

# Rewriting the lignin roadmap

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Considerable interest in lignin biosynthesis has been fueled by the many roles that lignin plays in development and in resistance to biotic and abiotic stress, as well as its importance to industry and agriculture. Although the pathway leading to the lignin polymer has been studied for decades, new insights into the enzymes of the pathway have required a complete re-evaluation of how we think lignin precursors are synthesized. Although free hydroxycinnamic acids have long been thought to be key intermediates, it has become apparent that many of the hydroxylation and methylation steps in the pathway occur instead at the level of hydroxycinnamic acid esters, and their corresponding aldehydes and alcohols.

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*Current Opinion in Plant Biology* 2002, 5:224–229

1369-5266/02/\$ – see front matter

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Published online 21 March 2002

### Abbreviations

C3H	<i>p</i> -coumarate 3-hydroxylase
CAD	cinnamyl alcohol dehydrogenase
CCoAOMT	caffeoyl CoA 3- <i>O</i> -methyltransferase
CCR	cinnamoyl CoA reductase
CoA	co-enzyme A
COMT	caffeic acid/5-hydroxyferulic acid <i>O</i> -methyltransferase
F5H	ferulate 5-hydroxylase
OMT	<i>O</i> -methyltransferase
P450	cytochrome-P450-dependent monooxygenase
ref	reduced epidermal fluorescence
SAD	sinapyl alcohol dehydrogenase

### Introduction

The phenylpropanoid pathway is responsible for the biosynthesis of a variety of products that include lignin, flavonoids, and hydroxycinnamic acid conjugates. Many intermediates and end products of this pathway play important roles in plants as phytoalexins, antiherbivory compounds, antioxidants, ultra-violet (UV) protectants, pigments, and aroma compounds [1–3]. The majority of carbon in the pathway, however, is channeled toward the synthesis of lignin, a complex three-dimensional polymer that is a principal structural component of plant cell walls.

Lignin has far-reaching impacts on agriculture, industry and the environment, making phenylpropanoid metabolism a globally important part of plant biochemistry. As the second most abundant polymer on earth, eclipsed only by cellulose, lignin is a major carbon sink in the biosphere, accounting for about 30% of the more than  $1.4 \times 10^{12}$  kg of carbon sequestered into terrestrial plant material each year [4]. As

a major polymer of cell walls, lignin also has a direct impact on wood characteristics, including the ease with which the undesirable lignin polymer can be removed from cellulose fibers during the pulping process. In addition, the mechanical support provided by lignin prevents lodging, a problem in many agronomically important plants. Lignin reduces the quality and digestibility of forage crops such as alfalfa, which in turn impacts the livestock industry [5]. Finally, the many functions of lignin and related products in resistance to biotic and abiotic stress make the phenylpropanoid pathway vital to the health and survival of plants.

To understand how plants respond to stress, a thorough knowledge of the various pathways that play a role in stress resistance is essential. This principle defines the utility of many new technologies. For example, the expression of tens of thousands of genes can be quantified by microarray analysis, but our incomplete knowledge of the metabolic pathways represented by these genes often limits the interpretation of the corresponding data. Similarly, the limiting factor in metabolic engineering efforts is often a lack of knowledge about the enzymes in a pathway, the steps that they catalyze, and the regulatory factors that control their expression. With this in mind, it is humbling to realize that despite decades of study, recent work has revealed many misconceptions in our understanding of the phenylpropanoid pathway. The focus of this review is on work that has eliminated these misconceptions by better defining the steps that lead to lignin precursors.

### The road less traveled?

The conventional model of the phenylpropanoid pathway includes a series of hydroxylation and methylation reactions in which cinnamic acid derived from phenylalanine is converted into a variety of hydroxycinnamic acids [6]. These hydroxycinnamic acids act as precursors for flavonoids, hydroxycinnamic acid conjugates, and lignin [6]. Activation of these acids to their corresponding co-enzyme A (CoA) thioesters, followed by two successive reductions, was conventionally thought to provide the monolignol building blocks for the lignin polymer (Figure 1a). This simple model was challenged by the identification of caffeoyl CoA 3-*O*-methyltransferase (CCoAOMT) as part of an elicitor-induced plant defense response [7–9]. Subsequent work suggested that this enzyme also functions in a route to lignin monomers in which *p*-coumaroyl CoA is converted to feruloyl CoA, thus providing an alternative to the conventional pathway involving free acids ([10–14]; Figure 1b). The significance of this pathway was demonstrated by the downregulation of CCoAOMT in transgenic tobacco [14]. In these transgenic plants, reduced CCoAOMT activity was associated with reduced syringyl and guaiacyl monomer production and dramatically lowered lignin content. These experiments provided the first indications that not all of the hydroxycinnamic acids were obligatory players in lignin biosynthesis.

**Figure 1**

Our understanding of the primary intermediates in lignin biosynthesis has evolved over the past two decades. (a) Early models of the phenylpropanoid pathway described the hydroxylation and methylation of phenylalanine (PHE)-derived cinnamic acids, followed by their reduction to guaiacyl (G) and syringyl (S) monolignols via CoA-activated intermediates [5]. (b) During the mid 1990s, a second pathway incorporating additional CoA intermediates that allowed an alternative route to guaiacyl monomers was discovered [6–13]. (c) Next, coniferaldehyde (CON ALD) and coniferyl alcohol (CON ALC) were identified as the primary precursors for syringyl lignin [17,18]. (d) Finally, *p*-coumaroyl quininate (*p*-COUM QUIN) and *p*-coumaroyl shikimate (*p*-COUM SHIK) were identified as likely intermediates in lignin biosynthesis [24]. 5OH CON ALC, 5-hydroxyconiferyl alcohol; 5OH CON ALD, 5-hydroxyconiferaldehyde; 5OH FER, 5-hydroxyferulate; CAF, caffeic acid; CAF CoA, caffeoyl CoA; CAF QUIN, caffeoyl quinic acid; CAF SHIK, caffeoyl shikimic acid; CINN, cinnamic acid; FER, ferulic acid; FER CoA, feruloyl CoA; *p*-COUM, *p*-coumaric acid; *p*-COUM CoA, *p*-coumaroyl CoA; SIN, sinapic acid; SIN ALC, sinapyl alcohol; SIN ALD, sinapaldehyde; SIN CoA, sinapoyl CoA.

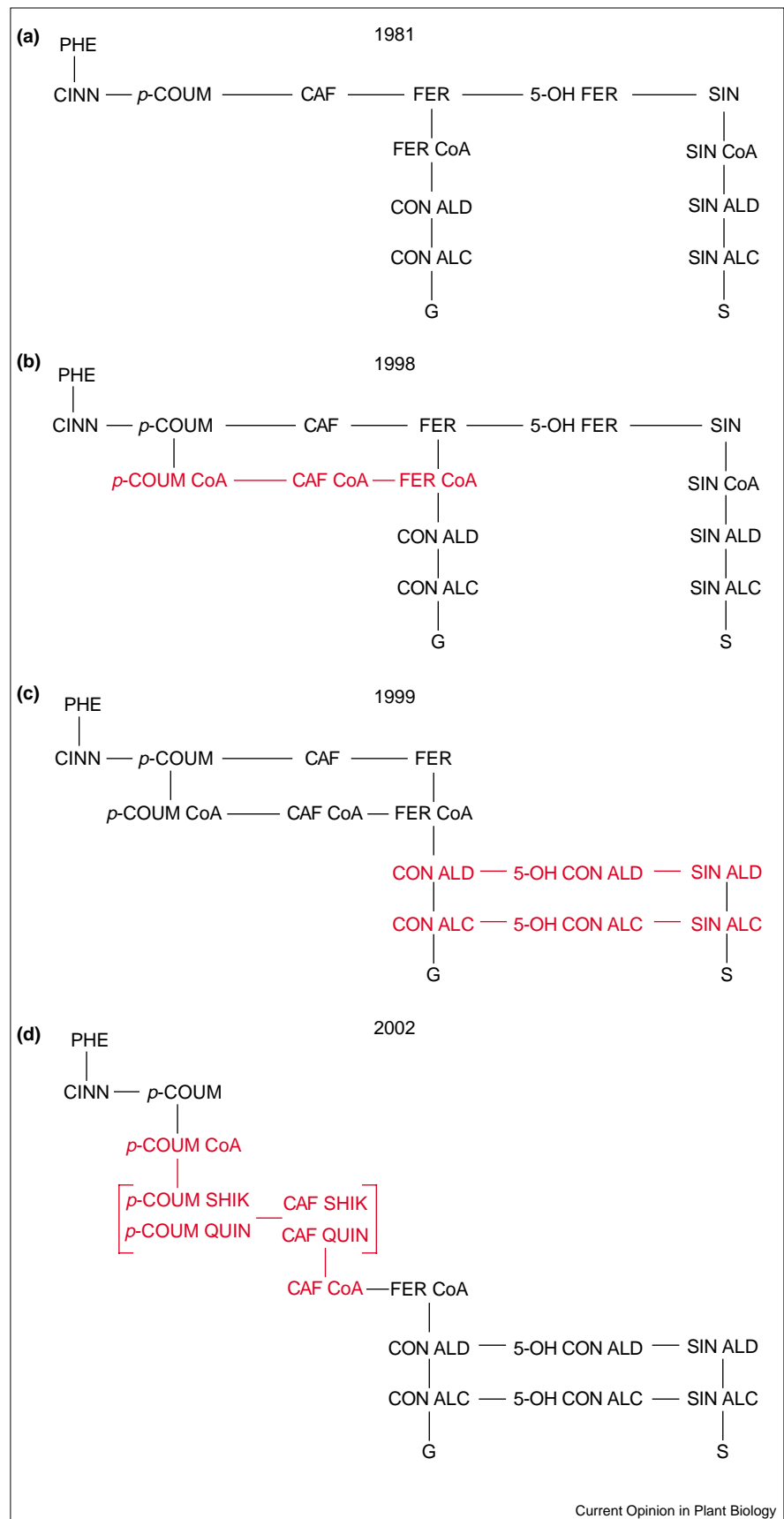
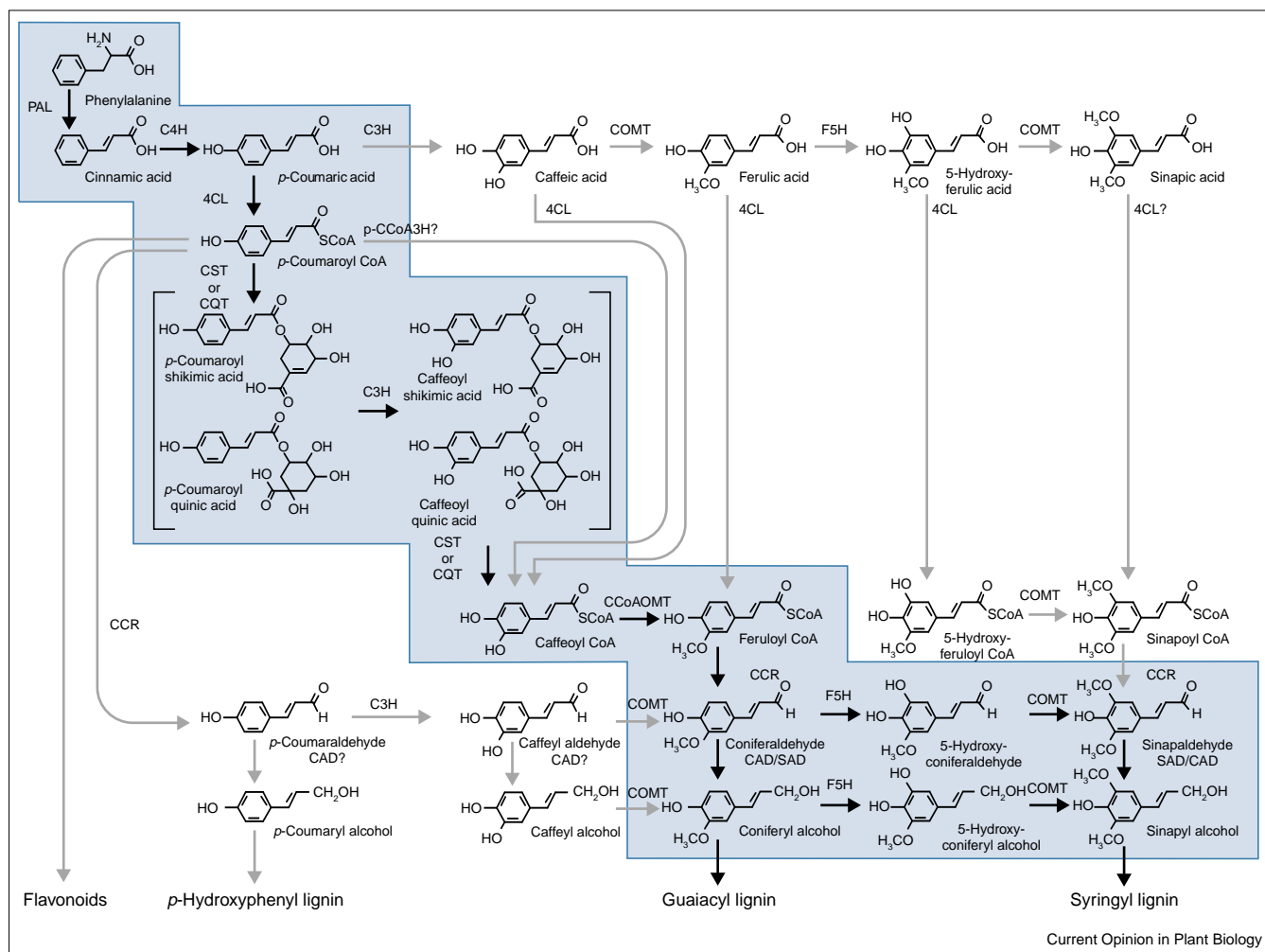


Figure 2



Revised model of the phenylpropanoid pathway leading to lignin biosynthesis. Reactions thought to be key in lignin biosynthesis are indicated with black arrows. Intermediate compounds and enzymes currently considered to form the prominent path to lignin are highlighted in blue. 4CL, 4-(hydroxycinnamoyl CoA) ligase; C3H, *p*-coumarate 3-hydroxylase; C4H, cinnamate 4-hydroxylase; CAD, cinnamyl alcohol dehydrogenase; CCoAOMT, caffeoyl CoA

*O*-methyltransferase; CCR, cinnamoyl CoA reductase; COMT, caffeic acid/5-hydroxyferulic acid *O*-methyltransferase; CQT, hydroxycinnamoyl CoA:quinic acid hydroxycinnamoyltransferase; CST, hydroxycinnamoyl CoA:shikimate hydroxycinnamoyltransferase; F5H, ferulate 5-hydroxylase; PAL, phenylalanine ammonia-lyase; *p*CCoA3H, *p*-coumaroyl CoA 3-hydroxylase; SAD, sinapyl alcohol dehydrogenase.

### Paving new roads to syringyl monomers

The precursors of guaiacyl and syringyl lignin in angiosperms are coniferyl alcohol and sinapyl alcohol, respectively. Ferulate 5-hydroxylase (F5H) is a cytochrome-P450-dependent monooxygenase (P450) that is required for the biosynthesis of syringyl monomers. For many years, its role in lignin biosynthesis was thought to be the conversion of ferulic acid to 5-hydroxyferulic acid, the precursor to sinapic acid and syringyl lignin ([15]; Figure 1a). More recently, two pieces of evidence suggested that F5H may function differently in syringyl lignin biosynthesis. First, when F5H-overexpressing *Arabidopsis*, tobacco, and poplar plants were analyzed, they were found to deposit lignin composed almost entirely of syringyl monomers [16,17]. As a model of the lignin biosynthetic pathway that incorporates

CCoAOMT provides an alternative route to guaiacyl monomers that would bypass F5H, it was difficult to rationalize how F5H overexpression could have such dramatic phenotypic effects if its *in vivo* substrate were actually ferulic acid. Second, labeled precursor feeding experiments in magnolia implicated coniferyl alcohol in sinapyl alcohol biosynthesis [18]. These issues were resolved by heterologous expression and subsequent enzymatic analysis of F5H. Kinetic analysis of the *Arabidopsis* F5H enzyme revealed that although F5H catalyzed the 5-hydroxylation of ferulic acid, it displayed a thousand-fold greater affinity for coniferaldehyde and coniferyl alcohol ([19]; Figure 2). Concurrent research demonstrated that recombinant sweetgum F5H exhibited a high affinity for coniferaldehyde, a substrate that also inhibited ferulate 5-hydroxylation [20].

Taken together, this work suggests that in lignin biosynthesis, F5H likely acts on coniferaldehyde and coniferyl alcohol, downstream in the phenylpropanoid pathway of its previously described substrate, ferulic acid (Figure 1c).

The identification of novel F5H activities immediately led to a search for an *O*-methyltransferase that is capable of converting the 'new' F5H products into syringyl-substituted monomers. Although caffeic acid/5-hydroxyferulic acid *O*-methyltransferase (COMT) was initially thought to be a bifunctional enzyme that used hydroxycinnamic acids as substrates [2], recombinant COMT from *Arabidopsis* [19], sweetgum [20], poplar [21], and alfalfa [22] have now all been demonstrated to have activity toward the novel F5H products (Figure 2). Further, the  $K_m$  (i.e. the Michaelis-Menten constant, the substrate concentration that allows the reaction to proceed at one half of its maximum rate) of recombinant poplar COMT for 5-hydroxyconiferaldehyde is five-fold lower than for 5-hydroxyferulate, and almost 30-fold lower than for caffeic acid [21]. Similarly, a recent extensive study of recombinant alfalfa COMT found that, in comparison to seven other possible substrates in the phenylpropanoid pathway, COMT exhibited the highest  $V_{max} K_m^{-1}$  values (where  $V_{max}$  is the rate of enzyme activity when saturated with substrate) for 5-hydroxyconiferaldehyde and the lowest for caffeic acid [22]. These *in vitro* data suggest that COMT acts after F5H in the conversion of coniferaldehyde and coniferyl alcohol to sinapaldehyde and sinapyl alcohol, respectively. This repositioning of COMT within the lignin biosynthetic pathway is also consistent with previous work with transgenic tobacco and poplar in which reduced COMT expression led to a marked reduction in syringyl lignin content but had little impact on total lignin [23,24]. It is also noteworthy that in this new model of lignin biosynthesis, COMT no longer has a function that is redundant with that of CCoAOMT, thus explaining how lignin phenotypes can be generated by CCoAOMT downregulation (Figure 2).

### Finding the bridge to caffeic acid derivatives

One of the first committed steps in the production of lignin is thought to be the meta-hydroxylation of *p*-coumaric acid or its derivatives. The long-standing model of lignin biosynthesis suggests that this step occurs at the level of the free acids, and that it is catalyzed by *p*-coumarate 3-hydroxylase (C3H) [6] (Figure 1a, Figure 2). Despite numerous attempts over the past thirty years to better understand C3H, until recently this hydroxylase was one of the last classic phenylpropanoid enzymes and genes that remained uncharacterized. Two research groups using different approaches have recently shed considerable light on this elusive enzyme [25••–27••].

Schoch *et al.* [25••] used a functional genomics approach to identify possible C3H candidates including CYP98A3, a P450. Although two reports had previously identified a P450 that uses the shikimate and quinate esters of *p*-coumarate as substrates [8,28], at the time, these compounds were not considered to be lignin biosynthetic intermediates.

Nevertheless, biochemical and kinetic characterization of CYP98A3 showed that this enzyme actively converts the 5-*O*-shikimate and 5-*O*-D-quininate esters of *p*-coumaric acid into their corresponding caffeic acid conjugates (Figure 2). With a turnover rate of over  $10\text{ s}^{-1}$  for the quinate ester, CYP98A3 has the highest turnover rate of any plant P450 characterized to date. Concurrent work by Franke *et al.* [27••] demonstrated the role of CYP98A3 in lignin biosynthesis. These authors described the identification of the *reduced epidermal fluorescence8* (*ref8*) mutant in *Arabidopsis*, which accumulates *p*-coumarate esters in place of the sinapoylmalate found in wild-type plants. Further phenotypic analysis showed that the *ref8* mutant has greatly reduced lignin levels, and that this lignin is not composed of wild-type guaiacyl and syringyl monomers but is produced mainly from *p*-coumaroyl alcohol. Positional cloning of the *REF8* gene showed that REF8 is CYP98A3 [26••]. The work of these two groups demonstrated that CYP98A3 is C3H, and provided direct evidence that *p*-coumaroyl shikimate and/or *p*-coumaroyl quinate are likely to be important intermediates in lignin biosynthesis (Figure 1d).

It is interesting that CYP98A3 is not active toward *p*-coumaroyl CoA [25••,26••], yet the *ref8* mutant shows that C3H is necessary for the synthesis of normal lignin and secondary metabolites in *Arabidopsis* [27••]. These data suggest that, if it exists, *p*-coumaroyl CoA 3-hydroxylase activity may not be quantitatively important, at least not in *Arabidopsis*. Taken together, this research further reinforces the theory that phenylpropanoid esters and thioesters play prominent roles in many steps of lignin biosynthesis that were previously thought to occur at the level of the free acids.

### Traffic control in monolignol biosynthesis

New insights into lignin biosynthesis have also come through antisense regulation of the dehydrogenases cinnamoyl CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD), enzymes that are thought to convert CoA-activated guaiacyl- and syringyl-substituted monomers into their corresponding aldehydes and alcohols, respectively (Figure 1a, Figure 2). Downregulation of CCR results in a 50% reduction in Klason lignin in tobacco, as well as in stunted growth, reduced apical dominance, abnormal leaf shape, and chlorosis [29]. Transgenic tobacco plants with downregulated expression of CAD, one step downstream in the phenylpropanoid pathway from CCR, show only a slight reduction in Klason lignin and discolored xylem tissue, but otherwise have a wild-type morphology [30]. One possible explanation for these seemingly contradictory results is that lignification is sufficiently plastic as to allow CAD-downregulated plants to form lignin directly from cinnamyl aldehydes [31,32]; whereas, insufficient lignin to support normal development is deposited when CCR expression is reduced.

Li *et al.* [33••] propose a different explanation. Conventional models of the phenylpropanoid pathway suggest that CAD

is a multifunctional enzyme with both guaiacyl- and syringyl-substituted substrates. A sinapyl alcohol dehydrogenase (SAD) in poplar has been identified that is co-expressed with F5H and COMT, and that co-localizes with syringyl lignin formation [33\*\*]. This implies that SAD, and not CAD, may be the primary enzyme responsible for the final step in syringyl monolignol biosynthesis, and that as a result, CAD downregulation has limited effects on plant morphology (Figure 2).

### Are there more twists in the road ahead?

Experiments with COMT continue to fuel discussion concerning its role in lignin biosynthesis. Recent work has shown that strong COMT downregulation in transgenic alfalfa not only results in the expected loss of syringyl lignin but also lowers guaiacyl lignin levels. In the same study, antisense downregulated CCoAOMT alfalfa showed no reduction in syringyl lignin and only a slight reduction in guaiacyl lignin [34]. Both of these results suggest that there may be some limited redundancy between COMT and CCoAOMT, which contradicts earlier results [12,13]. Part of the explanation for these results may lie in recent work that demonstrated that alfalfa COMT can methylate caffeoyl aldehyde and caffeoyl alcohol with  $V_{\max}$   $K_m^{-1}$  values close to those for 5-hydroxyconifer-aldehyde [22]. We also now know that although *Arabidopsis* C3H shows no detectable activity for *p*-coumaroyl alcohol, it does hydroxylate *p*-coumaric acid and *p*-coumaraldehyde, albeit at low rates [26\*\*]. This opens the possibility that C3H and COMT are involved in an additional pathway leading to coniferaldehyde (Figure 2). The kinetic analysis of plant *O*-methyltransferases (OMTs) is also being complemented by other experimental approaches. X-ray crystal structures of the first two plant OMTs have been solved recently, facilitating a structural analysis of the substrate discrimination of plant OMTs that may, in turn, help to clarify their role in lignin biosynthesis [35].

Finally, new work studying the simultaneous downregulation of CAD and CCR suggests that further clarification of the roles that these enzymes play in lignin biosynthesis may be needed. Transgenic tobacco with 32% of wild-type CCR activity and 12% of wild-type CAD activity had a nearly 50% reduction in lignin, discolored xylem, and increased levels of the phenolics ferulic acid and sinapic acid, much like plants downregulated only in CCR [36\*]. The phenotype of the CCR/CAD double transformant plants, however, was surprisingly similar to that of wild-type plants. This unexpected result was supported by nuclear magnetic resonance (NMR) analysis of lignin. This analysis revealed that, although clearly different from that of the wild-type, the lignin of the CCR/CAD double transformant had an NMR spectrum more like that of normal lignin than did the lignin produced by either CAD- or CCR-deficient plants. The explanation for these results is unclear, although it is known that reduction of total lignin does not necessarily have an adverse effect on plants in controlled conditions [37,38]. Changes in plant morphology that are caused by the manipulation of specific

enzymes in lignin biosynthesis may demonstrate that our understanding of this pathway is still incomplete.

### Conclusions

Our attempts to comprehend lignin biosynthesis and the phenylpropanoid pathway represent both an exciting challenge and a sobering example of the effort required to grasp only a small part of plant metabolism. This is particularly true when one considers that our knowledge of the phenylpropanoid pathway is considerably greater than that of many other areas of plant biochemistry. It is amazing that after decades of research, key metabolic steps in the biosynthesis of lignin precursors are still being discovered; that the true role of many of the enzymes we know to be involved in lignin biosynthesis are uncertain; and that we have only scratched the surface of understanding the regulation of lignin biosynthesis. Although each new result brings a clearer picture of the way(s) plants make lignin, considerable work is still needed before we have a reliable roadmap of the phenylpropanoid pathway.

In the scramble to define what post-genomic science will entail, one proposed goal is to understand the function of every protein in *Arabidopsis* by 2010. If lignin biosynthesis is representative of plant biochemistry in general, this goal is both daunting and vital.

### Acknowledgements

We thank Joanne Cusumano for critical reading of the manuscript. Work in CC's laboratory was supported by the National Science Foundation and by the US Department of Energy.

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