* (2003b) Down-regulation of the *AtCCR1* gene in *Arabidopsis thaliana*: effects on phenotype, lignins and cell wall degradability. *Planta*, **217**, 218-228.
- Goujon, T., Sibout, R., Pollet, B., *et al.* (2003a) A new *Arabidopsis thaliana* mutant deficient in the expression of *O*-methyltransferase impacts lignins and sinapoyl esters. *Plant Molecular Biology*, **51**, 973-989.
- Grabber, J.H., Ralph, J., Hatfield, R.D. & Quideau, S. (1997) *p*-Hydroxyphenyl, guaiacyl, and syringyl lignins have similar inhibitory effects on wall degradability. *Journal of Agricultural and Food Chemistry*, **45**, 2530-2532.

- Gross, G.G., Stockigt, J., Mansell, R.L. & Zenk, M.H. (1973) Three novel enzymes involved in reduction of ferulic acid to coniferyl alcohol in higher plants: ferulate:CoA ligase, feruloyl-CoA reductase and coniferyl alcohol reductase. *FEBS Letters*, **31**, 283-286.
- Guillaumie, S., Pichon, M., Martinant, J.P., Bosio, M., Goffner, D. & Barrière, Y. (2007) Differential expression of phenylpropanoid and related genes in *brown-midrib bm1*, *bm2*, *bm3*, and *bm4* young near-isogenic maize plants. *Planta*, **226**, 235-250.
- Guo, D., Chen, F., Inoue, K., Blount, J.W. & Dixon, R.A. (2001a) Down-regulation 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., Wheeler, J.S., *et al.* (2001b) Improvement of in-rumen digestibility of alfalfa forage by genetic manipulation of lignin O-methyltransferases. *Transgenic Research*, **10**, 457-464.
- Hahlbrock, K. & Grisebach, H. (1970) Formation of coenzyme A esters of cinnamic acids with an enzyme preparation from cell suspension cultures of parsley. *FEBS Letters*, **11**, 62-64.
- Halpin, C., Holt, K., Chojecki, J., *et al.* (1998) *Brown-midrib* maize (*bm1*)--a mutation affecting the cinnamyl alcohol dehydrogenase gene. *Plant Journal*, **14**, 545-553.
- Hoffmann, L., Besseau, S., Geoffroy, P., *et al.* (2004) Silencing of hydroxycinnamoyl-coenzyme A shikimate/quinate hydroxycinnamoyltransferase affects phenylpropanoid biosynthesis. *Plant Cell*, **16**, 1446-1465.
- Hoffmann, L., Maury, S., Martz, F., Geoffroy, P. & Legrand, M. (2003) Purification, cloning, and properties of an acyltransferase controlling shikimate ester intermediates in phenylpropanoid metabolism. *Journal of Biological Chemistry*, **278**, 95-103.
- Hu, W.-J., Harding, S.A., Lung, J., *et al.* (1999) Repression of lignin biosynthesis promotes cellulose accumulation and growth in transgenic trees. *Nature Biotechnology*, **17**, 808-812.
- Hu, W.-J., Kawaoka, A., Tsai, C.-J., *et al.* (1998) Compartmentalized expression of two structurally and functionally distinct 4-coumarate ligase genes in aspen (*Populus tremuloides*). *Proceedings of the National Academy of Sciences USA*, **95**, 5407-5412.

- Huang, J., Gu, M., Lai, Z., *et al.* (2010) Functional analysis of the Arabidopsis *PAL* gene family in plant growth, development, and response to environmental stress. *Plant Physiology*, **153**, 1526-1538.
- Humphreys, J.M., Hemm, M.R. & Chapple, C. (1999) Ferulate 5-hydroxylase from Arabidopsis is a multifunctional cytochrome P450-dependent monooxygenase catalyzing parallel hydroxylations in phenylpropanoid metabolism. *Proceedings of the National Academy of Sciences USA*, **96**, 10045-10050.
- Huntley, S.K., Ellis, D., Gilbert, M., Chapple, C. & Mansfield, S.D. (2003) Significant increases in pulping efficiency in C4H-F5H-transformed poplars: improved chemical savings and reduced environmental toxins. *Journal of Agricultural and Food Chemistry*, **51**, 6178-6183.
- Inoue, K., Sewalt, V.J.H., Balance, G.M., Ni, W., Sturzer, C. & 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 Physiology*, **117**, 761-770.
- Jackson, L.A., Shadle, G.L., Zhou, R., Nakashima, J., Chen, F. & Dixon, R.A. (2008) Improving saccharification efficiency of alfalfa stems through modification of the terminal stages of monolignol biosynthesis. *Bioenergy Research*, **1**, 180-192.
- Jones, L., Ennos, A.R. & Turner, S.R. (2001) Cloning and characterization of *irregular xylem4 (irx4)*: a severely lignin-deficient mutant of Arabidopsis. *Plant Journal*, **26**, 205-216.
- Jouanin, L., Goujon, T., deNadai, V., *et al.* (2000) Lignification in transgenic poplars with extremely reduced caffeic acid *O*-methyltransferase activity. *Plant Physiology*, **123**, 1363-1373.
- Jung, H.G. & Deetz, D.A. (1993) Cell wall lignification and degradability. In: *Forage cell wall structure and digestibility*, (eds. H.G. Jung, D.R. Buxton, R.D. Hatfield, & J. Ralph), pp. 315-346. ASA-CSSA-SSSA, Madison, WI.
- Jung, H.G., Sheaffer, C.C., Barnes, D.K. & Halgerson, J.L. (1997) Forage quality variation in the US alfalfa core collection. *Crop Science*, **37**, 1361-1366.
- Kajita, S., Hishiyama, S., Tomimura, Y., Katayama, Y. & 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 Physiology*, **114**, 871-879.

- Kajita, S., Katayama, Y. & Omori, S. (1996) Alterations in the biosynthesis of lignin in transgenic plants with chimeric genes for 4-coumarate:CoA ligase. *Plant and Cell Physiology*, **37**, 957-965.
- Kim, H., Ralph, J., Lu, F., *et al.* (2003) NMR analysis of lignins in CAD-deficient plants. Part 1. Incorporation of hydroxycinnamaldehydes and hydroxybenzaldehydes into lignins. *Organic and Biomolecular Chemistry*, **1**, 268-281.
- Kim, S.J., Kim, M.R., Bedgar, D.L., *et al.* (2004) Functional reclassification of the putative cinnamyl alcohol dehydrogenase multigene family in *Arabidopsis*. *Proceedings of the National Academy of Sciences USA*, **101**, 1455-1460.
- Kneusel, R.E., Matern, U. & Nicolay, K. (1989) Formation of *trans*-caffeoyl-CoA from *trans*-4-coumaroyl-CoA by Zn²⁺-dependent enzymes in cultured plant cells and its activation by an elicitor-induced pH shift. *Archives of Biochemistry and Biophysics*, **269**, 455-462.
- Knobloch, K.-H. & Hahlbrock, K. (1975) Isoenzymes of *p*-coumarate: CoA ligase from cell suspension cultures of *Glycine max*. *European Journal of Biochemistry*, **52**, 311-320.
- Knobloch, K.-H. & Hahlbrock, K. (1977) 4-Coumarate: CoA ligase from cell suspension cultures of *Petroselinum hortense* Hoffm. Partial purification, substrate specificity, and further properties. *Archives of Biochemistry and Biophysics*, **184**, 237-248.
- Ko, J.H., Kim, W.C. & Han, K.H. (2009) Ectopic expression of MYB46 identifies transcriptional regulatory genes involved in secondary wall biosynthesis in *Arabidopsis*. *Plant Journal*, **60**, 649-665.
- Koehler, L. & Telewski, F.W. (2006) Biomechanics and transgenic wood. *American Journal of Botany*, **93**, 1433-1438.
- Korth, K.L., Blount, J.W., Chen, F., Rasmussen, S., Lamb, C. & Dixon, R.A. (2001) Changes in phenylpropanoid metabolites associated with homology-dependent silencing of phenylalanine ammonia-lyase and its somatic reversion in tobacco. *Physiologia Plantarum*, **111**, 137-143.
- Koukol, J. & Conn, E.E. (1961) The metabolism of aromatic compounds in higher plants. IV. Purification and properties of the phenylalanine deaminase of *Hordeum vulgare*. *Journal of Biological Chemistry*, **236**, 2692-2698.

- Kuhnl, T., Koch, U., Heller, W. & Wellmann, E. (1989) Elicitor induced S-adenosyl-L-methionine: caffeoyl-CoA 3-O-methyltransferase from carrot cell suspension cultures. *Plant Science*, **60**, 21-25.
- Lacombe, E., Van Doorsselaere, J., Boerjan, W., Boudet, A.M. & Grima-Pettenati, J. (2000) Characterization of *cis*-elements required for vascular expression of the cinnamoyl CoA reductase gene and for protein-DNA complex formation. *Plant Journal*, **23**, 663-676.
- Lapierre, C., Pilate, G., Pollet, B., *et al.* (2004) Signatures of cinnamyl alcohol dehydrogenase deficiency in poplar lignins. *Phytochemistry*, **65**, 313-321.
- Lapierre, C., Pollet, B., Petit-Conil, M., *et al.* (1999) Structural alterations of lignins in transgenic poplars with depressed cinnamoyl alcohol dehydrogenase or caffeic acid *O*-methyltransferase activity have opposite impact on the efficiency of industrial Kraft pulping. *Plant Physiology*, **119**, 153-163.
- Laskar, D.D., Jourdes, M., Patten, A.M., Helms, G.L., Davin, L.B., Lewis & N.G. (2006) The Arabidopsis cinnamoyl CoA reductase *irx4* mutant has a delayed but coherent (normal) program of lignification. *Plant Journal*, **48**, 674-686.
- Lauvergeat, V., Lacomme, C., Lacombe, E., Lasserre, E., Roby, D. & Grima-Pettenati, J. (2001) Two cinnamoyl-CoA reductase (CCR) genes from *Arabidopsis thaliana* are differentially expressed during development and in response to infection with pathogenic bacteria. *Phytochemistry*, **57**, 1187-1195.
- Lee, D., Meyer, K., Chapple, C. & 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.
- Lee, Y., Chen, F., Dixon, R.A. & Voit, E.O. (2011) Integrative analysis of transgenic alfalfa (*Medicago sativa* L.) suggests new metabolic control mechanisms for monolignol biosynthesis. *PloS Computational Biology*, May 2011, 7 (5) e1002047.
- Leplé, J.C., Dauwe, R., Morreel, K., *et al.* (2007) Downregulation of cinnamoyl-coenzyme A reductase in poplar: multiple-level phenotyping reveals effects on cell wall polymer metabolism and structure. *Plant Cell*, **19**, 3669-3691.

- Li, L., Popko, J.L., Umezawa, T. & Chiang, V.L. (2000) 5-Hydroxyconiferyl aldehyde modulates enzymatic methylation for syringyl monolignol formation, a new view of monolignol biosynthesis in angiosperms. *Journal of Biological Chemistry*, **275**, 6537-6545.
- Li, L., Zhou, Y., Cheng, X., *et al.* (2003) Combinatorial modification of multiple lignin traits in trees through multigene cotransformation. *Proceedings of the National Academy of Sciences USA*, **100**, 4939-4944.
- Li, X., Bonawitz, N.D., Weng, J.K. & Chapple, C. (2010b) The growth reduction associated with repressed lignin biosynthesis in *Arabidopsis thaliana* is independent of flavonoids. *Plant Cell*, **22**, 1620-1632.
- Li, X., Ximenes, E., Kim, Y., *et al.* (2010a) Lignin monomer composition affects *Arabidopsis* cell-wall degradability after liquid hot water pretreatment. *Biotechnology for Biofuels*, **3**, 27.
- Lindermayr, C., Mollers, B., Fliegmann, J., *et al.* (2002) Divergent members of a soybean (*Glycine max* L.) 4-coumarate:coenzyme A ligase gene family. *European Journal of Biochemistry*, **269**, 1304-1315.
- Lu, F., Marita, J.M., Lapierre, C., *et al.* (2010) Sequencing around 5-hydroxyconiferyl alcohol-derived units in caffeic acid *O*-methyltransferase-deficient poplar lignins. *Plant Physiology*, **153**, 569-579.
- Ma, Q.H. & Xu, Y. (2008) Characterization of a caffeic acid 3-*O*-methyltransferase from wheat and its function in lignin biosynthesis. *Biochimie*, **90**, 515-524.
- MacKay, J.J., O'Malley, D.M., Presnell, T., *et al.* (1997) Inheritance, gene expression, and lignin characterization in a mutant pine deficient in cinnamyl alcohol dehydrogenase. *Proceedings of the National Academy of Sciences USA*, **94**, 8255-8260.
- Maher, E.A., Bate, N.J., Ni, W., Elkind, Y., Dixon, R.A. & Lamb, C.J. (1994) Increased disease susceptibility of transgenic tobacco plants with suppressed levels of preformed phenylpropanoid products. *Proceedings of the National Academy of Sciences USA*, **96**, 7802-7806.
- Mann, D.J.G., Labbé, N., Sykes, R., *et al.* (2009) Rapid assessment of lignin content and structure in switchgrass (*Panicum virgatum* L.) grown under different environmental conditions. *BioEnergy Research*, **2**, 246-256.
- Mansell, R.L., Gross, G.G., Stockigt, J., Franke, H. & Zenk, M.H. (1974) Purification and properties of cinnamyl alcohol-dehydrogenase from higher plants involved in lignin biosynthesis. *Phytochemistry*, **13**, 2427-2435.

- Marita, J.M., Ralph, J., Hatfield, R.D. & Chapple, C. (1999) NMR characterization of lignin in *Arabidopsis* altered in the activity of ferulate 5-hydroxylase. *Proceedings of the National Academy of Sciences USA*, **96**, 12328-12332.
- Marita, J.M., Ralph, J., Hatfield, R.D., Guo, D., Chen, F. & Dixon, R.A. (2003a) 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.
- Marita, J.M., Vermerris, W., Ralph, J. & Hatfield, R.D. (2003b) Variations in the cell wall composition of maize brown midrib mutants. *Journal of Agricultural and Food Chemistry*, **51**, 1313-1321.
- Marles, M.A., Vandenberg, A. & Bett, K.E. (2008) Polyphenol oxidase activity and differential accumulation of polyphenolics in seed coats of pinto bean (*Phaseolus vulgaris* L.) characterize postharvest color changes. *Journal of Agricultural and Food Chemistry*, **56**, 7049-7056.
- Martone, P.T., Estevez, J.M., Lu, F., *et al.* (2009) Discovery of lignin in seaweed reveals convergent evolution of cell wall architecture. *Current Biology*, **19**, 169-175.
- McCarthy, R.L., Zhong, R. & Ye, Z.H. (2009) MYB83 is a direct target of SND1 and acts redundantly with MYB46 in the regulation of secondary cell wall biosynthesis in *Arabidopsis*. *Plant and Cell Physiology*, **50**, 1950-1964.
- Meyer, K., Shirley, A.M., Cusumano, J.C., Bell-Lelong, D.A. & Chapple, C. (1998) Lignin monomer composition is determined by the expression of a cytochrome P450-dependent monooxygenase in *Arabidopsis*. *Proceedings of the National Academy of Sciences USA*, **95**, 6619-6623.
- Meyermans, H., Morreel, K., Lapierre, C., *et al.* (2000) Modifications 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. *Journal of Biological Chemistry*, **275**, 36899-36909.
- Millar, D.J., Long, M., Donovan, G., *et al.* (2007) Introduction of sense constructs of cinnamate 4-hydroxylase in transgenic tomato plants shows opposite effects on flux into stem lignin and fruit flavonoids. *Phytochemistry*, **68**, 1497-1509.

- Mir Derikvand, M., Sierra, J.B., Ruel, K., *et al.* (2008) Redirection of the phenylpropanoid pathway to feruloyl malate in Arabidopsis mutants deficient for cinnamoyl-CoA reductase 1. *Planta*, **227**, 943-956.
- Mitsuda, N., Iwase, A., Yamamoto, H., *et al.* (2007) NAC transcription factors, NST1 and NST3, are key regulators of the formation of secondary walls in woody tissues of Arabidopsis. *Plant Cell*, **19**, 270-280.
- Mitsuda, N., Seki, M., Shinozaki, K. & Ohme-Takagi, M. (2005) The NAC transcription factors NST1 and NST2 of Arabidopsis regulate secondary wall thickenings and are required for anther dehiscence. *Plant Cell*, **17**, 2993-3006.
- Mizutani, M., Ward, E., DiMaio, J., Ohta, D., Ryals, J. & Sato, R. (1993) Molecular cloning and sequencing of a cDNA encoding mung bean cytochrome P450 (P450C4H) possessing cinnamate 4-hydroxylase activity. *Biochemical and Biophysical Research Communications*, **190**, 875-880.
- Morreel, K., Ralph, J., Lu, F., *et al.* (2004) Phenolic profiling of caffeic acid *O*-methyltransferase-deficient poplar reveals novel benzodioxane oligolignols. *Plant Physiology*, **136**, 4023-4036.
- Musel, G., Schindler, T., Bergfeld, R., *et al.* (1997) Structure and distribution of lignin in primary and secondary cell walls of maize coleoptiles analysed by chemical and immunological probes. *Planta*, **201**, 146-159.
- Nakashima, J., Chen, F., Jackson, L., Shadle, G. & Dixon, R.A. (2008) Multi-site genetic modification of monolignol biosynthesis in alfalfa (*Medicago sativa*): effects on lignin composition in specific cell types. *New Phytologist*, **179**, 738-750.
- Nakatsubo, T., Kitamura, Y., Sakakibara, N., *et al.* (2008) At5g54160 gene encodes Arabidopsis thaliana 5-hydroxyconiferaldehyde *O*-methyltransferase. *Journal of Wood Science*, **54**, 312-317.
- Ni, W., Paiva, N.L. & Dixon, R.A. (1994) Reduced lignin in transgenic plants containing a caffeic acid *O*-methyltransferase antisense gene. *Transgenic Research*, **3**, 120-126.
- Niggeweg, R., Michael, A.J. & Martin, C. (2004) Engineering plants with increased levels of the antioxidant chlorogenic acid. *Nature Biotechnology*, **22**, 746-754.
- Oba, M. & Allen, M.S. (1999) Effects of brown midrib 3 mutation in corn silage on dry matter intake and productivity of high yielding dairy cows, *Journal of Dairy Science*, **82**, 135-142.

- O'Connell, A., Holt, K., Piquemal, J., *et al.* (2002) Improved paper pulp from plants with suppressed cinnamoyl-CoA reductase or cinnamyl alcohol dehydrogenase. *Transgenic Research*, **11**, 495-503.
- Oliver, A.L., Grant, R.J., Pedersen, J.F. & O'Rear, J. (2004) Comparison of *brown midrib-6* and *-18* forage sorghum with conventional sorghum and corn silage in diets of lactating dairy cows. *Journal of Dairy Science*, **87**, 637-644.
- Osakabe, K., Tsao, C.C., Li, L., *et al.* (1999) Coniferyl aldehyde 5-hydroxylation and methylation direct syringyl lignin biosynthesis in angiosperms. *Proceedings of the National Academy of Sciences USA*, **96**, 8955-8960.
- Pallas, J.A., Paiva, N.L., Lamb, C. & Dixon, R.A. (1996) Tobacco plants epigenetically suppressed in phenylalanine ammonia-lyase expression do not develop systemic acquired resistance in response to infection by tobacco mosaic virus. *Plant Journal*, **10**, 281-293.
- Parvathi, K., Chen, F., Guo, D., Blount, J.W. & Dixon, R.A. (2001) Substrate preferences of *O*-methyltransferases in alfalfa suggest new pathways for 3-*O*-methylation of monolignols. *Plant Journal*, **25**, 193-202.
- Patten, A.M., Cardenas, C.L., Cochrane, F.C., *et al.* (2005) Reassessment of effects on lignification and vascular development in the *irx4 Arabidopsis* mutant. *Phytochemistry*, **66**, 2092-2107.
- Patten, A.M., Jourdes, M., Brown, E.E., Laborie, M.P., Davin, L.B. & Lewis, N.G. (2007) Reaction tissue formation and stem tensile modulus properties in wild-type and p-coumarate-3-hydroxylase downregulated lines of alfalfa, *Medicago sativa* (Fabaceae). *American Journal of Botany*, **94**, 912-925.
- Patzlaff, A., McInnis, S., Courtenay, A., *et al.* (2003) Characterisation of a pine MYB that regulates lignification. *Plant Journal*, **36**, 743-754.
- Pilate, G., Guiney, E., Holt, K., *et al.* (2002) Field and pulping performances of transgenic trees with altered lignification. *Nature Biotechnology*, **20**, 607-612.
- Pincon, G., Maury, S., Hoffmann, L., *et al.* (2001a) Repression of *O*-methyltransferase genes in transgenic tobacco affects lignin synthesis and plant growth. *Phytochemistry*, **57**, 1167-1176.

- Piquemal, J., Chamayou, S., Nadaud, I., *et al.* (2002) Down-regulation of caffeic acid *O*-methyltransferase in maize revisited using a transgenic approach. *Plant Physiology*, **130**, 1675-1685.
- Piquemal, J., Lapierre, C., Myton, K., *et al.* (1998) Down-regulation in cinnamoyl-CoA reductase induces significant changes of lignin profiles in transgenic tobacco plants. *Plant Journal*, **13**, 71-83.
- Pu, Y., Chen, F., Ziebell, A., Davison, B.H. & Ragauskas, A.J. (2009) NMR characterization of C3H and HCT downregulated alfalfa lignin. *BioEnergy Research*, **2**, 198-208.
- Rae, A.L., Manners, J.M., Jones, R.J., McIntyre, C.L. & Lu, D.Y. (2001) Antisense suppression of the lignin biosynthetic enzyme, caffeate *O*-methyltransferase, improves *in vitro* digestibility of the tropical pasture legume, *Stylosanthes humilis*. *Australian Journal of Plant Physiology*, **28**, 289-297.
- Ralph, J., Akiyama, T., Kim, H., *et al.* (2006) Effects of coumarate 3-hydroxylase downregulation on lignin structure. *Journal of Biological Chemistry*, **281**, 8843-8853.
- Ralph, J., Hatfield, R.D., Piquemal, J., *et al.* (1998) NMR characterization of altered lignins extracted from tobacco plants down-regulated for lignification enzymes cinnamyl alcohol dehydrogenase and cinnamoyl-CoA reductase. *Proceedings of the National Academy of Sciences USA*, **95**, 12803-12808.
- Ralph, J., Kim, H., Lu, F., *et al.* (2008) Identification of the structure and origin of a thioacidolysis marker compound for ferulic acid incorporation into angiosperm lignins (and an indicator for cinnamoyl CoA reductase deficiency). *Plant Journal*, **53**, 368-379.
- Ralph, J., Lapierre, C., Marita, J.M., *et al.* (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., *et al.* (2001b) NMR evidence of benzodioxane structures resulting from incorporation of 5-hydroxyconiferyl alcohol into lignins of *O*-methyltransferase-deficient plants. *Journal of Agricultural and Food Chemistry*, **49**, 86-91.
- Ralph, J., Lundquist, K., Brunow, G., *et al.* (2004) Lignins: Natural polymers from oxidative coupling of 4-hydroxyphenyl-propanoids. *Phytochemical Reviews*, **3**, 29-60.
- Ralph, J., MacKay, J.J., Hatfield, R.D., O'Malley, D.M., Whetten, R.W. & Sederoff, R.R. (1997) Abnormal lignin in a loblolly pine mutant. *Science*, **277**, 235-239.

- Reddy, M.S.S., Chen, F., Shadle, G., Jackson, L., Aljoe, H. & Dixon, R.A. (2005) Targeted down-regulation of cytochrome P450 enzymes for forage quality improvement in alfalfa (*Medicago sativa* L.). *Proceedings of the National Academy of Sciences*, **102**, 16573-16578.
- Reichert, A.I., He, X.Z. & Dixon, R.A. (2009) Phenylalanine ammonia-lyase (PAL) from tobacco (*Nicotiana tabacum*): characterization of the four tobacco PAL genes and active heterotetrameric enzymes. *Biochemical Journal*, **424**, 233-242.
- Rhodes, M.J.C. & Woollorton, L.S.C. (1976) The enzymic conversion of hydroxycinnamic acids to the p-coumaroyl quinic and chlorogenic acids in tomato fruits. *Phytochemistry*, **15**, 947-951.
- Rohde, A., Morreel, K., Ralph, J., *et al.* (2004) Molecular phenotyping of the *pal1* and *pal2* mutants of *Arabidopsis thaliana* reveals far-reaching consequences on phenylpropanoid, amino acid, and carbohydrate metabolism. *Plant Cell*, **16**, 2749-2771.
- Ruel, K., Berrio-Sierra, J., Mir Derikvand, M., *et al.* (2009) Impact of *CCR1* silencing on the assembly of lignified secondary walls in *Arabidopsis thaliana*. *New Phytologist*, **184**, 99-113.
- Russell, D.W. (1971) The metabolism of aromatic compounds in higher plants. X. Properties of the cinnamic acid 4-hydroxylase of pea seedlings and some aspects of its metabolic and developmental control. *Journal of Biological Chemistry*, **246**, 3870-3878.
- Russell, D.W. & Conn, E.E. (1967) The cinnamic acid 4-hydroxylase of pea seedlings. *Archives of Biochemistry and Biophysics*, **122**, 256-258.
- Saathoff, A.J., Sarath, G., Chow, E.K., Dien, B.S. & Tobias, C.M. (2011) Downregulation of cinnamyl-alcohol dehydrogenase in switchgrass by RNA silencing results in enhanced glucose release after cellulase treatment. *PLoS One*. Jan 27;6(1):e16416.
- Saballos, A., Ejeta, G., Sanchez, E., Kang, C. & Vermerris, W. (2009) A genomewide analysis of the cinnamyl alcohol dehydrogenase family in sorghum [*Sorghum bicolor* (L.) Moench] identifies SbCAD2 as the brown midrib6 gene. *Genetics*, **181**, 783-795.

- Saballos, A., Vermerris, W., Rivera, L. & Ejeta, G. (2008) Allelic association, chemical characterization and saccharification properties of *brown midrib* mutants of sorghum (*Sorghum bicolor* (L.) Moench). *BioEnergy Research*, **1**, 193-204.
- Saka, S. & Goring, D.A.I. (1985) Localization of lignins in wood cell walls. In: *Biosynthesis and Biodegradation of Wood Component*s, (ed. T Higuchi), pp. 51-62. Academic, Orlando.
- Sarkanen, K.V. (1971) *Lignins: Occurrence, formation, structure and reactions*. (eds. K.V. Sarkanen & C.H. Ludwig), Wiley-Interscience, New York.
- Sattler, S.E., Funnell-Harris, D.L. & Pederson, J.F. (2010) Efficacy of singular and stacked brown midrib 6 and 12 in the modification of lignocellulose and grain chemistry. *Journal of Agricultural and Food Chemistry*, **58**, 3611-3616.
- Sattler, S.E., Saathoff, A.J., Haas, E.J., *et al.* (2009) A nonsense mutation in a cinamyl alcohol dehydrogenase gene is responsible for sorghum *brown midrib6* phenotype. *Plant Physiology*, **150**, 584-595.
- Schilmeller, A.L., Stout, J., Weng, J.K., Humphreys, J., Ruegger, M.O. & Chapple, C. (2009) Mutations in the cinnamate 4-hydroxylase gene impact metabolism, growth and development in Arabidopsis. *Plant Journal*, **60**, 771-782.
- Schoch, G., Goepfert, S., Morant, M., *et al.* (2001) CYP98A3 from *Arabidopsis thaliana* is a 3'-hydroxylase of phenolic esters, a missing link in the phenylpropanoid pathway. *Journal of Biological Chemistry*, **276**, 36566-36574.
- Sewalt, V.J.H., Ni, W., Blount, J.W., *et al.* (1997a) Reduced lignin content and altered lignin composition in transgenic tobacco down-regulated in expression of L-phenylalanine ammonia-lyase or cinnamate 4-hydroxylase. *Plant Physiology*, **115**, 41-50.
- Sewalt, V.J., Ni, W., Jung, H.G. & Dixon, R.A. (1997b) Lignin impact on fiber degradation: increased enzymatic digestibility of genetically engineered tobacco (*Nicotiana tabacum*) stems reduced in lignin content. *Journal of Agricultural and Food Chemistry*, **45**, 1977-1983.

- Shadle, G., Chen, F., Srinivasa Reddy, M.S., Jackson, L., Nakashima, J. & Dixon, R.A. (2007) Down-regulation of hydroxycinnamoyl CoA: shikimate hydroxycinnamoyl transferase in transgenic alfalfa affects lignification, development and forage quality. *Phytochemistry*, **68**, 1521-1529.
- Shen, H., Fu, C., Xiao, X., *et al.* (2009) Developmental control of lignification in stems of lowland switchgrass variety Alamo and the effects on saccharification efficiency. *BioEnergy Research*, **2**, 233-245.
- Shen, H., He, X., Poovaiah, C.R., *et al.* (2012) Functional characterization of the switchgrass (*Panicum virgatum*) R2R3-MYB transcription factor *PvMYB4* for improvement of lignocellulosic feedstocks. *New Phytologist*, **193**, 121-136.
- Sibout, R., Eudes, A., Mouille, G., *et al.* (2005) *CINNAMYL ALCOHOL DEHYDROGENASE-C* and *-D* are the primary genes involved in lignin biosynthesis in the floral stem of Arabidopsis. *Plant Cell*, **17**, 2059-2076.
- Sibout, R., Eudes, A., Pollet, B., *et al.* (2003) Expression pattern of two paralogs encoding cinnamyl alcohol dehydrogenases in Arabidopsis. Isolation and characterization of the corresponding mutants. *Plant Physiology*, **132**, 848-860.
- Song, J. & Wang, Z. (2011) RNAi-mediated suppression of the phenylalanine ammonia-lyase gene in *Salvia miltirrhiza* causes abnormal phenotypes and a reduction in rosmarinic acid biosynthesis. *Journal of Plant Research*, **124**, 183-192.
- Stewart, J.J., Akiyama, T., Chapple, C., Ralph, J. & Mansfield, S.D. (2009) The effects on lignin structure of overexpression of ferulate 5-hydroxylase in hybrid poplar. *Plant Physiology*, **150**, 621-635.
- Stewart, D., Yahiaoui, N., McDougall, G.J., *et al.* (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.
- Sutela, S., Niemi, K., Edesi, J., *et al.* (2009) Phenolic compounds in ectomycorrhizal interaction of lignin modified silver birch. *BMC Plant Biology*, **9**, 124.
- Tamagnone, L., Merida, A., Parr, A., *et al.* (1998) The AmMYB308 and AmMYB330 transcription factors from antirrhinum regulate phenylpropanoid and lignin biosynthesis in transgenic tobacco. *Plant Cell*, **10**, 135-154.

- Terashima, N., Fukushima, K., He, L.-F. & Takabe, K. (1993) Comprehensive model of the lignified plant cell wall. In: *Forage Cell Wall Structure and Digestibility*, (eds H.G. Jung, D.R. Buxton, R.D. Hatfield, J. Ralph), pp. 247-260. ASA-CSSA-SSA, Madison.
- Terashima, N., Nakashima, J. & Takabe, K. (1998) Proposed structure for protolignin in plant cell walls. In: *Lignin and lignin biosynthesis*, (eds N.B. Lewis, & S. Sarkanen), pp. 180-193. American Chemical Society Symposium Series 697. American Chemical Society, Washington, DC, USA.
- Teutsch, H.G., Hasenfratz, M.P., Lesot, A., *et al.* (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. *Proceedings of the National Academy of Sciences USA*, **90**, 4102-4106.
- Thévenin, J., Pollet, B., Letarnec, B., *et al.* (2011) The simultaneous repression of CCR and CAD, two enzymes of the lignin biosynthetic pathway, results in sterility and dwarfism in *Arabidopsis thaliana*. *Molecular Plant*, **4**, 70-82.
- Tiimonen, H., Aronen, T., Laakso, T., *et al.* (2005) Does lignin modification affect feeding preference or growth performance of insect herbivores in transgenic silver birch (*Betula pendula* Roth)? *Planta*, **222**, 699-708.
- Tsai, C.J., Popko, J.L., Mielke, M.R., Hu, W.J., Podila, G.K. & Chiang, V.L. (1998) Suppression of O-methyltransferase gene by homologous sense transgene in quaking aspen causes red-brown wood phenotypes. *Plant Physiology*, **117**, 101-112.
- Tu, Y., Rochfort, S., Liu, Z., *et al.* (2010) Functional analyses of caffeic acid O-methyltransferase and cinnamoyl-CoA-reductase genes from perennial ryegrass (*Lolium perenne*). *Plant Cell*, **22**, 3357-3373.
- Ulbrich, B. & Zenk, M.H. (1980) Partial purification and properties of *p*-hydroxycinnamoyl-CoA: shikimate *p*-hydroxycinnamoyl transferase from higher plants. *Phytochemistry*, **19**, 1625-1629.
- Umezawa, T. (2009) The cinnamate/monolignol pathway. *Phytochemical Reviews*, **9**, 1-17.
- Van der Rest, B., Danoun, S., Boudet, A.M. & Rochange, S.F. (2006) Down-regulation of cinnamoyl-CoA reductase in tomato (*Solanum lycopersicum* L.) induces dramatic changes in soluble phenolic pools. *Journal of Experimental Botany*, **57**, 1399-1411.

- Van Doorsselaere, J., Baucher, M., Chognot, E., *et al.* (1995) A novel lignin in poplar trees with a reduced caffeic acid / 5-hydroxyferulic acid *O*-methyltransferase activity. *Plant Journal*, **8**, 855-864.
- Vanholme, R., Ralph, J., Akiyama, T., *et al.* (2010) Engineering traditional monolignols out of lignin by concomitant up-regulation of *F5H1* and down-regulation of *COMT* in Arabidopsis. *Plant Journal*, **64**, 885-897.
- Vermerris, W. & Boon, J.J. (2001) Tissue specific patterns of lignification are disturbed in the *brown midrib2* mutant of maize (*Zea mays* L.) *Journal of Agricultural and Food Chemistry*, **49**, 721-728.
- Voelker, A.L., Lchenbruch, B., Meinzer, F.C., *et al.* (2010) Antisense down-regulation of 4CL expression alters lignification, tree growth and saccharification potential of field grown poplar. *Plant Physiology*, **154**, 874-886.
- Wadenbäck, J., von Arnold, S., Egertsdotter, U., *et al.* (2008) Lignin biosynthesis in transgenic Norway spruce plants harboring an antisense construct for cinnamoyl CoA reductase (CCR). *Transgenic Research*, **17**, 379-392.
- Wagner, A., Ralph, J., Akiyama, T., *et al.* (2007) Exploring lignification in conifers by silencing hydroxycinnamoyl-CoA: shikimate hydroxycinnamoyltransferase in *Pinus radiata*. *Proceedings of the National Academy of Sciences USA*, **104**, 11856-11861.
- Wagner, A., Tobimatsu, Y., Phillips, L., *et al.* (2011) CCoAOMT suppression modifies lignin composition in *Pinus radiata*. *Plant Journal*, **67**, 119-129.
- Wang, H., Avci, U., Nakashima, J., Hahn, M.G., Chen, F. & Dixon, R.A. (2010) Mutation of WRKY transcription factors initiates pith secondary wall formation and increases stem biomass in dicotyledonous plants. *Proceedings of the National Academy of Sciences USA*, **107**, 22338-22343.
- Weng, J.K., Banks, J. & Chapple, C. (2008a) Parallels in lignin biosynthesis: A study in *Selaginella moellendorffii* reveals convergence across 400 million years of evolution. *Communications in Integrative Biology*, **1**, 20-22.
- Weng, J.K., Li, X., Stout, J. & Chapple, C. (2008b) Independent origins of syringyl lignin in vascular plants. *Proceedings of the National Academy of Sciences USA*, **105**, 7887-7892.

- Weng, J.K., Mo, H. & Chapple, C. (2010) Over-expression of F5H in COMT-deficient Arabidopsis leads to enrichment of an unusual lignin and disruption of pollen wall formation. *Plant Journal*, **64**, 898-911.
- Wróbel-Kwiatkowska, M., Starzycki, M., Zebrowski, J., Oszmiański, J. & Szopa, J. (2007) Lignin deficiency in transgenic flax resulted in plants with improved mechanical properties. *Journal of Biotechnology*, **128**, 919-934.
- Xu, Z., Zhang, D., Hu, J., *et al.* (2009) Comparative genome analysis of lignin biosynthesis gene families across the plant kingdom. *BMC Bioinformatics* 10 (Suppl 11): S3.
- Yamaguchi, M., Kubo, M., Fukuda, H. & Demura, T. (2008) Vascular-related NAC-DOMAIN7 is involved in the differentiation of all types of xylem vessels in Arabidopsis roots and shoots. *Plant Journal*, **55**, 652-664.
- Yang, F., Mitra, P., Zhang, L., *et al.* (2013) Engineering secondary cell wall deposition in plants. *Plant Biotechnology Journal*, **11**, 325-335.
- Ye, Z.H., Kneusel, R.E., Matern, U. & Varner, J.E. (1994) An alternative methylation pathway in lignin biosynthesis in Zinnia. *Plant Cell*, **6**, 1427-1439.
- Zandbergen, N. (2006) Brown mid-rib corn showcased, *AgriNews Interactive*, vol 30, no.10, <http://www.agrinewsinteractive.com/archives/article-7899.htm>.
- Zeng, Y., Saar, B.G., Friedrich, M.G., *et al.* (2010) Imaging lignin-downregulated alfalfa using coherent anti-stokes Raman scattering microscopy. *BioEnergy Research*, **3**, 272-277.
- Zhang, K., Qian, Q., Huang, Z., *et al.* (2006) GOLD HULL AND INTERNODE2 encodes a primarily multifunctional cinnamyl-alcohol dehydrogenase in rice. *Plant Physiology*, **140**, 972-983.
- Zhao, Q. & Dixon, R.A. (2011) Transcriptional networks for lignin biosynthesis: more complex than we thought? *Trends in Plant Science*, **16**, 227-233.
- Zhao, Q., Gallego-Giraldo, L., Wang, H., *et al.* (2010a) A NAC transcription factor orchestrates multiple features of cell wall development in *Medicago truncatula*. *Plant Journal*, **63**, 100-114.

- Zhao, Q., Wang, H., Yin, Y., Xu, Y., Chen, F. & Dixon, R.A. (2010b) Syringyl lignin biosynthesis is directly regulated by a secondary cell wall master switch. *Proceedings of the National Academy of Sciences USA*, **107**, 14496-14501.
- Zhong, R., Demura, T. & Ye, Z.H. (2006) SND1, a NAC domain transcription factor, is a key regulator of secondary wall synthesis in fibers of Arabidopsis. *Plant Cell*, **18**, 3158-3170.
- Zhong, R. & Ye, Z.H. (2009) Secondary cell walls. In: *Encyclopedia of Life Sciences*. John Wiley Sons, Chichester, UK. CHAP ? PAGES ?
- Zhong, R., Morrison, W.H. 3rd, Himmelsbach, D.S., Poole, F.L. 2nd & Ye, Z.H. (2000) Essential role of caffeoyl coenzyme A O-methyltransferase in lignin biosynthesis in woody poplar plants. *Plant Physiology*, **124**, 563-578.
- Zhong, R., Morrison, W.H., Negrel, J. & Ye, Z.-H. (1998) Dual methylation pathways in lignin biosynthesis. *Plant Cell*, **10**, 2033-2046.
- Zhong, R., Richardson, E.A. & Ye, Z.H. (2007) The MYB46 transcription factor is a direct target of SND1 and regulates secondary wall biosynthesis in Arabidopsis. *Plant Cell*, **19**, 2776-2792.
- Zhong, R., & Ye, Z.H. (2007) Regulation of cell wall biosynthesis. *Current Opinion in Plant Biology*, **10**, 564-572.
- Zhong, R., Lee, C. & Ye, Z.H. (2010a) Evolutionary conservation of the transcriptional network regulating secondary cell wall biosynthesis. *Trends in Plant Science*, **15**, 625-632.
- Zhou, R., Jackson, L., Shadle, G., *et al.* (2010) Distinct cinnamoyl CoA reductases involved in parallel routes to lignin in *Medicago truncatula*. *Proceedings of the National Academy of Sciences USA*, **107**, 17803-17808.
- Zhou, J., Lee, C., Zhong, R. & Ye, Z.H. (2009) MYB58 and MYB63 are transcriptional activators of the lignin biosynthetic pathway during secondary cell wall formation in Arabidopsis. *Plant Cell*, **21**, 248-266.
- Ziebell, A., Gracom, K., Katahira, R., *et al.* (2010) Increase in 4-coumaroyl alcohol units during lignification in alfalfa (*Medicago sativa*) alters extractability and molecular weight of lignin. *Journal of Biological Chemistry*, **285**, 38961-38968.

Table and Figure Legends

Fig. 1.1 A scheme for monolignol biosynthesis in dicotyledonous angiosperms, including revisions encompassing the different biochemical activities of CCR forms in *Medicago truncatula* (Zhou *et al.*, 2010). See text for enzyme abbreviations.

Fig. 1.2 Transcriptional controls for the biosynthesis of monolignols in the context of secondary cell wall biosynthesis (based on Arabidopsis). Both NAC and MYB genes can activate the entire secondary cell wall biosynthesis pathway. F5H (ferulate 5-hydroxylase) is regulated by the NAC master-switch (which is also under autoregulatory control), whereas other lignin genes are regulated by MYB58/63/85 through AC elements in their promoters. MYB 4 is a lignin/phenylpropanoid pathway repressor.

Fig. 1.3 Cellular impacts of targeting monolignol biosynthesis in alfalfa through RNAi-mediated silencing of specific enzymatic steps. A. Micrographs showing cross sections through the 5th internodes of transgenic alfalfa plants down-regulated independently at each of the enzymatic steps shown. B. As in A, but showing a comparison of single down-regulation of COMT or CCoAOMT with the result of down-regulating both genes simultaneously. Reproduced from Nakashima *et al.*, 2008, with permission.

Fig. 1.4 Growth phenotypes of alfalfa and *M. truncatula* plants as a result of down-regulation (silencing or insertional mutagenesis) of monolignol biosynthetic enzymes or regulatory TFs. In each case, a corresponding wild-type control grown in parallel is included for comparison. A, B, RNAi-mediated down-regulation of HCT or C3'H, respectively, in alfalfa. C-E, transposon insertion mutants in CCR1, CCoAOMT and CAD, respectively, in *M. truncatula*. F, G, lignin autofluorescence in cross

sections of 6th internodes of wild-type *M. truncatula* and a line harboring a transposon insertion in the NST1 NAC transcription factor gene. H, growth phenotypes of the plants in F, G, showing lodging in the *nst1* mutant. I, J, lignin autofluorescence in cross sections of 6th internodes of wild-type *M. truncatula* and a line harboring a transposon insertion in the NST1 NAC transcription factor gene. K, growth phenotypes of the plants in I, J.

Fig. 1.1

Fig. 1.2

Fig. 1.3

Fig. 1.4