Recent Advances in Polyphenol Research

Volume 4

Edited by

Annalisa Romani,
Vincenzo Lattanzio
and Stéphane Quideau

WILEY Blackwell

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Recent Advances in Polyphenol Research
A series for researchers and graduate students whose work is related to plant phenolics and polyphenols, as well as for individuals representing governments and industries with interest in this field. Each volume in this biennial series focuses on several important research topics in plant phenols and polyphenols, including chemistry, biosynthesis, metabolic engineering, ecology, physiology, food, nutrition, and health.

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Dedication

This fourth volume of *Recent Advances in Polyphenol Ressearch* is dedicated to the memory of Edwin Haslam, Professor of Physical-Organic Chemistry at the University of Sheffield, UK, who peacefully passed away at his home in Exeter on October 3, 2013, aged 81. Professor Haslam had been a long-standing and faithful member of Groupe Polyphénols, and was for many of us a model, a helpful mentor, a great colleague, and a friend. His pioneering and outstanding contributions to the field of plant polyphenols were, still are, and will continue to be a great source of knowledge and inspiration.

Acknowledgments

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Preface

During the last 10 years there has been increasing interest in the study of plant polyphenols and their innumerable roles in a variety of very different contexts. Plant polyphenols are secondary metabolites and constitute one of the most common and widespread groups of substances in plants. Their structural diversity is likely the result of plant adaptive responses to natural selection.

Polyphenols express a large and diverse range of beneficial effects in plants and in humans consuming plant-derived food and beverages. For example, polyphenols are well known for their antioxidation activity, hormone-like behavior, and role as natural neurotransmitters, among many other biological activities. They also provide antimicrobial activity for the plant's own defense against invading pathogens.

The diversity of structures and activities of plant polyphenolic compounds has resulted in the emergence of numerous investigations in various and often interdisciplinary research areas, encompassing scientific domains as diverse as chemistry, biochemistry, biotechnology, ecology, physiology, nutrition and food chemistry, pharmacy and medicine, cosmetics, and textile technology, as well as in quality and environment controls and assessments.

It is thus the aim of the International Conference on Polyphenols, which is a biennial event that is organized under the auspices of Groupe Polyphénols, to provide scientists across disciplines with a forum for sharing new findings and for exchanging views and ideas on polyphenol research at large.

For the first time in its history, in 2012 the 26th International Conference on Polyphenols was organized in Florence, Italy. The interest in polyphenol science at the University of Florence involves many departments, including Pharmaceutical Sciences, Chemistry, Plant Sciences and Ecology, Food Science, and Medicine, as well as The Multidisciplinary Centre of Research on Food Sciences (CeRA – MCRFS) and the laboratory of Commodity Sciences and Quality Control, Environment Assessments and Certification. In these fields, particular attention has been dedicated to functional-food, nutraceutical, and cosmeceutical discoveries and applications.

At the 26th International Conference on Polyphenols, five different main topics were selected for the scientific program:

(1) *Phenols and Polyphenols Chemistry*: Covering (i) isolation and structural elucidation, and (ii) synthesis, reactivity, and physical-chemical properties.
(2) *Biosynthesis, Genetics, and Metabolic Engineering*: Dealing with biosynthesis and genetic manipulation.

(3) *Roles in Plants and Ecosystems*: Covering phenolic functions in plants and correlation with biotic and abiotic stresses.
(4) *Health and Nutrition*: Focusing on polyphenol metabolism and bioavailability, as well as cancer prevention and perspectives on gender-dependent human health effects.
(5) *Polyphenols and Drug Discovery*: Including new findings on sources of isolated and standardized polyphenolic fractions and novel epigenetic polyphenol mechanisms.

More than 400 scientists from 42 countries attended the conference in July 2012, with nearly 400 paper contributions, comprising 52 oral communications and 327 poster presentations (Fig. P.1).

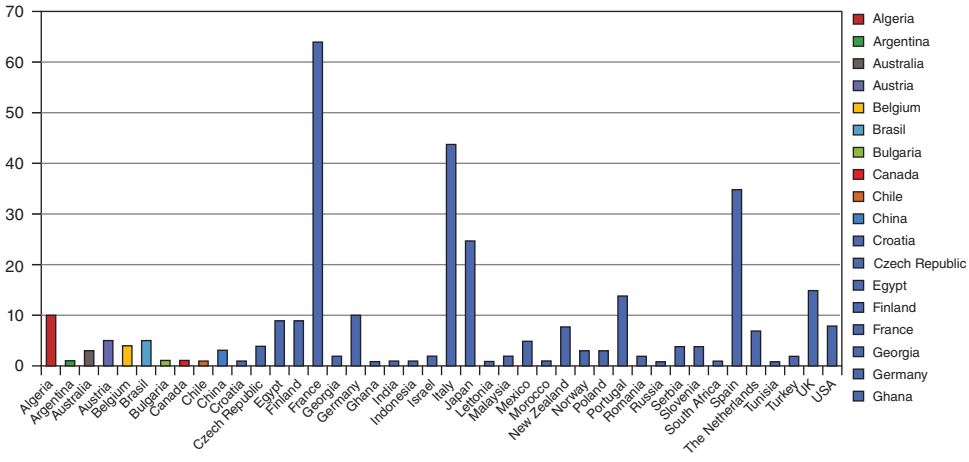

Fig. P.1 Contributions to the 26th International Conference on Polyphenols (number of papers presented) by country.

The success of this 26th edition of the International Conference on Polyphenols would not have been possible without the support of both public and private sponsors. The Scientific and Technological Pole and the Social Pole of the University of Florence, PIN of Prato, the National Council for Research, and several private-company sponsors (Agilent Technologies, BioTech Power, Indena, ISR Ecoindustria, Domus Olea, Force A, Biokyma, PhenoFarm, Dermaresia, Silva Team, Bioscen Future) are gratefully acknowledged.

All of the lectures, oral communications, and ensuing discussions and debates were broadcast live on RadioSpin, the University of Florence webradio, and through Ustation (the Italian university radio stations network), on the other connected university radios of the network. These radiophonic conference proceedings are available in podcasts on the RadioSpin Web site: www.radiospin.it.

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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-regulation of the expression of the enzymes of the monolignol pathway and down-regulation or over-expression of the 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

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1.1 Introduction

Lignin is a major component of plant secondary cell walls, and the second most abundant plant polymer on the planet. It 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 access to the sugar-rich cell-wall polysaccharides cellulose and hemicellulose for 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 informs the targets that have been selected for genetic modification of lignin content and composition in transgenic plants. Which gene is down- or up-regulated has a considerable effect on lignin content and composition. Equally, 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 can 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 basic 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 & 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 the secondarily thickened cell walls of plants, where it is critical to cell-wall structural integrity and gives strength to stems (Chabannes *et al.*, 2001b; Jones *et al.*, 2001). Lignin also imparts hydrophobicity to vascular elements for water transport. The lignin content of the 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 *et al.*, 2003). Fern

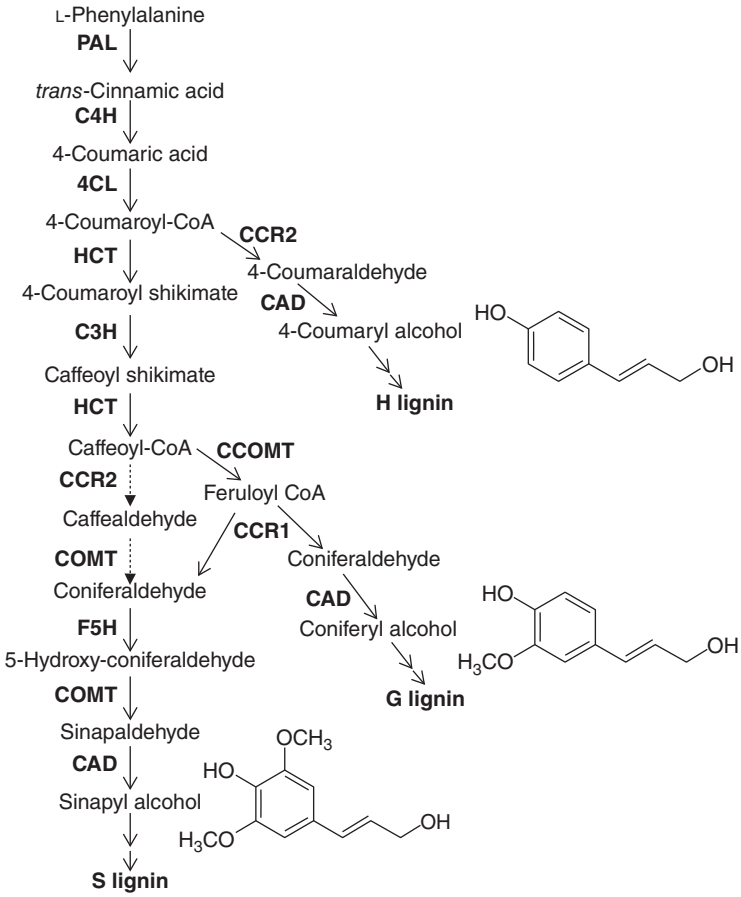

Fig. 1.1 Scheme for monolignol biosynthesis in dicotyledonous angiosperms, including revisions encompassing the different biochemical activities of cinnamoyl-CoA reductase (CCR) forms in *Medicago truncatula* (Zhou *et al.*, 2010). See text for enzyme abbreviations.

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 moellendorfii* (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 unit 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 the 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; this relationship has 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 the production of liquid biofuels through saccharification and fermentation (Chen & Dixon, 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, which differ in both function and 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 there (Donaldson, 2001; Boerjan *et al.*, 2003). Based on UV microscopy, the density of lignin is higher in the middle lamella and primary walls than in the 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. The lignin contents and compositions of switchgrass cv. Alamo grown in the field, in greenhouses, and in growth chambers

were 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 and then dramatically on progression from the elongation 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 eighth internode (Chen *et al.*, 2006). This was accompanied by an increase in S/G ratio from 0.087 to 0.640. The lignin contents (thioacidolysis yields) and lignin monomer compositions of individual cell types (vascular elements, phloem fibers, and vascular parenchyma) from the fifth internode were quite different (Nakashima *et al.*, 2008). Thioacidolysis yields were higher in vascular cells than in parenchyma and fiber cells (430, 267, and 76 μmol/g dry weight, respectively), with fiber and parenchyma cells enriched in S lignin units with an S/G ratio of 0.60 and 0.72 respectively, and vascular cells enriched in G lignin units with an 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 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 that 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 (TFs) 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.

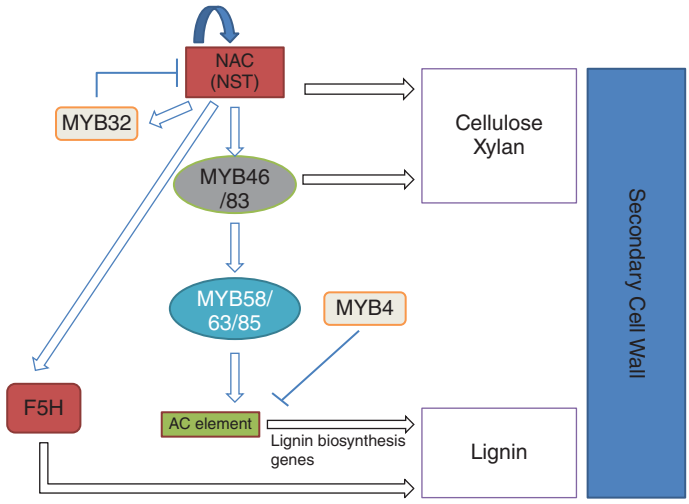

Fig. 1.2 Transcriptional controls for the biosynthesis of monolignols in the context of secondary cell-wall biosynthesis (based on Arabidopsis and *Medicago truncatula*). Both NAC and MYB genes can activate the entire secondary cell-wall biosynthesis pathway. In *M. truncatula*, F5H (ferulate 5-hydroxylase) 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.

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 studied extensively since the 1970s (Hahlbrock & Grisebach, 1970; Knobloch & Hahlbrock 1975, 1977). The enzyme converts hydroxycinnamic acids, preferably 4-coumaric acid, to the corresponding coenzyme-A thioesters. 4CL exists as a multigene family in those species studied to date (Hu *et al.*, 1998; Ehlting *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 knock-out 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 & Wooltorton, 1976; Ulbrich & Zenk, 1980), it was not originally appreciated that these reactions might 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 the 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 the acyl donor and shikimate or quinate as the acceptor, followed by 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 (Fig. 1.1). 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 did not change lignin content but led, respectively, to a decrease or an 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. Previously, 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)*

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

Cinnamoyl-CoA reductases (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).