PERGAMON

Phytochemistry 61 (2002) 221–294

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

Review

Trends in lignin modification: a comprehensive analysis of the effects of genetic manipulations/mutations on lignification and vascular integrity

Aldwin M. Anterola, Norman G. Lewis*

Institute of Biological Chemistry, Washington State University, Pullman, WA 99164-6340, USA

Received 13 June 2002

Abstract

A comprehensive assessment of lignin configuration in transgenic and mutant plants is long overdue. This review thus undertook the systematic analysis of trends manifested through genetic and mutational manipulations of the various steps associated with monolignol biosynthesis; this included consideration of the downstream effects on organized lignin assembly in the various cell types, on vascular function/integrity, and on plant growth and development. As previously noted for dirigent protein (homologs), distinct and sophisticated monolignol forming metabolic networks were operative in various cell types, tissues and organs, and form the cell-specific guaiacyl (G) and guaiacyl–syringyl (G–S) enriched lignin biopolymers, respectively. Regardless of cell type undergoing lignification, carbon allocation to the different monolignol pools is apparently determined by a combination of phenylalanine availability and cinnamate-4-hydroxylase/"*p*-coumarate-3-hydroxylase" (C4H/C3H) activities, as revealed by transcriptional and metabolic profiling. Downregulation of either phenylalanine ammonia lyase or cinnamate-4-hydroxylase thus predictably results in reduced lignin levels and impaired vascular integrity, as well as affecting related (phenylpropanoid-dependent) metabolism. Depletion of C3H activity also results in reduced lignin deposition, albeit with the latter being derived only from hydroxyphenyl (H) units, due to both the guaiacyl (G) and syringyl (S) pathways being blocked. Apparently the cells affected are unable to compensate for reduced G/S levels by increasing the amounts of H-components. The downstream metabolic networks for G-lignin enriched formation in both angiosperms and gymnosperms utilize specific cinnamoyl CoA *O*-methyltransferase (CCOMT), 4-coumarate:CoA ligase (4CL), cinnamoyl CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) isoforms: however, these steps neither affect carbon allocation nor H/G designations, this being determined by C4H/C3H activities. Such enzymes thus fulfill subsidiary processing roles, with all (except CCOMT) apparently being bifunctional for both H and G substrates. Their *severe* downregulation does, however, predictably result in impaired monolignol biosynthesis, reduced lignin deposition/vascular integrity, (upstream) metabolite build-up and/or shunt pathway metabolism. There was no evidence for an alternative acid/ester *O*-methyltransferase (AEOMT) being involved in lignin biosynthesis.

The G/S lignin pathway networks are operative in specific cell types in angiosperms and employ two additional biosynthetic steps to afford the corresponding S components, i.e. through introduction of an hydroxyl group at C-5 and its subsequent *O*-methylation. [These enzymes were originally classified as ferulate-5-hydroxylase (F5H) and caffeate *O*-methyltransferase (COMT), respectively.] As before, neither step has apparently any role in carbon allocation to the pathway; hence their individual downregulation/manipulation, respectively, gives either a G enriched lignin or formation of the well-known S-deficient *bm3* "lignin" mutant, with cell walls of impaired vascular integrity. In the latter case, COMT downregulation/mutation apparently results in utilization of the isoelectronic 5-hydroxyconiferyl alcohol species albeit in an unsuccessful attempt to form G-S lignin proper. However, there is apparently no effect on overall G content, thereby indicating that deposition of both G and S moieties in the G/S lignin forming cells are kept spatially, and presumably temporally, fully separate. Downregulation/mutation of further downstream steps in the G/S network [i.e. utilizing 4CL, CCR and CAD isoforms] gives predictable effects in terms of their subsidiary processing roles: while severe downregulation of 4CL gave phenotypes with impaired vascular integrity due to reduced monolignol supply, there was no evidence in support of increased growth and/or enhanced cellulose biosynthesis. CCR and CAD downregulation/mutations also established that a depletion in monolignol supply reduced both lignin contents and vascular integrity, with a concomitant shift towards (upstream) metabolite build-up and/or shunting.

* Corresponding author. Tel.: +1-509-335-8382; fax: +1-509-335-8206.
E-mail address: lewisn@wsu.edu (N.G. Lewis).

PII: S0031-9422(02)00211-X

The extraordinary claims of involvement of surrogate monomers (2-methoxybenzaldehyde, feruloyl tyramine, vanillic acid, etc.) in lignification were fully disproven and put to rest, with the investigators themselves having largely retracted former claims. Furthermore analysis of the well-known *bm1* mutation, a presumed CAD disrupted system, apparently revealed that *both* G and S lignin components were reduced. This seems to imply that there is no monolignol specific dehydrogenase, such as the recently described sinapyl alcohol dehydrogenase (SAD) for sinapyl alcohol formation. Nevertheless, different CAD isoforms of differing homology seem to be operative in different lignifying cell types, thereby giving the G-enriched and G/S-enriched lignin biopolymers, respectively. For the G-lignin forming network, however, the CAD isoform is apparently catalytically less efficient with all three monolignols than that additionally associated with the corresponding G/S lignin forming network(s), which can more efficiently use all three monolignols. However, since CAD does not determine either H, G, or S designation, it again serves in a subsidiary role—albeit using different isoforms for different cell wall developmental and cell wall type responses.

The results from this analysis contrasts further with speculations of some early investigators, who had viewed lignin assembly as resulting from non-specific oxidative coupling of monolignols and subsequent random polymerization. At that time, though, the study of the complex biological (biochemical) process of lignin assembly had begun without any of the (bio)chemical tools to either address or answer the questions posed as to how its formation might actually occur. Today, by contrast, there is growing recognition of both sophisticated and differential control of monolignol biosynthetic networks in different cell types, which serve to underscore the fact that complexity of assembly need not be confused any further with random formation. Moreover, this analysis revealed another factor which continues to cloud interpretations of lignin downregulation/mutational analyses, namely the serious technical problems associated with all aspects of lignin characterization, whether for lignin quantification, isolation of lignin-enriched preparations and/or in determining monomeric compositions. For example, in the latter analyses, some 50–90% of the lignin components still cannot be detected using current methodologies, e.g. by thioacidolysis cleavage and nitrobenzene oxidative cleavage. This deficiency in lignin characterization thus represents one of the major hurdles remaining in delineating how lignin assembly (in distinct cell types) and their configuration actually occurs.

Contents

1. Introduction: overall scope of the analysis

In just over a decade, a number of studies have been conducted to manipulate gene expression in the monolignol (**1**–**3**) pathway, with important ramifications for control of subsequent lignin assembly(ies) (see Fig. 1). These studies have usually been done with the overall aim of modifying lignin contents and/or their compositions in plants. The anticipated benefits to humanity are potentially economic and environmental, in terms of cheaper and more process-amenable trees for pulp and paper manufacture with less pollution, more readily digestible forage for livestock (Baucher et al., 1998) and improved feedstock for fuel/chemical production (Lorenz and Morris, 1995). However, meaningful interpretation of many of these studies has frequently been limited, in large part because of substantial difficulties in the methodologies currently used for lignin analyses and characterization.

Nevertheless, as described herein, these research endeavors, together with related transcriptional and metabolic profiling studies, are steadily yielding a growing treasury of scientific data. This includes gaining important insight into: (1) how genetic manipulations can predictably perturb and disrupt programmed lignin assembly, albeit in so doing often deleteriously weakening the vascular apparatus, and hence its ability to adequately function; (2) the dynamics of phenylpropanoid metabolism and how carbon flux through various pathways is differentially controlled; and (3) how specific segments of interconnected metabolic networks are differentially regulated and compartmentalized.

Before discussing the results of genetic down-regulation and mutant plant studies, an understanding of the dynamic factors affecting metabolic control of the phenylpropanoid pathway branch networks to the monolignols (**1**–**3**) is required. This necessitates taking into full account the *interconnectedness* of each biochemical step as it occurs in vivo, as opposed to analyzing each in isolation. Furthermore, although the biochemical properties of enzymes in the monolignol-forming pathway are quite well characterized (summarized in Lewis et al., 1999), this information cannot readily be extrapolated to identifying the actual regulatory (or even functional) roles of any of the steps in vivo. On the other hand, metabolic flux and transcriptional profiling analyses (Anterola et al., 1999, 2002), as well as the study of mutant and transgenic plants, help to reveal further the response of the whole metabolic system, when each of the steps is individually perturbed. As indicated throughout this contribution, some responses in different species share certain common features whereas others are more species-specific. Hence, there is the opportunity to infer from the resulting phenotypes a much more precise understanding of each of the enzymes' roles in vivo, and/or a particular species' ability to respond to such changes, e.g. through either deployment or activation of shunt pathways and the like.

This article thus considers the results of both mutations and transgenic experiments directed to lignin modification, and how differential control of carbon flux through the phenylpropanoid pathway towards the various monolignols (**1**–**3**) is effectuated. A primary goal is to apply findings from metabolic and transcriptional profiling studies for the prediction of trends with transgenic plants and mutants, i.e. with those subjected to either down- or up-regulation or mutation of the gene expression of various phenylpropanoid and/or related pathway enzymes. This analysis also addresses the effects of modulating both monomer supply and monomer type on lignin assembly, and, by extrapolation, on the resulting biomechanical properties of the vascular tissues.

2. Monolignol-derived lignification is highly conserved in the plant kingdom

2.1. The functional basis for organized heterogeneous lignin assembly

From an evolutionary perspective, the monolignol-derived lignins are found in the pteridophytes (ferns), gymnosperms and angiosperms. [Fig. 1 summarizes our current understanding of the biosynthetic pathway to the monolignols (**1**–**3**) from Phe (**4**) and/or Tyr (**5**).] With few exceptions, it has long been known (Lewis and Yamamoto, 1990) that the monolignol pathway affording the corresponding lignins in pteridophytes and gymnosperms only utilizes *p*-coumaryl (**1**) and coniferyl (**2**) alcohols, whereas in angiosperms, a third monolignol, sinapyl alcohol (**3**), is employed. That is, the monolignol pathway is remarkably highly conserved throughout the evolution of these vascular plant forms, although mutants in the pathway (discussed later) have been known and well-studied for almost eight decades. [Note that the various lignin aromatic components derived from **1**–**3** are known as *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) constitutents, respectively (see Fig. 1, inset).]

Monolignol deposition is, however, both spatially and temporally targeted differentially in a heterogeneous manner throughout various cell wall types and layers. In contemplating why this is the case, it is apparent that little consideration has yet been given as to the beneficial properties bestowed by this heterogeneous deposition, i.e. in achieving the required physiological properties and functions of a particular cell wall type within the vascular apparatus. Put in another way, various cell types biosynthesize distinct forms of lignified polymeric matrices in order to attain a *particular* form of vascular integrity, and hence achieve the overall physiological functions needed for specific tissues, e.g. for water/nutrient conduction in tracheary elements and to withstand the forces of compression in supporting tissues.

In gymnosperms, such as black spruce (*Picea mariana*), the lignin in the tracheary secondary wall is mainly guaiacyl rich (i.e. derived from coniferyl alcohol (**2**)), whereas *p*-coumaryl alcohol (**1**) is a significant component in the middle lamella regions (Whiting and Goring, 1982). On the other hand, the secondary walls of fibers and ray cells in birch (*Betula papyrifera*, an angiosperm) are guaiacyl-syringyl rich since they mainly contain sinapyl (**3**) and coniferyl (**2**) alcohol derived lignins, as do the middle lamella regions between the fibers. Vessel lignins, by contrast, are primarily coniferyl alcohol (**2**) derived (Fergus and Goring, 1970a,b). Grasses contain, in addition to the three monolignols (**1**–**3**), considerable amounts of covalently linked hydroxycinnamic acids (He and Terashima, 1990; Lewis and Yamamoto, 1990). This cell (wall) heterogeneity is thus considered to result in different biomechanical properties of the lignified matrix within the various cell wall layers. These observations alone imply that lignin assembly is a highly organized biochemical process, which can predictably differ according to cell type.

2.2. Challenges in studying lignification

Understanding the complexities involved in lignin assembly (e.g. how the process is initiated and how heterogeneous deposition of the various monomers is

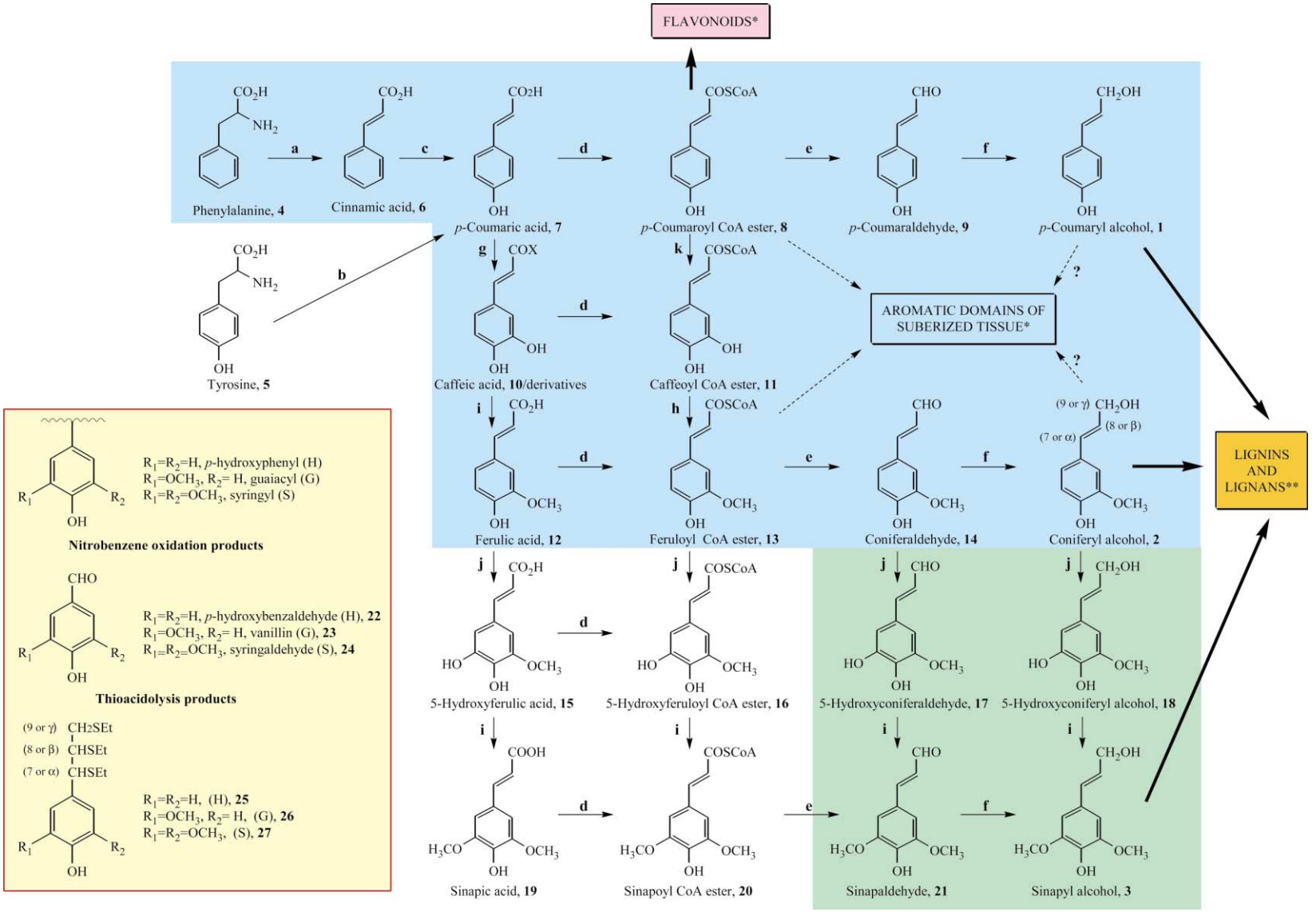
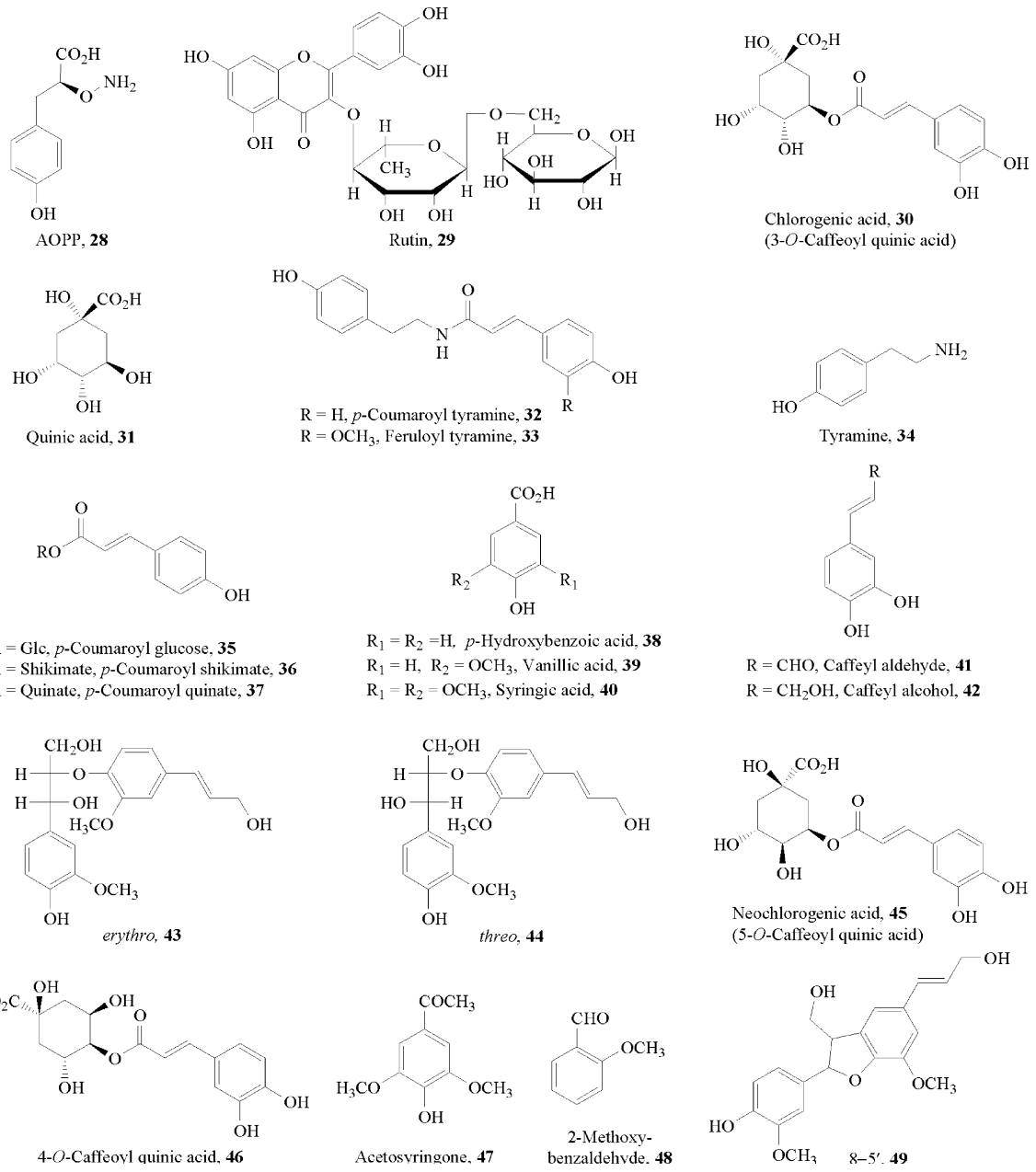


Fig. 1. Major segments of phenylpropanoid metabolism in vascular plants as currently understood: (a) Phenylalanine ammonia-lyase, PAL, (b) tyrosine ammonia-lyase, TAL (mainly believed to occur in grasses as a bifunctional PAL/TAL), (c) cinnamate-4-hydroxylase, C4H and its associated reductase, (d) 4-coumarate:CoA ligase, 4CL, (e) cinnamoyl-CoA reductase, CCR, (f) cinnamyl alcohol dehydrogenase, CAD, (g) *p*-coumarate 3-hydroxylase, C3H, (h) caffeoyl-CoA *O*-methyltransferase, CCOMT, (i) caffeate *O*-methyltransferase, COMT, (j) ferulate 5-hydroxylase, F5H and associated reductase and (k) *p*-coumaroyl CoA 3-hydroxylase, C3H and associated reductase. (Note: X = OH, quinate, shikimate, or an unknown functional group.) Inset shows the common denotation for unsubstituted (not cross-linked) aromatic rings in lignin polymers, as well as for the monomeric aldehyde products released by nitrobenzene oxidation and the monomeric thioethers obtained during thioacidolysis. Adapted from Lewis et al. (1999). Blue box displays provisional G-enriched lignin forming network; blue and green boxes display provisional G/S enriched lignin forming networks.



controlled) represents the main challenge in studying lignification today. Indeed, it is becoming increasingly clear that the formation and targetting of distinct monolignols (**1**–**3**) to regions of the cell walls, and to different cell types is at a much higher level of organization (and thus complexity) than previously supposed. Yet, surprisingly, a number of investigators believe that lignin macromolecular assembly, and consequently their structures, are completely random. This belief was recently boldly extended to encompass the view that if the traditional monomeric precursors are not available, other (monomeric) "non-traditional" metabolites can readily be incorporated. Indeed, to quote Boudet (1998), "Ralph suggests that plants simply require a polymer with specific mechanical properties, and that the actual composition of lignins is not particularly important." It was also claimed by Ralph (1997) that "perfectly viable plants are produced when traditional monomer production is inhibited and plants make lignins from 'abnormal' precursors". As the forthcoming pages consistently make clear, the experimental evidence in favor of this contention was not found at any level of inquiry.

The contributors of this article demonstrate further the existence of an exquisitely orchestrated biochemical process for lignin macromolecular assembly, in order to attain the requisite reinforcement of various cell types that are needed for vascular integrity of the tissues involved (Lewis and Davin, 1994; Lewis, 1999; Lewis et al., 1999; Croteau et al., 2000). This model not only

demands full biochemical control of macromolecular lignin assembly on a cell by cell basis, but it is also able to predict the deleterious effects on vascular integrity when assembly is perturbed in some manner. Lignification is thus within the realm of all other biochemical systems, in terms of it being both ordered and organized.

3. The control of monolignol ratio and lignin composition: metabolic and transcriptional profiling

Why do vascular plants consistently produce various types of cell walls with lignins from either two or three monolignols, and how does this occur in vivo? Furthermore, why are these moieties also differentially deposited into specific regions of developing cell wall types? To begin to answer such questions, two approaches have been taken: one is via metabolic flux and transcriptional profiling studies, and the second involves analysis of transgenic plants and/or mutants, where levels of particular pathway enzymes have been modified in some manner. Both have yielded valuable findings.

Metabolic and transcriptional profiling analyses in monolignol **1**–**3** biosynthesis were undertaken to ascertain the actual regulatory points in the phenylpropanoid pathway. [In this regard, the major "rate-determining" steps in a biochemical pathway are considered to be those catalyzed by enzymes with the highest flux control coefficient(s), with the latter being "*the fractional change in metabolic flux effected by a fractional change in amount (or activity) of the enzyme*" (Fell, 1997).] The experiments were hence designed to begin to systematically understand control of flux in monolignol biosynthesis, taking into account all of the steps in the pathway (Anterola et al., 1999). The approach employed *Pinus taeda* cell suspension cultures, whose undifferentiated cells could be indefinitely maintained on a growth medium containing 2,4-dichlorophenoxyacetic acid (Eberhardt et al., 1993). When transferred to a solution containing 8% sucrose (and 20 mM KI as an H_2O_2 scavenger), the cells essentially go into a static growth phase. However, the biochemical pathway (see Fig. 1) leading to *p*-coumaryl (**1**) and coniferyl (**2**) alcohols is induced, with both monolignols (**1**, **2**) being primarily excreted into the cell culture medium (Nose et al., 1995). [At present, cell cultures are the only means of effectively studying directly the biosynthesis of how monolignol composition and ratios are determined, i.e. without the complications associated with the assembly of different cell types within a growing plant form.]

The pool sizes of each intracellular phenylpropanoid metabolite were thus initially quantified during the course of monolignol pathway induction, in the presence and absence of exogenously supplied Phe (**4**) (Anterola et al., 1999). Addition of increasing amounts of Phe (**4**) to the point of metabolic saturation resulted in substantial increases in intracellular accumulation of cinnamic (**6**) and *p*-coumaric (**7**) acids up to 16- and 10-fold the basal level (Fig. 2A) (Anterola et al., 1999). None of the other metabolic intermediates to the monolignols in the cells were observed to increase in their levels despite Phe (**4**) saturation of the pathway. By contrast, the amounts of both *p*-coumaryl (**1**) and coniferyl (**2**) alcohols accumulating in the medium increased 26- and 3-fold, respectively, with preferential enhancement of the former, i.e. from relative ratios of ~1:8 when no Phe (**4**) was added, to ~1:1 at saturating levels (40 mM Phe) (Fig. 2B). Such fluctuations in monomeric content are also noted between lignins of "normal" and "reaction" woods of gymnosperms, respectively (Timell, 1986). This differential monolignol deposition, at least in part, gives cell walls very distinct functions, which help both to support the plant body weight and in achieving its orientation (Westing, 1965).

It was thus concluded that monolignol biosynthetic flux was controlled, not by a single rate-determining step, but

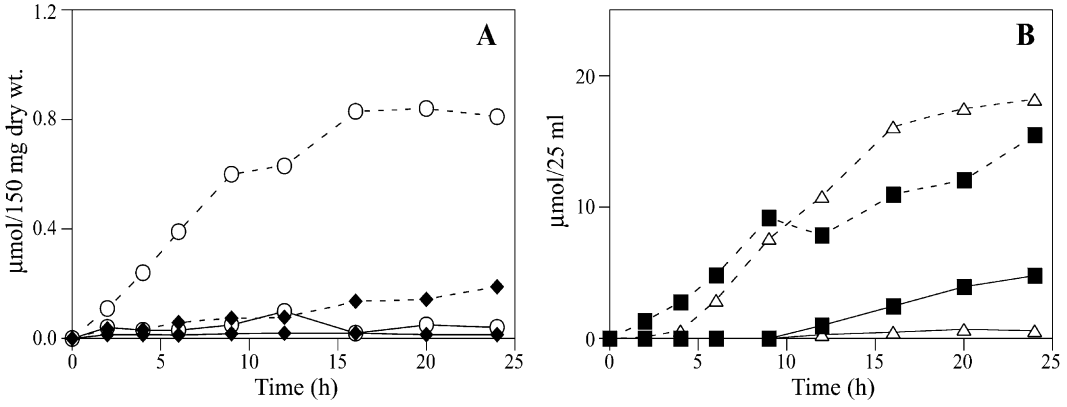


Fig. 2. Time course of metabolite accumulation in *P. taeda* cell suspension cultures: Intracellular accumulation of cinnamic (**6**, ○) and *p*-coumaric (**7**, ◆) acids in *P. taeda* cell suspension cultures incubated in 8% sucrose/20 mM KI solution (**A**), and corresponding levels of *p*-coumaryl (**1**, △) and coniferyl (**2**, ■) alcohol accumulation in the cell bathing medium (**B**). Solid and broken lines refer to the effects of 0 and 40 mM exogenously provided Phe (**4**), respectively. Adapted from Anterola et al. (2002).

by at least three factors, namely: Phe (**4**) supply, and the relative activities of cinnamate 4-hydroxylase (C4H) and *p*-coumarate 3-hydroxylase (C3H). There was no evidence obtained for any of the other downstream steps in the pathway exercising a *rate-determining* role in monolignol biosynthesis under the conditions employed. Additionally, when cinnamic (**6**), *p*-coumaric (**7**), caffeic (**10**) and ferulic (**12**) acids were administered to *P. taeda* cells, they were not metabolized further, but were instead converted into the corresponding glucosides. This suggested a channel pathway i.e. unlike Phe (**4**), the exogenously provided substrates were not accumulating in their correct location(s) within the cells, and that either shunt and/or detoxification pathways were being activated under such conditions.

Subsequent transcriptional profiling of the genes involved in monolignol biosynthesis reinforced these findings, i.e. that C4H and C3H were rate-limiting steps in the pathway (Anterola et al., 2002). Following cloning of all known genes encoding the phenylpropanoid pathway enzymes in *P. taeda*, quantitative real time PCR analyses were performed to measure transcript levels of each in the *P. taeda* cell cultures. It was found that the 8% sucrose/20 mM KI cell bathing solution coordinately induced all the genes required for monolignol formation, including phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), *p*-coumarate 3-hydroxylase (C3H), 4-coumarate:CoA ligase (4CL), caffeoyl-CoA *O*-methyltransferase (CCOMT), cinnamoyl-CoA reductase (CCR), and cinnamyl alcohol dehydrogenase (CAD), respectively.

Addition of exogenously provided Phe (**4**), which led to significant increases in intracellular cinnamic (**6**) and *p*-coumaric (**7**) acid levels, differentially upregulated further the expression of PAL, 4CL, CCOMT, and CCR, whereas C4H, C3H and CAD were only marginally affected. From these data, together with those of metabolic pool sizes, it appears that only C4H and C3H have true rate-limiting roles via transcriptional modulation, while the rest of the genes adjusted their respective transcript levels to accommodate higher metabolic flux. [CAD, apparently, has sufficient reducing potential, and therefore may not require much additional adjustment to increase metabolic flux.]

Another gene product, trivially called SAM:hydroxycinnamic acid/hydroxycinnamoyl CoA ester *O*-methyltransferase (AEOMT), and proposed as an alternative *O*-methyltransferase (OMT) involved in lignin biosynthesis in gymnosperms (Li et al., 1997) was not upregulated either in the absence or in the presence of Phe (**4**) in the induction medium; thus its proposed role in monolignol (**1**–**2**) biosynthesis in *P. taeda* is in doubt (Anterola et al., 2002). Given AEOMT's very low similarity to known caffeic acid and caffeoyl CoA OMTs (*circa* 34–41 similarity and 15–17% identity, respectively), its now presumed non-involvement in monolignol biosynthesis may not be surprising.

The metabolic and transcriptional profiling studies thus uncovered rudimentary aspects of phenylpropanoid pathway regulation. However, such investigations represent only one phase in defining the overall biological processes controlling monolignol formation, content and composition. There remains much to do. Other studies underway include: defining further signal transduction pathways that induces this differential regulation of gene expression in the various cell types involved; establishing what other "pathways" are coordinately up-regulated; and identifying the extent of post-trancriptional control of enzyme activities. Moreover, the identification of MYB (Tamagnone et al., 1998) and LIM (Kawaoka et al., 2000; Kawaoka and Ebinuma, 2001) transcription factors affecting phenylpropanoid metabolism represents yet another dimension in control of monolignol formation, whose import will need to be carefully factored in. Taken together, these findings permit the testing of a model for predicting results obtained with transgenic plants, modulated in their respective monolignol biosynthetic pathway enzyme levels. Much of the following article builds on the model, namely that Phe (**4**) supply, and relative activities of C4H and C3H, exercise decisive roles in establishing carbon allocation and differential metabolic flux to the various monolignols.

4. Technical limitations in current lignin analysis

Given that lignin deposition and composition can vary with cell wall layer, cell wall type and species, it is necessary to consider the efficacy and limitations of the lignin analytical procedures currently employed. Typically, these include methods of lignin quantification, estimates of lignin monomeric compositions, the isolation of lignin-derived preparations, and their spectroscopic analyses. None of these methods, however, are cell specific. Furthermore, each has serious limitations, most of which, while having been documented over several decades, are frequently either overlooked and/or ignored. This has been particularly true in the more recent analyses of transgenic plant tissues. However, as indicated below, the limitations are serious enough such that: (1) renewed efforts need to be launched to identify more suitable methods of lignin (fragment) analysis, including that of lignins within individual cell wall types; and (2) scientific reports in the future need to both specify, and be cognizant of, the limitations and assumptions in the lignin analyses undertaken.

4.1. Limitations in methods of lignin quantification (Klason, acetyl bromide, and thioglycolic acid analyses)

Two of the currently most popular analyses for apparently estimating lignin amounts are the Klason

(Klason et al., 1908) and acetyl bromide (Iiyama and Wallis, 1990) determinations; a less often utilized procedure is that of thioglycolic acid analysis (Brauns, 1952). The former method, developed for gymnosperm wood, involves the partial digestion of the plant material with 72% H_2SO_4 thereby leaving an insoluble (Klason lignin) residue. This method also releases smaller amounts of so-called soluble Klason lignin material, the quantities of which are significantly higher (up to 15% or so) when applied to the more acid-susceptible woody angiosperm lignins (Lai and Sarkanen, 1971). The procedure is, however, significantly compromised when used with herbaceous plants, this being believed to be due to overestimation because of co-precipitation of proteinaceous substances and the presence of non-lignin inorganic components (so-called ash constituents), i.e. depending upon the sample and development stage, values can be off by a factor of up to 2 and perhaps even higher (Lai and Sarkanen, 1971). Furthermore, the Klason procedure can also give overestimations when other non-lignin phenolics, such as those in woody bark tissues, are present. Accordingly, overestimations of lignin amounts due to contributions of non-lignin constituents can be sizeable, the extent of which needs to be determined and the lignin amounts revised accordingly (summarized in Lai and Sarkanen, 1971). For these reasons, Klason lignin data and their interpretations need to be both carefully collected and analyzed, particularly for transgenic herbaceous and juvenile (woody) plants.

Another popular lignin estimation method is the acetyl bromide determination, which relies upon solubilization of lignin fragments, with the amounts being quantified using an extinction coefficient of $\sim 20\ l\ g^{-1}\ cm^{-1}$. However, this method can result in significant lignin overestimations if non-lignin UV-absorbing substances are also solubilized. Another concern is that it has not been established whether this extinction coefficient can accurately be used for all lignins from all sources and developmental stages, including those derived from transgenic plants. The third and less often used method is that of thioglycolic acid extraction, which measures the absorbance at 280 nm of soluble materials released; of the three methods, as discussed later, this one can give very unreliable estimations.

4.2. Limitations in estimations of lignin monomeric compositions (pyrolysis GC/MS, nitrobenzene oxidation and thioacidolysis)

The three main methods used to estimate monomeric compositions of lignins include pyrolysis GC/MS (Ralph and Hatfield, 1991), nitrobenzene oxidation (Creighton et al., 1941) and thioacidolysis (Lapierre et al., 1986). There are several caveats to these analyses: (i) S/G values obtained by analysis of pyrolysis-GC/MS data do not currently give accurate quantification, and thus it is difficult to gauge how representative the reported S/G ratios are using this qualitative method. Hence, trends in S/G ratios by pyrolysis-GC/MS must be confirmed in other ways. (ii) S/G values obtained by all of the methods used currently are also surprisingly seldom reported in terms of actual percentages of lignin content. Moreover, the S/G amounts recovered, as estimated by the wet chemical methods (nitrobenzene oxidation and thioacidolysis), often represent only a very small proportion ($\sim$10–50% at best) of the overall estimated lignin present; Fig. 1 inset shows the products (**22**–**27**) obtained by these methods. [Of these, thioacidolysis is a procedure that releases monomeric adducts (**25**–**27**) from lignins, this being believed to occur primarily via cleavage of so-called β-*O*-aryl bonds. On the other hand, nitrobenzene oxidation, in strong alkali and at elevated temperatures, cleaves the side chains of various phenylpropanoids including those with unsubstituted (non-cross-linked) aromatic rings to yield the corresponding aldehydes (**22**–**24**). The thioacidolysis method is thought to be lignin specific, whereas the latter cleaves a variety of other phenylpropanoid constituents, e.g. hydroxycinnamic acids.] (iii) More often than not, the *p*-hydroxyphenyl (H) components of lignin cannot be detected by any of these methods. This is probably because the H-units are involved in other types of covalent interunit linkages which do not release appreciable levels of monomeric moieties (i.e. **22, 25**) during the tests employed. Thus, between 50 and 90% of the lignin cannot be detected using any of these methods. In short, *none* of the H/S/G analyses currently used can account for the bulk of the lignin being examined.

Hence, since lignin degradative analyses typically cannot provide an accurate assessment of both the presence and extent of H units, and even the G and S components are only recovered in amounts ranging from 10 to 50% of lignin apparently deposited, future major technological developments are urgently required to overcome these limitations. These are needed in order to advance further our understanding of the lignification process, i.e. as has already been done for the rapid progress in our ability to genetically manipulate each enzymatic step in lignin biosynthesis.

4.3. Limitations in lignin isolation procedures and spectroscopic analyses of lignin-derived fragments (e.g. ^{1}H and ^{13}C NMR spectroscopic analyses)

This review mainly uses three examples, described later in the text, to illustrate the serious limitations of both current "lignin" isolation procedures and application of various ^{1}H and ^{13}C NMR spectroscopic analyses in the study of such isolates. These limitations are discussed in detail in sections on 4-coumarate:CoA ligase, cinnamoyl CoA reductase, and cinnamyl alcohol dehydrogenase, respectively. It is first important for the

reader, however, to be cognizant of the fact that "native" lignin, regardless of specific cell type, cannot be isolated in an intact form. Additonally, even preparations originally described as "native" lignin by Brauns and coworkers (Brauns, 1939) were ultimately determined to be oligomeric polylignans, derived from a distinct biochemical pathway (Lewis and Davin, 1999) and involved in defense (Sakakibara et al., 1987). Moreover, the harsher "lignin" isolation procedures used today involve lengthy periods of hydrolysis (up to 11 or so days) in order to solubilize lignin fragments; however, neither the true extent of both bond-breaking and bond-making (artifact formation) is well understood, nor is the contribution of other non-lignin phenolic impurities, present in various cell and tissue types, accurately known. An additional concern is that such methods, originally developed for woody stem tissues, are now being routinely employed to non-woody species (juvenile and/or transgenic plants), even though the applicability of these technologies is largely both untested and unknown. Often, the so-called "lignin" isolates are dissolved in solvents such as dichloroethane–EtOH (2:1); such solubilization conditions are indicative of very low molecular weight components rather than polymeric moieties. For brevity, the limitations of these procedures and analyses are addressed in later sections.

5. Does perturbing lignin assembly always occur at the expense of vascular integrity?

To date, many transgenic plants and mutants have only been very preliminarily characterized, and thus the overall effects of genetic alteration are not well understood. This is especially true in terms of determining the effects on lignin assembly proper and the differential regulation of compartmentalized metabolic segments in the phenylpropanoid network. Nevertheless, it is instructive to consider what has been obtained thus far from studies directed towards lignin modification, and to identify some of the key information needed for the future. To the extent possible, these data include: (i) description of phenotypes obtained, including statistical analyses of results from same; (ii) the impact on lignin assembly and structure in particular cell types and the extent of their characterization (e.g. through comprehensive analyses of molecular weight profiles, and the application of various spectroscopic techniques); (iii) the effects on vascular integrity and on the biomechanical properties of the tissues so obtained; (iv) whether the pathways to the H, G, and S components of lignins are coordinately regulated in some manner or are maintained distinct; (v) the effects on pool sizes of metabolic intermediates in the phenylpropanoid pathway, as well as on levels of other Phe (**4**) derived metabolites; and (vi) the localization/expression of the various enzymes and genes.

Due to the limited (preliminary) information currently available, which mostly consists of changes in perceived gross lignin contents and variable syringyl/guaiacyl ratios (S/G, see inset, Fig. 1) (discussed below), only certain trends noted in these experiments can yet be discussed in any insightful manner. Indeed, some of the trends illustrated are based on only very few data points, and thus the statistical validity of several observations cannot be rigorously assessed at present.

In spite of such paucity of data, the transgenic plant and mutant studies are providing some inklings as to how lignin deposition and assembly differentially occur in cell walls, and the effects of perturbing and disrupting the lignin-forming system(s). Yet, fully understanding these effects at the molecular level remains difficult in large part due to lack of suitable methodologies. Indeed, as explained previously, current methodologies to study lignin deposition and structure remain seriously inadequate, and this is further complicated by the inability at present to effectively study lignification in individual cell wall assembly processes.

With these limitations in mind, a consideration of the analyses of various transgenic and mutant plant lines is given below.

5.1. Phenylalanine ammonia lyase (E.C. 4.3.1.5)

5.1.1. Questioning its role as a rate-limiting step

Being considered as the entry point enzyme to the phenylpropanoid pathway, the hypothesis that PAL served as a rate-determining step was initially reasonable (Rubery and Fosket, 1969). This hypothesis was based mainly on correlative studies of gene expression and enzyme induction with phenylpropanoid accumulation (Camm and Towers, 1973), as well as from teleological arguments (Rubery and Fosket, 1969). Thus, PAL was proposed as a rate-determining step in lignification mainly because it is a "metabolic branch-point" from which Phe (**4**) can give rise to lignins as well as to proteins (Rubery and Fosket, 1969). However, its role as a rate-determining step needed to be proven experimentally, not with correlative evidence.

Yet, in contrast, the previous discussion on both metabolic and transcriptional profiling in *P. taeda* indicated that PAL does not, in fact, serve as a rate-limiting step (Anterola et al., 1999, 2002). Furthermore, there may be distinct Phe (**4**) pools for specific purposes. That is, there may be a coordinated and specific upregulation of upstream pathways [leading to Phe (**4**)] for phenylpropanoid metabolism, including to specific branch pathways e.g. flavonoids. If correct, this would even remove PAL as being the "entry step", due to the existence of distinct and coordinated metabolic networks beginning from, for example, the pentose phosphate and glycolysis pathways and ending with the monolignols/lignins directly. In this regard, however, it is

readily acknowledged in many areas of inquiry, that our appreciation of transcriptional control, metabolic channeling and transport in living cells is grossly inadequate at present.

Nevertheless, various studies have been carried out to ascertain the effects of a general reduction of PAL activities in developing plants. This has been achieved through either the use of inhibitors or by generating transgenic plants with altered PAL activities. Such studies are particularly instructive when the precise effect(s) of down-regulation are examined, e.g. as regards the outcome on the vascular apparatus, as well as on build-up of associated metabolic intermediates, shunt metabolites and other end products.

The first manipulation of in vivo PAL activity was achieved with the use of L-α-aminooxy-β-phenylpropionic acid (L-AOPP, **28**), considered a specific inhibitor of PAL (Amrhein and Gödeke, 1977). Treatment with this inhibitor had many effects on various branches of phenylpropanoid metabolism. It reduced both the formation and the extent of accumulation of anthocyanins in *Ipomoea tricolor* and *Catharanthus roseus* flowers, red cabbage (*Brassica oleracea* var. *capitata*) seedlings, and buckwheat (*Fagopyrum esculentum*) hypocotyls (Amrhein and Holländer, 1979), as well as isoflavones in *Cicer arietinum* (Amrhein and Diederich, 1980), hydroxycinnamic acid esters in gherkin (*Cucumis sativus*) seedlings (Amrhein and Gerhardt, 1979), and lignins in mungbean (*Vigna radiata*) (Amrhein et al., 1983; Smart and Amrhein, 1985). The effects of L-AOPP (**28**) on mungbean development revealed that, upon comparison to the control (Fig. 3A), this treatment reduced the extent of lignin biosynthesis as evidenced by collapsed xylem vessels (Fig. 3B), due to weakening of the vascular apparatus. These studies could not, however, establish whether or not PAL quantitatively controlled metabolic flux towards any of these natural products. *Nevertheless, it has been known for almost 20 years that a reduction in lignin content had serious physiological consequences on the vascular apparatus.*

5.1.2. PAL downregulated transformants: reversion back to wild type-like phenotypes over successive generations

In most plant species, phenylalanine ammonia lyase exists as a multigene family (Lewis et al., 1999), and although not proven, it has been speculated that individual genes have distinct metabolic roles, e.g. to flavonoids, lignins, etc. (Liang et al., 1989). In tobacco (*Nicotiana tabacum*), for example, there are at least four PAL genes known (Fukasawa-Akada et al., 1996). However, the precise *physiological* roles of the corresponding enzymes have not yet been established in terms of specific involvement in any particular branch or network of phenylpropanoid metabolism. Nevertheless, transgenic tobacco plants with altered PAL activities and decreased lignin contents have been successively generated using antisense and sense suppression strategies.

Tobacco plants were first transformed using the PAL2 gene from French bean (*Phaseolus vulgaris*) in the sense and antisense orientations, under control of the constitutive CaMV 35S promoter (Elkind et al., 1990). The primary transformants (T_0) were selfed to afford the first generation progeny (T_1), with the second (T_2) up to the fifth (T_5) generation progenies being obtained by subsequent selfing. This resulted in sense transformants having either a reduction (through sense suppression) or an increase (from overexpression) in PAL activities, from as low as 1% to as high as 210% of control levels (Sewalt et al., 1997a).

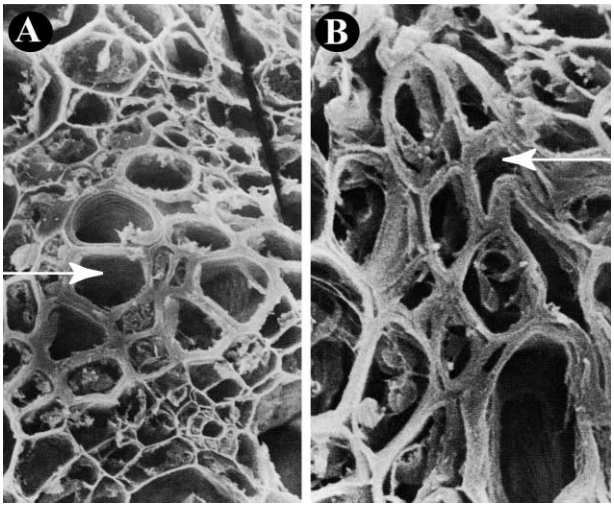


Fig. 3. Effect of PAL inhibitor L-AOPP (**28**) on vascular integrity of mungbean (*Vigna radiata*) hypocotyls. Normal and collapsed vessels of control (A) and L-AOPP (**28**)-treated (B) mungbean plants are indicated by arrows. Reproduced from Amrhein et al. (1983) with permission of Urban & Fischer Verlag.

This study made the assumption that introduction of a particular PAL gene from one species into another, under control of the CaMV 35S constitutive promoter, does not give phenotypes resulting from any other unintended effect(s) of heterologous gene transfer. (This matter is discussed later.) The phenotypes of T_0 transformants did, however, display a number of abnormalities, which included stunted growth, deformed flowers, leaf epinasty and localized lesions in the leaves (Bate et al., 1994) (see Fig. 4A and Table 1, where the latter summarizes the preliminary characterization of the plants completed to date). Note that given the many functions of PAL in different metabolic processes, it is not yet possible to fully ascertain which physiological effects are due to downstream reduction of lignin levels and which are not.

The transformants were also not stable, since from the T_0 to T_5 generations PAL activity was gradually restored to near wild type levels, with corresponding increases in the lignin levels measured. Moreover, the restoration of PAL activity afforded both somatic revertant and "over-expressor" phenotypes, while others remained PAL-downregulated (Korth et al., 2001). Interestingly, the analysis of xylem tissues of petiole cross-sections in the PAL-suppressed (Fig. 4B), somatic revertant (Fig. 4C), PAL overexpressors (Fig. 4D) and wild type (Fig. 4E) plants displayed significant differences, i.e. the overall vascular integrity was greatly compromised in the PAL-silenced tissues, as evidenced by the near absence of detectable vascular elements. By contrast, the over-expressors and somatic revertants had well-defined vascular elements of good apparent integrity (Korth et al., 2001).

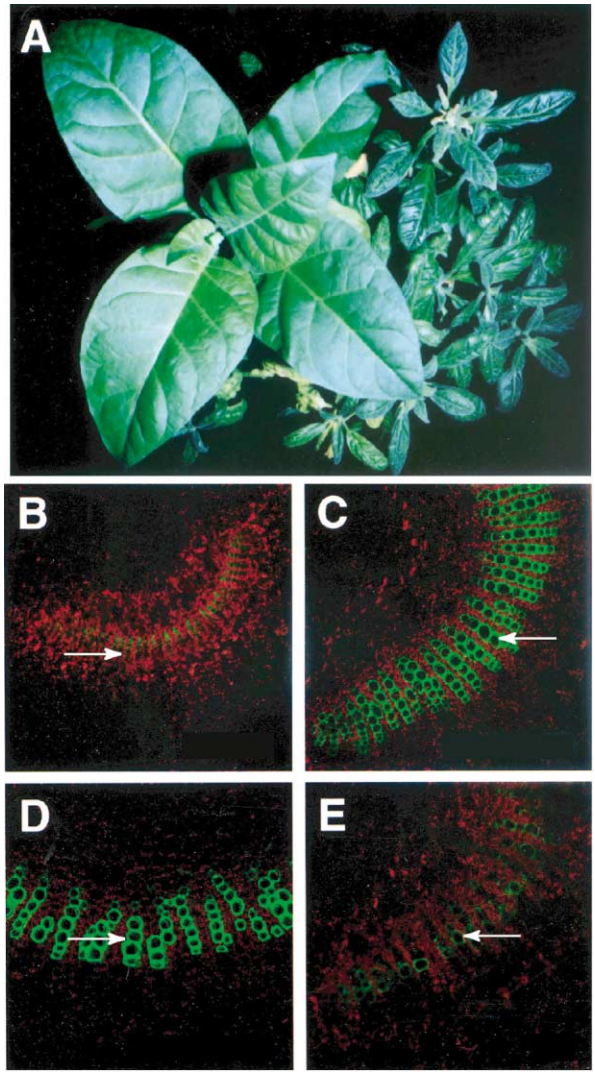


Fig. 4. Physiological effect of up- and down-regulation of PAL in tobacco (*Nicotiana tabacum* L. cv. Xanthi) plants. (A) Transgenic tobacco lines transformed with bean PAL2 gene, showing both somatic revertant (left) and PAL-downregulated (right) phenotypes in the same plant after five generations of selfing. UV fluorescence of stem cross-sections of antisense PAL suppressed (B), somatic revertant (C), PAL overexpressing (D), and wild type (E) plants. Reproduced from Korth et al. (2001) with permission of Blackwell Munksgaard. → = Vascular apparatus (lignified elements).

Table 1
Preliminary characterization of transgenic tobacco (T_1–T_5 generations) following introduction of sense PAL2 from bean (adapted from data from Elkind et al., 1990; Sewalt et al., 1997a)

Plant line	Generation[a]	Transgene orientation	Promoter	Gross morphological changes in phenotypes of primary (T_0) transformants*	Tissue analyzed for lignins	PAL activity (% relative to average activity of controls)	Lignin amounts[b] (% NDF)	S/G ratio[c]	Analytical techniques employed	Ultrastructural effects on cell wall types and patterns of lignin deposition
C17a	T_5	(Control)			Midstems	148	11.2	1.10	Pyrolysis-GC/MS to determine S/G ratio; Klason lignin analysis of neutral detergent fiber; histochemical staining; methoxyl group analysis	
C17c	T_5	(Control)			(10th–11th	103	10.8	n.d.[d]		
C17b	T_5	(Control)			internodes	49	10.9	n.d.[d]		
10–6a	T_1	Sense	35S	Stunted growth,	counting from the top)	210	11.2	1.22		
10–6c	T_1	Sense	35S	curled leaves,	from 5-week-old	199	8.6	n.d.[e]		
160P3b	T_2	Sense	35S	localized lesions, less	plants	26	8.0	n.d.[f]		Patterned wine-red/brown stain in xylem ray cells and sclerenchyma fibers (data not shown)
160P3a	T_2	Sense	35S	pollen and		2	5.7	1.88		
160P3c	T_2	Sense	35S	reduced viability,		1	3.4	n.d.[f]		
274T5b	T_5	Sense	35S	deformed		56	10.2	n.d.		
274T5c	T_5	Sense	35S	flowers, thinner		41	9.8	n.d.		
274T5a	T_5	Sense	35S	xylem, and less lignin		50	7.1	n.d.		

n.d. = not determined; NDF = neutral detergent fiber. The enzyme and gene expression localizations, as well as the frequency of interunit linkages in, and MW ranges of, lignins were not reported.

[a] The phenotypes of the T_1–T_5 progeny were not provided.

[b] Klason lignin contents are approximately half of that obtained by other methods. Neutral detergent fiber (NDF) lignin refers to a procedure for fractionation of tobacco plant material (Van Soest et al., 1991) prior to Klason lignin analysis.

[c] S/G ratios were estimated by pyrolysis-GC/MS; this method does not give accurate quantification.

[d] An average S/G ratio of 1.1 was estimated for control plants ($n = 3$).

[e] An average S/G ratio of 1.1 was estimated for PAL over-expressors ($n = 3$).

[f] An average S/G ratio of 1.6 was estimated for PAL sense-suppressed lines ($n = 3$).

5.1.3. *Effects on lignin content*

It was considered instructive to plot the reported reductions in lignin contents of T_1, T_2 and T_5 transformants against the corresponding PAL activities measured in vitro. These were plotted relative to the controls, with the latter being previously transformed plants in which the bean PAL2 gene could no longer be detected following segregation (after five generations of selfing) (Fig. 5A). The graph makes the provisional assumption that detectable PAL activities can in fact be compared with lignin contents, even though the data collection for each occurs only at the harvesting stage. That is, it is hoped that such correlations might reflect a comparable trend during overall growth and development, i.e. that are valid not only at the specific stage of plant growth when the data were collected. However, it must be emphasized that essentially all transgenic studies conducted thus far have the same gross limitations. Clearly, in the future what actually transpires (e.g. how PAL activities and lignin contents change as the plant matures) needs to be determined by more extensive analyses.

Nevertheless, a preliminary trendline has been drawn, based on nine out of the 11 data points, to represent the most likely effect of decreasing PAL activity on the production and deposition of lignin. The graph (Fig. 5A) suggests that significant reductions in lignin can indeed occur, but apparently only if PAL activity has been reduced below half that of the control plants. The actual method of lignin analysis used (acid insoluble Klason lignin of "neutral detergent fiber"), however, gave very low lignin contents (e.g. ~11% in wild type, see Table 1), relative to other methods; indeed, their values are almost half of those estimated using the other protocols, this perhaps being a consequence of the fiber fractionation process employed.

Yet, if the overall *trends* observed truly reflect the general relationship between PAL and lignin content at all stages of plant development, then there are at least four important implications for the role of PAL in vivo; however, it must be emphasized that caution needs to be exercised in making these extrapolations. Conclusions provisionally made must be more rigorously verified by additional and more detailed analysis of transgenic plant studies.

Firstly, the fact that lignin production is unaffected until PAL activity is apparently reduced to half its original amount *provisionally* suggests that there is more

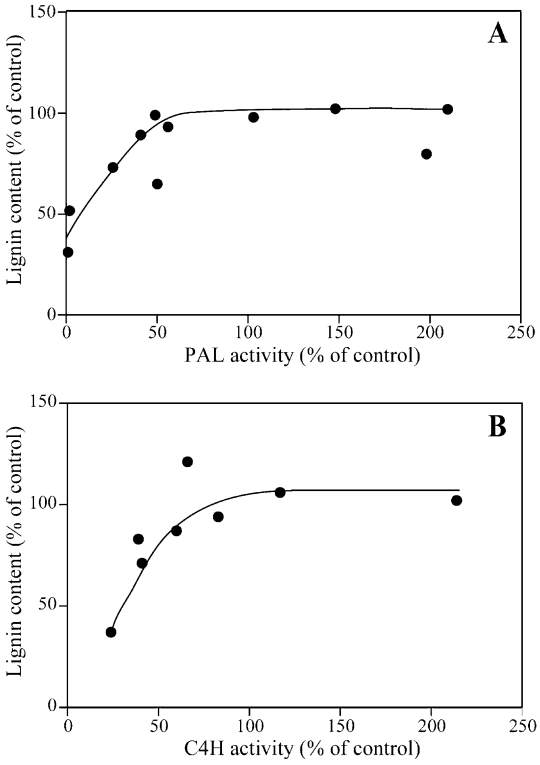


Fig. 5. Preliminary plots of lignin contents versus gross enzyme activities in transgenic tobacco plants with up- and down-regulated PAL (A) and C4H (B). Data recalculated from Sewalt et al. (1997b). Reported values were recalculated to obtain the percentages relative to the controls (see text for details).

PAL activity present in the control plants than "necessary" to support the carbon flow towards lignin biosynthesis. Thus, at least in the context of lignin biosynthesis, PAL cannot be rate-determining since there is apparently more of its activity than necessary. Conversely, the in vitro activities (measured from mid-stem sections) represent total activities of PAL enzymes that are not exclusively involved in lignin production, but which are also utilized in the biosynthesis of other phenylpropanoid products. It is unlikely, however, that the biosynthesis of any other phenylpropanoids would require the same levels of PAL activity as needed for lignins.

Secondly, since the amount of PAL present in control plants is apparently more than that necessary to support lignin biosynthesis, this implies that provision of more Phe (**4**) should result in an increased formation of downstream metabolites, at least up to cinnamic acid (**6**). Although this has not been demonstrated yet, such a finding would be consistent with the earlier investigations using *P. taeda* cell cultures where, as the amounts of available Phe (**4**) rose, both the intracellular pool of cinnamic acid (**6**) and the amounts of monolignols (**1** and **2**) formed increased (Anterola et al., 1999). This would yet be another line of evidence supporting further the role of increased Phe (**4**) supply in determining the amount of carbon allocated to the monolignol-forming pathway.

Thirdly, increasing further the activity of PAL did not result in any appreciable increase in lignins (as the trendline in Fig. 5A reveals). This again implies that PAL is not a rate-limiting step. Thus in order to increase lignin contents, it can be considered that the upstream steps controlling Phe (**4**) supply to the phenylpropanoid pathway may need to be upregulated, together perhaps with those of other slower steps downstream. Indeed, while it is recognized that the control points prior to Phe (**4**) deamination remain to be fully established, our previous studies had also indicated that N-recycling via the glutamine synthase/glutamate 2-oxoglutarate amino transferase (GS/GOGAT) pathway is involved in controlling Phe (**4**) supply (van Heerden et al., 1996; van Rensburg et al., 2000; Anterola et al., 2002).

Fourthly, the resulting plant material has significantly lower lignin levels and a weakened vasculature at highly repressed PAL activities. Indeed, as will become increasingly clear from this analysis, essentially all studies aimed at reduction of lignin typically resulted in ~50–60% lignin reduction at best, as determined by current analytical methods for quantification. Accordingly, this is the second line of evidence offered in this article to demonstrate that plants do not respond to reductions in lignin monomer supply by synthesizing other "non-traditional" (phenolic) metabolites to "compensate" for this reduction, and incorporating them into lignin.

5.1.4. Effects on lignin monomeric composition: evidence for differential control of G and G–S pathways?

One potentially interesting effect of PAL down-regulation, however, was the suggested increase in syringyl/guaiacyl (S/G) ratios in the lignified tissues from ~1.1 in the wild type to ~1.9 in the sense-suppressed line as the lignin contents decreased (see Table 1 and Fig. 1 inset). However, the study was not extended to the analysis of the various cell wall types in the tissue to establish if their relative proportions and/or lignification patterns had been altered. [In this regard, the S component in G–S lignin-forming cell walls is often considered to be mainly restricted to the fibers and is mainly laid down towards the end of lignin deposition in most mature tissues. This matter though needs to be investigated much more fully.]

The above data were taken by the investigators to *provisionally* suggest a preferential decrease in coniferyl alcohol (**2**) formation relative to that of sinapyl alcohol (**3**), which by extrapolation could suggest differential control of the G and G–S monolignol networks present in vessels (G) and fibers/ray cells (G–S), respectively; moreover, this might also extend to that of the H pathway as well, even though the latter was not detected by

the methods used and thus cannot be adequately quantified at this time. It should be emphasized though that only one transformant (160P3a) was reported as having this ~2-fold increase in S/G ratio (Table 1). The increase in S/G ratios of the other sense-suppressed lines was presumably not as large, since the reported average ratio was only 1.6 (Sewalt et al., 1997a). This trend was also qualitatively suggested by the use of the Wiesner (phloroglucinol-HCl) reagent; this test did not detect lignin in the PAL sense-suppressed line (160P3a), even though it still apparently contained ~50% of the wild type lignin content. The Wiesner test is often described as being "coniferaldehyde (**14**) end group" specific, and typically gives weak or no staining in the presence of syringyl units (Sarkanen and Ludwig, 1971). However, phloroglucinol-HCl is not absolutely specific, as often claimed, to coniferaldehyde (**14**) end groups.

5.1.5. Effects of Phe downregulation on other branches of phenylpropanoid metabolism: the existence of distinct metabolic networks?

The study using transgenic tobacco plants (Bate et al., 1994) also permitted examination of other effects of downregulating PAL on specific "branches" of phenylpropanoid metabolism, i.e. of rutin (**29**) and chlorogenic acid (**30**) levels in the leaves. Unlike lignin biosynthesis, the formation of these two phenylpropanoid metabolites seemed to be directly proportional to the residual PAL activities in the transformants, based on logarithmic plots of rutin (**29**) and chlorogenic acid (**30**) against PAL activity. It provisionally appears therefore that heterologous PAL gene repression affects distinct PAL-dependent pathways differentially, as would be expected. Furthermore, the chlorogenic acid (**30**) response curve had a steeper slope than that of rutin (**29**), which suggests that formation of the former was more sensitive to changes in PAL activity than that of the latter. If correct, these differences in the rate-determining capacity of PAL for distinct "branches" of the phenylpropanoid pathway can be considered as providing provisional support for the existence of distinct metabolic networks to each end product, as would be expected. Furthermore, the effect on chlorogenic acid (**30**) accumulation is of particular interest; PAL downregulation could potentially affect caffeic acid (**10**) formation but perhaps not that to the shikimate pathway intermediate, quinic acid (**31**), which is also needed for chlorogenic acid (**30**) formation. These data suggest, however, that PAL reduction has decreased caffeic acid (**10**) availability and hence the ability to form chlorogenic acid (**30**).

5.1.6. Special concerns as regards the use of tobacco for studying the effects of lignin downregulation

Plants in the Solanaceae, such as tobacco, produce other "mixed" metabolic products such as *p*-coumaroyl (**32**) and feruloyl (**33**) tyramines, particularly in response to "stress". These substances are, for example, found in both soluble (extractable) form and as cell wall bound entities in tobacco (Pearce et al., 1998, and references therein). It would thus be useful to establish the effect of downregulating PAL on the ability to accumulate tyramine (**34**), and its downstream *p*-coumarate and ferulate derivatives (**32**) and (**33**).

5.1.7. Challenges for the future

A major effect of severely repressing PAL activity (to ~1% wild type) through genetic downregulation was observed in the significant reduction in the total amounts of lignin formed (>50%), while there was no effect on lignin content by overexpression. Yet, in spite of such interesting results, these transgenics need to be more fully characterized, e.g. in terms of ascertaining potential differential effects on H, G, and G–S pathways, on lignin polymerization proper, on PAL isozyme localization and gene expression, and on ultrastructural analyses of the lignins in various cell types. For example, it is unknown as to what changes, if any, on the average lignin polymer molecular weight distributions and/or frequencies of inter-unit linkages have occurred upon PAL downregulation. Nor is it known what the physiological effects are of compromising the vascular apparatus on, for example, water and nutrient conduction. Note, however, that these limitations apply to the study of each enzymatic step in lignin formation.

It will also be important to statistically ascertain how compositional and quantitative changes in lignin biopolymer assembly are translated into different overall biomechanical properties of the vasculature. This could include different patterns of lignin deposition in distinct cell wall types and perhaps even on the frequencies of the resulting architectures encountered (e.g. number of vessels, fibers, etc.). Indeed, these data suggest that an examination of the effects of *progressively* downregulating PAL should be quite instructive.

Moreover, such studies will hopefully begin to shed light on the reasons for the abnormalities noted in growth and development. Clearly, such growth and developmental abnormalities represent important clues, and require full explanation in the future. For example, does the stunted growth reflect some sort of feedback mechanism? Or, what branches of metabolism are impacted other than those involving PAL? Furthermore, what changes occur in levels of mixed pathway intermediates, such as to the tyramine derivatives (**32** and **33**), and which cells are involved? Finally, it must also be cautioned that the use of the "constitutive" CaMV 35S promoter may also have inadvertent effects, due to its action not being restricted to the lignin forming vasculature.

Table 2
Preliminary characterization of transgenic tobacco (T_0) following introduction of sense and antisense C4H

	Plant line	Transgene orientation	Promoter	Tissues analyzed for lignins	% C4H activity (relative to average activity of controls)	Lignin amounts[a]	Estimated S and G recovery (% of lignin)	S/G ratio[b]	Analytical techniques employed
A.	11A	(Control)		Midstems	117	9.7	n.d.	n.d.	Pyrolysis-GC/MS;
	8A	(Control)		(8th–11th internodes	83	8.6	n.d.	0.6	Klason lignin analysis of neutral detergent
	201C	Sense	35S	counting from	214	9.3	n.d.	0.6	fiber; histochemical
	2C	Sense	35S	the top),	66	11.1	n.d.	n.d.	staining; methoxyl
	32C	Sense	35S	harvested 4	60	8.0	n.d.	n.d.	group analysis
	25C	Sense	35S	weeks after regrowth	39	7.6	n.d.	n.d.	
	72B	Antisense	35S		41	6.5	n.d.	0.1	
	13B	Antisense	35S		24	3.4	n.d.	0.0	
B.	C4H WT	(Control)		Xylem tissues from the fourth	n.d.	20.2	34.5	0.8	Thioacidolysis; acetyl bromide lignin
	C4H 32	Sense	35S	internodes	n.d.	20.3	35.6	1.1	determination;
	C4H 38	Sense	35S	of mature	n.d.	18.6	33.9	0.8	histochemical staining
	C4H 30	Sense	35S	plants	n.d.	14.7	33.4	0.8	
	C4H 49	Sense	35S		n.d.	17.9	29.1	0.8	
	C4H 45	Sense	35S		n.d.	20.1	29.3	0.7	
	C4H 34	Sense	35S		n.d.	18.4	27.4	0.5	
	C4H 10	Antisense	35S		n.d.	17.6	29.3	1.0	
	C4H 12	Antisense	35S		n.d.	19.0	34.7	0.9	
	C4H 2	Antisense	35S		n.d.	19.0	38.8	0.7	
	C4H 24	Antisense	35S		n.d.	19.2	34.9	0.7	

Transgenes used were alfalfa C4H (A) (Sewalt et al., 1997a; Blount et al., 2000) and French bean C4H (B) (Blee et al., 2001). The enzyme and gene expression localizations, the frequency of interunit linkages in and MW ranges of lignins, as well as the ultrastructural effects on cell wall types and patterns of lignin deposition were not determined (n.d.).

[a] Lignin amounts in entry (A) are shown as Klason lignin percentages of neutral detergent fiber (NDF), whereas those in the (B) entries are acetyl bromide lignin estimates as percentages of cell wall residue (CWR). Corrections were not included for protein and/or ash contents.

[b] Estimated by pyrolysis-GC/MS (A), and by thioacidolysis (B).

5.2. Cinnamate 4-hydroxylase (E.C. 1.14.13.11)

Cinnamate 4-hydroxylase (C4H) can exist in at least two forms depending upon the species, namely C4H-1 and C4H-1/C4H-2, based on studies of alfalfa (Fahrendorf and Dixon, 1993) and French bean (Nedelkina et al., 1999), respectively. C4H-1 has long been correlated with lignification (Lewis et al., 1999) and other phenylpropanoid pathway branchpoints, whereas the *physiological* role of C4H-2 still needs to be fully established.

Two preliminary studies have been directed to manipulating C4H levels and Table 2 and Figs. 5B, 6A to D summarize what is known thus far about these T_0 transformants and their lignin constituents. Interestingly, neither study reported any visible abnormalities in the growth and development of the phenotypes obtained (discussed later) in spite of the substantial reductions in estimated lignin contents achieved (see Table 2A and B); however, there is currently no information of effects on the vascular integrity and biomechanical properties of the C4H-downregulated lignified tissues (relative to the controls).

When the alfalfa C4H-1 gene was placed downstream of the CaMV 35S promoter in tobacco, the results obtained were of interest. Downregulation of C4H-1 via sense and antisense suppression (Sewalt et al., 1997a; Blount et al., 2000) apparently resulted in essentially a proportional decrease in lignin content, as indicated by Klason lignin analyses of the "neutral detergent fiber" as noted before; note again that this method gave lignin contents in tobacco that are, however, reduced by a factor $\sim$2, relative to other methods. Nevertheless, C4H activity over a wide range of detectable activities (24–214%) was plotted against lignin content as shown in Fig. 5B (controls employed transformants with an empty vector). The assumptions made in plotting C4H activity versus lignin content have the same limitations as for the PAL data analysis. Thus, assuming the *trend* of decreasing lignin content is correct, then this trendline would extend our prediction that C4H activity modulates metabolic flux in a rate-limiting manner, but cannot increase overall carbon allocation (Anterola et al., 1999, 2002). Indeed, increasing C4H activity did not result in more lignin being formed (Fig. 5B), in accordance

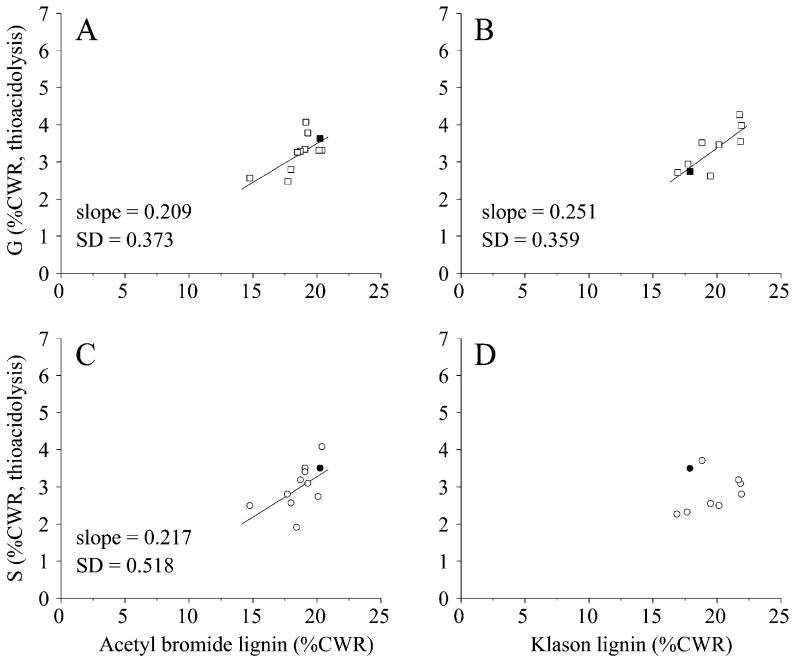


Fig. 6. Correlation of lignin in cell wall residues (CWR) of wild type (solid symbols) and C4H-transformed tobacco plants (open symbols) versus guaiacyl (G, ■, □) and syringyl (S, ●, ○) contents. Lignin estimates were determined by both the acetyl bromide (A, C) and Klason (B, D) methods. %G and %S amounts were determined by thioacidolysis analyses. SD = standard deviation. Adapted from Blee et al. (2001).

with Phe (**4**) supply being controlled by processes further upstream.

Besides the effects on overall lignin content, C4H downregulation reportedly also markedly affected the S/G composition of the transformants (Sewalt et al., 1997a). Using the non-quantitative method of pyrolysis-GC as discussed before for analysis of the mid-stems of 4-week old (from regrowth) transformed tobacco plants, the results obtained suggested significant decreases in the S/G ratio, i.e. from 0.6 in the control to 0 and 0.1 in the antisense-suppressed lines (Table 2A). The use of Wiesner staining also provisionally agreed with this observation, since the color reaction of lignified cross-sections remained essentially the same, except for its intensity being diminished due to the reduction in lignin content. Taken together, this is apparently the opposite trend to that reported for PAL. It was thus suggested that manipulation of C4H seems to exert greater control over metabolic flux to sinapyl alcohol (**3**), and hence potentially the G–S networks operative in fibers and ray cells. This explanation, however, assumes that (i) the data obtained is representative of S/G ratios and (ii) the C4H gene from alfalfa under control of the CaMV 35S promoter affects only expression of the endogenous C4H gene in the transformed tobacco.

In addition to the concerns raised previously about the limitations of the methodologies employed, an alternative possible explanation for the proposed decrease in the S/G ratios is that the expression of another P450 gene encoding ferulate 5-hydroxylase (F5H) may have been suppressed by introduction of the alfalfa C4H gene. Inadvertent epigenetic silencing of F5H (which is specifically involved in introducing the additional aromatic hydroxyl group before its methylation for sinapyl alcohol (**3**) biosynthesis) would be expected to cause a reduction in the S/G ratio. Hence, the effects of heterologous genetic silencing of C4H in tobacco on F5H levels should be examined in the future to establish if this indeed was adversely affected.

A second study (Blee et al., 2001) also examined the effects of C4H downregulation on lignification in tobacco, but this time using the French bean C4H-2 gene (of unknown physiological function) downstream of the CaMV 35S promoter as before (see Table 2B and Fig. 6A–D). In that study, a smaller decrease in the apparent lignin content [from ~20.2 to ~14.7% acetyl bromide (Table 2B, Fig. 6A and C) and ~21.9 to ~16.8% Klason (Fig. 6B and D) lignin, respectively], was noted, with the rate of lignification provisionally appearing to have been decelerated during growth and development; note that the Klason lignin estimates were uncorrected as before (see Section 4.1).

It was, nevertheless, considered instructive to individually plot the %G and %S contents (released by thioacidolysis) of the cell wall residues (CWR) for both transgenic and control plants versus their corresponding acetyl bromide and Klason lignin estimations, respectively, and to draw the best fitted line based on the least squares fit method (Freedman et al., 1978). From the limited data available, the plots of %G versus lignin contents (Klason lignin and acetyl bromide lignin, Fig. 6A and B) provisionally suggested an essentially

linear correlation with decreasing lignin amount, whereas the plots of %S versus lignin content (acetyl bromide and Klason lignin, Fig. 6C and D) were more erratic. However, only about 30–40% of the total lignin could be accounted for by the thioacidolysis technique, and thus the bulk of the lignin present was not detected (discussed later). Finally, the availability of only one control plant precluded a meaningful comparison of the lignin levels and monomeric compositional variations within the controls themselves.

Interestingly, the thioacidolysis analysis gave very slightly lower overall recoveries of S units relative to G; nitrobenzene oxidation, on the other hand, had an opposite effect (data not shown). The latter will be slightly overestimated by contributions from covalently bound tyramine (**34**), its hydroxycinnamic acid amides (**32** and **33**), and other hydroxycinnamic acids, which are also present in tobacco tissues in small amounts (discussed later). Additionally, based on the fitted line, G and S reductions do not appear to fully account for the decrease in total lignin amounts as judged by the projected intersection at the *X*-axis, assuming a linear trend (Fig. 6). If correct, this would imply one or more of the following: (i) that the earlier stages of lignin deposition either involve formation of substructures whose covalent linkages are not readily cleavable by thioacidolysis or nitrobenzene oxidation to give the monomeric derivatives (**22**–**27**); (ii) that lignin values are overestimated using the protocols employed; and/or (iii) a combination of both.

5.2.1. Challenges for the future

The data from downregulation of C4H-1 *provisionally* supports our interpretation that C4H is a major rate-limiting step whose downregulation apparently results in a progressive and quantitative reduction in lignin content. However, these data need to be extended using additional analyses such as those employed for the C4H-2 study. It will also be important to ascertain what other effects on metabolism have occurred, e.g. on formation of "mixed" pathway metabolites in tobacco, such as feruloyl (**33**) and *p*-coumaroyl (**32**) tyramine levels.

As for the PAL transformants, it will be important to correlate progressively decreasing C4H activities with cell wall developmental and morphological changes on a *statistical* basis, as well as in examining the effects on lignin composition (i.e. nature of inter-unit linkages, monomeric composition, and molecular weight distributions). It is anticipated that reductions in lignin will have profound effects on both lignin structure and cell wall morphology/biomechanical properties.

Additionally, it is not known whether the C4H downregulated phenotypes will revert back in subsequent generations or not, since all analyses to date were only carried out using T_0 transformants. This concern also applies to the other transformants discussed throughout the article. This raises another important point: in these two studies, as in many others, reports often indicate that there are no observable phenotypical changes, as determined by visual inspection. As will become increasingly clear in the article, many significant changes (such as in reductions in biomechanical tissue strength properties) can occur, even though they are not visible to the naked eye.

5.3. p-*Coumarate 3-hydroxylase*

p-Coumarate 3-hydroxylase is the name tentatively assigned to the enzyme responsible for introducing a hydroxyl group at the *meta*-position of phenylpropanoids (Lewis et al., 1999), i.e. presumably to produce caffeic acid (**10**) or a derivative thereof. In this regard, *p*-coumarate (**7**) and *p*-coumaroyl CoA (**8**) hydroxylating activities have been well documented in mungbean (Kojima and Takeuchi, 1989), sweet potato (*Ipomoea batatas*) (Tanaka and Kojima, 1991), and parsley (*Petroselinum crispum*) cell cultures (Kneusel et al., 1989). However, the enzymes were never purified to electrophoretic homogeneity. Polyphenol oxidases capable of hydroxylating *p*-coumaric acid (**7**), as well as those using *p*-coumaroyl CoA (**8**) and *p*-coumaroyl D-glucose (**35**) as substrates, have been purified from *I. batatas* (Tanaka and Kojima, 1991) and *Lithospermum erythrorhizon* (Wang et al., 1997), respectively. Yet, their involvement in lignification was considered unlikely, since inhibition of phenol oxidases in mungbean did not apparently affect activities involving the *meta*-hydroxylation of *p*-coumaric acid (**7**) (Duke and Vaughn, 1982).

A P450 gene from arabidopsis, known as CYP98A3, was recently found to encode a protein that, when expressed in yeast, catalyzes the 3-hydroxylation of both *p*-coumaroyl shikimate (**36**) and *p*-coumaroyl quinate (**37**), but not *p*-coumaric acid (**7**) itself or *p*-coumaroyl CoA (**8**) (Schoch et al., 2001). These activities have been previously reported in in vitro assays of microsomal preparations from carrot (*Daucus carota*) cell suspension cultures (Kühnl et al., 1987) but a link to lignin biosynthesis was not made. However, there is additional evidence for C3H (CYP98A3) being required for introduction of the 3-hydroxyl group, as well as a rate-limiting step in the monolignol pathway: (i) based on mRNA and protein localization in arabidopsis, its expression is correlated with the onset of lignification (Schoch et al., 2001); (ii) a mutation of the same gene in arabidopsis resulted in an *extremely dwarfed* phenotype whose lignin content was estimated to be considerably lower using thioglycolic acid extraction and Klason analyses, respectively (Franke et al., 2002). Furthermore, based on pyrolysis-GC/MS, nitrobenzene oxidation and DFRC (derivatization followed by reductive cleavage)

analyses, it was concluded to be essentially an H-derived lignin (Franke et al., 2002). In the thioglycolic acid extraction method, the lignin content was estimated to be ~20% of the control since the relative absorbance at 280 nm was reduced from 3.12 to 0.57; however, the extinction coefficient of an H-containing lignin is unknown and thus this measurement has little meaning at present. The Klason lignin content, by contrast, was also reduced in this case to ~40% of the original wild type (6.2 vs. 16.3%). Interestingly, in the mutant only H units were detected by nitrobenzene oxidation (~9.5% of Klason lignin content), whereas in the control wild type plants just traces of H units were detectable, in comparison to the G and S components which correspond to ~16.2 and 3.5% of the Klason lignin, respectively. These data are thus consistent with the pathways to both coniferyl (**2**) and sinapyl (**3**) alcohols apparently being blocked; and (iii) transcriptional profiling of *P. taeda* C3H was directly correlated with induction of monolignol biosynthesis in a rate-determining capacity (Anterola et al., 2002). Interestingly, in each of these plant species, as well as in *Sesamum indicum* (Ichikawa, Xia, Davin and Lewis, manuscript in preparation) and *Sorghum bicolor* (Bak et al., 1998), this gene apparently exists as a single copy.

5.3.1. The postulated importance of C3H downregulation in carbon allocation and lignin organization and assembly

Even though there remains the issue of the actual substrate being utilized, the above findings with C3H are fully consistent with it being a rate-limiting step (together with those of C4H and the factors controlling Phe (**4**) supply). Of particular interest are the data obtained with the C3H mutant: this apparently possessed an H-lignin, whose estimated Klason lignin content was ~40% relative to the wild type, with ~9.5% recovery of H units being obtained by nitrobenzene oxidation. In contrast, the actual percentage of the H component in arabidopsis wild type lignin has been difficult to determine by this oxidative degradation method. Such analyses, as described earlier, are currently restricted to the estimation of only non-cross-linked aromatic residues, e.g. *p*-hydroxybenzaldehyde (**22**) and the H corresponding thioacidolysis product (**25**), which are essentially undetectable in the wild type plants.

Importantly, this mutant phenotype apparently demonstrates that elimination of C3H activity has the effect of restricting carbon flow into the monolignol and lignin pathways, as well as on growth and developmental processes. This is because the overall levels of the H-constituents in the lignin did not increase correspondingly to act as surrogate precursors for G and S monolignols. A central question to be answered in the future is therefore why does H-lignification not compensate for their reduction?

One possibility is that normal growth and development is arrested in the absence of G and S constituents [which lead not only to G/S lignin but also to ferulate (**12**) and sinapate (**19**) wall bound esters]. In wild type plants, the deposition of the H-lignin component is currently viewed as being primarily involved in initiation of lignin assembly, with a preponderance being laid down in the middle lamella regions: the G and S components, by contrast, are considered to be laid down later on particularly at other locations. However, this pattern of deposition needs to be more comprehensively examined, particularly in the different cell types which undergo lignification. In particular, it will be very instructive to examine in detail the pattern(s) of lignin deposition in both mutant and wild type, as regards the requisite and presumed selective initiation sites for H-lignin assembly (Lewis et al., 1999).

Additionally, it also needs to be determined whether the full extent of cell wall assembly (so-called secondary thickening process) can even occur in the absence of the G and S components, including whether the H-lignin component can effectively penetrate the various regions of the cell wall that typically primarily contain the S + G constituents. Nevertheless, these data strongly suggest that C3H downregulation has a most significant effect on lignin content, its organization and assembly; this, in turn, raises other important questions including how this is (transcriptionally) controlled, and indeed to what extent the H, G, and G–S pathways are kept separate.

5.3.2. Challenges for the future

As mentioned before for PAL and C4H downregulation, much needs to be investigated: This includes determining how lignin assembly has been perturbed, and why its overall amount has decreased. Is there a feedback mechanism operative (e.g. acting on C4H and/or Phe (**4**) supply), when coniferyl (**2**) and sinapyl (**3**) alcohol formation is blocked or are there other consequences involving yet unknown (signaling) pathways? And what is the true H content of lignin in wild type arabidopsis, given the current limitations for both detection and quantification of the H components? Additionally, determining the effects this mutation has on, for example, growth and development, accumulation of non-lignin constituents (e.g. soluble phenolics), water/nutrient conduction and tissue mechanical strength (vascular integrity), should be most instructive to clarify further the nature of the rate-limiting (regulatory) role of C3H.

5.4. The consequences of manipulating the presumed non-rate-determining steps in monolignol and lignin pathways

A major assumption made in this analysis is that the rate-determining steps in carbon (i.e. phenylpropanoid)

allocation to the monolignol pathway involve Phe (**4**) availability (supply), and the relative levels of cinnamate 4-hydroxylase and *p*-coumarate 3-hydroxylase activities. Thus, restricting and/or downregulating subsequent downstream conversions can be anticipated to have no effect on overall *carbon allocation* to the pathway, unless, of course, there are other feedback and/or transcriptional control mechanisms operative that were not detected in any of the preceding studies.

Hence, downregulation of downstream steps to the monolignols (**1**–**3**) should have predictable consequences. These could include (i) build-up of pathway metabolites, including those upstream, in the particular cells impacted, (ii) metabolites being redirected (shunted) into competing biochemical pathways that also utilize these substrates, and/or (iii) non-lignin metabolites being transported into the wall where lignification would normally have occurred. If the latter happened during lignification, this would negatively impact upon lignin assembly proper. The reasons are straightforward: if lignin biosynthesis was perturbed by the presence of non-lignin monomers, lignin assembly which gives the needed biopolymeric matrix for the requisite vasculature properties would not occur and the corresponding cell-wall properties would be compromised. As emphasized earlier and now described below, the biochemical control of lignin assembly in the cell wall is clearly carefully orchestrated, including the need for spatially controlled lignin heterogeneity, to achieve the required physiological functions. Furthermore, the subsequent analyses of transgenic plants downregulated in each of the downstream pathway steps validate the above concept.

5.5. 4-Coumarate:CoA ligase (E.C. 6.2.1.12): dwarfed, normal and giant phenotypes?

4-Coumarate:CoA ligase (4CL) generally exists as a multigene family (Lewis et al., 1999). The isoforms typically have different substrate specificities and spatial/temporal expression patterns, which have long suggested distinct physiological roles (summarized in Lewis and Yamamoto, 1990). For example, in arabidopsis, At4CL1 and At4CL2 were believed to be involved in lignification, whereas At4CL3 is considered to function in flavonoid biosynthesis (Ehlting et al., 1999). Similarly, in aspen, it was proposed that Pt4CL1 was specifically involved in lignin biosynthesis, whereas Pt4CL2 participated in flavonoid formation (Hu et al., 1998). More recently, a 4CL isoform (Gm4CL1), capable of utilizing sinapate (**19**) as substrate, has been isolated from soybean (*Glycine max*), together with three others (Gm4CL2, Gm4CL3, Gm4CL4) (Lindermayr et al., 2002). Gm4CL1 and Gm4CL2 were proposed to be involved in growth and development (including lignin biosynthesis), whereas Gm4CL3 and Gm4CL4 were thought to respond to environmental stimuli. Thus far, 4CL downregulation has been carried out only on isoforms considered involved in lignin biosynthesis. It is not certain, however, whether these manipulations also affected any other 4CL isoforms (and hence other pathways), since, for example, the flavonoid levels in 4CL-suppressed plants were not analyzed (see below).

Genetic downregulation of 4CL was carried out using three different species, namely tobacco (Kajita et al., 1996), arabidopsis (Lee et al., 1997) and aspen (*Populus tremuloides*) (Hu et al., 1999); Tables 3–5 and Fig. 7A summarize what is currently known about the corresponding phenotypes and their lignin compositions. Yet even with the same enzymatic step being repressed, significant species-specific differences in the resulting transgenic plants were reported. These ranged, relative to the corresponding controls, from no visible phenotypic differences in arabidopsis, to dwarfing in tobacco, and to a report of spectacular enhanced growth in aspen. No explanation of any sort then or since has been provided for such dramatic differences.

Yet in spite of the reported species-specific growth and developmental differences, the effects on overall lignin content as a function of residual 4CL activity revealed a consistent trend for all three species examined (Fig. 7A). Although in these cases different species (tobacco, aspen, and arabidopsis) and different methods of lignin determination (acetyl bromide, Klason and thioglycolate, respectively) were used, the graph provisionally suggests relatively good agreement when the results were recalculated to express lignin contents and 4CL activities as percentages of the controls. A trendline based on all of the data points was drawn, illustrating the general effect of 4CL downregulation; controls employed either transformants with an empty vector in tobacco, or wild type plants in aspen and arabidopsis. The plots of lignin content versus 4CL activity suggest that significant reductions in lignin contents occur only after $>60\%$ reduction of 4CL activity. This again provisionally implies that there is more 4CL activity available in the wild type plants than necessary for the biosynthetic flux to the lignins. Following the same line of reasoning as for PAL, 4CL is thus considered *not* to be a rate-determining enzyme in lignin biosynthesis. This finding is consistent with that noted earlier for metabolic and transcriptional profiling in *P. taeda* (Anterola et al., 1999, 2002).

5.5.1. 4CL downregulation in tobacco

The most detailed analyses of the effects of downregulation of 4CL have been carried out with tobacco (Kajita et al., 1996). In those studies, the transformants were obtained using a near full-length 4CL cDNA constitutively expressed, under control of the CaMV 35S promoter, in either the sense or the antisense orientation. Nine transformants and three control plants (transformants with an empty vector) were grown until

Table 3
Preliminary characterization of transgenic tobacco following introduction of sense and antisense tobacco 4CL (adapted from Kajita et al., 1996, 1997a,b)

Plant line	Enzyme and gene expression localization	Transgene orientation	Promoter	Gross morphological changes in phenotypes of T_0 transformants	Tissue analyzed for lignins	% 4CL activity (relative to average activity of controls)	Lignin amounts[a] (% CWR)	Estimated S and G recovery (% of lignin)	S/G ratio	Analytical techniques employed
C1	See text	(Control)			Xylem	85	21.6	29.2	0.78	Nitrobenzene
C2	n.d.	(Control)			tissues	74	22.1	25.9	0.76	oxidation;
C3	n.d.	(Control)			(age of plants	141	21.3	28.5	0.86	acetyl bromide lignin
S3	n.d.	Sense	35S	Dwarfed, brown stem	were not	1	17.0	17.6	2.00	determination;
S2	n.d.	Sense	35S	Dwarfed, brown stem	specified)	19	17.3	15.6	1.87	histochemical
S4	See text	Sense	35S	Dark brown stem		1	16.8	20.6	0.79	staining;
S1	n.d.	Sense	35S	Dwarfed, brown stem		1	14.2	4.3	0.45	pyrolysis-GC/MS; NMR
A5	n.d.	Antisense	35S	Light brown stem		11	17.6	20.1	1.03	
A1	n.d.	Antisense	35S	None		42	20.5	24.6	0.87	
A4	n.d.	Antisense	35S	None		135	21.9	24.9	0.80	
A2	n.d.	Antisense	35S	None		101	22.9	26.1	0.76	
A3	n.d.	Antisense	35S	None		88	20.5	24.9	0.72	

Ultrastructural effects on cell wall types and patterns of lignin deposition were not studied. n.d. = not determined.
[a] Estimated by acetyl bromide method.

Table 4
Preliminary characterization of transgenic arabidopsis following introduction of antisense arabidopsis 4CL (adapted from Lee et al., 1997)

Plant line	Transgene orientation	Promoter	Gross morphological changes in phenotypes of T_0 transformants	Tissue analyzed for lignins	% 4CL activity (relative to average activity of controls)	Lignin amounts[a] (% CWR)	Estimated S and G recovery (% of lignin)	S/G ratio	Analytical techniques employed
RLD	(Control)			Bolting stems	100	15.0	62.1	0.48	Nitrobenzene
RLD:35S	Antisense	35S	None	that were 15	49	12.0	88.0	0.55	oxidation,
RLD:Pc4CL	Antisense	4CL1	None	cm in height	16	9.7	84.1	1.07	thioglycolate lignin
Columbia	(Control)				100	17.0	43.3	0.49	analysis,
Col:Pc4CL	Antisense	4CL1	None		22	12.0	48.0	0.88	histochemical
Col:35S	Antisense	35S	None		7	7.3	114.7	1.17	staining

The enzyme and gene expression localizations, the frequency of interunit linkages in, and MW ranges of lignins, as well as the ultrastructural effects on cell wall types and patterns of lignin deposition were not determined.
[a] Lignin contents were estimated by a measurement of relative absorbance at 280 nm, using the thioglycolic acid extraction method. This method does not quantify lignin relative to the cell wall residue. In this analysis, Columbia ecotypes were assumed to have ~17% lignin in the cell wall residue, with the amounts of lignin in the remaining lines being estimated according to relative absorbance levels. However, as explained in the text, these data are suspect.

flowering, and Table 3 summarizes the known effects on the phenotypes so obtained.

5.5.1.1. Phenotypes. Four of the five antisense plants were apparently morphologically indistinguishable from controls with lignin contents ~20.5–22.9% and S/G ratios ~0.7–0.9, whereas a fifth (~11% 4CL activity) had slightly brown stems, with an estimated lignin content of 17.6% and S/G ratio of 1. By comparison, all sense transformants had unusual brownish green stems, with brown and white cell walls, resulting in a mottled appearance and most were severely dwarfed. Moreover, the effects of 4CL downregulation (to ~1% of wild type 4CL activity) had a more pronounced effect on lignification: acetyl bromide lignin contents were reduced from ~22% in the wild type to ~14–17% in the sense transformants, with S/G ratios ranging from ~0.45 to ~2. However, even at such low levels of 4CL activity, the bulk of the carbon allocated to the pathway was still apparently directed towards lignin deposition, in accordance with this step not being rate-limiting.

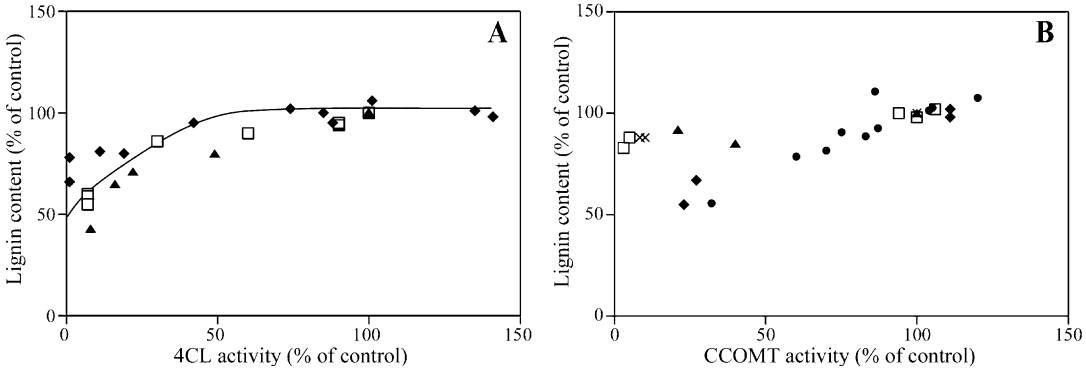


Fig. 7. Provisional plots of lignin contents versus gross enzyme activities in transgenic plants up- and down-regulated with: (A) 4CL; adapted from Kajita et al. (1996) (tobacco, ◆), Lee et al. (1997) (arabidopsis, ▲) and Hu et al. (1999) (aspen, □); (B) CCOMT; adapted from Zhong et al. (1998) (tobacco, ◆), Guo et al. (2001) (alfalfa, □), Pinçon et al. (2001) (tobacco, ▲), Meyermans et al. (2000) (poplar, ×), and Zhong et al. (2000) (poplar, ●).

Additionally, none of the analyses reported the amounts or compositions of the aqueous and organic solubles (so-called extractives) removed prior to lignin determination. Hence, no details of possible metabolic build-up as low molecular weight substances were disclosed. Such data are required in order to fully define both the nature and amounts of phenolics, or other substances, accumulating in the plant tissues when 4CL is downregulated.

One of the sense transformants (S4, see Table 3) was also arbitrarily subjected to a detailed morphological analysis of its stem tissues. This revealed an "uneven" gene silencing effect, as visualized by a "patchy" distribution of 4CL, using an immunolocalization approach (Kajita et al., 1997b). This patchiness only occurred in regions where vessel elements had partially collapsed, and where vascular integrity was compromised. On the other hand, the same cross-sections showed apparently intact vascular regions where 4CL was present; these data further underscore limitations in the gene-silencing approaches currently in use. Fig. 8 shows a cross-section of collapsed vessels (Fig. 8B) relative to controls (Fig. 8A).

5.5.1.2. Effects on lignification. The plots of G and S as percentages of the CWR versus corresponding percentage acetyl bromide lignin estimations were most instructive (Fig. 9A and B). Thus, the effect of decreasing 4CL activity (from ~100% in the wild type plants to ~1% in the transgenics) resulted in the precipitous reduction of detectable G and S fragments released during nitrobenzene oxidation, albeit possibly to slightly differing extents. That is, when lignin amounts were reduced to ~10–15% acetyl bromide lignin contents of the CWR, the G and S moieties were barely detectable, even though significant amounts of lignin were ostensibly still present. [Typically, the recoveries of the S and G nitrobenzene fragments recovered were ~25% of the estimated lignin levels, although one transformant had only ~5% recovery.] Additionally, in all cases, no H units were detected. These data thus clearly suggest that either the G and S components cannot be recovered (even with the nitrobenzene oxidation method) when lignin levels are reduced to <50%, and/or that the lignin determinations are overestimated. If the former, this would indicate that during the initial phases of lignin deposition, so-called "condensed"

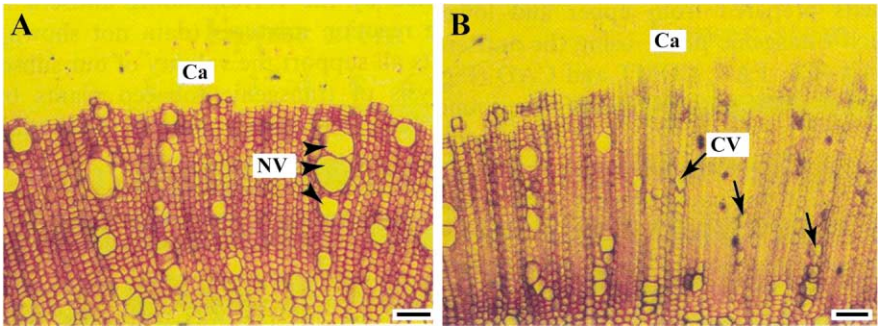


Fig. 8. Cross-sections of lower stem tissues of control (A) and 4CL-downregulated (B) tobacco plants. Ca = Cambium; NV = normal-shaped vessels; CV = collapsed vessels. Reproduced from Kajita et al. (1997b) with permission of Elsevier Science.

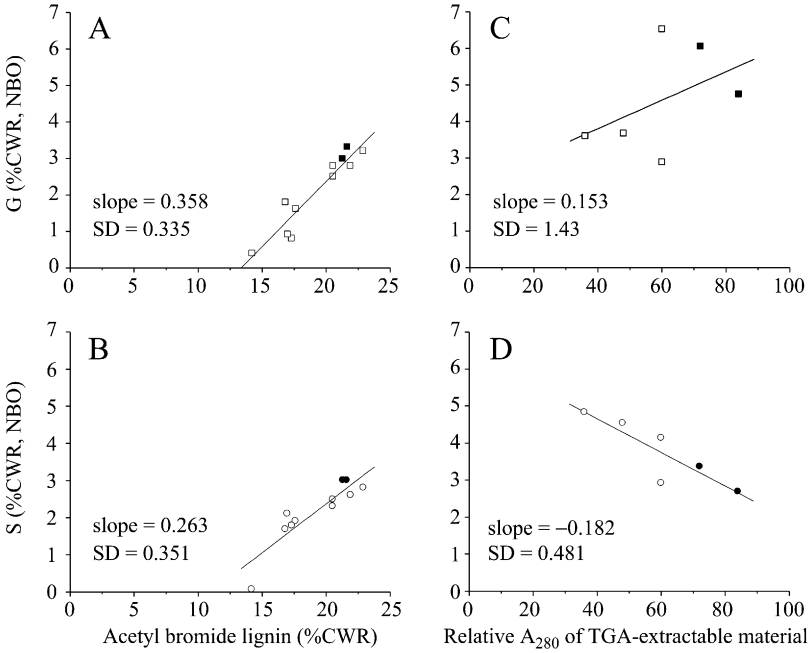


Fig. 9. Estimated lignin contents versus guaiacyl (G, ■, □) and syringyl (S, ●, ○) recoveries of cell wall residues (CWR) of control (solid symbols) and 4CL transgenic lines (open symbols) of tobacco (A, B) and arabidopsis (C, D). Lignin contents were determined using either the acetyl bromide or thioglycolic acid protocols, whereas G + S recoveries, from nitrobenzene oxidation (NBO), are calculated in terms of percentage of cell wall residue (%CWR). Data recalculated from Kajita et al. (1996) (A, B) and Lee et al. (1997) (C, D). SD = standard deviation.

structures are formed (e.g. the 8-5′ structure **49** as an example); these, however, cannot be detected and quantified using the degradative methods commonly employed.

Note, however, that the extent to which the H pathway is involved in the early phases of lignification is not well understood, although it is considered to be mainly involved in C–C rather than C–O linkage formation. Indeed, it is curious that in spite of the massive number of lignin degradative analyses (by nitrobenzene oxidation and thioacidolysis) conducted thus far, it is only now that the limitations of these techniques are exposed. If correct, these data imply that the alkali nitrobenzene oxidation cleavable moieties in lignin are formed only in the later stages of lignification. This immediately underscores the need to develop techniques to determine the precise chemical nature and the amounts of the lignins laid down in both the early and late stages of lignification.

5.5.1.3. NMR spectroscopic characterization of presumed lignin-derived fragments. In a related report (Kajita et al., 1997a), the 5-month old homozygous second generation (T_1) offspring was obtained by selfing of line S4. This transformant was further analyzed in terms of the presumed lignin and phenylpropenoic acid composition of its (4CL-downregulated) brownish xylem tissues: however, this now had a Klason lignin content very similar to that of wild type (~19.9 vs. 22.6%) and no estimation of its 4CL activity was provided.

Nevertheless, a "dioxane-soluble lignin" fraction from the brownish tissues of this transformant was isolated and compared with that obtained from the whitish xylem tissues of the corresponding control. This procedure, in both cases, only solubilized very minor amounts of the *presumed* lignin-derived substances in each tissue (~6 and ~9.6%), leaving 94 and 91.4% of the lignin unaccounted for, respectively. That is, the bulk of the lignin was either not solubilized or recoverable using this method. In any case, the ^{13}C NMR spectra of both samples (Fig. 10A and B) were nearly identical, except for four slightly larger resonances in the 4CL-downregulated transformant at δ ~170 ppm (peak 2), ~129 ppm (peak 9), ~115 ppm (peak 12) and ~36 ppm (peak 26), respectively, and slightly reduced intensities of signals (peaks 17–20, ~83–86 ppm) attributed mainly to β-*O*-4 linkages. The investigators, however, assigned the enhanced resonances at ~129 and 115 ppm to esterified *p*-coumaric (**7**) and ferulic (**12**) acids ester-linked to the lignin, even though alkaline hydrolysis had revealed that these were only present (in esterified and/or etherified form) in minute amounts (~0.25% of the cell wall residue). An alternative explanation is proposed herein: the resonances at ~129 (peak 9), ~115 (peak 12), and ~36 ppm (peak 26 which was unassigned by the authors) are detectable in both the NMR spectra of the transformant and that of the wild type (see Fig. 10A and B). These signals are more consistent with tyramine-linked amide linkages, i.e. such as in *p*-coumaroyl (**32**) and feruloyl (**33**) tyramines (Pearce

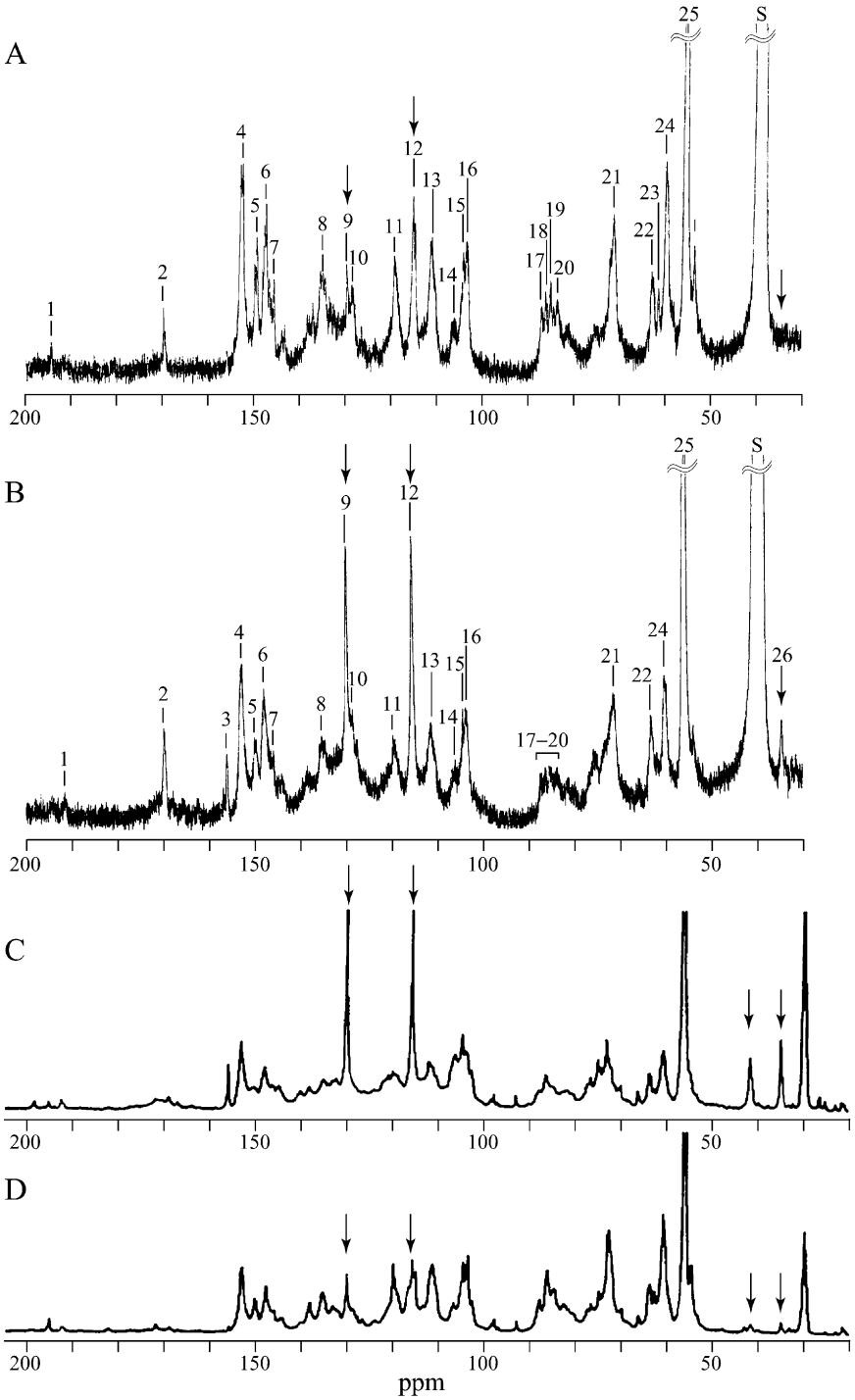


Fig. 10. ^{13}C NMR spectra of various isolates obtained using current lignin solubilization protocols, with preparations derived from control (wild type) and transgenic tobacco plants: control (A); sense 4CL-downregulated (B); antisense CCR downregulated (C); and wild type (D). Redrawn from Kajita et al. (1997a) (A, B) with permission of the American Society of Plant Biologists, and from Ralph et al. (1998) (C, D) with permission of the National Academy of Sciences, USA (Copyright 1998). Note: signal intensities do not reflect relative abundance. ↓ = Resonances indicating tyramine derivatives of unknown cellular origin.

et al., 1998). [The remaining presumed methylene group on the tyramine side-chain at C-8 is not detected as it is masked by the solvent peak (~40 ppm).] In any event, based on these spectral data, it is difficult to establish whether levels of the putative tyramine-derived derivatives **32** and **33** had actually increased (due to metabolic build-up) or were more prominent due to the small reduction in lignin content.

At this juncture, several points again need to be raised regarding the most serious limitations in the "lignin"

isolation and NMR spectroscopic characterization procedures currently used: First, relative peak sizes in NMR spectra do not reflect relative abundance when, as in this case, spectra are not acquired under conditions for quantification. Indeed, it is very well known that minor impurities and/or highly mobile components in polymeric preparations can display disproportionately large signals when present in very small amount. In this case, the resonances at ~129, 115 and 36 ppm are relatively sharp, which further suggests that the tyramine-derived moieties are part of very low molecular weight compounds and thus would display disproportionately large signals.

Additionally, the procedures for lignin isolation, as described earlier, are most inadequate: the procedure employed in this case involved "ball-milling" of dried extractive-free plant tissue in toluene for 4 days, extracting the "lignin" with dioxane/water (96:4) for an additional 7 days, then concentrating the solution so obtained and precipitating the organics in diethyl ether. The resulting precipitates were then individually dissolved in ~90% acetic acid, and diluted ~20-fold in water in order to reprecipitate the "milled tobacco lignin" preparation. This procedure thus only provides a means for recovery of ~6–10% of the presumed lignin, and gives no indication of molecular size and/or the extent of unrelated (non-lignin) substances co-precipitating; i.e. preparations obtained in 9.6 and 6.0% yields, respectively, cannot be taken as being representative of the bulk lignin present. Nor does this method distinguish between the different cell types in the plant species examined, and thus cannot be viewed as being lignin specific. Nor does this lengthy procedure establish where, for example, the tyramine (**34**) components have originated from, since their specific cellular origins are unknown.

An even more serious limitation lies within the solubility of the "lignin" preparations, these being dissolved in dichloroethane/ethanol (2:1) prior to NMR spectral analyses. Lignins are, however, not soluble in such solvents, and in order to do so would have to be greatly degraded (chemically modified) during the grinding and solubilization procedures used. Thus, at best, the substances observed by NMR spectroscopic analyses are of very low molecular weight, the origins of which are not well understood in terms of cell types involved. As an additional concern, the "lignin" isolation conditions were conducted under mild (including grinding and milling steps) to strongly acidic conditions, for very lengthy periods of time; these favor not only bond-breaking but also bond-making processes which can lead to artifacts.

5.5.1.4. Challenges for the future. These data, when taken together, clearly emphasize the need for more detailed analyses on the effects of 4CL downregulation: i.e. on both the nature and amounts of the soluble components (so-called extractives), as well as on the "bulk" and more readily cleavable lignin constitutents, and how and when they are deposited; in the identification and quantification of the cell wall chemical moieties to which the putative tyramine (**34**) derivatives are linked; what shunt and/or competing biochemical pathway metabolites are formed; and whether their presence in any way results in disruption of normal lignin assembly. The overall effects on the vasculature also need to be more fully understood, in terms of water/nutrient conduction and on the strength properties of the corresponding tissues.

5.5.2. 4CL downregulation in arabidopsis

In arabidopsis, the antisense transformants were generated using a near full-length 4CL gene, placed downstream of either a CaMV 35S or a parsley 4CL promoter (Lee et al., 1997). The transformants (containing either construct) consequently had reduced levels of 4CL transcripts in the leaves, but not in the other genes examined, which included 6-phosphogluconate dehydrogenase, 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase, 5-enolpyruvyl-shikimate-3-phosphate synthase, PAL and C4H. Immunoblot analysis and spectrophotometric enzyme assays further revealed reduced levels of 4CL in the stems of the antisense lines. These data were taken to suggest that only 4CL activity was being affected.

Based on transcript, protein and enzyme activity levels, 4CL downregulation did not appear to be dependent on the promoter used, although the uniformity of 4CL repression among the different cell types and regions within the leaves and stems was not examined for each. The effect of 4CL downregulation on lignin composition of arabidopsis transformants did, however, display a quite different trend from that in tobacco. Firstly, there were apparently no visible phenotypic changes in the transformants relative to the wild type, even though the estimated thioglycolic acid (TGA) lignin contents dropped significantly: from ~15 to 9.7% in the RLD lines, and from ~17 to 7.3% in the Columbia lines (Table 4). Enzymatic activities relative to controls were also reduced down to ~7–16% of the corresponding controls. If these reductions in estimated lignin contents are correct, this would suggest that the vasculature has been significantly weakened. Unfortunately, no tests were conducted either to establish to what extent the vascular apparatus had been compromised.

Secondly, the plots of %G and %S in the CWR relative to the absorbance measured at 280 nm (corresponding to TGA-extractable lignin) was instructive: Although the variability in the data is very large, the results suggest that a progressive decrease in %G content occurs whereas the S levels increase relative to the decrease in absorbance (i.e. TGA lignin) (Fig. 9C and D, and Table 4). Overall, however, the total amounts

recovered of both the G and S components in the CWR remained generally the same; while these data might suggest a differential effect on either the G or G–S monolignol forming networks in the various cell types, the nitrobenzene oxidation method does not establish the chemical identity of the S and G components (e.g. whether it is monolignol or hydroxycinnamic acid derived).

Thirdly, the trends, at least in terms of S + G recoveries by nitrobenzene oxidation relative to TGA-extractable lignin isolation require re-evaluation, as they are not in apparent agreement with each other. If the controls were assumed to have ~17% lignin (as estimated by other methods) and the lignin contents of the transformants were estimated proportionally based on relative absorbances at 280 nm of the TGA-extractable components, the S + G recoveries in three of the transformants would range between 80 and 115% rather than the 10–50% found in all other lignin degradation analyses. Accordingly, these data thus strongly suggest that the thioglycolic acid estimated lignin amounts cannot be accurately correlated with the nitrobenzene oxidation data.

There are several possibilities to account for the observations made, which can include one or more of the following: (i) the lignin determination analyses using the thioglycolic acid procedure are inaccurate as suggested earlier for the C3H study; (ii) due to reduction in 4CL activity, increased metabolism into other metabolites, such as cell-wall bound (unlignified) hydroxycinnamic acids occurs which overestimate the results obtained by nitrobenzene oxidation analyses; (iii) different CoA ligases may be differentially affected; and (iv) the various transformants may even be at different developmental stages: S levels are considered to typically increase with increasing maturity.

5.5.3. 4CL downregulation in aspen

5.5.3.1. Phenotypes: reassessment of increased cellulose biosynthesis. The findings regarding 4CL downregulation of aspen differed greatly from those of the other two studies. In these investigations (Hu et al., 1999), the reverse of the coding sequence of the 4CL1 gene, which was considered to be lignin-specific (Hu et al., 1998), was constitutively expressed in transformants using a duplicated enhancer CaMV 35S promoter. The aspen transformants so obtained were reported to have grown much faster than the controls (wild type plants), i.e. being about 50% taller after 10 weeks growth. While no additional report on this proposed growth enhancement has since been forthcoming, the observations made could not have been directly related to either lignin reduction or 4CL downregulation as stated. Plants with only ~6% reduction in overall lignin content (20.4 versus 21.6% wild type) and having ~90% residual 4CL activity grew to the same height as those with ~45% reduction in lignin content (11.8% versus 21.6% wild type) with residual 4CL activity <7% (see Tables 5 and 6 and Fig. 11). Consequently, the proposed growth enhancement, if correct, is not explicable based on the reasoning and data given.

It was also reported (Hu et al., 1999) that the rate of cellulose formation and its accumulation in aspen transformants had increased, in order to compensate for lignin reduction. However, the authors did not address whether the putatively higher cellulose contents of the 4CL-suppressed lines were in fact due to an elevated production of the latter, or simply a consequence of decreasing the amounts of one structural component (in this case, lignin) in the whole tissue. Table 6 thus recalculates (adjusts) the cellulose content of each aspen transformants on the basis of the measured lignin

Table 5
Preliminary characterization of transgenic aspen following introduction of antisense aspen 4CL1 (adapted from Hu et al., 1999)

Plant line	Enzyme and gene expression localization	Transgene orientation	Promoter	Gross morphological changes in phenotypes of T_0 transformants	Tissue analyzed for lignins	% 4CL activity (relative to average activity of controls)	Lignin amounts[a] (% CWR)	S/G ratio	Analytical techniques employed
Control	Developing xylem	(Control)			Wood from 10-month old trees	100	21.6	2.3	Thioacidolysis, Klason lignin[a] analysis, NMR
A12	n.d.	Antisense	35S	Taller than control		90	20.6	1.8–2.2	
A11	n.d.	Antisense	35S	Taller than control		90	20.4	1.8–2.2	
A9	n.d.	Antisense	35S	Taller than control		60	19.4	1.8–2.2	
A15	n.d.	Antisense	35S	n.r.		30	18.6	1.8–2.2	
A5	n.d.	Antisense	35S	n.r.		<7	13.0	1.8–2.2	
A8	n.d.	Antisense	35S	n.r.		<7	12.9	1.8–2.2	
A4	n.d.	Antisense	35S	n.r.		<7	12.8	1.8–2.2	
A6	n.d.	Antisense	35S	Taller than control		<7	11.8	1.8–2.2	

The frequency of interunit linkages in and MW ranges of lignins, as well as ultrastructural effects on cell wall types and patterns of lignin deposition were not determined (n.d.). n.r. = not reported.

[a] Lignin determinations/values were apparently not corrected for proteinaceous and/or ash-forming substances.

Table 6
Recalculation of cellulose contents of aspen transformants adjusted for reductions in lignin contents, relative to observed values (recalculated from data in Hu et al., 1999)

Plant line	Observed lignin content [% (w/w) of dry wood]	Calculated[a] cellulose content [% (w/w) of dry wood]	Observed cellulose content [% (w/w) of dry wood]	Percentage difference between observed and calculated cellulose contents
Control	21.62	44.23	44.23	0.00
A12	20.60	44.81	46.55	3.89
A11	20.40	44.92	45.95	2.30
A9	19.40	45.48	47.49	4.41
A15	18.60	45.93	45.98	0.10
A5	13.02	49.08	49.74	1.34
A8	12.90	49.15	48.14	−2.06
A4	12.83	49.19	48.35	−1.71
A6	11.84	49.75	50.83	2.17

[a] To account for % decrease in lignin amounts, cellulose contents, C′, were calculated as follows: $C' = C/(100-D) \times 100$, where C is cellulose content (% of dry wood) of the control, D is the decrease in lignin per 100 g of dry wood. $D = L-R$, where L is the lignin content (% of dry wood) of the control, R is the lignin content that remained per 100 g of dry wood, normalized to the control. $R = [(100-L) \times O]/(100-O)$, where O is the observed lignin content. Example (for A12): $R = [(100-21.62) \times 20.60]/(100 \times 20.60) = 20.34$; $D = 21.62-20.34 = 1.28$; $C' = 44.23/(100-1.28) \times 100 = 44.81$.

reductions, and compares these with the observed values. The recalculation clearly demonstrates that there were only modest changes (2–4% increase or decrease) in cellulose content, *with the largest "increase" occurring in the less severely lignin-depleted transformants* (A12, A11 and A9). Thus, it is concluded that there was no increased production in cellulose in contrast to the original reports by Hu et al. (1999).

5.5.3.2. Effects on lignification. The 4CL-downregulated aspen tissues were also subjected to NMR spectroscopic and nitrobenzene oxidation/thioacidolysis analyses. Thus, milled wood lignins (MWLs) were prepared from both the wild type and the transformant A6, which had ~45% lignin reduction and less than 7% residual 4CL activity (Table 5); details were not provided on the methods employed or yields obtained. Additionally, while the HSQC-TOCSY and HMQC spectral data were not provided for evaluation, both these and the HSQC spectra of both transgenic and wild type lignin samples were reportedly similar: unlike tobacco, however, no hydroxycinnamic acids were apparently evident. On the other hand, these researchers reported increased levels of *non-lignin* cell-wall associated alkali extractable *p*-coumaric (**7**), ferulic (**12**) and sinapic (**19**) acids in the aspen A6 transformant relative to the wild type, i.e. increasing from 199 to 2145, 510 to 2431, and 0 to 2452 nmol/g dry wood, respectively. While this observation is consistent with phenylpropenoic acids accumulating, they account for only ~0.14% of dry wood, which even if associated with lignin would represent only ~0.63% of the latter.

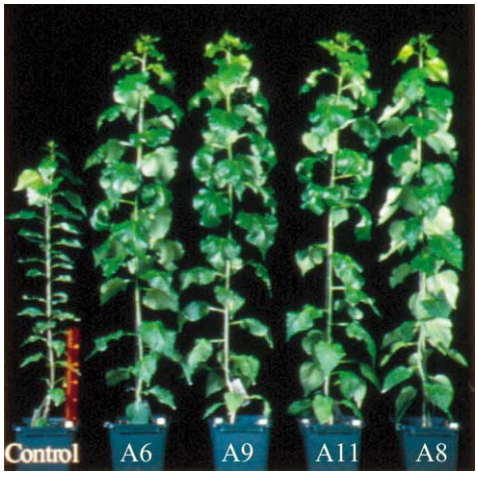


Fig. 11. Putative enhanced growth of 4CL-downregulated aspen relative to the wild type. Ten-week old control (left) beside four transgenic aspen plants (right) of the same age (see text for details). Reproduced from Hu et al. (1999) with permission of Nature America.

The S/G ratios (~1.8 to ~2.2) of transgenic aspen plants were also reported to be essentially the same as that of wild type, an observation again at variance with the findings with tobacco and arabidopsis. However, no additional comment can be made with respect to these S/G values, since the yields (on a CWR basis) were not provided, relative to the controls. Based on the data obtained in the previous and subsequent analyses, the recoveries would be expected to be low if lignin reductions to the extent reported are observed. Unfortunately, the extent of any vasculature collapse in the tissues was not determined. Nor was any testing of the mechanical properties of the transformants reported in order to gauge the effects of the downregulation described on vascular integrity.

5.6. *Caffeoyl-CoA* O-*methyltransferases (EC 2.1.1.104)*

The first cloning of a gene encoding a CCOMT was reported using parsley (Schmitt et al., 1991). Later, CCOMT was reported to be involved in lignification in zinnia, tobacco, tomato (*Lycopersicon esculentum*), soybean, alfalfa and forsythia (*Forsythia suspensa*) (Ye, 1997). CCOMT cDNAs have also been isolated from a number of species, including arabidopsis (Zou and Taylor, 1994), aspen (Meng and Campbell, 1998), *Stellaria longipes* (Zhang et al., 1995), *Vitis vinifera* (Busam et al., 1997), tobacco (Martz et al., 1998), and poplar (*Populus trichocarpa*) (Chen et al., 1998). Typically, CCOMT exists as a multigene family (Lewis et al., 1999): for example, tobacco has at least six known CCOMT isoforms, zinnia has at least five and both poplar and arabidopsis have two each. However, to date, there is only one known CCOMT isoform in aspen, alfalfa, parsley, *V. vinifera* and *S. longipes*.

CCOMT, when heterologously expressed in *E. coli* and assayed in vitro, is considered specific for caffeoyl (**11**) and 5-hydroxyferuloyl (**16**) CoA, and generally does not efficiently use hydroxycinnamic acids as substrates (Busam et al., 1997; Inoue et al., 1998; Martz et al., 1998). There is as yet no report of the activity of this enzyme towards the aldehydes and alcohols. Thus, the known in vitro properties of CCOMT lead to at least three possible physiological functions during monolignol biosynthesis: (i) methylation of caffeoyl CoA (**11**) to form feruloyl CoA (**13**), (ii) methylation of 5-hydroxyferuloyl CoA (**16**) to form sinapoyl CoA (**20**), and (iii) methylation of both substrates (see Fig. 1). Whatever substrate is actually used, downregulation of CCOMT would again not be expected to decrease the amount of carbon allocated to the phenylpropanoid pathway, since the corresponding regulatory steps are considered to be situated further upstream.

Five studies have been reported thus far examining the effects of suppressing the expression of various caffeoyl CoA *O*-methyltransferases (CCOMTs), these being performed in tobacco (Zhong et al., 1998; Pinçon et al., 2001), alfalfa (Guo et al., 2001) and poplar (Meyermans et al., 2000; Zhong et al., 2000). In each case, the transgene was overexpressed under the control of the CaMV 35S promoter, except for alfalfa, which utilized the bean PAL2 promoter to drive expression of CCOMT in either sense or antisense orientations. The results of these experiments are summarized in Tables 7 and 8 and Figs. 7B, 12A–D and 13A–D, respectively; again significant differences were noted.

As mentioned before, the relative lignin contents from CCOMT downregulation experiments were recalculated as percentages of the controls, these being plotted as a function of either CCOMT protein levels or CCOMT activities (Fig. 7B). Lignin estimations employed the Klason procedure, whereas CCOMT levels were quantified either through immunoblot analyses in the case of tobacco (Pinçon et al., 2001) and poplar (Meyermans et al., 2000), or by using caffeoyl CoA (**11**) as a substrate for the enzyme assays in tobacco (Zhong et al., 1998), poplar (Zhong et al., 2000) and alfalfa (Guo et al., 2001). Although it would be preferable to estimate relative CCOMT activities by both methods, CCOMT immunoblotting may possibly be a more accurate way of evaluation. This is because endogenous COMTs are also capable of methylating caffeoyl CoA (**11**), i.e. it was reported that an alfalfa COMT expressed in *E. coli* can methylate caffeoyl CoA (**11**) to about half of that for caffeic acid (**10**) (Inoue et al., 1998). Hence, CCOMT activities measured in plant extracts using caffeoyl CoA (**11**) may be overestimated due to the presence of endogenous COMTs.

5.6.1. *CCOMT differential downregulation in tobacco*

The two studies on CCOMT downregulation in tobacco gave most different results, and thus both the design of each experiment and the analytical methodologies employed are discussed. First, Zhong et al. (1998) isolated four different CCOMT cDNAs from tobacco stem cDNA library, and classified them into two groups, in which the members of each group shared >94% nucleotide sequence identity. However, it must be emphasized that the precise physiological role of each is unknown, e.g. as regards temporal/spatial expression and physiological function in a particular cell type. [One or more may, for example, be specifically used for feruloyl tyramine (**33**) formation.] Nevertheless, CCOMT 1 and 2 were assigned to Group 1, whereas CCOMT 3 and 9 were allocated to Group 2. Antisense CCOMT1 and CCOMT9 sequences were then used to downregulate both isoforms in Groups 1 and 2, respectively. On the other hand, Maury et al. (1999) isolated six CCOMT genes and grouped them into three classes, of which CCOMT 1, 2, 3 and 4 were in Class 1, CCOMT5 was in Class 2, and CCOMT6 was in Class 3: Pinçon and associates (Pinçon et al., 2001)

Table 7
Preliminary characterization of transgenic tobacco and alfalfa following introduction of homologous CCOMT transgene

Plant line	Enzyme and gene expression localization	Transgene orientation	Promoter	Gross morphological changes in phenotypes of T_0 transformants	Tissue analyzed for lignins	CCOMT levels (% relative to average of controls)	Lignin amounts[a] (% CWR)	Estimated S and G recovery (% of lignin)	S/G ratio	Analytical techniques employed
A. Tobacco, Zhong et al. (1998)										
XT1	Protein localized	(Control)			"Mature"	100	17.3	17.2	0.70	Pyrolysis-MS;
XT2	mainly in xylem	(Control)			stems from	100	18.1	16.8	0.79	Klason lignin analysis;
1+9–123	ray cells, some in	Antisense	35S	None reported	plants of	28	11.9	13.0	0.91	NaOH hydrolysis;
9–164	phloem and xylem fibers	Antisense	35S	None reported	unspecified age	23	9.7	17.4	1.05	histochemical staining
B. Tobacco, Pinçon et al. (2001)										
Control	n.d.	(Control)			Stems of 12-	100	17.4	50.7	0.92	Thioacidolysis[b];
AS27	n.d.	Antisense	35S	Dwarfed stem,	week old	40	14.8	47.0	0.90	Klason lignin
AS30	n.d.	Antisense	35S	narrow leaves, short stamen	plants	21	16.0	52.0	0.84	analysis; histochemical staining
C. Alfalfa, Guo et al. (2001)										
1	mRNA localized	(Control)			Sixth to ninth	106	17.9	50.6	0.47	Thioacidolysis[b];
2	in xylem in 2nd	(Control)			internodes	100	17.2	51.3	0.52	Raney nickel
48	internode, xylem	(Control)			from stems	94	17.6	47.1	0.56	desulfurization;
ACC315	and phloem in 3rd	Antisense	PAL2	None reported	of the same	5	15.5	50.5	0.67	Klason lignin
ACC305	and 5th internodes, some in xylem in 7th internode	Antisense	PAL2	None reported	development stage (age not specified)	3	14.6	41.5	1.06	analysis; NMR; histochemical staining

The frequency of interunit linkages in and MW ranges of lignins, as well as the ultrastructural effects on cell wall types and patterns of lignin deposition were not determined (n.d.).

[a] Lignin amounts were not corrected for proteinaceous and/or ash-forming constituents.

[b] Assumes that all thioacidolytic products came from β-*O*-4 linked coniferyl (**2**) and sinapyl (**3**) alcohols in the lignin polymers.

Table 8
Preliminary characterization of transgenic poplar following introduction of homologous CCOMT transgene

Plant line	Enzyme and gene expression localization	Transgene orientation	Promoter	Gross morphological changes in phenotypes	Tissue analyzed for lignins	CCOMT levels (% relative to average of controls)	Lignin amounts[a]	Estimated S and G recovery (% of lignin)[b]	S/G ratio	Analytical techniques employed
A. Zhong et al. (2000)							(% of average of controls)			
AV1	Protein localized	(Control)			Stems from	87	98	n.d.	n.d.	Klason;
AV2	mainly in xylem	(Control)			9-month old	104	102	n.d.	n.d.	histochemical
ACoA1	parenchyma cells and	Antisense	35S	None	plants	105	102	n.d.	n.d.	staining;
ACoA2	phloem in top part of	Antisense	35S	None		120	107	n.d.	n.d.	pyrolysis-MS;
ACoA3	stem; in lower part of	Antisense	35S	None		60	78	n.d.	n.d.	diffusion
ACoA4	stem, found in xylary	Antisense	35S	None		83	88	n.d.	n.d.	reflectance
ACoA5	fibers, xylem ray	Antisense	35S	None		86	110	n.d.	n.d.	infrared Fourier
ACoA6	parenchyma cells,	Antisense	35S	None		75	90	n.d.	n.d.	transform
ACoA7	phloem fibers and	Antisense	35S	None		70	81	n.d.	n.d.	spectral
ACoA8	phloem	Antisense	35S	Light orange wood		32	55	n.d.	n.d.	analysis
B. Meyermans et al. (2000)							(% CWR)			
Wild type	mRNA localized in xylem in top	(Control)				100	19.9	42.6	1.77	
sccoaomt-16	internodes, xylem and phloem in	Sense	35S	Pink-red xylem	Stems from 7-month old plants	8.4	17.6	40.6	1.97	Thioacidolysis; Klason lignin analysis; NMR;
sccoaomt-29	middle and lower internodes	Sense	35S	Pink-red xylem		10.2	17.5	41.4	1.91	histochemical staining

The frequency of interunit linkages in and MW ranges of lignins, as well as the ultrastructural effects on cell wall types and patterns of lignin deposition were not determined (n.d.).

[a] Meyermans et al. (2000) reported Klason lignin as %CWR; Zhong et al. (2000) reported Klason lignin as % relative to average of controls. Lignin estimations were not corrected for proteinaceous and/or ash-forming constituents.

[b] Assumes that all thioacidolytic products came from β-*O*-4 linked coniferyl (**2**) and sinapyl (**3**) alcohols in the lignin polymers.

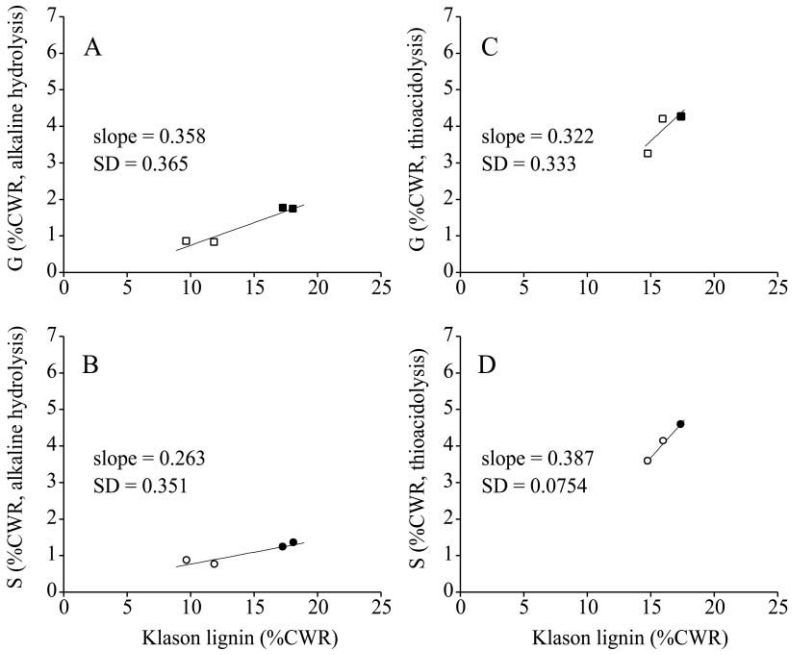


Fig. 12. Estimated lignin contents versus guaiacyl (G, ■, □) and syringyl (S, ●, ○) recoveries of cell wall residues (CWR) of control (solid symbols) and CCOMT transgenic lines (open symbols) in tobacco. Klason lignin contents and G (A, C) and S (B, D) recoveries, following alkaline hydrolysis (A, B) and thioacidolysis (C, D), are estimated in terms percentage of cell wall residue (% CWR). Data recalculated from Zhong et al. (1998) (A, B) and Pincon et al. (2001) (C, D). SD = standard deviation.

used the CCOMT2 to downregulate CCOMTs in tobacco. Thus, all of the experiments were designed in a manner that would not be expected to yield incisive results regarding the physiological role of each isoform.

5.6.1.1. Phenotypes. Both tobacco CCOMT down-regulation studies (Zhong et al., 1998; Pinçon et al., 2001) gave very different findings, including phenotypes which were quite distinct: Zhong et al.'s transgenic

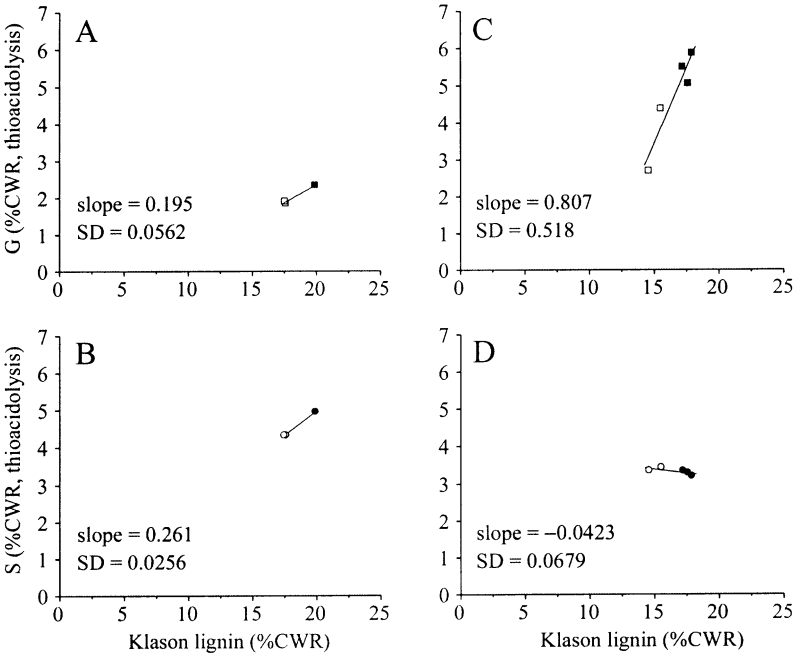


Fig. 13. Estimated lignin contents versus guaiacyl (G, ■, □) and syringyl (S, ●, ○) recoveries of cell wall residues (CWR) of control (solid symbols) and CCOMT transgenic lines (open symbols) in poplar (A, B) and alfalfa (C, D). Klason lignin contents and G (A, C) and S (B, D) recoveries from thioacidolysis are estimated in terms of percentage cell wall residue (% CWR). Data recalculated from Meyermans et al. (2000) (A, B) and Guo et al. (2001) (C, D). SD = standard deviation.

plants grew normally, whereas those of Pinçon et al. were dwarfed. Of the 109 antisense plants produced by Zhong et al., two (see Table 7A, lines 1 + 9–123 and 9–164 and Fig. 12A and B) were selected for further study, those having 28 and 23% residual CCOMT activities, and estimated lignin contents reduced by ~33 and 45%, respectively, relative to the controls.

Despite the changes in lignin content and compositions, however, both transgenic lines apparently grew to the same height at the same rate, and flowered at the same time as the wild type. Tissue analyses also revealed that some of the vessel elements in both the primary and secondary xylem were deformed (crushed and collapsed inward), as expected from the reduction in lignin contents and, hence, compromised vascular integrity. The ages (i.e. maturity) of the plants were not described in this study.

On the other hand, Pinçon et al. (2001) generated 27 antisense plants, of which only two (see Table 7B, lines AS27 and AS30, and Fig. 12C and D) were selected for further analysis. These mature (12-week old) transformants had more moderate reductions in both CCOMT activities and Klason lignin contents (~15 and 8%, respectively), with essentially no effect on S/G ratios (~0.9) relative to the controls.

It is also noteworthy that, in the latter study, the level of CCOMT inhibition apparently also varied depending on the developmental stage of the plant. Plants having <40% residual CCOMT activity at the in vitro plantlet stage attained CCOMT activities comparable to (or even greater than) the controls after 6 weeks of growth, but were again reduced to <60% of the controls after another 6 weeks. However, it is uncertain whether the activity detected was due to resurgence of CCOMT levels or to (transient) upregulation of other OMTs. Nevertheless, these results emphasize the need to measure directly and unambiguously the *actual* protein levels at different developmental stages of the plant, before any correlation between enzyme suppression and lignin biosynthesis can be made.

5.6.1.2. Effects on lignification. Fig. 12A and B plot the %G and %S recoveries in the CWR versus the Klason lignin content of the Zhong et al. tobacco transformants, with G and S recoveries being estimated by alkaline hydrolysis of each line. There are two noteworthy features, although the number of data points is very small: first, both G and S levels appear to have decreased in roughly comparable ways, relative to the lowering of the lignin contents. However, the overall G + S recoveries using this method, were much lower than that typically attained, and thus this alkaline hydrolysis method may not give an accurate depiction of the overall effect. Second, the S/G *ratios* apparently increased slightly from ~0.70–0.79 in the wild type to 0.91–1.05 in the transformants (Table 7A). While this is perhaps indicative of differential control over G and G–S monolignol-forming pathway networks in different cell types and/or differential hydrolytic properties of lignin fragments in the transgenic plants, the data are too few to draw any statistically valid conclusions. Furthermore, no thioacidolysis determinations were carried out to exclude interference from non-lignin moieties, such as cell wall-bound hydroxycinnamic acids. Clearly, it would be instructive to determine the precise effects on G and S compositions and recoveries by studying each CCOMT individually.

In the Pinçon et al. (2001) contribution, by comparison, the S and G recoveries once again decreased relatively uniformly with the reduction in estimated Klason lignin content (Fig. 12C and D). Note in this case, however, that the corresponding G and S recoveries were more than 2–3-fold higher than that of the Zhong et al. study. As emphasized earlier, the Klason lignin values in both studies may not be accurate for reasons of both overestimation (due to proteinaceous and ash-forming components) and underestimation (due to more readily hydrolysable angiosperm lignins). Furthermore, although the data are insufficient to extrapolate both G and S recoveries as lignin contents are decreased further, they provisionally suggest that thioacidolytic recoveries may be undetectable at about 30% of the estimated overall Klason lignin levels. Yet, in spite of the considerable differences in both phenotypes observed and analytical methodologies employed, the Zhong et al. (1998) and Pinçon et al. (2001) studies displayed the same overall trend, in terms of reducing S + G recoveries and lignin amounts.

5.6.1.3. Challenges for the future. One question that remains unanswered is why did both studies generate such vastly different phenotypes, in terms of growth and development, as a consequence of differential downregulation of arbitrarily selected CCOMT gene family members. Unfortunately, due to the very small number of fully characterized transformants selected, it is not possible at this time to discern what trend, if any, is representative. Indeed, the apparently contradictory results obtained again emphasize the need for statistically valid data using many more transformants, as well as studying the effects of progressive downregulation. The requirement for more comprehensive analyses becomes even more important considering the variability of CCOMT expression across the different developmental stages of the plant, and interference from other OMTs. Accordingly, much more needs to be done with each of the CCOMTs *individually* to determine unambiguously what role each has, what substrate is used, and in what tissues. Could the observations being made result from different, albeit related, CCOMT functions in different cell types/tissues being differentially affected?

Additonally, neither study reported the presence of any other phenylpropanoid constituents accumulating in either the cell wall residue or in the "extractable" portions of the transgenic tissues; these analyses are needed in order to account for the carbon no longer directed to lignin assembly. Mechanical testing is also required in order to help quantify the weakening of the vascular apparatus, as is comprehensive analysis of the lignins present in these tissues.

5.6.2. CCOMT downregulation in poplar

Two studies have also been reported on CCOMT downregulation in poplar (Meyermans et al., 2000; Zhong et al., 2000). Zhong et al. generated eight transgenic *Populus tremula* × *Populus alba* plants (see Table 8A, lines ACoA1–8) using CCOMT cDNA from same (sequence not reported), expressed in the antisense orientation using the CaMV 35S promoter. Of these, only line ACoA8 had significant reductions in both CCOMT activity (68%), and in lignin contents (45% reduction), relative to the controls (transformed plants with empty vector). The other transformants had smaller reductions in lignin levels.

In the work of Meyermans et al. (2000), two isoforms of CCOMT were obtained from *Populus trichocarpa*, of which one was arbitrarily selected to downregulate CCOMT in *Populus tremula* × *Populus alba*, using both sense and antisense constructs. Of the transformants obtained, only those with the sense construct had strongly reduced CCOMT levels (~8–10% of control, see Table 8B).

5.6.2.1. Phenotypes. Line ACoA8 was used by Zhong et al. for further characterization, and while no abnormal growth patterns or lesions were observed, some vessels were again slightly deformed. The wood also displayed a light orange color, which was resistant to extraction with 50% aqueous methanol. Gas–liquid chromatographic analysis of the extracted phenolics showed no significant increase in caffeic acid (**10**) levels, but an ~2- to ~3-fold increase in minute levels of *p*-hydroxybenzoic acid (*p*HBA, **38**) was observed (data was not shown and exact amounts were not reported). Note however that *p*HBA (**38**) is a well-known minor "extractive" constituent of poplar (*Populus tremula*) wood (Smith, 1955) (discussed later). Unfortunately, S and G recoveries relative to decreasing lignin contents were not provided, thereby limiting the analysis of this study.

Two sense transformants (see Table 8B and Fig. 13A and B, lines sccoaomt-16 and sccoaomt-29) were chosen by Meyermans et al. (2000) for further analysis: these had ~8.4 and ~10.2% residual CCOMT levels relative to wild type, respectively. Both transformants, with only moderate reductions in estimated lignin contents (ca. 12% reductions in total), had pink-red xylem, which fluoresced upon UV microscopic analysis. One antisense line generated by Meyermans et al. had a mottled pink-red coloration (data was not shown). This particular transgenic plant also had reduced CCOMT levels in the pink-red xylem region, whereas normal levels of CCOMT were present in the neighboring whitish xylem, as estimated by immunoblot analysis. Thus, similar to what was observed in the 4CL-downregulation of tobacco, gene-silencing seemed not to be uniformly distributed throughout the xylem. (No other details regarding this and other antisense lines were reported.)

5.6.2.2. Effects on lignification. It is unknown as to whether or not the particular CCOMT isoforms used by either Zhong et al. (2000) or Meyermans et al. (2000) are those which are specifically involved in lignification, and again the effects on individual isoforms need to be thoroughly investigated. Nevertheless, the plots of %G and %S of the CWR, in relation to estimated Klason lignin contents, showed relatively comparable reductions in G and S amounts with reduced lignin levels (Fig. 13A and B). This effect on both G and S recoveries would be expected if CCOMT was utilized for biosynthesis of both guaiacyl and syringyl moieties. (However, from the extent of downregulation noted, the effects on lignin biosynthesis overall were also quite minimal, indicating that it is not a rate-limiting step.) Additional attempts to detect thioacidolysis dimers (β-1, β-β, β-5, 4-*O*-5, 5-5, and α-β), which are apparently released during thioacidolysis in trace amounts, showed no difference between transformants and controls. As before, there is insufficient data (number of transformants analyzed) to make statistically valid conclusions.

To investigate whether other possible changes had occurred, the investigators isolated preparations, following lengthy lignin isolation procedures, and subjected them to NMR spectroscopic analyses (Meyermans et al., 2000). The procedure used, however, had many of the limitations noted previously in 4CL downregulation: in each case, the extractive-free cell wall material was "ball-milled, suspended in 50 mM acetate buffer (pH 5.0), and treated with 30 mg of cellulase (Cellulysin) per 1 g of ball-milled material. Cell wall digestions ran for 8 days with fresh enzyme and buffer being added after 2.5 and 5 days of incubation at 30 °C. The resulting lignin polysaccharide complex was subjected to fractionation in 96:4 (v/v) dioxane/water."

The ^{13}C NMR spectra (see Fig. 14A and B, peaks P_1–P_7) of the crude dioxane isolates (reported to account for 73 and 72% of wild type and sccoaomt-16 Klason lignins, respectively: no details given) were similar. These data apparently demonstrate that CCOMT repression did not result in either the possible build up of intermediates such as caffeic acid (**10**), caffeyl aldehyde (**41**) or caffeyl alcohol (**42**), and/or their incorporation into the lignin. However, there were ten

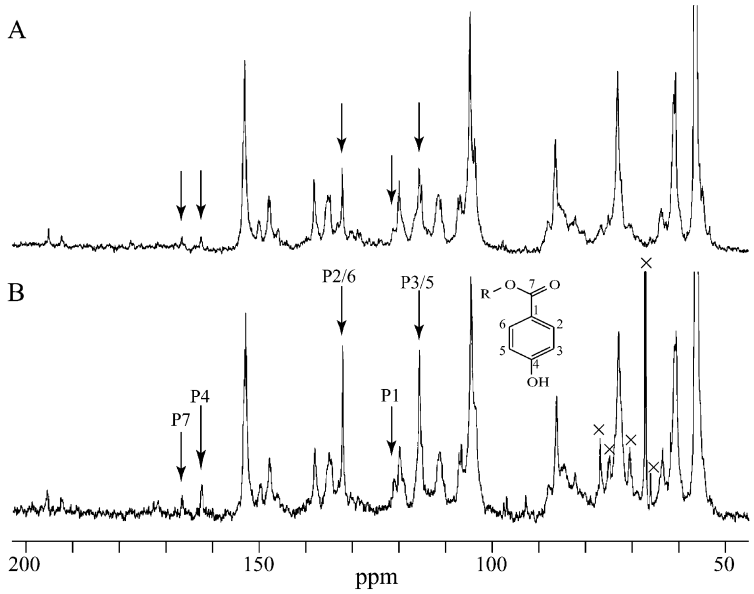


Fig. 14. ^{13}C NMR spectra of dioxane-soluble isolates from wild type (A) and CCOMT-downregulated poplar, sccoaomt-16 (B), following application of a lengthy lignin isolation procedure (see text). Components depicted as P1 to P7 (↓) are estimated to represent 0.1–0.2% of the cell wall residue. × = impurities in the sample. Reproduced from Meyermans et al. (2000) with permission of the American Society for Biochemistry and Molecular Biology via Copyright Clearance Center.

additional resonances in the transformants: five were ascribed to undefined impurities in the preparation (designated ×) and the remainder corresponded to signals of well-known esterified *p*HBA (**38**) (derivatives). It is not known though whether both sets of resonances (i.e. × and P1–P7) are interrelated or not, based on the data provided. Yet, this is a possibility since all of the additional signals are increased in intensity relative to wild type.

As indicated earlier in the 4CL downregulation section, care must be exercised in interpreting the spectral data for the following reasons: (i) mobile components on a polymer backbone (e.g. side-chain attachments) or low molecular weight impurities in same can result in disproportionately large NMR signals; (ii) as for feruloyl tyramine (**33**) derivatives, the cellular and cell (type) origin of the *p*HBA (**38**) constituents is unknown and their co-isolation with a lignin fraction may simply be coincidental; (iii) with the small reduction in lignin levels observed (presumed to be ~12%), the relatively small increases in *p*HBA (**38**) signal intensities may solely be due to this decrease in lignin content and/or to a minor form of metabolic shunting. Indeed, quantification of the levels of *p*HBA (**38**) in the poplar cell wall residue, using GC/MS verified that its levels had only increased marginally, i.e. from about 0.12% of the cell wall residue of wild type to 0.19–0.22% in the transformants.

Initially, (Smith, 1955) had also attributed the presence of *p*HBA (**38**) moieties as being minor constituents of poplar lignin (~0.8%); however, much of it was removed during cold methanol extraction, which neither solubilizes lignin nor efficiently removes extractives. At that time, it was also incorrectly believed that various "extractives" in different plant species represented traces of native lignin, which had not been fully polymerized. It is now known that various metabolites are primarily defense compounds laid down, for example, during heartwood formation and the like (Sakakibara et al., 1987; Gang et al., 1998). Furthermore, their presence in so-called lignin preparations could occur in at least two ways other than for lignification: (i) via artifactual generation (e.g. through transesterification under acidic conditions during the lengthy isolation procedures), and/or (ii) by being deposited into *prelignified* wood, via parenchyma cell excretion, as occurs during heartwood formation in other tree species (Chattaway, 1952). Indeed, a portion of such substances can become immobilized in the lignified matrix, even though they are not part of the lignified structural assembly. Thus, even though poplar does not form heartwood, the constituents to which the *p*HBA (**38**) moieties are covalently linked needs to be determined, as does how, when, and if these moieties are excreted into cell walls, and if so, into what type. [Indeed, whether the increase in *p*HBA (**38**) moieties is a direct effect of CCOMT downregulation or part of the plant's stress response also remains unknown at this point.]

5.6.2.3. Accumulation of shunt metabolites. Analysis of the methanol extracts of xylem tissues from transgenic lines sccoaomt-16 and sccoaomt-29 also established a minute increase in apparent metabolic shunting into non-lignin products, i.e. in terms of increased amounts

Table 9
Preliminary characterization of transgenic arabidopsis plants following introduction of the CYP84 transgene (Meyer et al., 1998; Marita et al., 1999)

Plant line	Transgene orientation	Promoter	Gross morphological changes in phenotypes of T_0 transformants	F5H activity	Lignin amounts[a] (% CWR)	S and G recovery[a] (% of CWR)	Estimated S and G recovery[a] (% of lignin)	S/G ratio	Proposed frequency of interunit linkages in lignins (estimated by NMR analysis with less than 20% lignin recovered)	Analytical techniques employed[a]
wt	(Control)		Blue-green under UV	n.d.	17	3.81	22.4	0.22	8-5′, Dibenzodioxocins, 8-*O*-4′, 8-8′	Nitrobenzene oxidation;
fah1-2	(Mutant)		Red under UV	n.d.	19	4.90	25.8	0.00	More 8-5′, dibenzodioxocins, and 8-*O*-4′, but fewer 8-8′	Klason lignin analysis; NMR; DFRC method;
A	Sense	35S	None reported	n.d.	19	6.34	33.4	0.05	Trace 8-5′, trace	histochemical
B	Sense	35S	None reported	n.d.	19	4.50	23.7	0.16	dibenzodioxocins,	staining
C	Sense	35S	None reported	n.d.	19	4.69	24.7	0.24	*erythro* 8-*O*-4′	
G	Sense	35S	None reported	n.d.	19	4.45	23.4	0.24	predominates over	
E	Sense	35S	None reported	n.d.	19	6.53	34.4	0.29	*threo* 8-*O*-4′	
F	Sense	35S	None reported	n.d.	19	7.22	38.0	0.34	(80% *erythro*),	
D	Sense	35S	None reported	n.d.	19	4.77	25.1	0.36	more 8-8′	
H	Sense	35S	None reported	n.d.	19	4.14	21.8	0.37		
I	Sense	35S	None reported	n.d.	19	4.57	24.1	0.40		
L	Sense	C4H	None reported	n.d.	11	7.47	67.9	0.8	Trace 8-5′,	
K	Sense	C4H	None reported	n.d.	11	7.36	66.9	0.9	trace dibenzodioxins,	
M	Sense	C4H	None reported	n.d.	11	7.91	71.9	2.4	*erythro* 8-*O*-4′	
O	Sense	C4H	None reported	n.d.	11	6.63	60.2	3.5	predominates over	
J	Sense	C4H	None reported	n.d.	11	7.33	66.7	4.8	*threo* 8-*O*-4′	
P	Sense	C4H	None reported	n.d.	11	9.05	82.2	5.8	(80% *erythro*),	
N	Sense	C4H	None reported	n.d.	11	8.84	80.3	8.9	more 8-8′	
R	Sense	C4H	None reported	n.d.	11	8.49	77.2	9.0		
Q	Sense	C4H	None reported	n.d.	11	6.95	63.2	11.4		

n.d. = not detected.

[a] Stem tissues of 5-week old plants were used for nitrobenzene oxidation (NBO) and derivatization followed by reductive cleavage (DFRC) analyses; stem sections of 8-week old plants were employed for Klason lignin determination and NMR spectral analyses. Lignin estimations were not corrected for proteinaceous and/or ash-forming constituents. The enzyme and gene expression localizations were not carried out.

of glucosides of caffeic (**10**) (~1.2–2.5 μg/g), sinapic (**19**) (~4–20 μg/g) and vanillic (**39**) acids (~0.2–0.4 μg/g), which together though represent only ca. 0.0023% of the woody tissue. The increased accumulation of glucosides is, however, consistent with a portion of the corresponding substrates for CCOMT being shunted into other metabolic pathways, when the requisite processing for lignification does not occur. Indeed, although not proven, the pink-red coloration noted in the tissues is likely a consequence of oxidation of catechols present, such as caffeic acid (**10**), or of related phenolics that build up. However, when considered together, these substances only account for a minuscule amount of the lignin depletion noted. Accordingly, it will be useful to much more comprehensively examine the transformants in terms of effects on lignin polymerization (i.e. molecular weight distribution), on integrity of the vascular tissues, and on the full range of the chemical constituents within the soluble extractives. Nevertheless, on the basis of the very limited reduction in lignin content noted, the results are generally consistent with what would be expected for downregulation of a non-rate-limiting step.

5.6.3. CCOMT downregulation in alfalfa

A fifth CCOMT downregulation study was performed using alfalfa (Guo et al., 2001), which apparently only has one CCOMT; its downregulation had only a moderate effect on overall estimated Klason lignin contents even at highly repressed levels. In that study, transformants were generated by expressing the full-length alfalfa CCOMT gene in the sense or antisense orientation, under control of the bean PAL2 promoter. Of the 20 sense and 14 antisense plants obtained, two sense (SCC16 and SCC20) and two antisense (ACC315 and ACC305) lines were severely depleted in CCOMT activities (< 10% of the wild type).

5.6.3.1. Phenotypes. There was no report of any developmental abnormalities in the transgenic lines. Only the two antisense lines, however, were analyzed further, and these had low residual CCOMT activities (~3–5% of the wild type, see Table 7C) and almost undetectable CCOMT transcripts and proteins, as revealed by RNA and protein gel blot analyses. [By comparison the COMT protein levels increased ~2-fold in ACC305, but not in ACC315.] Additionally, both phloroglucinol-

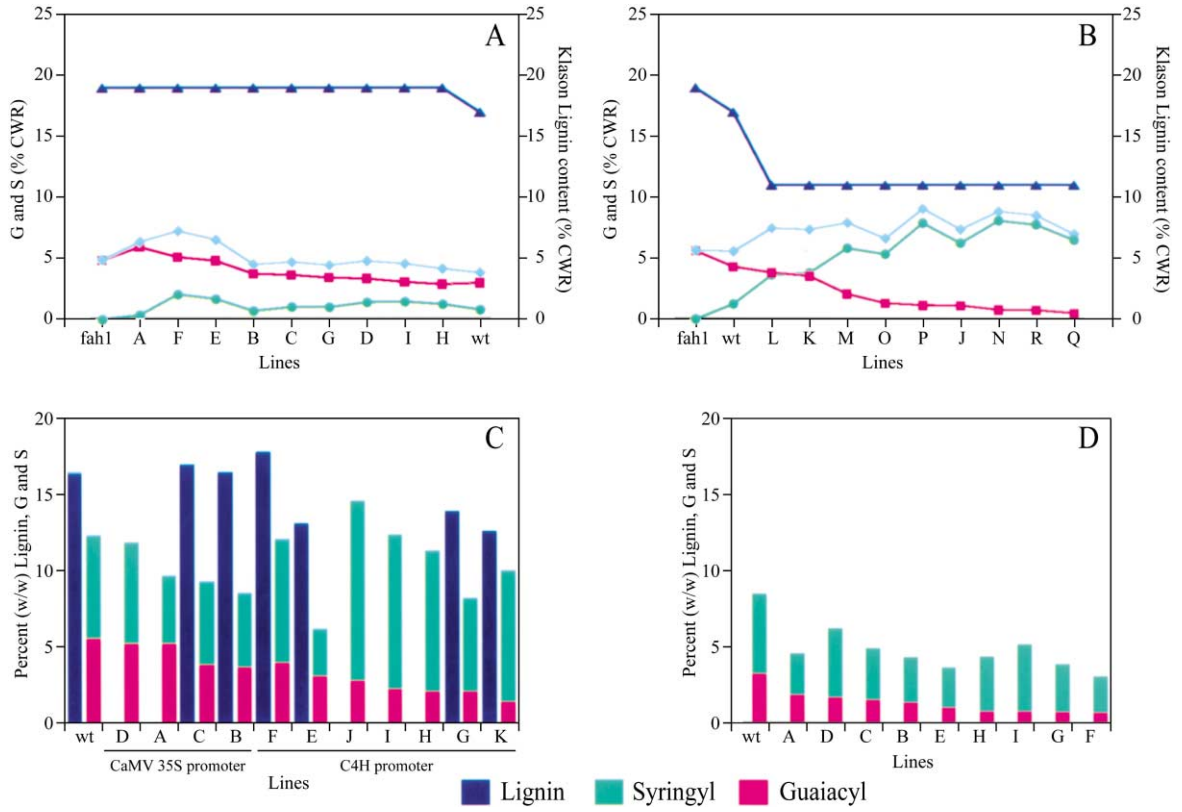


Fig. 15. Graphical analyses of reported lignin, guaiacyl and syringyl contents in up- and down-regulated arabidopsis, tobacco and poplar plants. Arabidopsis *fah1* mutant was transformed with either 35S-F5H (A), or C4H promoter-F5H (B) constructs (Meyer et al., 1998), whereas tobacco (C) and poplar (D) plants were transformed with the arabidopsis C4H promoter-F5H construct (Franke et al., 2000). Legend for (A) and (B): ■ = G; ● = S; ◆ = G + S; ▲ = Klason lignin.

HCl and Maüle reagent tests were essentially indistinguishable from the wild type.

5.6.3.2. Effects on lignification and accumulation of shunt metabolites. Fig. 13C and D plots the %G and %S thioacidolysis recoveries of the CWR as a function of estimated Klason lignin contents in the various control and transformant lines as before. Again there are too few data points for statistically valid conclusions to be drawn. The data obtained thus far, however, suggest that regardless of the estimated small reductions in lignin contents of both transformants, there may be a trend towards a differential reduction in the recovery of G fragments. On the other hand, S recovery is apparently for the most part unchanged. Furthermore, extrapolation of the data (based only on two points) would suggest that G components would be undetectable if estimated lignin levels were reduced to ca. 10–11% lignin in the CWR (~60% of the original lignin). (Trace amounts of thioacidolysis dimers were also detected but no significant differences were noted in comparison to the controls.) While these data suggest exclusive involvement of CCOMT in formation of the G units, and that S biosynthesis does not require CCOMT (which is in conflict with the previous findings in tobacco and poplar), it should not be ignored that in this alfalfa study the CCOMT gene was targeted using a heterologous bean PAL2 promoter (rather than the 35S CaMV constitutive or the native CCOMT promoters). It is therefore possible that the apparent specificity of the effect of CCOMT downregulation towards G-lignin biosynthesis may simply be due to the synchronized activity of the bean PAL2 promoter with the G-forming network. Nonetheless, the data provisionally suggest that the G and G–S pathways are kept distinct.

Additionally, in a somewhat analogous manner to the poplar study, the analysis of soluble phenolics in the CCOMT-downregulated alfalfa plants revealed an increased accumulation of caffeoyl-β-D-glucoside (with a caffeic acid (**10**) aglycone, whose exact structure is unknown) albeit in trace amounts, i.e. from <0.000005 to 0.000025% cell wall residue. While these data are consistent with increased formation of shunt metabolites, the amounts accounted for, relative to lignin depletion levels, are too minute to draw any meaningful conclusions.

5.6.3.3. Challenges for the future. The effect of CCOMT downregulation on lignification in alfalfa may be more considerable than what Fig. 13C and D suggest, since

for almost 70 years it has been known that herbaceous plants and grasses contain significant levels of proteins, which are rendered insoluble during the Klason lignin analyses (summarized in Lai and Sarkanen, 1971). Indeed, alfalfa is grown, in large part, for its high protein contents and is thus a prime candidate for overestimated lignin contents using the Klason method. These data thus once again underscore the need to fully characterize the bulk of the material currently being classified as lignin, and to determine whether actual lignin levels are indeed being correctly assessed for each plant system studied.

5.7. Hydroxylation at C-5 (previously ferulate 5-hydroxylase specific)

Ferulate 5-hydroxylase (F5H) introduces the final hydroxyl group at C-5 on the aromatic ring, thereby ultimately giving the substitution pattern necessary for S unit formation. The enzyme was initially detected in poplar (*Populus* × *euramericana*) (Grand, 1984), whose crude microsomal extracts were reported capable of converting ferulic acid (**12**) into 5-hydroxyferulic acid (**15**) ($K_m = 63$ μM). [The first report of 5-hydroxyferulic acid (**15**) as a natural product came from a study of maize phenolics some 15 years ago (Ohashi et al., 1987).] The gene CYP84, presumed to encode F5H was subsequently cloned from arabidopsis, and is considered responsible for the final aromatic hydroxylation step, which ultimately affords sinapyl alcohol (**3**) (Chapple et al., 1992). It was identified through mutational studies using an ethane methylsulfonate-mutagenized *sin1* arabidopsis mutant, which was reportedly devoid of sinapate esters and lignin syringyl units. When this mutant was administered [U-^{14}C]-Phe (**4**), conversion into radiolabeled ferulic (**12**) but not sinapic (**19**) acids occurred, whereas the wild type accumulated both. Furthermore, providing 5-hydroxyferulate (**15**) to the mutant restored some capability for formation of sinapic acid (**19**). Taken together these data suggested that the mutation(s) had blocked the conversion of ferulic acid (**12**) into 5-hydroxyferulic acid (**15**).

Proof of the role of F5H in sinapate ester formation came from studies of genetic complementation with the F5H gene of the T-DNA *fah1* arabidopsis mutant (Chapple et al., 1992), with the latter being similar to *sin1* in that it lacked sinapate esters and S lignins. Complementation of *fah1* with F5H restored the formation of sinapate esters, which proved that F5H was involved in sinapate ester biosynthesis.

5.7.1. Substrate specificity questions

In a later comprehensive review of the enzymes and proteins involved in monolignol biosynthesis and lignin assembly (Lewis et al., 1999), it was noted that there had been insufficient demonstration in vitro of the substrate specificity of CYP84. This cautionary remark was made in part because of other reports by Fukushima et al. (1997) which suggested the preferred substrate for hydroxylation was coniferyl alcohol (**2**) rather than ferulic acid (**12**). This in turn implied that the pathway to sinapyl alcohol (**3**) in lignin formation was via coniferyl (**2**) and 5-hydroxyconiferyl (**18**) alcohols (Fukushima et al., 1997; Chen et al., 1999). Those same investigators later demonstrated that administration of pentadeuterated [γ,γ'-$^2H_2$, OC^2H_3]-coniferyl alcohol (**2**) to *Magnolia kobus* resulted in its *intact* conversion into [γ,γ'-$^2H_2$, OC^2H_3]-sinapyl alcohol (**3**), albeit in low overall conversion (~1%) (Matsui et al., 2000). The data thus suggested that both hydroxylation and methylation reactions occurred at the hydroxycinnamyl (coniferyl) alcohol level; however, these researchers did not rule out the possibility that hydroxylation/methylation might also (partially) occur with the aldehyde.

This galvanized the subsequent functional expression of recombinant CYP84 from arabidopsis in yeast by others, which in turn revealed a preference for the NADPH-dependent 5-hydroxylation of both coniferyl alcohol (**2**) ($K_m = 3$ μM, $V_{max} = 6$ pkat/mg) and coniferyl aldehyde (**14**) ($K_m = 1$ μM, $V_{max} = 5$ pkat/mg) in vitro, rather than with ferulic acid (**12**) ($K_m = 1$ mM, $V_{max} = 4$ pkat/mg) (Humphreys et al., 1999). At about the same time, it was reported that a CYP84 from sweetgum also preferentially catalyzed hydroxylation of coniferyl aldehyde (**14**) rather than either ferulic acid (**12**) or coniferyl alcohol (**2**), and that its activity towards ferulic acid (**12**) was inhibited by coniferyl aldehyde (**14**) (Osakabe et al., 1999). Furthermore, in vitro hydroxylation and methylation activities, which were required for the formation of 5-hydroxyconiferyl (**17**) and sinapyl (**21**) aldehydes, respectively, were demonstrated as being present in several angiosperms: these included aspen, acacia, basswood, black ash, hophor bean, red maple, sugar maple, sweetgum, white birch and yellow birch (Li et al., 2000). Yet, given that many plant systems can accumulate sinapic acid (**19**) derivatives, both the physiological substrates being utilized by CYP84 in vivo, and whether more than one form of CYP84 with different substrate specificities exists, still need to be established.

5.7.2. Implications of manipulating syringyl levels in transgenic plants

In spite of unresolved issues regarding the actual physiological substrates, interesting studies have been conducted on manipulating F5H (CYP84) expression in arabidopsis (Meyer et al., 1998), tobacco (Franke et al., 2000) and poplar (Franke et al., 2000). Indeed, the findings are of considerable potential utility in defining further how the pathway to sinapyl alcohol (**3**) is distinctly organized, although the results obtained for each species were very different as explained below.

Unfortunately to date, no biomechanical testing of any of the F5H transformants has yet been done, in order to attempt to estimate the changes (weakening or strengthening) to the vascular apparatus that may have occurred.

Thus, since sinapyl (S) alcohol (**3**) units are typically viewed as being deposited during the later stages of lignification (e.g. during fiber development), the overexpression of F5H in all cell types would presumably have important consequences. For example, the coniferyl alcohol (**2**) formed in the early stages of lignin deposition, could be anticipated to be converted into 5-hydroxy analogs. Thus, the question of the metabolic fate of the presumed hydroxy analogs is of considerable interest, particularly since (as discussed later), there is a specific COMT involved in introducing the second methyl group in the biosynthesis of sinapyl alcohol (**3**).

5.7.3. Overexpression of F5H in arabidopsis

The CYP84 gene was first overexpressed in the arabidopsis null mutant *fah1*, a mutant rich in guaiacyl units, with the gene being linked to either the CaMV 35S or C4H promoters. The latter has the advantage of being targeted to every cell where C4H is expressed, whereas the former is considered to be non-specifically targeted to most cell types. A rationale for using the null mutant in the transformation was to avoid potential epigenetic silencing due to endogenous CYP84 in the wild type.

Based on the analysis of the transgenic data obtained for PAL, C4H, 4CL and CCOMT, it might be expected that F5H, while initiating the branch point conversion to sinapyl alcohol (**3**), would have no effect on overall *carbon allocation* to the phenylpropanoid pathway. If this assumption is correct, then there are at least two possibilities that can be considered as regards the effects of F5H overexpression. One is that this would yield increased levels of sinapyl alcohol (**3**) and thus overall increase the total lignin amounts due to the addition of an extra methoxyl group. A second possibility is that if both coniferyl (**2**) and sinapyl (**3**) alcohol pathways are truly kept distinct, both within and among different cell types, then addition of an extra hydroxyl group to a guaiacyl moiety might afford a catechol (5-hydroxylated monomer); the latter, if transported into the cell wall, would presumably have a deleterious effect on lignin assembly proper, since catechols can readily undergo both quinone formation and nucleophilic attack and thus would be unable to polymerize properly (discussed later in section on COMTs).

5.7.4. Effects on lignification: arabidopsis

The effects of the genetic manipulation of CYP84 expression in arabidopsis were preliminarily examined by determining the guaiacyl and syringyl components released through nitrobenzene oxidation of solvent-extracted and cell wall enriched preparations (Meyer et al., 1998), and by measurement of Klason lignin contents of same (Marita et al., 1999). However, these determinations were surprisingly not conducted at the same time: nitrobenzene oxidations were conducted on 5-week old plants, whereas Klason lignin estimates were on 8-week old arabidopsis stems. The results obtained are summarized in Table 9 and Fig. 15. Note that in Fig. 15 the levels of recoverable guaiacyl and syringyl constituents (through nitrobenzene oxidation) are not plotted as a function of ferulate 5-hydroxylase activity, because activity was not detected (Meyer et al., 1998). For illustrative purposes, the transgenic lines are generally aligned in order of decreasing guaiacyl (G) content, following the null mutant (*fah1*) and wild type controls.

At this point, it is important to note that most inconsistent data was subsequently reported for the lignin contents of both wild type and *fah1* mutants of arabidopsis. That is, in another study by the same investigators (Jung et al., 1999), the Klason lignin values reported (of ash-free residues, with no data provided for the latter) were very high, and thus cannot be viewed as being representative of lignin amounts without additional proof: the reported values were of 30.9% (wild type) and 31.6–35.8% for the *fah1* mutants, these being in excess of lignin levels in most woody gymnosperm tissues! Furthermore, while nitrobenzene oxidation analyses of these tissues were apparently carried out, no data was provided to correlate H, G and S recoveries as a function (percentage) of either cell wall residues and/or putative lignin contents. Such data again underscore the need for caution in interpretion of putative effects on lignification/lignin assembly, and the necessity to overcome the technical obstacles in lignin analyses.

However, in the Meyer et al. (1998) and Marita et al. (1999) studies, using the CaMV 35S promoter, there was apparently a very small increase in the Klason lignin contents (~19%) of both the *fah1* and the CYP84-overexpressing arabidopsis transformants A–H when compared with the wild type (~17%) (Table 9 and Fig. 15A); however, the statistical significance, if any, of this cannot be evaluated with only one data point reported for the wild type plant. Nevertheless, based on the Klason lignin determinations and the limitations described earlier, the amount of carbon allocated to the phenylpropanoid pathway towards the lignins was apparently unchanged by overexpression of CYP84 in arabidopsis, as predicted from the previous metabolic flux and transgenic plant studies. Thus, assuming CYP84 indeed encodes for ferulate 5-hydroxylase, it seems that this step is not a flux control point for carbon allocation in lignin biosynthesis.

Furthermore, overexpression of CYP84 in arabidopsis using the 35S promoter did not lead to any significant increase in syringyl moieties relative to that of wild type (Fig. 15A), although small increases (up to ~2.5-fold) were noted in transformant F; note also that

the S and G recoveries as a percentage of the putative lignin present ranged from 21.8 to 38% in the transformants. However, S/G ratios only varied from ~0.2 to ~0.4, except for one transgenic line (A), which actually had a lower S/G ratio (0.05). These data may mean that the CaMV 35S promoter does not efficiently target cells involved in sinapyl alcohol (**3**) biosynthesis. Why the CaMV 35S promoter had this effect is unknown at present, but if this is correct, it signifies difficulties in using promoters of this type that may not drive the expression in the targeted manner desired. Additionally, why the wild type had a very low syringyl (S) content was also surprising [cf. 4CL downregulation in arabidopsis, where the previous wild type plants with the same investigators had S/G ratios ~0.5 (Lee et al., 1997)].

On the other hand, when CYP84 was placed under control of the arabidopsis C4H promoter, there were two notable effects: the first was that the measurable insoluble Klason lignin content was reduced substantially (to ~11% of wild type control) (Fig. 15B). The second effect was that the syringyl recoveries from the cell wall residues increased from ~1.2% in the wild type to as high as ~8% in the three transformants (lines P, N and R), as revealed by nitrobenzene oxidation analysis. This was also accompanied by a concomitant decrease in guaiacyl constituents from ~25% to as low as ~4% of Klason lignin in line Q.

The first effect, i.e. measured lignin content of ~11%, has been potentially attributed to less cross-linking of the syringyl units in the lignin polymers so formed, and thus to greater losses experienced during the Klason lignin test (Marita et al., 1999). Although these researchers imply that the lignin contents may actually be higher (e.g. 17 or 19% or even more), this needs to be verified experimentally. A second effect (see Fig. 15B) is the substantial increase in the amount of syringaldehyde (**24**) released following nitrobenzene oxidation of the extractive-free cell wall enriched material. Based on the ~11% lignin content, the recoveries of S and G units would approximately correspond up to ~80% of the estimated lignin content (Table 9). Alternatively, if the lignin contents are ~19%, then the recoveries would be closer to that expected (~40%). It is not possible, using the nitrobenzene oxidation method, to gauge to what extent, for example, cell wall based hydroxycinnamic acids are also skewing the data obtained.

The increases in observed syringyl residues should thus also be verified by thioacidolysis, and the polymeric nature of the lignins established through, for example, molecular weight determinations. Hence, while it appears to be a reasonable assumption that the guaiacyl components of cells that produce guaiacyl–syringyl lignins are mainly converted into syringyl lignins, the data reported do not yet fully prove this, e.g. given the discrepancies between recoveries of Klason lignin and nitrobenzene oxidation results.

Preliminary characterization of the partially solubilized portion of the presumed lignin fractions of arabidopsis was also carried out using NMR spectroscopic analysis (Marita et al., 1999). In that study, the "lignin" recoveries from the tedious, time-consuming, procedures employed were again very low: wild type plants (~17% total Klason lignin, 24.6% of which was recovered for analysis); the *fah1-2* mutant (19% of lignin, 17.4% recovery); 35S–CYP84 construct (17% lignin, 29.7% recovery) and C4H–CYP84 construct (11% lignin, 27.5% recovery). In the latter case, if the lignin contents are indeed ~17–19% as implied by these investigators, then the recovery is even lower ($\leqslant$17%).

The acetylated solubilized lignin sample, derived from the C4H promoter–CYP84 construct and presumed to account for <17% of the total lignin solubilized, was reported to contain S and G units in a ratio of ~9:1 (Fig. 16A, compare peaks labeled S and peaks labeled G). Spectroscopic analysis also suggested that the β-*O*-4 linkages substantially predominated (% not reported), in which ~80% had an *erythro* (**43**) stereochemistry (Fig. 16A, peaks *e* and *t*). By contrast, the acetylated *fah1-2* preparation (predominantly consisting of guaiacyl moieties) had β-*O*-4 linkages in the *erythro* (**43**)/*threo* (**44**) stereochemistries, in approximately equal ratios (Fig. 16B, peaks *e* and *t*), with syringaresinol-like residues (peak $S_{2/6}$) being evident to a much lesser extent. Fig. 16C displays, for comparative purposes, the NMR spectrum of the acetylated lignin of the wild type control. Clearly, it needs to be established whether (i) the bulk of the lignin yet uncharacterized (presumed to constitute ~83% of the total) displays these same characteristics or not; (ii) how the regioselectivity is attained to predominantly afford the β-*O*-aryl linkage and (iii) whether the true lignin content of the C4H–CYP84 construct is 11, 17, 19% or something else.

5.7.5. Tobacco CYP84 transformants

The CYP84 gene from arabidopsis was also transformed into tobacco under the control of the CaMV 35S and arabidopsis C4H promoters (Franke et al., 2000). The results are summarized in Table 10 and Fig. 15C with the transformants again being ordered in terms of decreasing guaiacyl recoveries next to the wild type (only one control reported for C4H construct). Again no ferulate 5-hydroxylase activities were reported for any substrates, and Klason lignin determinations were obtained only for selected transformants (Fig. 15C). Once again the nitrobenzene oxidation and Klason lignin determinations were apparently conducted at different stages of growth and development, and this may partially account for why S and G recoveries relative to estimated lignin contents were uncharacteristically high (64.6% for wild type versus 111.2% for transformants). On the other hand, S and G recoveries remained relatively similar (~10–12% CWR) for most transformants,

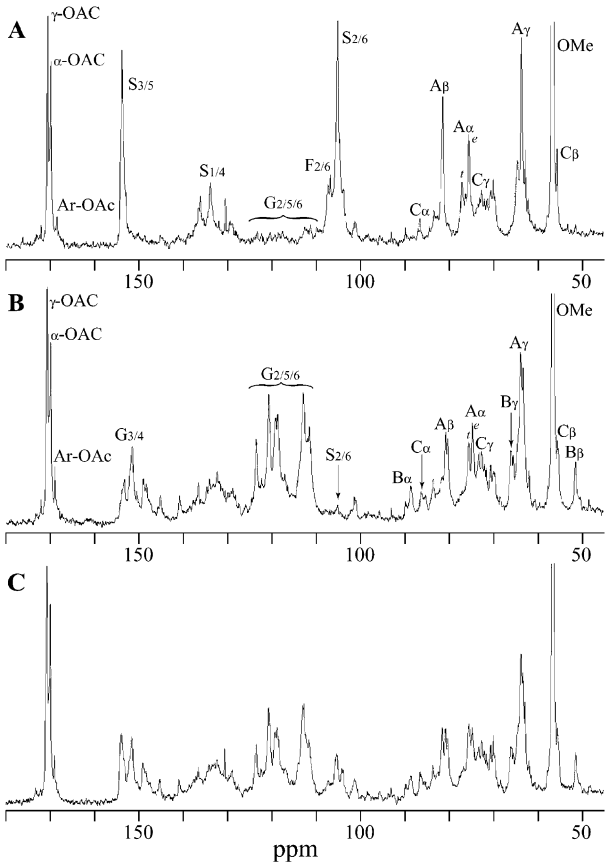


Fig. 16. ^{13}C NMR spectra of acetylated dioxane-soluble lignin-enriched preparations from C4H promoter-F5H transgenic (A), *fah1-2* mutant (B) and wild type (C) arabidopsis. Lignin recoveries as percentage of estimated Klason lignin were: (A) 24.6; (B) 17.4; and (C) $\leqslant$ 17%. G = guaiacyl unit; S = syringyl unit. Reproduced from Marita et al. (1999) with permission of the National Academy of Sciences, USA (Copyright 1999).

Table 10
Preliminary characterization of transgenic tobacco following introduction of the arabidopsis CYP84 transgene (Franke et al., 2000)

Plant line	Transgene orientation	Promoter	Gross morphological changes in phenotypes of transformants	F5H activity	Klason lignin contents (% CWR)[a]	S and G recovery (% of CWR)[a]	Estimated S and G recovery (% of lignin)[a]	S/G ratio	Analytical techniques employed[a]
wt				n.d.	16.5	10.7	64.6	0.97	Nitrobenzene
A	Sense	35S	None reported	n.d.	n.d.	9.7	n.d.	0.74	oxidation;
D	Sense	35S	None reported	n.d.	n.d.	11.8	n.d.	1.08	Klason lignin
B	Sense	35S	None reported	n.d.	17.0	8.5	49.8	1.13	analysis;
C	Sense	35S	None reported	n.d.	17.8	9.3	52.2	1.21	thioglycolate lignin
wt				n.d.	16.5	12.3	74.7	1.04	determination;
F	Sense	C4H	None reported	n.d.	n.d.	12.0	n.d.	1.75	DFRC method;
E	Sense	C4H	None reported	n.d.	16.5	6.3	38.0	0.89	
G	Sense	C4H	None reported	n.d.	13.9	8.1	58.3	2.58	
J	Sense	C4H	None reported	n.d.	13.1	14.5	111.2	3.64	
H	Sense	C4H	None reported	n.d.	n.d.	11.3	n.d.	3.88	
I	Sense	C4H	None reported	n.d.	n.d.	12.4	n.d.	3.93	
K	Sense	C4H	None reported	n.d.	12.6	9.9	79.1	5.37	

The enzyme and gene expression localizations, the frequency of interunit linkages in, and MW ranges of, lignins as well as the ultrastructural effects on cell wall types and patterns of lignin deposition were not determined (n.d.).

[a] Stems and petioles from 3–6 month old tobacco plants were used: one set from T_0 plants (age not specified) for NBO and another set from younger T_1 plants (age not specified) for Klason, thioglycolic acid (TGA) and derivatization followed by reductive cleavage (DFRC) analyses. Lignin estimations were not corrected for proteinaceous and/or ash-forming constituents.

further indicating that the Klason lignin levels cannot be compared with G and S recoveries in this manner.

With the CaMV 35S promoter (Fig. 15C, entries A–D), there was little apparent effect on either Klason lignin content relative to the wild type control, or on syringyl/guaiacyl ratios, as previously noted for arabidopsis (see Table 10, entries B and C). With the arabidopsis C4H promoter linked to CYP84 (entries F–K), it was found that the syringyl derivatives in the cell wall residue increased almost 2-fold from 7% in the wild type to as high as 12% of the cell wall residue in one transformant (J). However, the recovery values of the guaiacyl and syringyl components accounted for essentially all, or in some cases even exceeded, the amount of Klason lignin recovered for the few cases reported (Fig. 15C, see entries J and K). Note, however, that surprisingly again each of the lignin and S/G analyses were carried out on different tissues at different stages of maturity. Thus, as for arabidopsis, it is important that a convincing experimentally based explanation for these discrepancies be obtained. In spite of these concerns, and while it provides no insight into how lignin assembly is effectuated, it appears that there is an increase in S units with a concomitant decline in G. Again, it will be important to identify the cell types that are involved in these changes.

5.7.6. Poplar CYP84 transformants

Lastly, CYP84 from arabidopsis was expressed in poplar using the arabidopsis C4H promoter (Franke et al., 2000), and the transgenic lines obtained relative to the wild type are again arranged in order of decreasing guaiacyl residues based on nitrobenzene oxidation analysis (Table 11 and Fig. 15D). Again, no correlation with ferulate 5-hydroxylase activities was reported, and Klason lignin contents were not determined. (Additionally, the limitations of using a heterologous arabidopsis promoter rather than the native one are currently unknown.) Nevertheless, these data revealed an overall reduction in both guaiacyl and syringyl derived constituents relative to wild type, but with no corresponding increase in syringyl units being noted. Instead, only a decrease in overall G recovery levels was observed, this perhaps being due to a failure to generate S moieties from those originally designated as G in those cell types. No additional analysis of these transformants can be given since, for example, Klason lignin estimations were not reported.

5.7.7. Challenges for the future: implications for lignin assembly

Taken together, while these results are of considerable interest, the analyses are as yet too preliminary to draw any conclusions as regards the effects on *overall* lignin and/or cell wall assembly processes, and on the vascular integrity of the various cell types. However, with phenotypes rich in H (C3H), G (*fah1*) and S (C4H-promoter–F5H) components, these should be of considerable utility in the future to begin to study the distinct phases of the overall lignification and cell wall assembly processes, at least in arabidopsis. This is because the various phenotypes should provide essential information on how lignin initiation and penetration into the distinct cell wall layers of various cell types are effectuated in each phenotype. As discussed earlier, the role currently believed to be assumed by the H-units in overall lignin assembly involves H-lignin initiation at specific sites on the periphery of the S_1 layer, which then eventually progress into the middle lamella. On the

Table 11
Preliminary characterization of transgenic poplar following introduction of the arabidopsis CYP84 transgene (Franke et al., 2000)

Plant line	Transgene orientation	Promoter	Gross morphological changes in phenotypes of transformants[a]	F5H activity	Klason lignin contents (% CWR)[a]	S and G recovery (% of CWR)	S/G ratio	Analytical techniques employed[a]
wt	(Control)			n.d.	n.d.	8.30	1.44	Nitrobenzene oxidation; DFRC method
A	Sense	C4H	None reported	n.d.	n.d.	4.43	1.32	
B	Sense	C4H	None reported	n.d.	n.d.	4.24	1.99	
C	Sense	C4H	None reported	n.d.	n.d.	4.73	2.11	
D	Sense	C4H	None reported	n.d.	n.d.	6.10	2.44	
E	Sense	C4H	None reported	n.d.	n.d.	3.53	2.50	
F	Sense	C4H	None reported	n.d.	n.d.	2.97	3.56	
G	Sense	C4H	None reported	n.d.	n.d.	3.74	4.23	
H	Sense	C4H	None reported	n.d.	n.d.	4.19	4.39	
I	Sense	C4H	None reported	n.d.	n.d.	4.99	5.57	

The enzyme and gene expression localizations, the frequency of interunit linkages in, and MW ranges of, lignins as well as the ultrastructural effects on cell wall types and patterns of lignin deposition were not determined (n.d.).

[a] Stems and petioles from 3–6 month old plants were used for these analyses: 1 set from T_0 plants for nitrobenzene oxidation analysis, 1 set from T_1 plants for DFRC (derivatization followed by reductive cleavage). Lignin estimations were not corrected for proteinaceous and/or ash-forming constituents.

other hand, the G constituents are laid down initially at specific (initiation) sites in the S_1 sublayer and then the G-lignin extends inwards towards the plasma membrane, whereas the S units are currently viewed to be restricted mainly to late stages of lignification, particularly in the fibers. However, all of this needs to be examined carefully, and on a cell by cell and species by species basis.

Nevertheless, both the G (*fah1*) and S (C4H-promoter–F5H) phenotypes should be particularly useful in the study of G and S lignin initiation and its progression throughout the cell walls of the various cell types. This matter is potentially important as regards our proposal of lignin assembly utilizing proteins harboring discrete arrays of monolignol (radical) dirigent binding sites with subsequent template polymerization (Lewis et al., 1998, 1999; Sarkanen, 1998; Lewis, 1999; Croteau et al., 2000). However, two notes of caution need to be taken into consideration of these phenotypes: (i) in the G- and S-rich arabidopsis phenotypes, both the H-contents and their relative disposition in the lignin of the cell wall are unknown, and (ii) in the S-rich phenotype, the presence of ~10–12% G units may be of critical importance for overall lignin assembly.

Accordingly, all of the transformants need to be much more thoroughly characterized in terms of: (i) the enzymatic step(s) that was (were) perturbed; (ii) the true (Klason) lignin contents of each transformant including that of the soluble portions; (iii) further verification of monomeric composition (e.g. as estimated by thioacidolysis); (iv) how lignin biosynthesis is initiated and progresses in each of the aforementioned phenotypes; (v) the frequencies of interunit linkages in all of the lignin, not just the small percentages more readily isolable; (vi) to what extent, if any, catechol (5-hydroxyguaiacyl) moieties are formed in the cell types that originally generated G lignins, but were not recovered, or, alternatively, which OMT introduces the second methyl group into the aromatic ring to give the S-unit in these cells; (vii) the molecular weight ranges of the lignins obtained; (viii) the nature and amount of the "extractive" components; and (ix) what deleterious effects, if any, on vascular integrity and plant growth/development have resulted. Such studies are particularly needed in order to generate useful information with regards to how lignification is initiated and proceeds.

5.8. Methylation of the C-5 hydroxyl group [previously caffeic acid O-methyltransferase specific (E.C. 2.1.1.68)] and the bm3 mutation

5.8.1. The bm3 mutation: weakening of the vascular apparatus and stalk lodging

Prior to analyzing the effects of downregulation of "caffeic acid *O*-methyltransferases", a brief discussion of the so-called brown-midrib mutations is provided for needed historical context, particularly since these have long been studied. In 1931, a paper entitled "Brown midrib in maize and its linkage relations" (Jorgenson, 1931) described the discovery of a maize mutation in 1924 in Minnesota whose trait was inherited as a simple Mendelian recessive. A brown pigmentation was noted in the lignified tissues of the mutants' stems, roots, leaves, tassels, and cobs (but not in pollen grains or kernels). Although no details were provided, the author indicated that lignification preceded pigmentation, and that pigmentation occurred only in the trachea but not in the tracheid. Interestingly, when initially deposited, the pigment had only a light yellowish color but on standing became dark orange-brown, indicative of further oxidative transformations. This mutant has since been designated as the *bm1* mutation (Kuc and Nelson, 1964), and has been reported as affecting CAD (Halpin et al., 1998, described later). Several other types of brown-midrib (*bm*) mutations have also been described (Kuc et al., 1968), one of which is the *bm3* mutant first described in 1935 (Emerson et al., 1935).

The so-called *bm3* mutant has, relative to the "normal" phenotype, lower amounts of cell-wall constituents, acid detergent fiber and acid detergent lignin (Muller et al., 1971), as well as an increased forage digestibility (Barnes et al., 1971). These conclusions were made based on an assessment of the properties of the tissues obtained during an extensive extraction and fractionation procedure. However, although the underlying reasons for these changes were not understood at the molecular level, it is quite perplexing that the clues that emerged have essentially been ignored by most of the recent investigations. In addition to these changes, it was also found that the *bm3* mutation has an adverse effect on stem stalk strength (17–26% less crushing strength and 8–14% less stalk-section weight). That is, the *bm3* mutation resulted in maize plants being more prone to stalk lodging (falling down and collapsing) (Zuber et al., 1977), due to a weakening of vascular integrity, perhaps due to an increased brittleness. In any event, this weakening was viewed as a significant disadvantage during maize growth and development and in its subsequent handling.

Given the apparent decrease in lignin content as determined initially using these methods (described later), subsequent crude enzyme analyses of extracts from "normal" and "*bm3*" maize were carried out at 10, 20 and 30 days of growth and development to determine if any of the monolignol pathway enzymes were affected, i.e. specifically PAL, OMT, 4CL, CCR and CAD (Grand et al., 1985). [At that time, it was considered that OMT was bifunctional for both caffeic (**10**) and 5-hydroxyferulic (**15**) acids.] However, only the enzyme activities of OMT in the mutant were lower than that of the "normal" line, with PAL, 4CL, CCR and CAD remaining essentially unchanged, i.e. the mutant had

~10% of OMT activity of the "normal" line, suggesting that a lignin specific *O*-methyltransferase was downregulated (Grand et al., 1985). Shortly thereafter, thioacidolysis of "normal" and *bm3* lignified tissues by others also revealed a substantial (3-fold) decrease in detectable S units, in addition to the presence of trace amounts of an unknown 5-hydroxyguaiacyl component (Lapierre et al., 1988). These data suggested that the mutation was specifically involved in sinapyl alcohol (**3**) biosynthesis, which would thus largely affect cells forming G–S lignins. At the same time, it was proposed that 5-hydroxyconiferyl alcohol (**18**) (an isoelectronic species) might be deployed as a "replacement" lignin precursor instead of sinapyl alcohol (**3**) (Lapierre et al., 1988). Yet, based on the known phenotypes, clearly lignification proper had been adversely affected. Nevertheless, it was of interest that the isoelectronic 5-hydroxyconiferyl alcohol (**18**), lacking the second methyl group, could apparently undergo monomer transport into the cell wall, when the methylation step was disrupted via mutation.

*5.8.2. The bm3 mutation and the second methylation step in sinapyl alcohol (**3**) formation*

With this historical context, it nevertheless took several years to establish a role for the enzyme originally described as COMT (caffeic acid *O*-methyltransferase); it is now established that it is a 5-hydroxyguaiacyl *O*-methyltransferase (Lewis et al., 1999) and that the *bm3* mutation occurs in its encoding gene (Vignols et al., 1995). However, the actual physiological substrate(s) used by this enzyme in vivo still needs further clarification since its various recombinant forms (from tobacco, aspen and alfalfa, respectively), when heterologously expressed in *E. coli*, are reportedly able to utilize 5-hydroxyferulic acid (**15**), 5-hydroxyferuloyl CoA (**16**), 5-hydroxyconiferaldehyde (**17**), 5-hydroxyconiferyl alcohol (**18**), and even the corresponding caffeyl moieties (**10**, **11**, **41**, **42**). For example, the tobacco *E. coli*-expressed COMT-1, which is the isoform proposed to be involved in lignification, has reportedly surprisingly low V_{max}/K_m values of 0.02, 0.03, 0.04, 0.04, and 0.02 nkat mg^{-1} protein μM^{-1} for caffeic acid (**10**), 5-hydroxyferulic acid (**15**), caffeoyl CoA (**11**), 5-hydroxyferuloyl CoA (**16**), and 5-hydroxyconiferyl alcohol (**18**), respectively (Maury et al., 1999). The activity against 5-hydroxyconiferyl aldehyde (**17**) was not reported. On the other hand, a recombinant COMT from aspen, which was also expressed in *E.coli*, has V_{max}/K_m values of 0.12, 0.73 and 3.85 nkat mg^{-1} protein μM^{-1} for caffeic acid (**10**), 5-hydroxyferulic acid (**15**), and 5-hydroxyconiferyl aldehyde (**17**), respectively, with no significant methylating activity apparently being obtained when 5-hydroxyconiferyl alcohol (**18**) was used as substrate (Li et al., 2000); thus these investigators concluded that 5-hydroxyconiferyl aldehyde was the preferred substrate in vivo. By contrast, *E. coli*-expressed alfalfa COMT has V_{max}/K_m values of 0.1, 0.6, 2.8, 3.1, 1.8, and 1.4 nkat mg^{-1} protein μM^{-1} for caffeic acid (**10**), 5-hydroxyferulic acid (**15**), caffeyl aldehyde (**41**), 5-hydroxyconiferyl aldehyde (**17**), caffeyl alcohol (**42**) and 5-hydroxyconiferyl alcohol (**18**), respectively (Parvathi et al., 2001). Clearly based on the rather divergent data, it would be premature to unambigously assign a physiological substrate until this is proven in vivo.

The effect of COMT downregulation on lignification became the subject of numerous studies since 1994 using a variety of plant species. As early as 1995 (Atanassova et al., 1995), it was definitively shown that the role of COMT was indeed in the final methylation step ultimately affording the syringyl units of lignins. Tables 12–14 and Figs. 17–20 summarize the results of the various transgenic experiments on downregulating COMT in tobacco (Dwivedi et al., 1994; Atanassova et al., 1995; Zhong et al., 1998), poplar (Van Doorsselaere et al., 1995a; Jouanin et al., 2000), aspen (Tsai et al., 1998) and alfalfa (Guo et al., 2001). As before, the data reported from each study were recalculated to attempt to correlate the estimated lignin contents and the corresponding COMT activities relative to controls (set at 100%); the latter employed wild type plants (Dwivedi et al., 1994; Atanassova et al., 1995; Jouanin et al., 2000), transformants with an empty vector in tobacco (Zhong et al., 1998), aspen (Tsai et al., 1998), and alfalfa (Guo et al., 2001), as well as plants transformed with the β-glucuronidase gene in poplar (Van Doorsselaere et al., 1995a,b). These data (i.e. Klason lignin analyses) are, however, not corrected for losses due to more readily soluble phenolics (through COMT downregulation), or the contribution of various proteinaceous and inorganic components (rendered by ashing the tissues). COMT activities in those studies were also measured using caffeic (**10**) and/or 5-hydroxyferulic (**15**) acids as substrates, rather than 5-hydroxyconiferyl aldehyde (**17**)/alcohol (**18**). Moreover, the conclusions made assumed that measured COMT activities approximate only that involved in lignin-related COMT, and not in other forms of metabolism (e.g. to sinapate esters, or other methylated products, etc.). In the future, COMT studies should measure protein levels by immunohybridization assays, and compare the results obtained with the corresponding extractable enzymatic activities.

From the data plotted (Fig. 17A), COMT suppression apparently had either fairly moderate to no effect on overall lignin content, with one striking exception (Ni et al., 1994) in tobacco. That is, in all the other studies, COMT downregulation had little effect, if any, on carbon flux to lignin, as to be anticipated from the preceding rationale on carbon allocation/metabolic flux. From these three sets of experimental data, more than 70% of the carbon flux into lignin apparently still occurred, even at 3% COMT activity relative to wild type controls.

Table 12
Preliminary characterization of transgenic tobacco plants following introduction of homologous COMT transgene

	Plant line	Enzyme and gene expression localization	Transgene orientation	Promoter	Gross morphological changes in phenotypes of T_0 transformants	Tissue analyzed for lignins	COMT activity (% relative to average of controls)[d]	Lignin amounts[e] (% CWR)	Estimated S, G and 5-OHG recovery (% of lignin)	S/G ratio	Analytical techniques employed
A.[a]	C1		(Control)	35S		Woody	105	23.8	36.6	0.81	Nitrobenzene
	C2	mRNA	(Control)	35S		tissue	95	23.9	37.1	0.76	oxidation;
	A2	localized in	Antisense	35S	None	from stems	73	22.6	35.9	0.79	Klason lignin
	A4	young xylem	Antisense	35S	None	of 3-month	86	22.0	36.6	0.75	analysis
	A17	cells in the	Antisense	35S	None	old plants	76	21.3	36.4	0.69	
	A3	stem; xylem and phloem in	Antisense	35S	None		54	21.1	37.1	0.64	
B.[b]	A2	the petiole;	Sense				322	n.d	n.d.	1.0	
	T12	vascular	(Control)	35S		Stems from	191	n.d	n.d.	0.93	Thioacidolysis;
	T11	bundles of	(Control)	35S		plants of	172	17.7	8.1	1.07	Klason lignin
	T19	leaves;	(Control)	35S		unspecified	80	19.1	7.6	0.96	analysis;
	T7	upper and	(Control)	35S		age	69	n.d	n.d.		histochemical
	B18	lower	Antisense	35S	None		12	18.7	5.0	0.29	staining
	A17	epidermis of	Sense	35S	None		2	18.5	4.3	0.09	
	B10	leaves;	Antisense	35S	None		8	18.8	4.8	0.07	
	B1	epidermis and xylem	Antisense	35S	None		6	17.6	4.3	0.07	
C.[c]	XT1	of roots	(Control)	35S		"Mature"	110	17.3	17.2	0.60	Pyrolysis-MS;
	XT2		(Control)	35S		stems from	94	18.1	16.8	0.68	Klason lignin
	TMTG		Antisense	35S	None	plants of	27	16.3	11.6	0.10	analysis; NaOH
	TMT5		Antisense	35S	None	unspecified age	30	16.3	10.0	0.05	hydrolysis; histochemical staining

The frequency of interunit linkages in, and MW ranges of, lignins, and the ultrastructural effects on cell wall types and patterns of lignin deposition were not determined (n.d.).

[a] Adapted from Dwivedi et al. (1994).

[b] Adapted from Atanassova et al. (1995).

[c] Adapted from Zhong et al. (1998).

[d] Except for Dwivedi et al., controls refer to wild type population used by the investigators, which are not presented in the table.

[e] Lignin estimations were not corrected for proteinaceous and/or ash-forming constituents.

In the apparent exception (Ni et al., 1994) to this trend, the COMT gene from alfalfa was used to transform tobacco using the CaMV 35S promoter. The data obtained suggested a steady decline in lignin content, as estimated from the absorbances of solubles released by thioglycolic acid extraction. A later report on the same transformants (Sewalt et al., 1997b) confirmed this apparent trend in lignin reduction using the combined techniques of neutral detergent fiber isolation (NDF) with Klason lignin determination of the latter. These data thus suggested that significant changes had occurred in terms of polyphenol and lignin extractability using the thioglycolic acid method, and in the overall properties of the corresponding fractionated fibers, as had previously been noted by Muller et al. (1971) with the *bm3* mutants.

Confirmation of the role of COMT came from correlation of estimates of guaiacyl and syringyl contents versus relative COMT activities, with the former being determined by both thioacidolysis and nitrobenzene oxidation analyses, respectively. These data revealed that, regardless of level of COMT repression, there was either essentially no reduction in guaiacyl content or the levels actually increased (Fig. 17B). In contrast, there was a drastic reduction in syringyl levels when overall COMT activities were reduced by ~30% (Fig. 17C). This in turn revealed a specific role for COMT in methylation of the 5-hydroxyl group. Thus, the COMT and CCOMT data appear to suggest distinct metabolic roles for each, with the former definitively affording sinapyl (**3**) alcohol (and S units), and the latter probably being involved in formation of coniferyl alcohol (**2**) (i.e. G units, and perhaps also in the first methylation step of S units, if a different CCOMT isoform is employed; however, this is not proven). Note also that overexpression of COMT did not result in changes in S/G ratios and recoveries, in accordance with it having no role in carbon allocation to the pathway.

Since there also appears to be no significant reduction in syringyl content until below 30% residual COMT activity, this also implies that COMT is not a rate-determining step for sinapyl alcohol (**3**) biosynthesis, in spite of its specific involvement in the latter. An interesting pattern in the graph are the two distinct clusters

Table 13
Preliminary characterization of transgenic poplar plants following introduction of homologous COMT transgene

	Plant line	Transgene orientation	Promoter	Gross morphological changes in phenotypes of T_0 transformants	Tissue analyzed for lignins	Relative COMT activity (%)	Lignin amounts[d] (% CWR)	S,G and 5-OHG recovery (% of lignin)	S/G ratio	Analytical techniques employed
A.[a]	PBI 121	(Control)	35S		Phloem &	100	20.4	31.4	2.13	Thioacidolysis;
	PBI 121	(Control)	35S		xylem from	100	19.0	33.5	1.42	Klason lignin
	ASB 6A	Antisense	35S	Pale	upper stem	52	19.1	35.5	1.55	analysis; acetyl
	ASB 4A	Antisense	35S	rose	of 3-month	58	19.3	31.1	1.60	bromide method
	ASB 10B	Antisense	35S	discoloration	old plants	4.5	17.1	24.6	0.32	
	ASB 2B	Antisense	35S	of the xylem		6	16.8	22.2	0.23	
B.[a]	PBI 121	(Control)	35S		Phloem and	100	19.4	38.5	1.94	Thioacidolysis;
	PBI 121	(Control)	35S		xylem from	100	19.6	34.3	1.57	Klason lignin
	ASB 4A	Antisense	35S	Pale	upper stem	58	19.3	43.0	1.46	analysis; acetyl
	ASB 6A	Antisense	35S	rose	of 6-month	52	19.2	33.0	0.93	bromide method
	ASB 10B	Antisense	35S	discoloration	old plants	4.5	19.2	26.6	0.47	
	ASB 2B	Antisense	35S	of the xylem		6	19.3	25.4	0.25	
C.[b]	Control	(Control)	35S		Xylem from	100	19.7	50.0	1.96	Thioacidolysis;
	SOMT-1	Sense	35S	Brownish	6-month	101	19.5	44.9	2.02	Klason lignin
	EuSOMT1	Sense	35S	discoloration	old plants	156	19.2	42.6	2.07	analysis;
	EuSOMT2	Sense	35S	of the		115	18.0	46.8	1.83	histochemical
	70SOMT3	Sense	35S	xylem		2	16.4	22.4	0.07	staining; Kraft pulping assays
D.[c]	Control	(Control)	35S		Wood from	n.d.	19.0	43.0	2.03	Thioacidolysis; Wiesner
	asomt10B	Antisense	35S	Pale rose xylem	2-year	n.d.	19.3	30.6	0.48	reaction; Klason lignin
	asomt2B	Antisense	35S	Pale rose xylem	old plants	n.d.	19.5	27.9	0.45	analysis; kraft pulping assays

The ultrastructural effects on cell wall types and patterns of lignin deposition were not determined (n.d.).
[a] Adapted from Van Doorssealaere et al. (1995a).
[b] Adapted from Jouanin et al. (2000).
[c] Adapted from Lapierre et al. (1999).
[d] Lignin estimations were not corrected for proteinaceous and/or ash-forming constituents.

of points, suggesting an on-and-off switching mode in shutting down syringyl biosynthesis, i.e. in contrast to the gradual decreases observed for PAL, C4H and 4CL downregulation. This, in turn, suggests some form of feedback control in this part of the phenylpropanoid network, i.e. COMT is apparently completely switched off at ~70% reduction, whereas the other OMTs not involved in sinapyl alcohol (**3**) formation remain active.

It was also instructive to examine more fully the trends obtained for each of the COMT downregulation studies by individually plotting the effects on both lignin contents and S/G recoveries as before (Figs. 18A–F, 19A–F and 20A and B). In each case, the %G and %S recoveries of the CWR are plotted against the estimated % Klason lignin contents of the CWR's. Thus, in the original report by Dwivedi et al. (1994) using a heterologous system, where a *Populus* COMT gene was employed in tobacco, the nitrobenzene oxidation data suggested that the G levels in the cell wall residue had slightly decreased relative to the estimated lignin content (slope = 0.08), whereas the decreases in S content were more slightly pronounced (Table 12A, Fig. 18A and B) (slope = 0.29). However, with only two data points for the control (wild type) plants and relatively similar results for the transformants, no statistically valid conclusions on either G or S biosynthesis were possible from this data.

A specific role for COMT emerged from the work of Atanassova et al. (1995) in tobacco (Fig. 18C and D), with a tobacco COMT now being used for down-regulation and whose activity was repressed down to as little as 2–8% (Table 12B). The estimated Klason lignin contents, however, remained essentially unchanged, with the %G amounts apparently increasing marginally; however, the S components were either much reduced or were absent relative to the controls. Comparable findings in tobacco were later reported by Zhong et al. (1998) (Fig. 18E and F and Table 12C), and subsequently in poplar (Fig. 19A–F and Table 13A–D) (Van Doorsselaere et al., 1995a; Lapierre et al., 1999; Jouanin et al., 2000), and aspen (not plotted since lignin contents of each transformant were not reported, but see Table 14A) (Tsai et al., 1998). Somewhat similar observations were noted in alfalfa, although in this case, a significant decline in lignin levels apparently occurred, particularly at low COMT levels (~4% of controls); however, as before the S levels were greatly reduced whereas the G levels decreased only slightly (Fig. 20A

Table 14
Preliminary characterization of transgenic aspen (Tsai et al., 1998) and alfalfa (Guo et al., 2001) plants following introduction of homologous COMT transgene

	Plant line	Enzyme and gene expression localization	Transgene orientation	Promoter	Gross morphological changes in phenotypes of T_0 transformants	Tissue analyzed for lignins	%COMT activity	Lignin amounts[a] (% CWR)	S,G and 5-OHG recovery (% of lignin)	S/G ratio	Analytical techniques employed
A.	*Aspen*	n.d.									Thioacidolysis;
	C		(Control)			Not specified	100	20.56[b]	43.8	2.12	Klason lignin
	S10		Sense	35S	Mottled red-brown wood		126	20.56[b]	44.3	1.55	analysis;
	S7		Sense	35S	Mottled red-brown wood		119	20.56[b]	45.3	1.44	Wiesner reaction;
	S12		Sense	35S	Mottled red-brown wood		108	20.56[b]	41.4	1.75	acetyl bromide
	S14		Sense	35S	Mottled red-brown wood		89	20.56[b]	37.2	1.65	method; UV-
	S3		Sense	35S	Mottled red-brown wood		68	20.56[b]	34.6	1.67	spectrophotometry
	S13		Sense	35S	Completely red wood		28	20.56[b]	29.2	0.56	
B.	*Alfalfa*										
	1	mRNA	(Control)			Sixth to ninth	87	17.2	51.3	0.47	Thioacidolysis;
	2	localized in	(Control)			internodes	95	17.9	50.6	0.52	Raney nickel
	48	xylem cells in	(Control)			from stems	119	17.6	47.1	0.56	desulfurization;
	SC52	the 2nd, 3rd	Sense	35S	None reported	of the same	17	14.2	31.2	0.08	Klason lignin
	SC4	and	Sense	35S	None reported	development	15	12.7	35.2	0.05	analysis; NMR;
	SC5	5th internodes,	Sense	35S	None reported	stage	18	12.5	38.4	0.03	histochemical
	AC310	and barely visible in the xylem of the 7th internode; not in the phloem	Antisense	35S	None reported	(age not specified)	4	15.3	29.2	0.00	staining

The frequency of interunit linkages in, and MW ranges of, lignins, and the ultrastructural effects on cell wall types and patterns of lignin deposition were not determined (n.d).

[a] Lignin estimations were not corrected for proteinaceous and/or ash-forming constituents.

[b] Lignin contents in aspen transformants were reportedly not different from the control; only the average was provided (Tsai, et al., 1998).

and B and Table 14B) (Guo et al., 2001). Again, while there are very few data points for the controls (i.e. to better understand the levels of S/G and lignin variation), and for the transformants, it is evident that the content of S has been dramatically reduced.

Furthermore, the first important provisional implication from these data is that S-lignin biosynthesis and assembly in cells forming G/S lignin are apparently kept fully separate from G-lignin formation in the same cells. This is because recovery of the fragments of the latter appear to be essentially unaffected by COMT downregulation. However, this is a provisional conclusion given that only a fraction of the lignin content (~10–50%) can be accounted for by thioacidolysis degradation. Nevertheless, this raises again the long-considered possibility of the existence of regions within cell walls that contain only homogenous S and G lignin biopolymers, respectively.

In order to explain further the reductions in syringyl levels, various analyses were conducted to ascertain whether *isoelectronic* 5-hydroxy species, (e.g. 5-hydroxyferulate (**15**), 5-hydroxyconiferaldehyde (**17**) or 5-hydroxyconiferyl alcohol (**18**)) were formed instead. While thioacidolysis studies had previously revealed relatively low levels of 5-hydroxyderivatives (Lapierre et al., 1988), these researchers, nevertheless, had suggested that 5-hydroxyconiferyl alcohol (**18**) rather than sinapyl alcohol (**3**) was being formed. However, in comparison to sinapyl alcohol (**3**) derived components, the recovery of 5-hydroxyguaiacyl (5-OHG) moieties would be expected to be low. This is because even if this *isoelectronic species* participated in active transport into specific regions of the cell wall, where sinapyl alcohol (**3**) should be targeted, its participation in ordered lignin assembly would not be expected to duplicate that of the latter. Yet, regardless of its possible targeting to, and relative disposition in, the developing wall, the catechol nature of 5-hydroxyconiferyl alcohol (**18**) would be expected to favor oxidation into quinones (from the catechols), which can then undergo nucleophilic attack. Moreover, the presence of an additional phenolic group in **18** can be presumed to add further complications, when considering the consequences of free-radical formation and coupling. Indeed, it is thus not surprising that these downregulated plants have highly colored (brown) tissues, with little recoverable 5-hydroxyguaiacyl components (e.g. by thioacidolysis) and possessing phenolic constituents that are even more difficult to remove in Kraft alkaline pulping trials (Lapierre et al., 1999). Accordingly, much remains to be done to fully understand how ordered lignin assembly is disrupted during COMT downregulation.

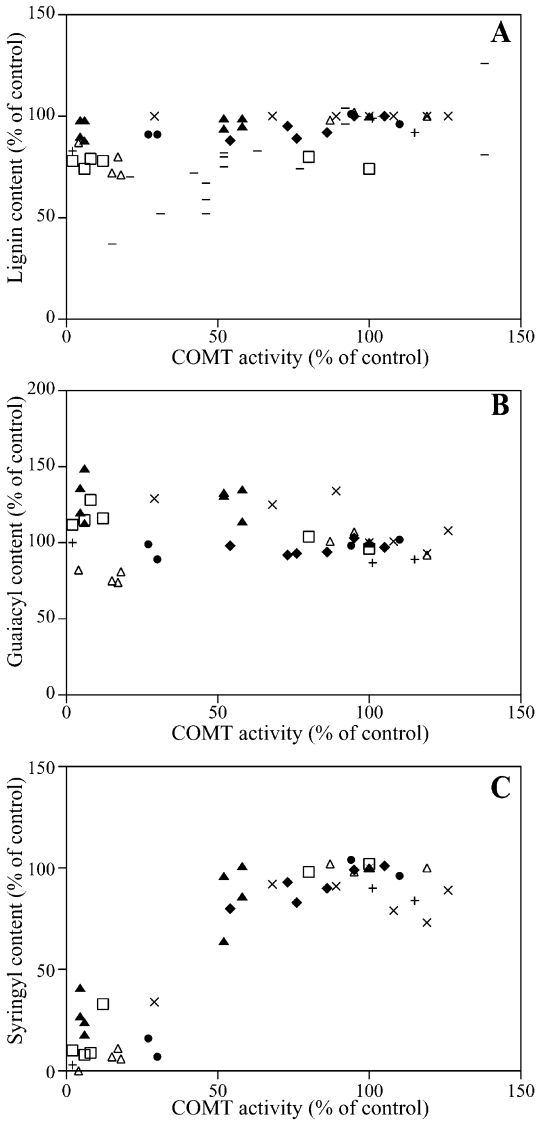


Fig. 17. Preliminary plots of lignin (A), guaiacyl (B) and syringyl (C) contents in cell wall residue versus gross enzyme activity in COMT up- and down-regulated transgenic plants. Adapted from Dwivedi et al. (1994) (◆, tobacco), Atanassova et al. (1995) (□, tobacco), Van Doorsselaere et al. (1995a) (▲, poplar), Tsai et al. (1998) (×, aspen), Zhong et al. (1998) (●, tobacco), Guo et al. (2001) (△, alfalfa), Jouanin et al. (2000) (+, poplar), and Ni et al. (1994) (–, tobacco).

5.8.3. COMT downregulation: implications for lignin assembly and the vascular apparatus

With one exception (Ni et al., 1994), none of the research studies reported thus far apparently considered and applied the findings by others during the nineteen-seventies on the effect of the *bm3* mutation (COMT dowregulation) on cell wall properties, polyphenol extractability and in differences in fiber composition and properties. These represented important clues, however, that will ultimately help define further how lignin assembly has been adversely affected, and in what cell types and where (subcellular location). Thus, in many ways, while the role of the gene is now unambiguously clarified, the overall effects on lignin assembly, polyphenol extractability and vascular properties remains as poorly understood as it was almost 30 years ago.

Not unexpectedly, the body of data so far indicates that COMT downregulated plants also display substantial weakening of the vasculature, as was observed with the *bm3* mutants. COMT downregulated poplar tissues had reduced work of fracture relative to controls ($\sim$0.026 versus 0.037 nJ/mm^2), as well as alterations in work needed for specific cracking, using the double cantilever beam test (Huang et al., 1999). Together, these preliminary data are consistent with a weakened vascular apparatus in both the COMT transformants and mutants, this presumably being solely due to the inability to form sinapyl alcohol (**3**) (S) units and incorporate them into lignin proper.

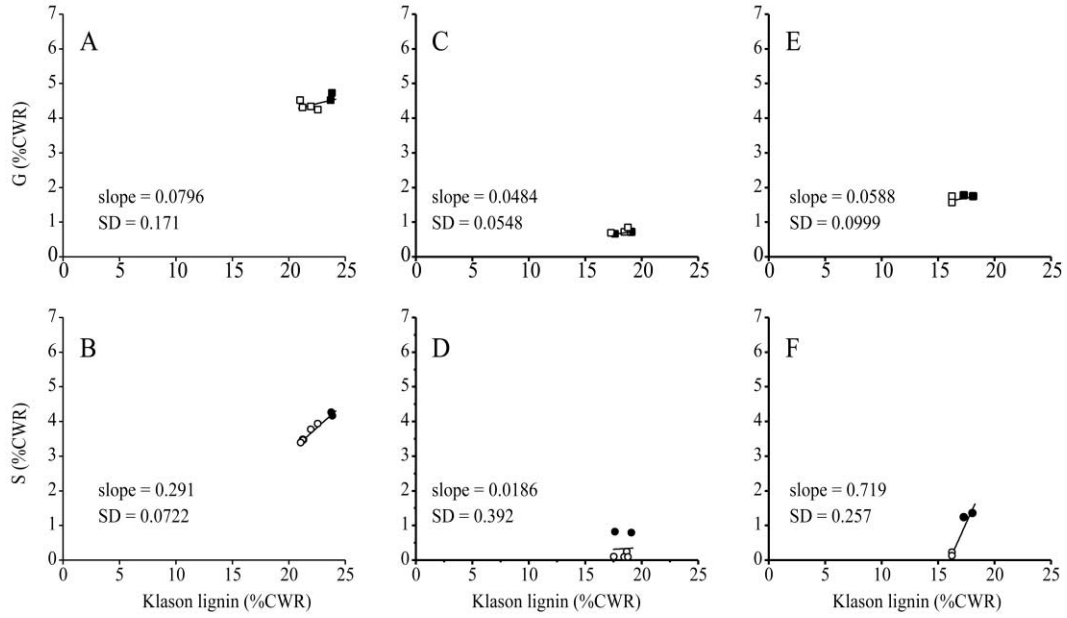


Fig. 18. Estimated lignin contents versus guaiacyl (G, ■, □) and syringyl (S, ●, ○) recoveries of cell wall residues (CWR) of control (solid symbols) and COMT transgenic lines (open symbols) in tobacco. Klason lignin contents and G (A, C, E) and S (B, D, F) recoveries from nitrobenzene oxidation (A, B), thioacidolysis (C, D) and alkaline hydrolysis (E, F) are estimated in terms of percentage cell wall residue (%CWR). Data recalculated from Dwivedi et al. (1994) (A, B), Atanassova et al. (1995) (C, D), and Zhong et al. (1998) (E, F). SD = standard deviation.

Additionally, from the data summarized in Figs. 18–20, one reason for the weakening of the vasculature observed is the moderate decrease in Klason lignin recovery with increasing COMT downregulation. Yet, if carbon allocation to the pathway is indeed unchanged, then this general moderate reduction in gross Klason lignin contents may result from at least one or more of the following: first, a portion of the carbon originally designated for S in G–S lignin forming cells, was metabolically shunted away from lignin biosynthesis; and second, the 5-hydroxyguaiacyl units (rather than S) were transported into the cell wall, but were (as expected)

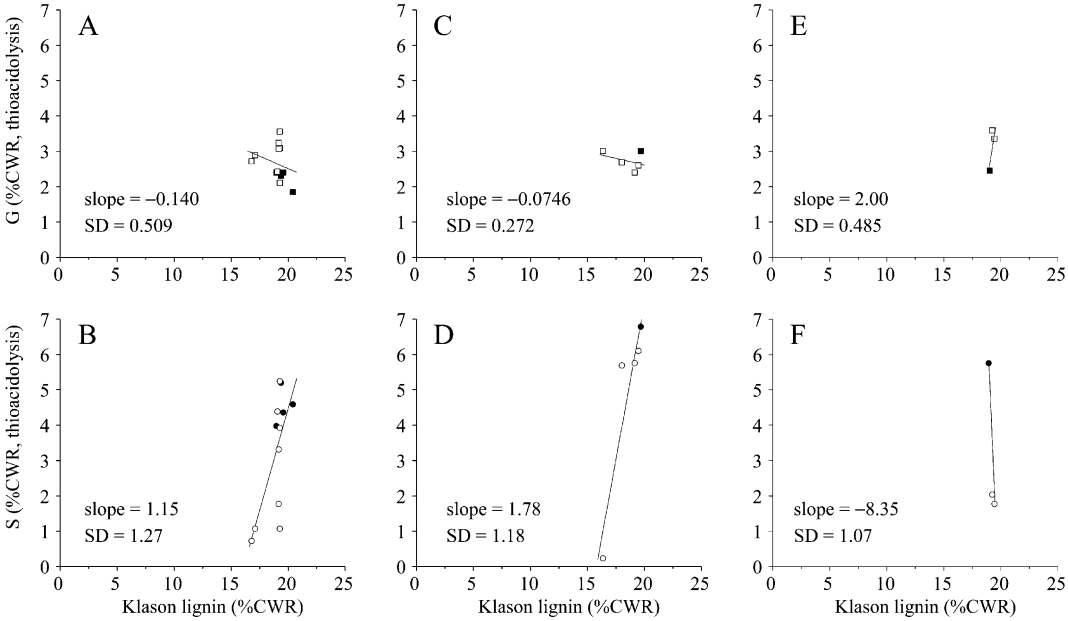


Fig. 19. Estimated lignin contents versus guaiacyl (G, ■, □) and syringyl (S, ●, ○) recoveries of cell wall residues (CWR) of control (solid symbols) and COMT transgenic lines (open symbols) in poplar. Klason lignin contents and G (A, C, E) and S (B, D, F) recoveries from thioacidolysis are estimated in terms of percentage cell wall residue (%CWR). Data recalculated from Van Doorsselaere et al. (1995a) (A, B), Jouanin et al. (2000) (C, D), and Lapierre et al. (1999) (E, F). SD = standard deviation.

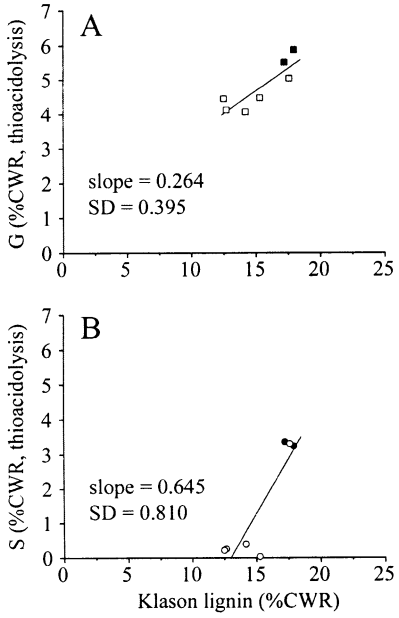


Fig. 20. Estimated lignin contents versus guaiacyl (G, ■, □) and syringyl (S, ●, ○) recoveries of cell wall residues (CWR) of control (solid symbols) and COMT transgenic lines (open symbols) in alfalfa. Klason lignin contents and G (A) and S (B) recoveries from thioacidolysis are estimated in terms of percentage cell wall residue (%CWR). Data recalculated from Guo et al. (2001). SD = standard deviation.

unable to effectively participate in lignin assembly. This could occur either via an attempted (disrupted) polymerization, whereby the number of cross-links formed and/or macromolecular assembly are compromised. Indeed, the latter situation would readily explain the reductions in stem strength, the altered tissue fractionation properties and more facile polyphenol/lignin extractability, the discoloration of the lignified tissue, the limited thioglycolic acid extraction (Ni et al., 1994) and the difficulties experienced in kraft pulping. Clearly, these data do not, in any manner, support the claims by Ralph (1997) that "the actual composition of lignins is not particularly important." Furthermore, nor does the inclusion of 5-hydroxyconiferyl alcohol (**18**) into the lignified tissue (Ralph et al., 2001), a situation known previously for almost 15 years (Lapierre et al., 1988) support the earlier claim (Ralph et al., 1997) that phenols other than monolignols can be integrated into the lignin polymer. This is because 5-hydroxyconiferyl alcohol (**18**) is isoelectronic to sinapyl alcohol, whereas the other molecules previously proposed as surrogate lignin monomers (see Sections 5.9 and 5.10) were not. As described in the next two sections, these putative "surrogates" were ultimately shown *not* to be involved in lignin biosynthesis.

As for the previous steps in monolignol biosynthesis, it is evident that much remains still to be done with respect to understanding the full effects of COMT downregulation and the nature of the *bm3* mutants. This is particularly true since the incorporation of sinapyl alcohol (**3**) units is often believed to occur at late stages of lignification during growth and development. Thus, further studies are needed to determine, amongst other things: (i) which cell types are actually involved in deposition of the S units, and how, when and why they are deposited; (ii) what changes in patterns of lignin deposition and polyphenol formation result when S formation in G/S lignin forming networks is compromised by COMT downregulation; (iii) the nature of the "on–off" switch involved in forming 5-hydroxyguaiacyl rather than S units; (iv) the nature of the changes in monomer binding, orientation and oxidation during macromolecular assembly that occur if S units are replaced by those of 5-OHG, and (v) the effects on the molecular weight distributions of guaiacyl, 5-hydroxyguaiacyl and syringyl components, and ultimately on the properties of the vasculature itself.

5.9. Cinnamoyl CoA reductase (E.C. 1.2.1.44)

Cinnamoyl CoA reductase (CCR) is often viewed as catalyzing the NADPH-dependent conversion of *p*-coumaroyl CoA (**8**), feruloyl CoA (**13**), and sinapoyl CoA (**20**) into the corresponding aldehydes **9**, **14** and **21** (Fig. 1). Yet, with at least three different possible substrates, there has been some discussion as to whether either distinct CCR isoforms exist that specifically utilize different CoA esters and/or whether different isoforms have broader substrate specificities, e.g. in angiosperms, for the additional reduction of sinapoyl CoA (summarized in Lewis and Yamamoto, 1990; Davin and Lewis, 1992; Lewis et al., 1999). Indeed, even if the pathway to sinapyl alcohol (**3**) proceeded via either 5-hydroxyconiferaldehyde (**17**) or 5-hydroxyconiferyl alcohol (**18**) (Chen et al., 1999; Matsui et al., 2000), there still could be a distinct CCR isoform in a specific cell type forming sinapyl alcohol (**3**) other than that or those required for the G and H lignin components in other cell types, respectively; however, this has not been established.

Nevertheless, two isoforms have been isolated from arabidopsis [AtCCR1 and AtCCR2, (Jones et al., 2001; Lauvergeat et al., 2001)] and maize [ZmCCR1 and ZmCCR2, (Pichon et al., 1998)], although only one of the isoforms in each of these species is thought to be involved in lignification. Based on homology searches of the arabidopsis database, there are, however, eight putative CCR-like proteins with more than 40% identity and 50% similarity to AtCCR1. On the other hand, in eucalyptus, poplar, tobacco, sugarcane and loblolly pine, only one CCR gene thus far has been found. Thus, if distinct isoforms or alleles exist specifically for H, G, S, or G–S lignin formation in different cell types, either they are encoded by genes of lower homology to the known CCR, and/or the genes are expressed only transiently, thus eluding detection.

5.9.1. Weakening the vasculature through reduced monolignol supply

From the rationale stated earlier in this analysis, CCR should not be a rate-limiting step controlling carbon allocation into the monolignol-forming pathway, unless there is some form of feedback control. Yet as recently as 1998 (Piquemal et al., 1998), CCR was indeed proposed as regulating the carbon flux to the monolignols **1**–**3**. The viewpoint was expressed that CCR was the first *lignin-specific* step in the phenylpropanoid pathway, and hence assumed a regulatory function. However, monolignols **1**–**3** are not deployed exclusively for lignin biosynthesis, since they are also used for formation of lignans and other cinnamaldehyde (**14, 21**) and monolignol (**1**–**3**) derived products (Lewis and Davin, 1999). Moreover, if direct metabolic networks to the monolignols are fully activated as proposed, e.g. from glycolysis and pentose phosphate pathways (Anterola et al., 2002), then the CCR step need have no regulatory role whatsoever.

Yet severe downregulation of CCR would have significant and deleterious physiological consequences, as would the marked reduction of enzymatic activity of any step in the monolignol (**1**–**3**) forming pathway. For example, if CCR activity was totally suppressed, the pool sizes of the corresponding cinnamoyl CoA esters that might accumulate would be expected to be very small, given the limited availability of CoA. While such cinnamoyl CoA ester intermediates would presumably not be transferred into lignifying cell walls, depletion of CoA availability may have other effects on overall cellular metabolism (unless this branch of the phenylpropanoid pathway is fully compartmentalized). Thus, assuming that the carbon intended for the phenylpropanoid pathway is still allocated, then either build-up of intermediates (including CoA esters) and/or their metabolism into shunt products would be expected. Accordingly, a reduction in monolignol (**1**–**3**) supply would predictably result in a significant weakening of the vascular apparatus, as noted previously for PAL and 4CL downregulation. Indeed, the studies completed thus far on CCR downregulation are in full agreement with this prediction.

5.9.2. Phenotypes

The first study, which was conducted in two phases, employed an antisense strategy using tobacco (Piquemal et al., 1998; Chabannes et al., 2001) (see Table 15) whereas the second used a mutation that incorrectly splices CCR mRNA transcripts in arabidopsis (Jones et al., 2001). In the first study, tobacco plants were initially heterologously transformed with an antisense CCR gene obtained from eucalyptus, this being overexpressed under control of the CaMV 35S promoter. In terms of visible phenotypic changes, the transformant CCR.H (B3) was unusual, being severely stunted with spoon-like leaves that were much darker than wild type, whereas the other transformants appeared "normal". In agreement with the estimated reductions in lignin contents (discussed below), cross-sections of the stem tissues of CCR.h (B3H) revealed that several vessel elements had collapsed due to a weakening of the vasculature (Fig. 21). Tensile testing of CCR-modified tobacco stem tissue also provided further support that the vasculature had been weakened (Hepworth, et al, 1998). These observations were strengthened by the report (Jones et al., 2001) of an *irx4* mutant (lacking the presumed lignin pathway CCR) which resulted in an only ~50% reduction in estimated lignin content (determined by thioglycolate analysis). In this mutant, the stem tissues were much more susceptible to mechanical stress, with bending modulus with maximum stress at yield being significantly compromised due to a weakening of the vasculature, as to be expected.

5.9.3. Effects on lignification

Table 15 summarizes the properties of various CCR downregulated tobacco transformants and their progeny, and Fig. 22A and B plots the very few examples obtained of %G and %S recoveries in the CWR versus the Klason lignin estimations. As before, there are insufficient control experiments to determine the true variation levels within wild type plants. Nevertheless, the data demonstrate that when CCR activity is severely repressed (to ~3% of wild type), monolignol formation and supply is reduced as expected and the estimated lignin contents decrease accordingly in this case to just under $\leqslant$50% of wild type (control) values, i.e. from ~19.4% wild type to ~10.2% B3H [designated CCR.h in a subsequent report (Chabannes et al., 2001)] and from 22.2% wild type to 10.7% CCR.H [designated B3 in a previous report (Piquemal et al., 1998)]. However, once again the Klason lignin analyses do not take into account factors that may overestimate or underestimate the true lignin contents, particularly in the transformants. As noted, in the other studies described earlier, the ability to genetically reduce the *estimated* lignin content below 40–50% of the wild type value is apparently either difficult to attain, or the lignin amounts are substantially in error.

The data obtained also suggest that the trends in reduction of both S and G levels upon CCR downregulation are very similar, if not identical. That is, these CCR downregulation studies have given no convincing evidence for independent control of either the S or G pathway (Fig. 22A and B). Furthermore, the data imply that both S and G moieties would be undetectable at about 70–80% reduction of the original wild type lignin contents. Whether these trends are correct, however, or whether the data obtained largely result from a heterologous downregulation approach and/or limitations in the lignin determinations currently employed cannot satisfactorily be judged at this time. However, it

Table 15
Preliminary characterization of transgenic tobacco plants following introduction of *Eucalyptus* CCR

Plant line	Enzyme and gene expression localization	Transgene orientation	Promoter	Gross morphological changes in the phenotypes	Tissue analyzed for lignins	% CCR activity	Lignin amounts[d] (% CWR)	Estimated S and G recovery (% of lignin)	S/G ratio	Analytical techniques employed	Ultrastructural effects on cell wall types and patterns of lignin deposition
Wild type[a]	Differentiating xylem[b]	(Control)			Whole stems from early flowering plants acclimatized after 7 weeks	n.d.	19.4	25.5	1.13	Thioacidolysis; Klason lignin analysis; mild alkaline hydrolysis; histochemical analysis	n.d.
Azygous[a]	n.d.	(Control)				n.d.	20.9	30.2	0.73		n.d.
D2H[a]	n.d.	Antisense	35S	Brown xylem		n.d.	17.6	23.6	0.96		n.d.
B6–8[a]	n.d.	Antisense	35S	Brown xylem		n.d.	21.0	26.1	1.52		n.d.
D12–4[a]	n.d.	Antisense	35S	Brown xylem		n.d.	20.0	n.d.	n.d.		n.d.
B3H[a]	n.d.	Antisense	35S	Same as B3		n.d.	10.2	12.2	1.64		Collapsed vessels
Wild type[c]	Differentiating xylem[b]	(Control)	35S		Base of the stems from 2.5 month old plants	100	22.2	21.3	0.91	Thioacidolysis; Klason lignin; histochemical analysis; fluorescence microscopy; alkaline hydrolysis; HPLC-MS	n.d.
CCR.H[c] (a.k.a. B3)	n.d.	Antisense	35S	Stunted growth; spoon-like leaves; delayed flowering; intensely brown xylem		3	10.7	15.2	3.22		Collapsed vessels
CCR.h[c] (a.k.a. B3H)	n.d.	Antisense	35S	Same as B3		11	15.1	18.8	1.40		n.d.

The frequency of interunit linkages in, and MW ranges of, lignins and the ultrastructural effects on cell wall types and patterns of lignin deposition were not determined (n.d.).

[a] Adapted from Piquemal et al. (1998).

[b] Adapted from Lacombe et al. (1997).

[c] Adapted from Chabannes et al. (2001).

[d] Lignin estimations were not corrected for proteinaceous and/or ash-forming constituents.

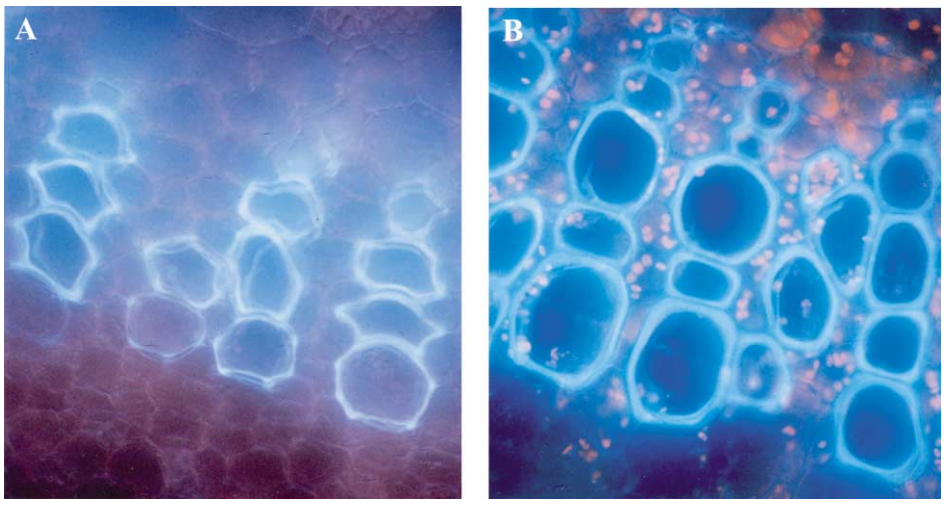


Fig. 21. Stem cross-sections of CCR-downregulated tobacco line CCR.H (A) and wild type (B) as seen under a fluorescent microscope. A displays collapsed vessels, whereas B shows those of normal shape. Reproduced from Piquemal et al. (1998) with permission of Blackwell Publishing.

was also noteworthy that an equivalent reduction in lignin content (~50%) was achieved with the *irx4* mutant, again perhaps indicating that either more than one CCR is involved and/or that the lignin analyses are overestimated.

5.9.4. *The question of "non-traditional" phenolics being incorporated into lignin during CCR repression is resolved*

Given the significant extent of CCR down regulation obtained (~97% activity suppression relative to controls), one question—particularly if CCR is not a rate-limiting step—involves the metabolic fate of the so allocated carbon. A possible explanation to this, however, emerged with determination of the quantities of plant material removed upon aqueous and organic extraction: the hemizygous offspring (B3H, ~10.2% Klason lignin) obtained by back-crossing CCR.H with the wild type (19.4% Klason lignin) had ~36% of its dry weight as organic and aqueous solvent extractable relative to ~21% of wild type. This difference could by itself account for the quantity of lignin downregulated. However, the soluble substances have not yet been fully identified and/or quantified, in order to understand further what effects CCR downregulation actually has on the pathway(s) to the monolignols **1**–**3** *and/or related upstream metabolism*. To date, the CCR.H transformant

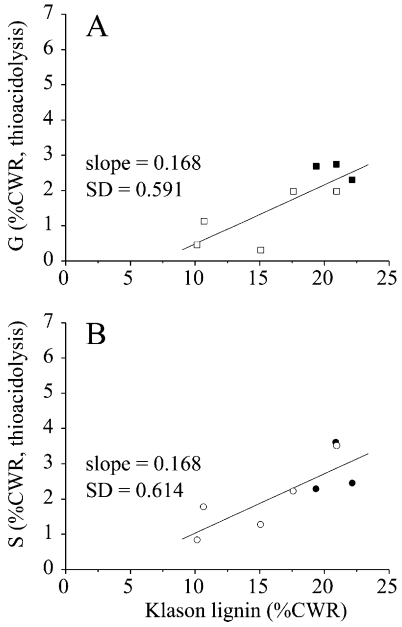


Fig. 22. Estimated lignin contents versus guaiacyl (G, ■, □) and syringyl (S, ●, ○) recoveries of cell wall residues (CWR) of control (solid symbols) and CCR transgenic lines (open symbols) in tobacco. Klason lignin contents and G (A) and S (B) recoveries from thioacidolysis are estimated in terms of percentage cell wall residue (%CWR). Data recalculated from Piquemal et al. (1998) and Chabannes et al. (2001). SD = standard deviation.

was found to have only minute increases (exact quantities not reported) in the levels of solvent extractable neochlorogenic (**45**), 4-*O*-caffeoyl quinic (**46**) and chlorogenic (**30**) acids (Chabannes et al., 2001); note also that it is not known whether these three substances **45**, **46** and **30** are artifacts arising from intramolecular acyl migration during their isolation or if they are distinct natural products.

In previous articles (Boudet, 1998; Ralph et al., 1998), it was repeatedly reported that when CCR activity was suppressed, the corresponding B3(H) tobacco transformant(s) compensated for the reduction in monolignol (**1**–**3**) supply by incorporating other "non-traditional" phenolics into the lignifying cell wall. This gave support to the view which espoused that "the actual composition of lignins is not particularly important" (Ralph, 1997). However, neither the analysis of CCR-downregulated tissue cross-sections nor their mechanical testing in any way supports this view. In contrast, as discussed and shown earlier, the vasculature was instead predictably severely weakened and compromised by downregulating monolignol (**1**–**3**) supply.

Initially, the "non-traditional" phenolics reported to be incorporated into lignin included ferulic (**12**) and sinapic (**19**) acids, as well as acetosyringone (**47**) (Boudet, 1998). It was also reported that feruloyl tyramine (**33**) was "heavily incorporated (copolymerized) into lignin as a consequence of CCR downregulation" (summarized in Boudet, 1998; Ralph et al., 1998; Chabannes et al., 2001), and that this moiety represented a chemical "signature" for CCR downregulation (Chabannes et al., 2001). It should be noted, though, that no explanation was given as to what "heavily incorporated (copolymerized)" actually meant in quantifiable amounts, nor was any proof provided that it was in fact copolymerized with lignin.

Not unexpectedly, a more detailed analysis of the composition and relative amounts of the *alkali-releasable phenolics* present in wild type, CCR.H and CCR/CAD double transformants, has subsequently removed further consideration of such "non-traditional phenolics" as being involved in lignification. Indeed, they are now classified as "non-lignin phenolics" by the same investigators (Chabannes et al., 2001). Actually, the overall combined totals of the hydroxycinnamic acids **12** and **19**, and C_6C_1 (**23**, **24**, **39**, **40**, **47**) derivatives remained essentially unchanged regardless of the plant line examined: For illustrative purposes, Fig. 23 tabulates the individual and combined amounts of the alkali-releasable phenolics, such as vanillin (**23**), syringaldehyde, (**24**) acetosyringone (**47**), syringic (**40**), vanillic (**39**), ferulic (**12**) and sinapic (**19**) acids as a percentage of CWR, in the wild type, CCR downregulated, and CCR/CAD double transformants, respectively. While the levels of acetosyringone (**47**) and ferulic acid (**12**) fluctuated the most, the overall minuscule total of their combined amounts in extractive-free cell wall residues accounts for only between ~0.04 and 0.07% dry wt of the various plant lines. Thus, not only are they very *minor* constituents, but their levels *were not upregulated* in any meaningful manner to compensate for the massive reduction in monolignol **1**–**3** levels. Thus they can be excluded from having any significant role in lignin deposition, as originally claimed. Indeed, it is difficult to fathom why these investigators had proposed that non-lignin metabolites would replace the monolignols in lignin, given that an arbitrary replacement of physiological substrate(s) would be contrary to the formation of any other known biopolymer. Nevertheless, from a biosynthetic perspective, it will be worthwhile in the future to determine the nature of the moieties (presumably hemicelluloses) to which the aromatic constituents **12**, **19**, **23**, **24**, **39**, **40**, and **47** are actually attached, as well as to how and why they are formed.

Feruloyl tyramine (**33**) and its derivatives were also reported as "non-traditional phenol" replacements for lignin biosynthesis in tobacco (Ralph et al., 1998). However, as noted earlier in the analysis of 4CL downregulated transformants as well as with wild type tobacco (Fig. 10A and B), these moieties were also present in *all* of the tobacco lines examined. Furthermore, the spectra of both the 4CL downregulated lignin-derived preparation and that of the CCR-downregulated transformant are strikingly similar (cf. Fig. 10B and C), if not (essentially) identical. Perhaps the most important difference is that each preparation contains minor amounts of various impurities, which are apparently unique to the actual isolation procedure employed. [Indeed, even the controls have distinct but minor impurities, as noted by close inspection of the spectra of each (Fig. 10A and D). In any event, these data establish that the presence of feruloyl tyramine (**33**) (and related moieties) is not a chemical "signature" for CCR downregulation as claimed.

However, the presence of feruloyl tyramine (**33**)-derived moieties in the presumed lignin-derived fractions again requires reappraisal of the "lignin" isolation procedures being employed, including full consideration of the extremely low yields obtained. In the CCR-downregulation studies, these preparations were obtained as follows: cell wall residues, following removal of organic and aqueous solubles, were "ball-milled, suspended in acetate buffer and treated with Cellulysin cellulase before extraction with 96:4 dioxane: water" (Ralph et al., 1998; Chabannes et al., 2001). Additional insight into the procedure cannot be made, as none of the papers included essential information on the treatments as regards time, temperature, concentration and pH of buffer, and amounts and duration of cellulase and dioxane:water treatment; nor were details provided to verify that the NMR spectra were being recorded under conditions for quantification. The

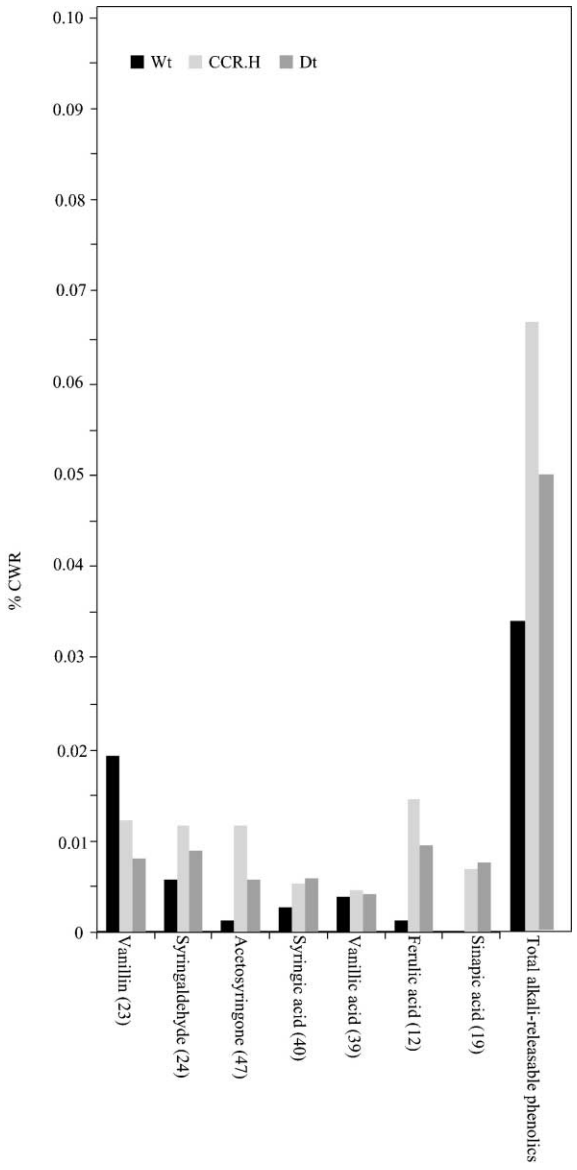


Fig. 23. Amounts of alkali-releasable phenolics from cell walls of wild type (Wt), and CCR downregulated (CCR.H) and CCR/CAD (Dt) tobacco plants (recalculated from Chabannes et al., 2001). CWR = cell wall residue.

reported yields of the lignin-derived preparations were, however, extremely low: i.e. wild type controls and CCR-downregulated transformants gave ~7 and 8.5% by weight of the original Klason lignin (Ralph et al., 1998) and ~6 and 1.5% by weight of cell wall residues obtained (Chabannes et al., 2001), respectively. In the latter study, the procedures employed were further complicated by the presence of contaminating iron filings in the tissues, this apparently resulting from the ball-milling process. The actual reported yields were thus perhaps even lower as the investigators also estimated that $\leqslant$15% polysaccharides were present as additional impurities (Ralph et al., 1998). In any event, greater than 93% of the original lignin content, as estimated by these investigators, was unaccounted for. Additionally, to what extent these actual lignin values are even overestimated cannot be adequately gauged until the true lignin contents are determined, i.e. in the absence of coprecipitating proteins, for example. It is thus important that the remaining ~93% of the putative lignin present in these transformants is appropriately characterized.

As for 4CL downregulation (Fig. 10B), the relatively sharp signals of the feruloyl tyramine (**33**) derived components in the NMR spectra (Fig. 10C) of the CCR-downregulated lignin-derived isolate do not constitute proof that they were either co-polymerized with lignin or were even present within the same cell wall types as the lignins. In contrast, the relative sharpness of the feruloyl tyramine (**33**) resonances suggests that they are low molecular weight entities, as previously concluded in our analysis of the NMR spectra of the 4CL isolates

(Fig. 10A and B), which were soluble in dichloroethane–ethanol (2:1). Additionally, although it was reported that the "feruloyl tyramine (**33**) was heavily copolymerized into lignin," a subsequent report appears to substantially modify, if not withdraw, that claim as well. In that report, while the actual levels of feruloyl tyramine (**33**) derivatives in the transformants were not provided, they were estimated as only being ~3× that of wild type (data not provided) (Ralph et al., 1998). If this is in fact correct, and given that the estimated lignin contents *also* decreased by about one-half, then this suggests that the levels of feruloyl tyramine (**33**) derivatives may actually not have increased at all. Their presence in these preparations as minor constituents does, however, emphasize the need for caution to be exercised when using procedures developed for isolation from lignins for woody plants to other essentially non-woody systems, as has been previously well documented in the older lignin literature (Lai and Sarkanen, 1971). In summary, however, it can be concluded that the claims of non-traditional phenols being incorporated into lignin as a consequence of CCR-downregulation have not been substantiated.

5.10. Cinnamyl alcohol dehydrogenase (E.C. 1.1.1.195)

It has long been recognized that gymnosperm CAD, such as isolated from either spruce (*Picea abies*) (Lüderitz and Grisebach, 1981) or loblolly pine (*Pinus taeda*) (O'Malley et al., 1992), displays a strong substrate preference for both *p*-coumaraldehyde **9** and coniferaldehyde **14**. Moreover, in the gymnosperms, at least in loblolly pine, there is some preliminary evidence to suggest that only a single gene encoding CAD exists (O'Malley et al., 1992) thereby indicating that one form of this enzyme utilizes both substrates **9**/**14**. Sinapaldehyde **21**, by contrast, is not an effective substrate for gymnosperm CAD (Galliano et al., 1993), in harmony with the recognition that gymnosperms produce guaiacyl-enriched lignins. On the other hand, angiosperm CAD exists in various isoforms, which can display an enhanced substrate specificity preference for sinapyl aldehyde **21** (Wyrambik and Grisebach, 1975), in accordance with angiosperms being capable of forming guaiacyl–syringyl lignins in certain cell types.

The properties of CAD, together with the proposed catalytic mode of action, substrate specificities from different plant sources, and what was hitherto known about tissue specificity to this point, were comprehensively summarized elsewhere and need not be repeated here (Lewis et al., 1999). To date, most research on CAD (homologs) has been conducted on tobacco (Knight et al., 1992), loblolly pine (O'Malley et al., 1992), eucalyptus (Goffner et al., 1992; Grima-Pettenati et al., 1993), poplar (Van Doorsselaere et al., 1995b), alfalfa (Van Doorsselaere et al., 1995b; Brill et al., 1999), maize (Halpin et al., 1998), and aspen (Li et al., 2001), respectively. Of these, alfalfa has two CAD-like proteins, MsaCAD1 and MsaCAD2, both of which are considered to be involved in lignin biosynthesis (Brill et al., 1999), and having ~50 and ~77% amino acid sequence identity, respectively, to the eucalyptus CAD (pEuCAD2) present in developing xylem. In a somewhat analogous manner, aspen also has two CAD homologs, trivially named PtSAD and PtCAD (Li et al., 2001), which display ~54 and 80% identity to the aforementioned eucalyptus CAD (Grima-Pettenati et al., 1993), and ~68 and ~85% identity to MsaCAD1 and MsaCAD2, respectively. Additionally, in maize, poplar and tobacco, various CAD homologs have been described, with each being ~75, 80, 77 and 80% (tobacco has two CAD isoforms) identical to the eucalyptus CAD. Moreover, with the completion of the arabidopsis genome sequencing project (The Arabidopsis Genome Initiative, 2000), some 16 distinct CAD/CAD-like proteins have been identified, whose physiological roles now need to be fully and unambiguously determined. [These homologs provisionally suggest the involvement of various metabolic networks in various aspects of cinnamyl aldehyde metabolism.]

CAD is often reported as a key enzymatic step in lignin biosynthesis (Baucher et al., 1996) and has been the subject of many investigations using a variety of CAD mutants and CAD downregulated transformants. However, as indicated earlier, monolignols can have various metabolic fates, e.g. into lignans as well as lignins, and thus an exclusive role cannot be arbitrarily assigned. Moreover, metabolic and transcriptional profiling data revealed that CAD is neither a regulatory nor key enzyme, in terms of carbon allocation to the pathway (Anterola et al., 1999, 2002). Indeed, nor does CAD designate H, G or S character, as this is determined by upstream metabolic processes. Thus the CAD isoforms essentially fulfill downstream processing roles, reducing the various aldehydes to the monolignols as they are generated in the different cell types. Nevertheless, downregulation of CAD in different plant species, as for any enzymatic step in the monolignol pathway, would be anticipated to have a deleterious effect on lignification in terms of depletion of monomer supply. This could potentially be manifested as build-up of substrate cinnamaldehydes (**9**, **14**, **21**) and/or their upstream metabolites, as well as competitive branch pathway conversions into non-lignin products. Indeed, most of the results obtained for CAD downregulation/mutation studies seem to favor, perhaps exclusively, metabolism *away* from the lignin-forming pathway.

Yet, the data summarized in Fig. 24 could provisionally be interpreted to suggest that substantial reduction in CAD activity (down to ~7% of wild type) in various plant species has little effect on total amounts of lignin being formed, as estimated by Klason, acetyl bromide and TGA extraction analyses. [This apparent lack of an

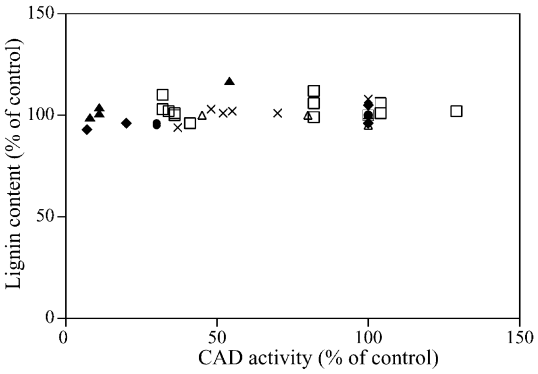


Fig. 24. Provisional plots of lignin contents versus gross CAD activities in transgenic plants with up- and down-regulated CAD. Adapted from Halpin et al. (1994) [tobacco, ◆], Baucher et al. (1996) [poplar, □], Yahiaoui et al. (1998) [tobacco, ▲], Baucher et al. (1999) [alfalfa, ×], Lapierre et al. (1999) [poplar, ●], and Hibino et al. (1995) [tobacco, △]. Reported values were recalculated from the Klason lignin amounts to obtain the percentages relative to the controls, except for the data from Hibino et al. (1995), who described only the results of acetyl bromide determinations (expressed as percentage of control).

effect on lignin amounts is fully consistent, however, with no regulatory role for CAD in carbon allocation to the pathway.] At a cursory glance, therefore, these data would imply that lignification has neither been affected nor impaired (e.g. through reduction of monolignol **1**–**3** supply) and that, by extrapolation, the requisite structural integrity of the corresponding vascular tissues was attained.

A more detailed analysis of these same data in this review, however, leads to a very different interpretation, in terms of the reductions in overall lignin biosynthesis and weakening of the vasculature that are actually achieved (discussed below). As before for COMT, a brief historical review of the *bm1* mutation though is first needed for context, followed by consideration of the various results obtained with the CAD-down-regulated transformants.

5.10.1. CAD mutations in maize and the negative traits of the bm1 mutant

The first report of a naturally occurring genetic modification in the lignin biosynthetic pathway was made more than 70 years ago by Jorgensen (1931), who described the brown midrib (*bm1*) mutation in maize. Later, the visual differences noted between the wild type and *bm1* mutant plants, in terms of the reddish brown coloration of the lignified tissue of the latter, gave rise to the term "colored or abnormal lignins" (Kuc and Nelson, 1964; Kuc et al., 1968). However, the differences observed by Kuc and Nelson (1964) between "normal" and *bm1* mutants also led them to conclude that the distinctions were such that "a random series of condensations and esterifications would not explain the esterification primarily in *p*-hydroxycinnamic acid onto the lignin core, the difference in *p*-hydroxycinnamic acid content in 'normal' and *bm1* lignin, and the differences in the core themselves." Today, this conclusion is just as valid as it was in 1964.

From a commercialization perspective, in spite of the many studies directed towards possible usage of the brown midrib maize for dairy silage, the disadvantages still outweigh the advantages. Some of the disadvantages in the growth and development of these mutants for animal (dairy) feed include: lower grain and silage yields; lodging susceptibility; poor early season vigor; delayed flowering and delayed early season growth rates (Weller et al., 1985; Gentinetta et al., 1990). Such disadvantages are presumably quite deleterious to the mutated maize itself, as they appear to be neither useful nor desirable traits. Moreover, the defense functions of the mutants may also be compromised.

[As an aside, in the non-woody Graminaceous species, the anatomy of the lignified tissues is very different from those of truly woody plants and, indeed, the former are more readily biodegradable. Moreover, in addition to the monolignols **1**–**3**, there are *p*-coumarate (**7**) and ferulate (**12**) esters linked to the lignin "core", and various monolignol hydroxycinnamate esters have been implicated as directly involved in lignin formation in the Gramineae (Higuchi et al., 1994; Ralph et al., 1998). At some point in the future, it will be most useful to systematically investigate the biochemical processes involved in lignin assembly in the various cell types of their vascular tissues. This must include establishing unambiguously the nature of the precursors undergoing polymerization, since this has been largely ignored to this point, as is how this polymerization process is controlled.]

Halpin et al. (1998) recently concluded that the *bm1* mutation in maize affects expression of a cinnamyl alcohol dehydrogenase (CAD) gene, this having ~75% identity with the aforementioned eucalyptus CAD. Of the mutants examined, whose CAD activities were apparently reduced to ~14–60% of the wild type plants, the Klason lignin contents of the mature stems were estimated to be only ~20% lower than that of wild type controls (15.3±0.1% wild type versus

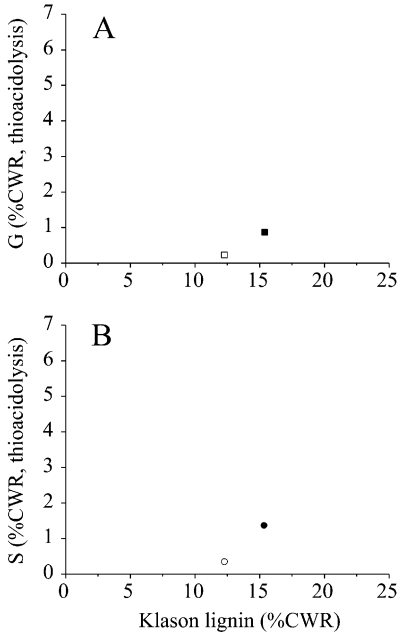


Fig. 25. Estimated lignin contents versus guaiacyl (G, ■, □) and syringyl (S, ●, ○) recoveries of cell wall residues (CWR) of control (solid symbols) and *bm1* mutant lines (open symbols) in maize. Klason lignin contents and G (A) and S (B) recoveries from thioacidolysis are estimated in terms of percentage cell wall residue (%CWR). Data recalculated from Halpin et al. (1998).

12.2±0.1% mutant relative to CWR) (Fig. 25). Assuming that only one CAD gene is impacted in the mutant, which need not be the case, the 14–60% residual CAD activity may thus be misleading, e.g. due to the presence of other CAD homologs and/or other alcohol dehydrogenases present in the cell free extracts, which are capable of utilizing coniferyl aldehyde (**14**) as a substrate. Additionally, the Klason lignin values may also be overestimated, for the reasons discussed earlier.

Interestingly, the thioacidolysis products of the *bm1* mutant and wild type maize had essentially identical S/G ratios (*bm1*, 1.37 versus wild type, 1.40). However, these products were recovered in uncharacteristically low yields relative to the putative lignin amounts present, i.e. the combined G+S recoveries of the controls represented only ~2.1% of the CWR (~14% of the estimated Klason lignin content), whereas in the *bm1* mutant, the recoveries were further reduced to ~0.5% of CWR (~4% of the estimated Klason lignin). Moreover, the data in Fig. 25A and B—each of which only contains two points and are thus not statistically valid—suggest that the G and S units would apparently be undetectable when Klason lignin amounts decrease from ~15% (wild type) to below ~10–12% of the CWR. Further analyses of the *bm1* mutant tissues also indicated that the cinnamyl aldehydes **9**, **14**, and **21** did not apparently accumulate, since thioacidolysis failed to detect the corresponding thioketal derivatives.

When taken together, these observations thus raise three interesting possibilities: (i) that the basis and reliability of the Klason lignin determinations are again questionable; (ii) alternatively, that the stages of early lignin assembly only contain substructures that do not release S and G units by thioacidolysis treatment; and (iii) that the CAD mutation affects *both* S and G formation, thereby suggesting that the pathways to coniferyl (**2**) and sinapyl (**3**) alcohols are *not* independently modulated. This, of course, assumes that only one CAD gene is affected by the *bm1* mutation, which has not been proven. If this is in fact the case, however, then the mutant data suggests that even if there were specific CAD isoforms for the aldehydes **9**, **14** and **21**, this level of control can be overridden.

Provan et al. (1997) also examined a subset of the phenolic constituents present in *bm1* and wild type maize, this specifically being some of those released from mature internodes 1–4. In this case, the estimation of "lignin" (described in that study as total phenolic constituents) by the acetyl bromide method, gave much lower "lignin" values (~8–9% w/w wild type *versus* ~7.4–8.6% w/w *bm1* mutant), as compared to those of Halpin et al. 1994, 1998 (15.3 and 12.2% w/w, respectively). [In spite of those differences, however, both studies did suggest that there was a *decrease* in the amounts of lignin being produced, albeit quite modest in extent (~4–20% reduction).] Additionally, a portion of the phenolics (estimated at ~90% of the acetyl bromide solubles) was readily saponifiable, following overnight treatment of the cell wall residue with 1 N NaOH at ambient temperature. These alkali solubles were next acidified (pH 2) to give precipitates, thereby affording so-called "alkali lignin" preparations upon extraction with dioxane–water (96:4). The resulting isolates from both wild type and *bm1* mutant plants were

then subjected to NMR spectroscopic analyses, with the "lignins" being dissolved in acetone–water (9:1) for this purpose; these, however, only represented about 27% of the total phenolics in the *bm1* mutant versus ~38% of those in the wild type. The differences in recovery presumably reflect the consequences of less monolignols **1**–**3** being formed and being directed towards lignin assembly in the *bm1* mutant. However, since the carbon allocation to the pathway has remained unchanged, there is an increase in formation of non-lignin lower molecular weight entities via competitive branch pathway metabolism; however, whether this occurs at the hydroxycinnamaldehyde level or at an upstream metabolic step has not been resolved (discussed later).

Nevertheless, for the phenolic constituents actually isolated, the spectra of both wild type and mutant isolates were very similar (Fig. 26), except mainly for the presence of three small aldehydic resonances (1–3) and two other sharper signals (12, 16), which are presumed to correspond to *p*-hydroxycinnamic acid moieties. It is unknown whether these additional resonances are even part of the lignin biopolymer-derived fragments, or are instead lower molecular weight components isolated under the protocol utilized. However, based on the similarities of both spectra, it would provisionally appear that the lignin-enriched component in each isolate has remained essentially unaltered, even though the absolute amount in the mutant was reduced.

Considering together the results of both studies (Provan et al., 1997; Halpin et al., 1998), it is clear that caution needs to be exercised in interpreting the data obtained for the following reasons: (i) there is a significant discrepancy between the estimated Klason and acetyl bromide (total phenolic content) lignins in both studies, which requires a full explanation; (ii) at the very minimum, 62–73% of the phenolic constituents remain unaccounted for, and are thus uncharacterized; (iii) the solubility of the lignin-enriched phenolic preparation [examined by NMR spectroscopic analysis in acetone–water (9:1)] indicates that they are of very low molecular weight; and (iv) the data provide no insight into the possible cellular origins of the alkali-soluble components, e.g. whether they are from lignified cell walls or not, and whether the constituents are covalently bonded to the lignin or not. Accordingly, until more exhaustive and definitive analyses are comprehensively conducted,

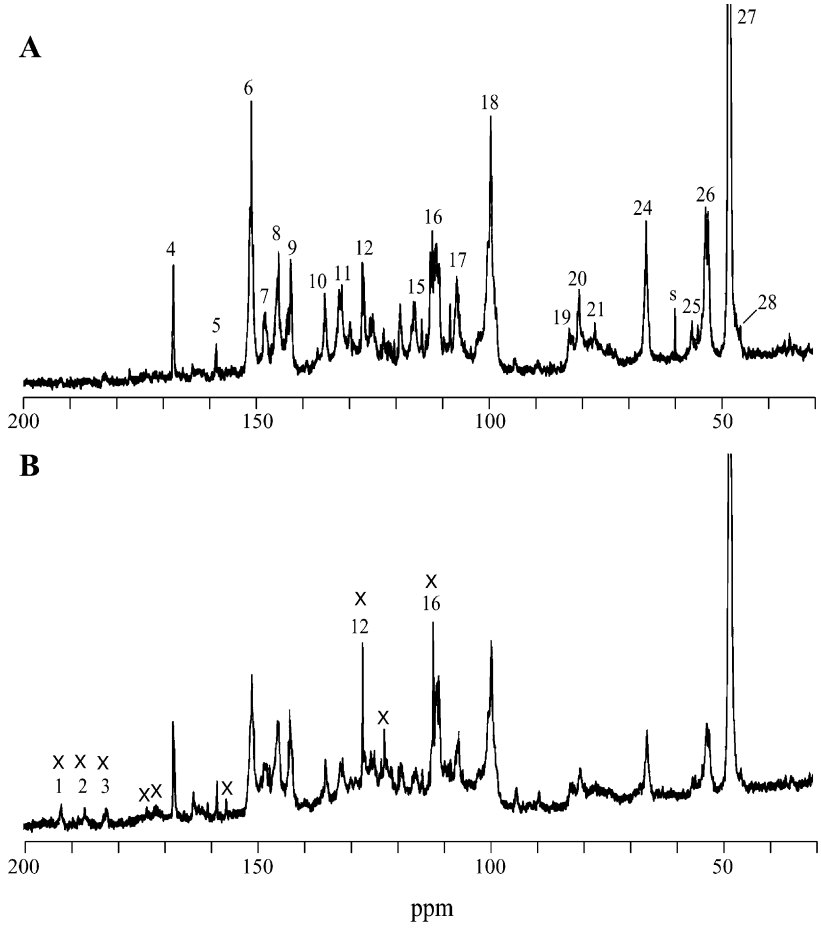


Fig. 26. ^{13}C NMR spectra of "alkali-soluble lignin" preparations derived from normal (A) and *bm1* mutant (B) maize plants, harvested at anthesis plus 5 days. Reproduced from Provan et al. (1997) with permission from the American Chemical Society. Differences noted in the recorded resonances are indicated by 'X'.

there is very little that can be concluded about the effects that the CAD mutation has on the *bm1* mutant, other than provisionally that of small (~4–20%) decreases in lignin amounts.

5.10.2. CAD mutations in loblolly pine

A loblolly pine mutation, described as CAD$^-$, has also been reported in the literature. Initially, it was claimed to contain 2-methoxybenzaldehyde (**48**) (Ralph et al., 1997) (another "non-traditional" phenolic) in its presumed lignin, but this has since been challenged (Gang et al., 1998; Lewis, 1999). The assignments were subsequently retracted as the NMR signals were instead apparently associated with coniferyl (**14**) and sinapyl (**21**) aldehydes, and had, in fact, been misassigned (Ralph et al., 1998). This mutation is not included in this analysis, given that it is unknown to what extent additional mutations are present in the so-called CAD$^-$-plant (personal communication from L. Pearson, Westvaco, to N.G. Lewis). The loblolly pine mutation and its characteristics have been discussed elsewhere (Gang et al., 1998; Lewis, 1999), and will be addressed again in the future.

5.10.3. Preliminary evidence for distinct G and G–S lignin forming CAD networks in different cell types

From the preceding discussion on "COMT" (Section 5.8), the observation had been made (Fig. 17) that %G recoveries remained essentially unchanged when "COMT" was significantly downregulated, whereas S recoveries decreased precipitously. This provisionally suggested that deposition of the S-component of lignin (in the different cell types impacted) occurred independently of that of G, and thus indicated that the S-biopolymer was being laid down in both a *homogeneous and targeted* (cell wall region specific) *manner*.

In this regard, it has long been known that syringyl units in angiosperm lignins are incorporated into phloem fibers, as well as in parenchyma secondary cell walls (Fergus and Goring, 1970b), whereas the vessels mainly contain guaiacyl-rich lignins. In accordance with this observation, COMT mRNA's (leading to S lignin), as evidenced by tissue printing, were found to be mainly detectable in the differentiating phloem fibers, and then as maturation continues in the differentiating xylem cells (Ye and Varner, 1995). CCOMT gene expression, by contrast, displayed an opposite effect. Together, these expression patterns suggested that formation of both G and S units were under differential control—even though this did not affect the overall carbon allocation to the pathway.

Moreover, in the original work by the groups of Zenk (Mansell et al., 1976) and Grisebach (Wyrambik and Grisebach, 1975), it had been recognized that CAD existed in various forms in angiosperms, which led to consideration of distinct isoforms displaying different substrate specificities. However, this was not pursued to any great extent until quite recently, albeit with mixed results.

In a study by Brill et al. (1999) using alfalfa (lucerne), two CAD isoforms, CAD-1 and CAD-2, were obtained (MsaCAD1 and MsaCAD2, respectively). CAD-2, as indicated earlier, had ~77% identity to that of the eucalyptus lignin-specific CAD, whereas CAD-1 displayed ~50% identity. Interestingly, these investigators speculated that both were lignin-specific, and noted that CAD-1 was expressed most highly in stem internodal sections 1–3 (from the apex) whereas CAD-2 appeared to be most strongly expressed in internodal sections 4/5.

In that particular study, recombinant lucerne CAD-1 and CAD-2 were also expressed in *E. coli* (using the pET vector), with crude homogenates—rather than purified proteins—being assayed for relative CAD activities; the assays used cinnamaldehyde (rather than *p*-coumaraldehyde **9** which was unavailable), and coniferaldehyde **14** and sinapaldehyde **21** as potential substrates. Kinetic data and product identifications were also obtained indirectly, since only NADPH cofactor oxidation (monitored at 340 nm) was examined. From these data, the investigators nevertheless concluded that both coniferaldehyde **14** and sinapaldehyde **21** could be used as substrates, with CAD-2 apparently being more active than CAD-1; note, however, of the substrates tested, cinnamaldehyde apparently served as the best substrate in both cases. Thus, this study neither examined the actual substrate specificities of the purified proteins nor unambiguously identified the enzymatic products so obtained. [As an aside, most crude *E. coli* preparations display non-specific alcohol dehydrogenase activities (unpublished observations).]

A second study (Li et al., 2001) examined the presumed CAD-1 and CAD-2 homologs present in aspen, trivially named PtSAD (68% identical to MsaCAD-1) and PtCAD (79% identical to MsaCAD-2), respectively. As before, aspen CAD-1 was most strongly expressed in internodes 1–3 and to a decreasing extent in 4–9, whereas CAD-2 was most evident in internodes 4–9. Interestingly, CAD-1 was localized to both phloem and xylem tissues, whereas CAD-2 was mainly (but not exclusively) detectable in the xylem-forming tissues. Recombinant CAD-1 and CAD-2 were next expressed in *E. coli* with the corresponding proteins being purified to apparent homogeneity (no details provided). Substrate specificity was again examined using *p*-coumaryl aldehyde **9**, caffeyl aldehyde **41**, coniferyl aldehyde **14**, 5-hydroxyconiferyl aldehyde **17** and sinapyl aldehyde **21** as potential substrates (see Table 16), with product analysis using HPLC/MS. In this way, CAD-2 displayed good substrate specificity for coniferyl aldehyde **14** and *p*-coumaryl aldehyde **9** with sinapyl aldehyde **21** being somewhat more slowly converted. Nevertheless, CAD-2 was capable of converting all three aldehydes **9**, **14**, **21** into the monolignols **1**–**3**.

Table 16
Reported kinetic properties of recombinant CAD1 and CAD2 from aspen (*Populus tremuloides*), expressed in *E. coli* (Li et al., 2001)

Substrates	K_m (μM)	V_{max} (nmol/min/μg)	V_{max} (nkat/mg)	V_{max}/K_m (nkat/mg/μM)	V_{max}/K_m (%)
CAD 2 (PtCAD)					
p-Coumaryl aldehyde **9**	6.2	0.17	2.83	0.46	30.07
Caffeyl aldehyde **41**	37.0	0.15	2.50	0.07	4.45
Coniferyl aldehyde **14**	2.3	0.21	3.50	1.52	100.00
5-OH Coniferyl aldehyde **17**	17.5	0.17	2.83	0.16	10.65
Sinapyl aldehyde **21**	9.1	0.10	1.67	0.18	12.05
CAD 1 (PtSAD)					
p-Coumaryl aldehyde **9**	15.6	2.90	48.33	3.10	27.52
Caffeyl aldehyde **41**	140.0	2.00	33.33	0.24	2.11
Coniferyl aldehyde **14**	12.7	2.30	38.33	3.02	26.81
5-OH Coniferyl aldehyde **17**	36.1	3.80	63.33	1.75	15.58
Sinapyl aldehyde **21**	7.4	5.00	83.33	11.26	100.00

Recombinant CAD-1, however, was a much more efficient (faster) enzyme with **9**, **14**, **17**, and **21** substrates than recombinant CAD-2 (although this was not discussed in the article). That is, the overall conversions of *p*-coumaryl aldehyde **9** and 5-hydroxyconiferyl aldehyde **17** were ~7- and ~10-fold better, whereas with coniferyl **14** and sinapyl **21** aldehydes increases of ~2- and ~60-fold were observed. Thus, with the provisional assumption that recombinant protein kinetic data accurately reflects that of the corresponding native counterparts, CAD-1 is a more active enzyme than CAD-2 for all of the substrates examined. Clearly, since all substrates can be used, the overall physiological significance remains to be fully established, since it is unknown as to how the H, G and S monomer supply to either CAD isoform, prior to reduction, is controlled.

Nevertheless, in an analogous way as for COMT and CCOMT gene expression, these investigators sought to examine the patterns of CAD-1 and CAD-2 protein localization in developing aspen internodes with different patterns again being noted: at the 3rd internode, CAD-2 was associated with the guaiacyl-rich metaxylem, whereas with progressive maturation (this and older internodes) it was detected in the xylem fusiform initials, then in the (G-rich) vessels, as well as in the developing ray cells. In these cell types (vessels excluded), CAD-2 preceded detection of CAD-1 with the latter being strongly detectable in GS-rich radial and axial ray cells. The patterns of CAD-1 and CAD-2 expression were, however, quite different in the phloem region; CAD-1 was detected in protophloem parenchyma cells (internode 3), and then in the primary phloem fibers (e.g. internode 6). However, by the stage of development of internodes 8–12, the CAD-2 was now mainly present, although its intensity of labeling progressively decreased as lignification began to subside. These data thus further suggest, as already established for COMT and CCOMT, differential control over the G and GS lignin forming networks during development of the various cell (wall) types.

The major conclusion from the Li et al. (2001) study was the hypothesis of unique physiological roles for both CAD-1 and CAD-2, i.e. as being sinapyl aldehyde **21** and coniferaldehyde **14** specific. However, this was not proven, and indeed, the different isoforms could just as readily instead be differentially employed for the biosynthesis of G *and* G-S lignins in the various cell wall types and corresponding wall layers. Furthermore, there is considerable circumstantial evidence against a unique role for CAD-1 in sinapyl alcohol **3** formation: first, the CAD maize mutation of a *presumed* CAD-2 gene [which, according to Li et al. (2001) is coniferyl aldehyde **14** specific] affects both G and S levels of the resulting lignin biopolymers. Secondly, all CAD down-regulation studies—which targeted the proposed coniferaldehyde **14** specific CAD-2 homologs—resulted in both small reductions in lignin levels as well as *decreases* in S/G ratios. These data, when taken together with that of protein localization profiles do not, therefore, support the concept of substrate-unique roles for CAD-1 and CAD-2, as proposed by Li et al. (2001). Instead, the data reveal a complex pattern of control of lignin assembly, which needs to be systematically and fully dissected at the individual cell and cell wall layer level. Indeed, how CAD-1 and CAD-2 evolved to achieve their apparently different substrate preferences for the three monolignol aldehydes **9**, **14**, and **21** cannot yet be gauged. Another aspect of the Li et al. (2001) paper that requires some consideration is that of the proposal that angiosperm lignin is a consequence of *heterologous* guaiacyl–syringyl lignin deposition; however, these investigators gave no insight (if this was the meaning) as to whether CAD-1 had resulted from introduction of a foreign gene during angiosperm evolution or not.

5.10.4. Reassessment of effects of CAD downregulation in transgenic alfalfa, poplar and tobacco

CAD downregulated transformants have been obtained with different species including alfalfa, poplar and tobacco. In tobacco, only antisense strategies were applied, with both heterologous (Higuchi et al., 1994; Hibino et al., 1995) and homologous (Halpin et al., 1994; Yahiaoui et al., 1998) genes being used. On the other hand, homologous genes were employed for both sense and antisense experiments in poplar (Baucher et al., 1996; Lapierre et al., 1999), and an antisense approach was utilized in alfalfa (Baucher et al., 1999). In all of the studies, the transgenes were either overexpressed or downregulated under control of the CaMV 35S promoter. Each study is discussed in detail below.

5.10.4.1. Alfalfa. Although alfalfa contains two CAD-like proteins, MsaCAD1 and MsaCAD2, (Brill et al, 1999) only the latter was used for downregulation of CAD activity in alfalfa (Baucher et al., 1999), this having ~77% identity to the developing xylem (lignin-specific) eucalyptus CAD. This resulted in apparent reductions of ~30–60% CAD activity relative to that of the controls, presumably in part because one of the CAD isoforms (MsaCAD1) was only partially (if at all) downregulated. Table 17 summarizes what is known about these transformants, and Fig. 27A and B again plot %G and %S thioacidolysis recoveries versus the corresponding estimated Klason lignin contents of the CWR. As for maize, the S and G recoveries were low (~20%) relative to the Klason lignin estimates, but slightly higher (~20–30%) when compared to the acetyl bromide lignin measurements (data not shown): as before, the reliability of both lignin and CAD activity estimations cannot be rigorously assessed for reasons stated earlier.

In this study, there was also noted a small reduction in overall S/G ratios as detectable CAD activity decreased relative to the controls, i.e. from ~0.5 control versus ~0.3–0.5 in the transformants, but with only the one line 2-3H having a S/G ratio of ~0.27 (Table 17). Given that there are at least two CAD homologs in alfalfa, however, and that these are presumably differentially expressed in most cell types (as noted for aspen), this result is perhaps not unexpected. That is, even if the CAD homolog targetted (CAD-2) was mainly involved in G rather than G–S lignin formation, the 35S CaMV promoter would nevertheless be expected to target all cell types. Hence, at higher levels of CAD repression, the corresponding CAD homolog (CAD-1), presumed to generate G–S lignin, would also perhaps be downregulated to some extent in addition to CAD-2 (even when they are expressed in the same cell types). Indeed, this could provisionally explain the fluctuations in S/G ratios and S/G recoveries observed. On the other hand, if CAD-1 and CAD-2 are differentially expressed at different stages of cell wall development in particular cell wall types as noted earlier and if only CAD-2 is downregulated, then the effects could simply also result from such perturbation on organized lignin assembly. [Note that these investigators also examined whether the red-brown coloration of the CAD downregulated phenotypes displayed was due to a build-up of the substituted cinnamaldehyde (**9**, **14**, **21**); however, they were not detected.]

Selected transgenic lines were also field cultivated together with the corresponding controls, where it was observed that the relative CAD activities in the trans-

Table 17
Preliminary characterization of transgenic alfalfa plants following introduction of a CAD transgene (adapted from Baucher et al., 1999)

Plant line	Enzyme and gene expression localization	Transgene orientation	Promoter	Gross morphological changes in the phenotypes	Tissue analyzed for lignins	% CAD activity	Lignin content[a] (% CWR)	Estimated S and G recovery (% of lignin)	S/G ratio	Analytical techniques employed
C1	n.d.	(Control)	–	–	Xylem ring from 3-month old plants	100	17.8	20.7	0.50	Thioacidolysis; acetyl bromide; Klason lignin analysis; Jarrige lignin determination; alkaline hydrolysis
C2	n.d.	(Control)	35S			89	17.5	22.7	0.54	
C3	n.d.	(Control)	35S			96	17.9	22.4	0.50	
C4	n.d.	(Control)	35S			122	17.3	21.8	0.49	
C5	n.d.	(Control)	35S			96	18.5	20.6	0.53	
C6	n.d.	(Control)	35S			100	18.0	23.1	0.52	
9-2D	n.d.	Antisense	35S	Red xylem		70	18.3	21.7	0.50	
9-1E	n.d.	Antisense	35S	Red xylem		48	17.9	22.0	0.47	
2-4A	n.d.	Antisense	35S	Red xylem		52	18.1	21.8	0.41	
2-8C	n.d.	Antisense	35S	Red xylem		55	18.0	18.2	0.47	
2-3H	n.d.	Antisense	35S	Red xylem		37	16.7	15.0	0.27	
9-7I	n.d.	Antisense	35S	Red xylem		44	19.2	17.8	0.38	

The frequency of interunit linkages in, and MW ranges of, lignins, as well as ultrastructural effects on cell wall types and patterns of lignin deposition were not determined (n.d.).

[a] Lignin estimations were not corrected for proteinaceous and/or ash-forming constituents.

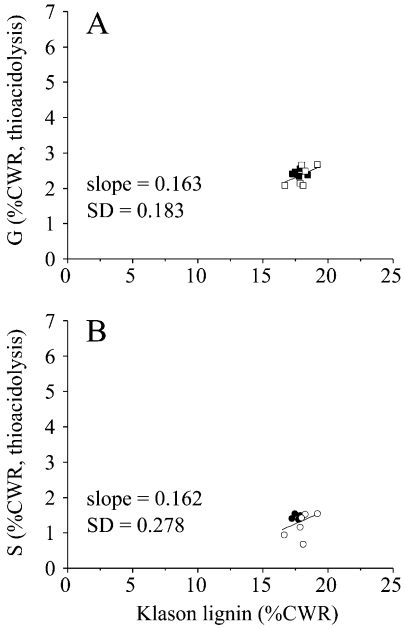


Fig. 27. Estimated lignin contents versus guaiacyl (G, ■, □) and syringyl (S, ●, ○) recoveries of cell wall residues (CWR) of control (solid symbols) and CAD-downregulated lines (open symbols) in alfalfa. Klason lignin contents and G (A) and S (B) recoveries from thioacidolysis are estimated in terms of percentage cell wall residue (%CWR). Data recalculated from Baucher et al. (1999). SD = standard deviation.

formants had declined nearly 3-fold. While this was attributed to environmental conditions influencing the amplitude of the antisense effect, this explanation needs to be reconsidered, since the controls had declined approximately 3-fold as well. Nor were there any significant changes in the levels of "alkali soluble" lignins between the controls and the transformants, since the mean values for each were ~97.7% in agreement; yet the investigators concluded that two lines (9-7I and 2-3H) had significant increases in alkali-solubility behaviour. Of the two single data points given (no standard deviation provided), line 9-7I had a reduced amount of cell wall residue prior to saponification. However, the reduction in alkali insoluble residue recovery was apparently due to an *increased* extractive content and not to alkali solubility per se. Additionally, in the various lines examined, there were only trace amounts of *p*-coumaric (**7**) and ferulic (**12**) acids, vanillin (**23**) and syringaldehyde (**24**) released following mild alkali treatment (1 N NaOH, 20 h, 20 °C). Overall, the data are consistent with CAD having no rate-limiting role in carbon allocation to the pathway, and little to no effect on lignin deposition at the levels of CAD activity repression achieved.

5.10.4.2. Poplar. Two rather conflicting studies were reported by the same investigators on the effects of CAD downregulation in poplar, using CAD-downregulated transformants harvested after 3 months (Baucher et al., 1996) and 2 years (Lapierre et al., 1999) growth, respectively. As noted with *bm1* maize and CAD-downregulated alfalfa, poplar transformants with significantly reduced CAD levels (estimated at ~30% wild type activities) displayed a red coloration in the xylem tissues; this was not observed in either control or sense transformants. As for alfalfa, the CAD downregulated gene showed high homology to that of the eucalyptus CAD (80% amino acid identity).

In the first study, 3-month old juvenile wood was harvested from each plant line with both controls and transformants having ~23–32% of their dry weight removed by organic and aqueous solvent extraction procedures (details not provided). Fig. 28A and B plots %G and %S recoveries versus estimated Klason lignin contents in the CWR of various plant lines obtained, and Table 18 summarizes the preliminary characterization of the controls (pBI121), antisense (e.g. line ASCAD8) and sense transformants (e.g. line CAD23). These data suggest that CAD downregulation actually led to small increases, rather than decreases, in measurable lignin content. However, juvenile wood is well known not to give accurate lignin determinations using the Klason lignin method for reasons stated earlier (e.g. proteins coprecipitating, as described in Lai and Sarkanen, 1971). Moreover, based on the variability in the data obtained, and the presence of only one control, no meaningful conclusions can be drawn as regards trends in S/G ratios, although a greater variability in S recoveries was noted for the specimens examined (see Fig. 28A and B), suggesting perhaps that S formation was more greatly affected at this developmental stage. This would again not be an unexpected result, even if the CAD-2 being targetted was guaiacyl-lignin specific. This is because as before it cannot be fully gauged at this stage, using the CaMV 35S promoter, as to what

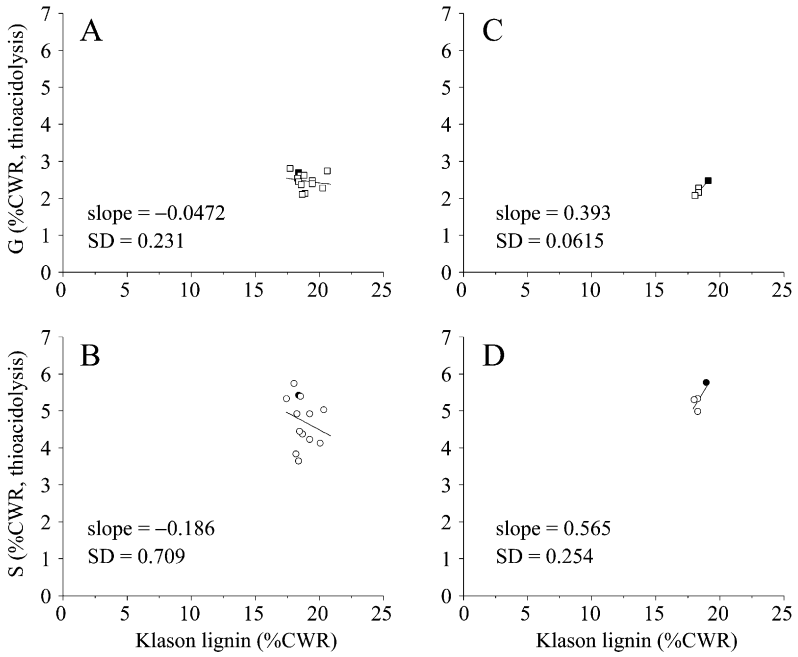


Fig. 28. Estimated lignin contents versus guaiacyl (G, ■, □) and syringyl (S, ●, ○) recoveries of cell wall residues (CWR) of control (solid symbols) and CAD-downregulated lines (open symbols) in poplar. Klason lignin contents and G (A, C) and S (B, D) recoveries from thioacidolysis are estimated in terms of percentage cell wall residue (%CWR) for 3-month (A, B) and 2-year old (C, D) tissues, respectively. Data recalculated from Baucher et al. (1996) and Lapierre et al. (1999). SD = standard deviation.

Table 18
Preliminary characterization of transgenic poplar plants following introduction of a CAD transgene

Plant line	Enzyme and gene expression localization	Transgene orientation	Promoter	Gross morphological changes in the phenotypes	Tissue analyzed for lignins	% CAD activity	Lignin amounts[c] (% CWR)	Estimated S and G recovery (% of lignin)	S/G ratio	Analytical techniques employed
PBI121[a]	n.d.	(Control)	–			100	18.4	44.8	1.71	Thioacidolysis;
ASCAD49[a]	n.d.	Antisense	35S	Patchy red	Stems from	41	17.7	45.7	1.64	Klason lignin
ASCAD8[a]	n.d.	Antisense	35S	None	3-month	82	20.6	37.6	1.58	analysis; alkali
ASCAD3-2[a]	n.d.	Antisense	35S	None	old plants	104	18.5	40.6	1.61	extraction; UV-
ASCAD10[a]		Antisense	35S	Patchy red		82	18.3	45.1	1.95	Vis; histochemical
ASCAD14[a]	n.d.	Antisense	35S	Intense red xylem		82	19.5	37.6	1.71	staining
ASCAD21-2[a]	n.d.	Antisense	35S	Intense red xylem		36	18.4	34.1	1.36	
ASCAD3–1[a]	n.d.	Antisense	35S	None		104	19.5	33.8	1.50	
ASCAD21-1[a]	n.d.	Antisense	35S	Intense red xylem		36	18.6	32.2	1.32	
ASCAD52-1[a]	n.d.	Antisense	35S	Intense red xylem		32	20.3	31.4	1.56	
ASCAD52-2[a]	n.d.	Antisense	35S	Intense red xylem		32	18.9	34.1	1.77	
CAD23[a]	n.d.	Sense	35S	None		129	18.8	42.5	1.77	
CAD9[a]	n.d.	Sense	35S	None		115	n.d.	n.d.	1.76	
CAD1[a]	n.d.	Sense	35S	Red xylem		34	18.7	34.9	1.82	
Control[b]		(Control)	–		Wood from	100	19.0	41.2	2.03	Thioacidolysis;
ASCAD52[b]		Antisense	35S	Red xylem	2-year old	n.d.	18.3	38.7	2.04	Klason lignin
ASCAD21[b]	n.d.	Antisense	35S	Intense red xylem	plants	30	18.3	40.7	2.03	analysis; alkali extraction; Kraft
SCAD1[b]	n.d.	Sense	35S	Red xylem		30	18.0		2.23	pulping; Wiesner; Ni desulfurization

The frequency of interunit linkages in, and MW ranges of, lignins and the ultrastructural effects on cell wall types and patterns of lignin deposition were not determined (n.d.).

[a] Adapted from Baucher et al. (1996).

[b] Adapted from Lapierre et al. (1999).

[c] Lignin estimations were not corrected for proteinaceous and/or ash-forming constituents.

extent the G and G–S lignin-forming networks in different cell types are differentially affected. Moreover, as noted in essentially all of the downregulation studies discussed so far, it would be instructive to employ the native promoter for each gene being specifically downregulated.

Attempts to trap the corresponding cinnamyl aldehydes **9**, **14**, and **21**, which might build up during CAD repression were also unsuccessful, since the corresponding thioacidolysis products were again not detected. Moreover, the overall yields of alkali-releasable phenolics following 2 N NaOH treatment for 24 h at 35 °C gave similar amounts of vanillin (**23**), syringaldehyde (**24**), and *p*-hydroxybenzoic acid (**38**) (i.e. these represented ~0.24% of the CWR of the controls versus ~0.28–0.63% in the corresponding transformants).

A possible difference between the 3-month old controls and the most heavily downregulated CAD transformants (ASCAD21 and ASCAD52), however, was again in the alkaline solubility behaviour following 2 N NaOH treatment for 24 h at 35 °C. These approximately corresponded to ~28–44% in the CAD-downregulated plants versus ~20% in the control, when tabulated as a percentage of the estimated lignin content of each line. However, none of the alkali-releasable constituents were characterized further in order to establish their identity, and thus no further consideration can be given to them at this stage.

Yet, since one of the reasons for CAD downregulation was to obtain more readily pulpable wood, the pulping characteristics of 3-month old ASCAD52 and ASCAD21 transformants were also examined. As to be expected, there was some variability in the measured Klason lignin contents of each line: ASCAD21 (or lines thereof) had average estimated lignin contents of 18.4±0.5%, ASCAD52 (or lines thereof) had values ranging from 17.6±0.1 to 20.3±0.2%, and the controls varied from 18.3±0.1 to 19.2% (Lapierre et al., 1999).

Representatives of all three lines were subjected to kraft pulping, which in the article was actually incorrectly described as a lignin sulfonation process. [Kraft pulping utilizes Na_2S and NaOH at elevated temperatures, and typically at full charge gives low pulp yields (40–55%); sulfonation, on the other hand, is generally carried out at low pH with sodium or ammonium sulphite (Lewis et al., 1983; Lewis and Yean, 1985; Padmapriya et al., 1985; Bialski et al., 1986; Luthe and Lewis, 1986).] Nevertheless, in the kraft pulping trial undertaken, the pulp yields for ASCAD52, ASCAD21 and pBI121 (control) were 43.4, 47.5 and 47.9%, with kappa numbers for the corresponding pulps being 18.8, 19.2. and 20.8, respectively. [No standard deviations were provided for any of the analyses. Indeed, as in many of the studies undertaken thus far for transgenic tissues, the variability within the controls and transformants was not statistically determined.]

The relationship between kappa number and lignin content is estimated as % lignin = 0.147 × kappa number. Accordingly, the above data revealed that the corresponding pulps had very similar residual lignin contents of 2.76, 2.82 and 3.05%, respectively. When corrected for differences in pulp yield, however, the data indicated that the pulping characteristics of the two CAD transformants were only slightly better than the corresponding control line, this presumably reflecting increased formation of more non-lignin alkali-labile components and thus less lignin as monolignol supply is reduced.

When the corresponding plant lines had further matured (~2 years growth), the lines ASCAD21 and ASCAD52 were re-examined. The estimated Klason lignin levels were now apparently slightly lower than before (Fig. 28C and D) with concomitant reductions in both %G and %S recoveries. Although there are only very few data points reported for both the controls and transformants, the preliminary data obtained gave no conclusive indication that G and S pathways were independently modulated. That is, levels of both were apparently reduced more or less to the same extent, and thus the S/G ratios remained very similar. Each line was also subjected to mild alkaline hydrolysis (2 N NaOH, 37 °C, overnight) to examine the levels of *p*HBA (**38**), vanillin (**23**), and syringaldehyde (**24**) released. While some fluctuations in their overall levels were observed, they constituted a very small portion of the ASCAD21 tissue (~0.45% of CWR and ~0.33% of CWR of the control). Moreover, while attempts to detect the thioketal derivatives of the aldehydes were now successful, they were present at minuscule levels.

An additional pulping evaluation was conducted using the lines ASCAD21 and ASCAD52, relative to the control (pBI121) at three "active alkali" charges (15, 17 and 20%, respectively) (Lapierre et al., 1999), the results of which are summarized in Table 19. However, in that trial, ASCAD52 now functioned no better than the control, and ASCAD21 was marginally better. Surprisingly, the investigators attributed this marginal increase in pulping efficacy as a consequence of a higher frequency of free phenolic groups in G lignin (see Table 19), whose frequencies were estimated as being 33.6% for ASCAD21, 30.5% for ASCAD52 and 26.4% for control, respectively. However, since no comparable trend in pulping efficacy was observed between the three samples (i.e. the control, ASCAD52 and ASCAD21 lines), nor were variabilities in the control lines documented, this explanation has little or no experimental validity at present.

Together the data obtained thus give little evidence that CAD downregulation in poplar, at the levels attained, was particularly beneficial for the reasons originally envisaged (e.g. for pulp and paper applications). The data did, however, clearly demonstrate that, as

Table 19
Pulp characteristics of control and two year-old CAD transgenic poplar, and estimations of molar percentages of G and S units with free phenolic groups in their lignins prior to pulping (Lapierre et al., 1999)

Plant line	Klason lignin contents[a]	15% Active alkali charge		17% Active alkali charge		20% Active alkali charge		G with free phenol[b] (%molar)	S with free phenol[b] (%mol)
		Pulp yield[b]	kappa no.	Pulp yield[b]	kappa no.	Pulp yield[b]	kappa no.		
Control	19.4±0.2	49.7	20.5	48.2	15.1	46.9	11.9	26.4	3.0
ASCAD52	19.4±0.2	49.2	19.4	48.0	14.7	46.9	12.5	30.5	3.5
ASCAD21	18.7±0.1	50.1	16.1	49.1	15.0	47.5	12.4	33.6	4.5

[a] Lignin estimations were not corrected for proteinaceous and/or ash-forming constituents.
[b] No standard deviations provided.

carried out in this study, CAD has no rate-limiting capacity in overall carbon allocation to the monolignol pathway, and thus may not be a particularly useful biotechnological target. Furthermore, there is currently no *comprehensive* understanding of the mechanical integrity of tissues from CAD downregulated poplar transformants, which can also be presumed to be adversely affected if lignin assembly in any way has been partially impaired due to a reduction in monolignol supply. However, preliminary investigations on CAD downregulated plants have indicated that the biomechanical properties of these transformants were compromised, indicative of some weakening of the vasculature (Huang et al., 1999).

5.10.4.2. Tobacco. Several studies were undertaken to examine the effects of gross CAD downregulation in tobacco, using the CAD isoforms most homologous (77 and 80% amino acid sequence identity) to that of the eucalyptus CAD (see Table 20). Thus, in the initial study by Halpin et al. (1994), various antisense lines were obtained (using an ~1-kb fragment of the tobacco CAD gene expressed under control of the CaMV 35S promoter) with CAD activities apparently being reduced to circa 20 and 7% of wild type levels, respectively (Table 20A). As before, the lines severely repressed in CAD activity had reddish-brown colored xylem tissue; however, there were no other visible phenotypical changes reported, in terms of growth and development.

The antisense lines, relative to the controls, apparently showed only slightly reduced lignin levels, based on Klason and acetyl bromide estimations (Table 20A), whereas thioglycolic acid treatment displayed substantive (13–50%) increases in the overall UV absorbing (λ 280 nm) solubilized components in the transformants; no explanation was provided as to why this was the case. [A plot of lignin versus thioacidolysis S and G recoveries cannot be made in this case because the latter were not reported.] Nevertheless, the S/G ratios of the thioacidolysis components of the cell wall residues were apparently reduced from 0.85–0.83 (control) to ~0.75–0.46 in the antisense lines; however, there was again no evidence of build-up of sinapyl aldehyde (**21**), since no trace of the S-derived thioketal was detected. A comparable effect on S/G ratios at high levels of CAD reduction was also noted by Yahiaoui et al. (1998) (Table 20C), but not with Hibino et al. (1995), who were unable to achieve transformants with significantly reduced CAD activity (Table 20B).

Additional studies (Bernard-Vailhé et al., 1996) with the same severely CAD-downregulated transformants as that of Halpin et al. (1994) revealed mixed behaviour of tissues in their alkali solubility, i.e. when treated with 1 N NaOH (2 h, 35 °C), the most extensively CAD-downregulated line (AS50, 7% CAD activity of control) had ~37.7% of the cell wall components removed, relative to ~32.5% of the control. However, another line possessing ~20% residual CAD activity (AS40) was essentially identical to the control.

The most strongly downregulated CAD line and its control were subjected to the lengthy "Björkman lignin" isolation procedure (Björkman, 1954), the yields of which were not reported. This involved grinding tissues in a vibrating mill (using porcelain balls) at 4 °C for 10 days, extracting the resulting residues with dioxane-H_2O (96:4) for 24 h, centrifuging the corresponding suspensions, and precipitating the lignin-enriched preparation in sodium sulfate solution to remove low molecular weight contaminants. The precipitates so obtained were washed with water, dissolved in acetic acid and again precipitated using diethyl ether. Interestingly, this procedure removed the reddish-brown colored components characteristic of the CAD down-regulated plants, thereby implying that these constituents were *not* part of the lignin biopolymer. However, the actual lignin recoveries (yields) were not reported.

5.10.5. Reconsideration of NMR spectroscopic analyses of CAD-downregulated tobacco lignin derived isolates

The ^{13}C NMR solution spectra of the lignin-derived precipitates from the CAD-downregulated and corresponding control plants were, however, most instructive (Fig. 29A and C, respectively). Both spectra (recorded under conditions for quantification of resonances) were essentially identical, except for one minor signal at ~110 ppm (marked by an ×), possibly due to an

Table 20
Preliminary characterization of transgenic tobacco plants following introduction of a CAD transgene

Plant line	Enzyme and gene expression localization	Transgene orientation	Promoter	Gross morphological changes in the phenotypes	Tissue analyzed for lignins	% CAD activity	Lignin amounts[f] (% CWR)			Estimated S and G recovery (% of lignin)	S/G ratio	Analytical techniques employed
							KL	AcBr	TGA[g]			
A. Control 40[a]	n.d.	(Control)	–	–	95- and 120-day old plants for staining; for other methods, age not specified	100	18.5	21.2	281	n.d.	0.85	Thioglycolate; Klason lignin analysis; acetyl bromide; pyrolysis-MS; histochemical staining
Control 50[a]	n.d.	(Control)	–	–		100	16.9	19.7	279	n.d.	0.83	
AS 40[a]	n.d.	Antisense	35S	None		20	16.9	21.1	317	n.d.	0.75	
AS 50[a]	n.d.	Antisense	35S	Red-brown xylem		7	16.4	19.0	421	n.d.	0.46	
B. Control[b]	n.d.	(Control)	–	–	Stems of 2-month old plants	100	n.d.	100[e]	n.d.	20.1	0.96	Thioacidolysis; acetyl bromide; histochemical staining; FT–IR
acad-8[b,d]	n.d.	Antisense	35S	None		80	n.d.	100[e]	n.d.	17.4	0.87	
acad-4[b,d]	n.d.	Antisense	35S	Brown stem		45	n.d.	100[e]	n.d.	18.0	1.02	
acad-5[b,d]	n.d.	Antisense	35S	None		95	n.d.	95[e]	n.d.	n.d.	n.d.	
C. Control[c]	n.d.	(Control)	–	–	Xylem ring from 3-month old plants	100	18.1	n.d.	n.d.	21.5	0.96	Thioacidolysis; acetyl bromide; Klason lignin analysis
3P27[c]	n.d.	Antisense	35S	Red patches		54	21.2	n.d.	n.d.	17.3	0.95	
7TP5[c]	n.d.	Antisense	35S	Red stripes		11	18.8	n.d.	n.d.	17.3	0.75	
7TP42[c]	n.d.	Antisense	35S	Red stripes		11	18.3	n.d.	n.d.	14.8	0.60	
T37[c]	n.d.	Antisense	35S	Red xylem		8	17.9	n.d.	n.d.	22.1	0.38	

The frequency of interunit linkages in, and MW ranges of, lignins and the ultrastructural effects on cell wall types and patterns of lignin deposition were not determined (n.d.).

[a] Adapted from Halpin et al. (1994).

[b] Adapted from Higuchi et al. (1994) and Hibino et al. (1995).

[c] Adapted from Yahiaoui et al. (1998).

[d] Heterologous transformants harboring the *Aralia* CAD gene; the rest of the transformants used tobacco CAD.

[e] Expressed as percentage of the control. Klason lignin contents were not reported.

[f] Lignin estimations were not corrected for proteinaceous and/or ash-forming constituents.

[g] Thioglycolic acid extractable lignin expressed as the absorbance at 280 nm per gram of tissue.

impurity introduced during isolation. The NMR-estimated S/G ratios of the isolates were also very similar (0.69 vs. 0.73 by NMR), and the minuscule levels of aldehydic resonances detected (circa 185–190 ppm) did not change very significantly in relative intensities in either sample. Moreover, assuming that the samples are somewhat representative of the *actual lignins* present in their tissues, the data would suggest that neither S nor G pathways had been differentially affected in any significant manner, in contrast to the trend suggested by thioacidolytic analyses. Clearly, based on the information herein, the CAD-downregulated sample did *not* contain increased levels of non-traditional phenols either, such as the corresponding aldehydes **9**, **14**, or **21**, or other ''abnormal'' lignin components in the lignin-enriched isolates.

Other studies were carried out on the CAD downregulated tobacco lines (Ralph et al., 1998; Yahiaoui et al., 1998; Chabannes et al., 2001). Thus, Fig. 30A and B plots the %G and %S recoveries relative to Klason lignin contents as a percentage of the CWR, and these data (using transformants with CAD activities of $\leqslant 11\%$ relative to the control) suggest that the relative lignin amounts in the transformants had actually *increased* slightly. Furthermore, although the G and S recoveries were low (3–4% CWR, combined), the gross data indicated that the S recovery levels appear to be more greatly reduced relative to G at the highest levels of CAD suppression (see Table 20C). This could again be due to one or more of the reasons stated earlier. Once again, however, it is not possible to ascertain whether the effects on G and G–S forming networks are a consequence of the non-specific 35S CaMV promoter being used, rather than the native promoter driving the gene of interest.

It was additionally instructive to tabulate the Klason lignin estimates in this analysis in the above control and transformant lines as a function of decreasing CAD activity, prior to and following saponification (2 N NaOH, 2 h, 25 °C) (Fig. 31). This revealed that saponification removes circa 25% of the putative ''lignin'' (alkali soluble phenolics, together with hemicelluloses, etc.) in the controls and that this amount remains essentially unchanged until CAD is downregulated to $<20\%$ residual activity. That is, in the wild type plants, some 25% of the ''lignin'' is removed in this manner,

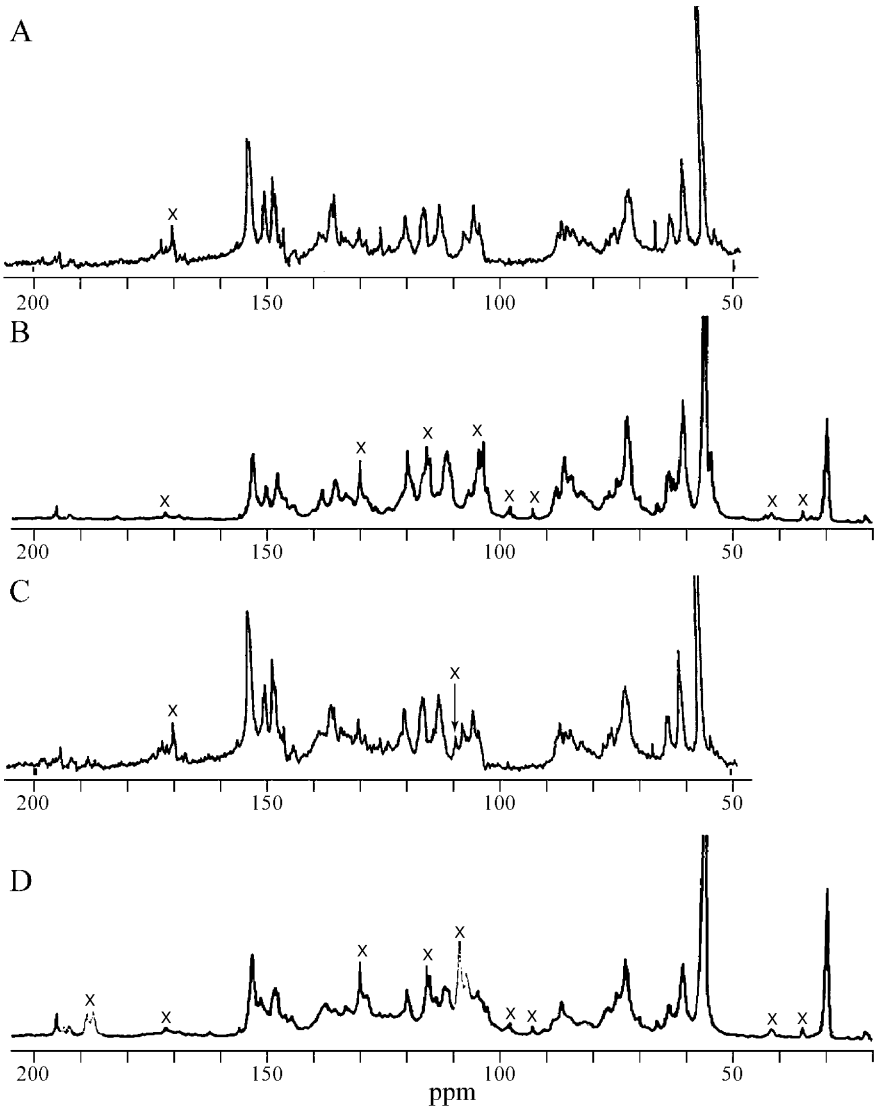


Fig. 29. ^{13}C NMR spectra of various lignin-enriched isolates obtained using current lignin solubilization protocols, with preparations derived from wild type [A: Bernard-Vailhé et al. (1996) and B: Ralph et al. (1998)] and CAD transgenic tobacco plants [C: Bernard-Vailhé et al. (1996) and D: Ralph et al. (1998)]. Redrawn from Bernard-Vailhé et al. (1996) (A, C) with permission of the American Chemical Society (Copyright 1996), and from Ralph et al. (1998) (B, D) with permission of the National Academy of Sciences, USA (Copyright 1998). "X" indicates additional resonances either absent or greatly reduced in the respective comparison spectra, i.e. A vs. B, and C vs. D.

thereby revealing the presence of a metabolic branchpoint affording lignin (~75%) in one case, and an uncharacterized alkali soluble phenolic fraction (~25%) in the other. At highly repressed CAD levels, however, more than 50% of the "lignin" was now removed by cold alkali treatment further indicating that the carbon distribution into metabolic branchpoints giving the lignin and alkali soluble components, respectively, had been altered. Yet, even though the alkali labile moieties have not been adequately chemically characterized, the data reveal an increased activity in the metabolic branchpoint when monolignol **1**–**3** supply is reduced, i.e. lignin formation can no longer occur and instead increased metabolism into these alkali-labile components occurs instead. Clearly, much needs to be done in order to understand the biochemical and physiological consequences of this metabolic branchpoint, including which of the CAD homologs are actually involved.

The spectroscopic characterization of the lignin-enriched isolates from these CAD-downregulated transformants was also carried out as before: this used as a tissue source, stems from the most heavily downregulated CAD transformants (<7% CAD activity relative to the control) (Ralph et al., 1998; Chabannes et al., 2001) with yields of the lignin-enriched preparations isolated totaling ~17% (antisense CAD, T37) versus ~7% for the control, respectively, i.e. from 83 to 93% of the presumed lignin amounts were not recovered. However, the spectroscopic data obtained and the

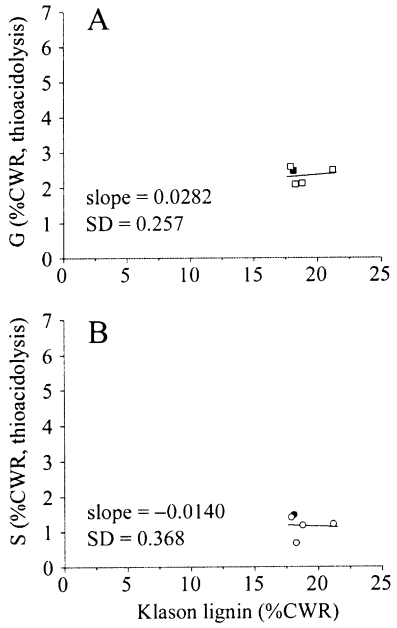


Fig. 30. Estimated lignin contents versus guaiacyl (G, ■, □) and syringyl (S, ●, ○) recoveries of cell wall residues (CWR) of control (solid symbols) and CAD-downregulated lines (open symbols) in tobacco. Klason lignin contents and G (A) and S (B) recoveries from thioacidolysis are estimated in terms of percentage cell wall residue (%CWR). Data recalculated from Yahiaoui et al. (1998). SD = standard deviation.

interpretations offered differed substantially from that of the previous study by Bernard-Vailhé et al. (1996), even though the CAD-downregulated transformants were ostensibly the same. Firstly, as noted for the CCR downregulation study (Chabannes et al., 2001), there were difficulties encountered in sample purification, with $\leqslant$15% polysaccharide being detected as impurities in the latter study. Secondly, a direct comparison of the Bernard-Vailhé et al. (1996) and Chabannes et al. (2001) NMR spectra of the controls (Fig. 29A and B, respectively) revealed a number of additional resonances in the latter (marked by ×'s in Fig. 29B) that are not evident in Fig. 29A; this strongly suggests the presence of non-lignin impurities in the latter sample. Indeed, of these, the signals at ~36, 42, 115 and 129 ppm are again consistent with hydroxycinnamoyl tyramine moieties (**32** and **33**) [previously described in the 4CL and CCR downregulation sections (5.5.1 and 5.9)], whereas the other extraneous signals cannot yet be assigned. Interestingly, none of these resonances appears to be present in the Bernard-Vailhé et al. (1996) control tissue preparations, this again suggesting that the extraneous signals in Fig. 29B are due to low molecular weight impurities in the Chabannes et al. (2001) sample. Thirdly, discrepancies in the spectral profiles are even more marked when each of the two ^{13}C NMR spectra of the two CAD downregulated specimens are compared directly (Fig. 29C and D, respectively); these would have been anticipated to be essentially identical, given that both are derived from transformants with a comparable level of downregulation of the same CAD gene. However, once again several additional resonances are

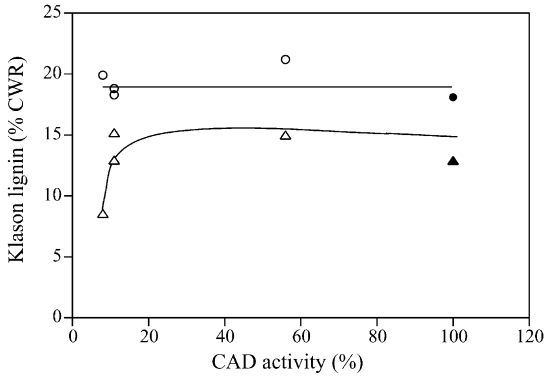


Fig. 31. Alkali solubility of lignin in control (solid symbols) and CAD-downregulated lines (open symbols) in tobacco. Klason lignin contents are estimated in terms of cell wall residue (CWR). Legend: Klason lignin in unsaponified residue: ●, ○; and Klason lignin in saponified residue: △, ▲. Data recalculated from Yahiaoui et al. (1998).

observed in the Ralph et al. (1998) spectrum (see Fig. 29D), which are not present in that of Fig. 29C (Bernard-Vailhé et al., 1996). These additional resonances marked by ×'s (Fig. 29D) can again be ascribed to impurities, including hydroxycinnamoyl tyramine residues (**32, 33**, see above) as well as aldehydic components (185–190 ppm) reported as being sinapyl aldehyde (**21**) derived. Thus, given the discrepancies between the Bernard-Vailhé et al. (1996) and Ralph et al. (1998) spectroscopic data, it is concluded that the additional resonances either correspond to impurities in the Ralph et al. preparations, and/or were artifacts introduced during isolation. Yet it was these additional resonances that were mainly used in support of "non-traditional phenolics" and "metabolic plasticity" in lignin structure. Surprisingly, these claims followed that of the earlier report by Bernard-Vailhé et al. (1996), who had essentially analyzed the same controls and transformants. Clearly, their spectra did not contain such moieties, a situation further complicated by lack of reference by Ralph et al. (1998) and Chabannes et al. (2001) to the earlier work of Bernard-Vailhé et al. (1996).

5.10.6. Preliminary biomechanical properties of CAD downregulated tobacco transformants

Several studies (Hepworth and Vincent, 1998, 1999; Hepworth et al., 1998, 1999; Huang et al., 1999) examined the effects of CAD downregulation on the biomechanical properties of transgenic (antisense) tobacco stem tissues, relative to the controls. The preliminary data reported indicated that CAD downregulation indeed predictably results in weakening of the vascular apparatus, although most of the papers gave little specific information as to the nature and type of transgenic plants analyzed. For example, the study by Hepworth et al. (1998), which also indicated that the control tobacco stems had incongruously high lignin contents of 40–50%, reported their findings in a way that did not effectively distinguish between the CAD and CCR downregulated transformants used. A second study (Hepworth and Vincent, 1998) then concluded that antisense CAD transformants had reduced longitudinal tensile stiffness and lower shear and Young's modulii, respectively, to that of the controls; a third investigation (Hepworth and Vincent, 1999) also indicated that the CAD downregulated transgenic plants were able to respond to periodic flexural bending of their stems during growth and development by increasing the thickness of the xylem tissue cylinder in a compensatory manner. However, in both of these studies, no particulars on the transformants were provided. A more recent analysis (Huang et al., 1999) which in this case specified the transgenic lines examined, demonstrated that the various CAD downregulated transgenic tobacco stems used required less cutting work in the transverse and longitudinal directions, again indicative of a weakened vascular apparatus. [Note also that, in this study, the acetyl bromide lignin contents of control and CAD downregulated transformants were again estimated to be incongruously high, i.e. 33.35 and 30.44%, respectively.] Taken together, these data predictably support the view that severe CAD downregulation weakens the vasculature apparatus, due to decrease in monolignol supply and hence in lignin amount. It was therefore perhaps not surprising that CAD downregulated plants also displayed enhanced cell wall degradability (Chabannes et al., 2001).

5.10.7. Challenges for the future

The effects of CAD downregulation on lignin biosynthesis are apparently only substantial when extensive downregulation (<11% CAD activity) is achieved, in accordance with the non-regulatory downstream processing role of this enzymatic step in monolignol biosynthesis. Nevertheless, when substantial repression of CAD activity is achieved, a considerable portion of the carbon designated for monolignol biosynthesis is instead assimilated via a metabolic branchpoint towards formation of uncharacterized e.g. [alkali soluble] components. The structures of these components now need to be fully determined, including what they are formed from, and where and when they are deposited, including the cell types involved. Another important question to be resolved is that of the physiological roles of different CAD isoforms in G and G-S lignin deposition in different cell types. The analyses of plant tissues from *bm1* mutants (presumably a CAD-2 mutation) do not support the concept of different cinnamyl alcohol dehydrogenases with unique specificity for coniferaldehyde **14** and sinapaldehyde **21**, respectively. Nor do the results obtained from downregulation studies of CAD, which thus far have targetted CAD2 isoforms, support an unique role either. Hence, it will be most instructive to establish the different (and/or) supporting roles that each isoform has during distinct phases of cell wall lignification, including deposition in cell wall regions and layers. As for all the other studies, it will be worthwhile to examine the effects of downregulating the various CAD isoforms using the actual promoters associated with the various homologs.

6. Concluding remarks

This comprehensive analysis summarizes what is known thus far as regards the preliminary characterization of plant tissues, following either up- or downregulation and/or mutation of various steps in the monolignol-forming pathway. The data obtained reveal a much more complex pattern of lignin assembly in different cell types and cell wall layers than hitherto recognized. No evidence, at any level of inquiry, indicated

that the process of lignification, including biopolymer assembly, involved the oft claimed but unproven model of random (coupling) formation. However, it was beyond the scope of this analysis to discuss additional topics, i.e., monolignol transport mechanisms to the plasma membrane, targeting of various monolignols **1**–**3** to specific regions in developing cell walls, and in how ordered lignin assembly is achieved in different cell wall types; these will be treated separately elsewhere.

The analysis of the genetic and mutational manipulation of steps involved in monolignol biosynthesis did, however, give fully predictable results for the proposed model of multisite modulation, wherein phenylalanine **4** availability, and the activities of C4H and C3H are rate-limiting. As expected, suppression of non-rate-determining steps downstream in the pathway did not alter the stipulated carbon flux towards lignin, although targetting to monolignol biosynthesis could be predictably reduced. Furthermore, no evidence was obtained in support of "non-traditional" phenolics being incorporated into lignin proper. Instead, depletion of monolignol supply adversely affected vascular integrity and function, and in some instances perhaps even growth and development of the plant as well.

Thus, in summation, the data obtained to date are fully consistent with strict biochemical control being exercised over formation of the H, G and S components of lignin, the complex control of which is necessary in different cell types to achieve the requisite physiological properties within the vascular apparatus. Moreover, the recent ability to obtain plants with enriched H, G and S levels in their lignins, relative to that of wild type, will be of important utility in studying many of the processes that are involved in, and which control, lignin assembly. However, this will also require the development of new technologies to study such questions, including even that for lignin analysis itself. Clearly, the recent biotechnological advances made are only now just beginning to facilitate the systematic dissection of the lignification process, and hence as to how it is formed in vivo.

Acknowledgements

The authors wish to thank the Department of Energy (DE-FG03-97ER20259), the National Aeronatutics and Space Administration (NAG2-1198 and NAG-1513), the US Department of Agriculture (99-35103-8037), McIntire-Stennis and the G. Thomas Hargrove and Lewis and Dorothy B. Cullman for generous support of the studies related to this review. The authors are also indebted to Drs. Richard A. Dixon and Jacquline Grima-Pettenati for PAL and CCR figures, respectively, and to Dr. Clint Chapple for provision of C3H papers prior to publication. The authors particularly wish to thank Dr. Laurence B. Davin (Washington State University) for substantial help and provision of needed insight during preparation of this review, and to Dr. Simo Sarkanen (University of Minnesota) for generous and far-reaching discussions on the material within the review. Thanks are also extended to Michael G. Paice (PAPRICAN) and to Dr. Ragai Ibrahim (Concordia University) for critically reading the manuscript.

References

Amrhein, N., Diederich, E., 1980. Turnover of isoflavones in *Cicer arietinum* L. Naturwissenschaften 67, 40–41.

Amrhein, N., Frank, G., Lemm, G., Luhmann, H.-B., 1983. Inhibition of lignin formation by L-α-aminooxy-β-phenylpropionic acid, an inhibitor of phenylalanine ammonia-lyase. European Journal of Cell Biology 29, 139–144.

Amrhein, N., Gerhardt, J., 1979. Superinduction of phenylalanine ammonia-lyase in gherkin hypocotyls caused by the inhibitor, L-α-aminooxy-β-phenylpropionic acid. Biochimica et Biophysica Acta 583, 434–442.

Amrhein, N., Gödeke, K.-H., 1977. α-Aminooxy-β-phenylpropionic acid-a potent inhibitor of L-phenylalanine ammonia-lyase in vitro and in vivo. Plant Science Letters 8, 313–317.

Amrhein, N., Holländer, H., 1979. Inhibition of anthocyanin formation in seedlings and flowers by the enantiomers of α-aminooxy-β-phenylpropionic acid and their *N*-benzyloxycarbonyl derivatives. Planta 144, 385–389.

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

Anterola, A.M., van Rensburg, H., van Heerden, P.S., Davin, L.B., Lewis, N.G., 1999. Multi-site modulation of flux during monolignol formation in loblolly pine (*Pinus taeda*). Biochemical and Biophysical Research Communications 261, 652–657.

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

Bak, S., Kahn, R.A., Nielsen, H.L., Møller, B.L., Halkier, B.A., 1998. Cloning of three A-type cytochromes P450, CYP71E1, CYP98, and CYP99 from *Sorghum bicolor* (L.) Moench by a PCR approach and identification by expression in *Escherichia coli* of CYP71E1 as a multifunctional cytochrome P450 in the biosynthesis of the cyanogenic glucoside dhurrin. Plant Molecular Biology 36, 393–405.

Barnes, R.F., Muller, L.D., Bauman, L.F., Colenbrander, V.F., 1971. *In vitro* dry matter disappearance of brown midrib mutants of maize (*Zea mays* L.). Journal of Animal Science 33, 881–884.

Bate, N.J., Orr, J., Ni, W., Meromi, A., Nadler-Hassar, T., Doerner, P.W., Dixon, R.A., Lamb, C.J., Elkind, Y., 1994. Quantitative relationship between phenylalanine ammonia-lyase levels and phenylpropanoid accumulation in transgenic tobacco identifies a rate-determining step in natural product synthesis. Proceedings of the National Academy of Sciences of the United States of America 91, 7608–7612.

Baucher, M., Bernard-Vailhé, M.A., Chabbert, B., Besle, J.-M., Opsomer, C., Van Montagu, M., Botterman, J., 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., Van Doorsselaere, J., Tollier, M.-T., Petit-Conil, M., Cornu, D., Monties, B., Van Montagu, M.,

Inzé, D., Jouanin, L., Boerjan, W., 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 Sciences 17, 125–197.

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

Bialski, A.M., Luthe, C.E., Fong, J.L., Lewis, N.G., 1986. Sulphite-promoted delignification of wood: identification of paucidisperse lignosulphonates. Canadian Journal of Chemistry 64, 1336–1344.

Björkman, A., 1954. Isolation of lignin from finely divided wood with neutral solvents. Nature 174, 1057–1058.

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

Blount, J.W., Korth, K.L., Masoud, S.A., Rasmussen, S., Lamb, C., 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.

Boudet, A.-M., 1998. A new view of lignification. Trends in Plant Sciences 3, 67–71.

Brauns, F.E., 1939. Native lignin. I. Its isolation and methylation. Journal of the American Chemical Society 61, 2120–2127.

Brauns, F.E., 1952. The Chemistry of Lignin. Academic Press, New York.

Brill, E.M., Abrahams, S., Hayes, C.M., Jenkins, C.L., Watson, J.M., 1999. Molecular characterisation and expression of a wound-inducible cDNA encoding a novel cinnamyl-alcohol dehydrogenase enzyme in lucerne (*Medicago sativa* L.). Plant Molecular Biology 41, 279–291.

Busam, G., Junghanns, K.T., Kneusel, R.E., Kassemeyer, H.-H., Matern, U., 1997. Characterization and expression of caffeoyl-coenzyme A 3-*O*-methyltransferase proposed for the induced resistance response of *Vitis vinifera* L.. Plant Physiology 115, 1039–1048.

Camm, E.L., Towers, G.H.N., 1973. Phenylalanine ammonia lyase. Phytochemistry 12, 961–973.

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

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

Chattaway, M., 1952. The sapwood-heartwood transition. Australian Forestry 16, 25–34.

Chen, C., Meyermans, H., Van Doorsselaere, J., Van Montagu, M., Boerjan, W., 1998. A gene encoding caffeoyl coenzyme A 3-*O*-methyltransferase (CCoAOMT) from *Populus trichocarpa* (Accession No. AJ223621). Plant Physiology 117, 719.

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

Creighton, R.H.J., McCarthy, J.L., Hibbert, H., 1941. Studies on lignin and related compounds. LIX. Aromatic aldehydes from plant materials. Journal of the American Chemical Society 63, 3049–3052.

Croteau, R., Kutchan, T.M., Lewis, N.G., 2000. Natural products (secondary metabolites). In: Buchanan, B.B., Gruissem, W., Jones, R.L. (Eds.), Biochemistry and Molecular Biology of Plants. American Society of Plant Physiologist, Rockville, MD, pp. 1250–1318.

Davin, L.B., Lewis, N.G., 1992. Phenylpropanoid metabolism: biosynthesis of monolignols, lignans and neolignans, lignins and suberins. In: Stafford, H.A., Ibrahim, R.K. (Eds.), Phenolic Metabolism in Plants, Vol. 26. Plenum Press, New York and London, pp. 325–375.

Duke, S.O., Vaughn, K.C., 1982. Lack of involvement of polyphenol oxidase in ortho-hydroxylation of phenolic compounds in mung bean seedlings. Physiologia Plantarum 54, 381–385.

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

Eberhardt, T.L., Bernards, M.A., He, L., Davin, L.B., Wooten, J.B., Lewis, N.G., 1993. Lignification in cell suspension cultures of *Pinus taeda*. *In situ* characterization of a gymnosperm lignin. The Journal of Biological Chemistry 268, 21088–21096.

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

Elkind, Y., Edwards, R., Mavandad, M., Hedrick, S.A., Ribak, O., Dixon, R.A., Lamb, C.J., 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 of the United States of America 87, 9057–9061.

Emerson, R. A., Beadle, G. W., Fraser, A. C., 1935. A Summary of Linkage Studies in Maize. Cornell University Agricultural Experimental Station Memoir 180.

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.

Fell, D., 1997. Understanding the Control of Metabolism. Portland Press, Ltd, London.

Fergus, B.J., Goring, D.A.I., 1970a. The distribution of lignin in birch wood as determined by ultraviolet microscopy. Holzforschung 24, 118–124.

Fergus, B.J., Goring, D.A.I., 1970b. The location of guaiacyl and syringyl lignins in birch xylem tissue. Holzforschung 24, 113–117.

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

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

Freedman, D., Pisani, R., Purves, R., 1978. Statistics. Norton & Company, Inc, New York.

Fukasawa-Akada, T., Kung, S.-D., Watson, J.C., 1996. Phenylalanine ammonia-lyase gene structure, expression, and evolution in *Nicotiana*. Plant Molecular Biolgy 30, 711–722.

Fukushima, K., Matsui, N., Taguchi, S., Yasuda, S., 1997. On the roles of monolignol glucosides and monolignol pathway (coniferyl alcohol—5-hydroxyconiferyl alcohol—sinapyl alcohol) in lignin biosynthesis of *Magnolia kobus*. In: 9th International Symposium on Wood and Pulping Chemistry, 9–12 June 1997, Montreal, Quebec, Canada 28-(21–24).

Galliano, H., Heller, W., Sandermann, H.J., 1993. Ozone induction and purification of spruce cinnamyl alcohol dehydrogenase. Phytochemistry 32, 557–563.

Gang, D.R., Fujita, M., Davin, L.B., Lewis, N.G., 1998. The "abnormal lignins": mapping heartwood formation through the lignan biosynthetic pathway. In: Lewis, N.G., Sarkanen, S. (Eds.), Lignin and Lignan Biosynthesis, Vol. 697. ACS Symposium Series, Washington, DC, pp. 389–421.

Gentinetta, E., Bertolini, M., Rossi, I., Lorenzoni, C., Motto, M., 1990. Effect of brown midrib-3 mutant on forage quality and yield in maize. Journal of Genetics and Breeding 44, 21–26.

Goffner, D., Joffroy, I., Grima-Pettenati, J., Halpin, C., Knight, M.E., Schuch, W., Boudet, A.M., 1992. Purification and characterization of isoforms of cinnamyl alcohol dehydrogenase from eucalyptus xylem. Planta 188, 48–53.

Grand, C., 1984. Ferulic acid 5-hydroxylase: a new cytochrome P-450-dependent enzyme from higher plant microsomes involved in lignin synthesis. Federation of European Biochemical Societies Letters 169, 7–11.

Grand, C., Parmentier, P., Boudet, A., Boudet, A.M., 1985. Comparison of lignins and of enzymes involved in lignification in normal and brown midrib (*bm*$_3$) mutant corn seedlings. Physiologie Végétale 23, 905–911.

Grima-Pettenati, J., Feuillet, C., Goffner, D., Borderies, G., Boudet, A.M., 1993. Molecular cloning and expression of a *Eucalyptus gunnii* cDNA clone encoding cinnamyl alcohol dehydrogenase. Plant Molecular Biology 21, 1085–1095.

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

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

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

He, L., Terashima, N., 1990. Formation and structure of lignin in monocotyledons. III. Heterogeneity of sugarcane (*Saccharum officinarum* L.) lignin with respect to the composition of structural units in different morphological regions. Journal of Wood Chemistry and Technology 10, 435–459.

Hepworth, D.G., Vincent, J.F.V., 1998. The mechanical properties of xylem tissue from tobacco plants (*Nicotiana tabacum* 'Samsun'). Annals of Botany 81, 751–759.

Hepworth, D.G., Vincent, J.F.V., 1999. The growth response of the stems of genetically modified tobacco plants (*Nicotiana tabacum* 'Samsun') to flexural stimulation. Annals of Botany 83, 39–43.

Hepworth, D.G., Vincent, J.F.V., Schuch, W., 1998. Using viscoelastic properties of the woody tissue from tobacco plants (*Nicotiana tabacum*) to comment on the molecular structure of cell walls. Annals of Botany 81, 729–734.

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

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

Hu, W.-J., Harding, S.A., Lung, J., Popko, J.L., Ralph, J., Stokke, D.D., Tsai, C.-J., Chiang, V.L., 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., Lung, J., Osakabe, K., Ebinuma, H., Chiang, V.L., 1998. Compartmentalized expression of two structurally and functionally distinct 4-coumarate:CoA ligase genes in aspen (*Populus tremuloides*). Proceedings of the National Academy of Sciences of the United States of America 95, 5407–5412.

Huang, X., Jeronimidis, G., Vincent, J.F.V., 1999. Mechanical properties of wood from transgenic poplar trees with modified lignification. The 2nd Symposium of Chinese Youth Scholars on Material Science and Technology 10, 1–9.

Humphreys, J.M., Hemm, M.R., Chapple, C., 1999. New routes for lignin biosynthesis defined by biochemical characterization of recombinant ferulate 5-hydroxylase, a multifunctional cytochrome P450-dependent monooxygenase. Proceedings of the National Academy of Sciences of the United States of America 96, 10045–10050.

Iiyama, K., Wallis, A.F.A., 1990. Determination of lignin in herbaceous plants by an improved acetyl bromide procedure. Journal of the Science of Food and Agriculture 51, 145–161.

Inoue, K., Sewalt, V.J.H., Ballance, G.M., Ni, W., Stürzer, 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.

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

Jorgenson, L.R., 1931. Brown midrib in maize and its linkage relations. Journal of the American Society of Agronomy 23, 549–557.

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

Jung, H.-J.G., Ni, W., Chapple, C.C.S., Meyer, K., 1999. Impact of lignin composition on cell-wall degradability in an *Arabidopsis* mutant. Journal of the Science of Food and Agriculture 79, 922–928.

Kajita, S., Hishiyama, S., Tomimura, Y., Katayama, Y., Omori, S., 1997a. 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:coenzyme A ligase. Plant Cell Physiology 37, 957–965.

Kajita, S., Mashino, Y., Nishikubo, N., Katayama, Y., Omori, S., 1997b. Immunological characterization of transgenic tobacco plants with a chimeric gene for 4-coumarate:CoA ligase that have altered lignin in their xylem tissue. Plant Science 128, 109–118.

Kawaoka, A., Ebinuma, H., 2001. Transcriptional control of lignin biosynthesis by tobacco LIM protein. Phytochemistry 57, 1149–1157.

Kawaoka, A., Kaothien, P., Yoshida, K., Endo, S., Yamada, K., Ebinuma, H., 2000. Functional analysis of tobacco LIM protein Ntlim1 involved in lignin biosynthesis. The Plant Journal 22, 289–301.

Klason, P., von Heidenstam, G., Norlin, E., 1908. Teoretiska undersökningar rörande kolning af ved. II. Torrdestillation af furu-, gran-, björk- och bokved. Arkiv för Kemi, Mineralogi och Geologi 3, 1–17.

Kneusel, R.E., Matern, U., Nicolay, K., 1989. Formation of *trans*-caffeoyl-coA from *trans*-4-coumaroyl-CoA by Zn^{2+}-dependent enzymes in cultured plant cells and its activation by an elicitor-induced pH shift. Archives of Biochemistry and Biophysics 269, 455–462.

Knight, M.E., Halpin, C., Schuch, W., 1992. Identification and characterisation of cDNA clones encoding cinnamyl alcohol dehydrogenase from tobacco. Plant Molecular Biology 19, 793–801.

Kojima, M., Takeuchi, W., 1989. Detection and characterization of *p*-coumaric acid hydroxylase in mung bean, *Vigna mungo*, seedlings. The Journal of Biochemistry 105, 265–270.

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.

Kuc, J., Nelson, O.E., 1964. The abnormal lignins produced by the *brown-midrib* mutants of maize. I. The *brown-midrib-1* mutant. Archives of Biochemistry and Biophysics 105, 103–113.

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

Kühnl, T., Koch, U., Heller, W., Wellmann, E., 1987. Chlorogenic acid biosynthesis: characterization of a light-induced microsomal 5-*O*-(4-coumaroyl)-D-quinate/shikimate 3′-hydroxylase from carrot (*Daucus carota* L.) cell suspension cultures. Archives of Biochemistry and Biophysics 258, 226–232.

Lacombe, E., Hawkins, S., Van Doorsselaere, J., Piquemal, J., Goffner, D., Poeydomenge, O., Boudet, A.-M., Grima-Pettenati, J., 1997. Cinnamoyl CoA reductase, the first committed enzyme of the lignin branch biosynthetic pathway: cloning, expression and phylogenetic relationships. The Plant Journal 11, 429–441.

Lai, Y.Z., Sarkanen, K.V., 1971. Isolation and structural studies. In: Sarkanen, K.V., Ludwig, C.H. (Eds.), Lignins. Occurence, Formation, Structure and Reactions. Wiley Interscience, New York, pp. 165–240.

Lapierre, C., Monties, B., Rolando, C., 1986. Thioacidolysis of poplar lignins: identification of monomeric syringyl products and characterization of guaiacyl-syringyl lignin fractions. Holzforschung 40, 113–118.

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

Lapierre, C., Tollier, M.-T., Monties, B., 1988. Mise en évidence d'un nouveau type d'unité constitutive dans les lignines d'un mutant de maïs *bm3*. Comptes Rendus de l'Académie des Sciences. Série III, Sciences de la Vie 307, 723–728.

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. The Plant Cell 9, 1985–1998.

Lewis, N.G., 1999. A 20th century roller coaster ride: a short account of lignification. Current Opinion in Plant Biology 2, 153–162.

Lewis, N. G., Davin, L. B., 1994. Evolution of lignan and neolignan biochemical pathways. In: Nes, W. D., (Ed.), Isopentenoids and Other Natural Products: Evolution and Function. ACS Symposium Series No. 562, pp. 202–246.

Lewis, N.G., Davin, L.B., 1999. Lignans: biosynthesis and function. In: Barton, D.H.R., Sir, Nakanishi, K., Meth-Cohn, O. (Eds.), Comprehensive Natural Products Chemistry, Vol. 1. Elsevier, London, pp. 639–712.

Lewis, N.G., Davin, L.B., Sarkanen, S., 1998. Lignin and lignan biosynthesis: distinctions and reconciliations. In: Lewis, N.G., Sarkanen, S. (Eds.), Lignin and Lignan Biosynthesis, Vol. 697. ACS Symposium Series, Washington, DC, pp. 1–27.

Lewis, N. G., Davin, L. B., Sarkanen, S., 1999. The nature and function of lignins. In: Barton, D. H. R., Sir, Nakanishi, K., Meth-Cohn, O., (Eds.), Comprehensive Natural Products Chemistry, Vol. 3, pp. 617–745.

Lewis, N.G., Goring, D.A.I., Wong, A., 1983. Fractionation of lignosulphonates released during the early stages of delignification. Canadian Journal of Chemistry 61, 416–420.

Lewis, N.G., Yamamoto, E., 1990. Lignin: occurrence, biogenesis and biodegradation. Annual Review of Plant Physiology and Plant Molecular Biology 41, 455–497.

Lewis, N.G., Yean, W.Q., 1985. High performance size-exclusion chromatography of lignosulphonates. Journal of Chromatography 331, 419–424.

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

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. The Journal of Biological Chemistry 275, 6537–6545.

Li, L., Popko, J.L., Zhang, X.-H., Osakabe, K., Tsai, C.-J., Joshi, C.P., Chiang, V.L., 1997. A novel multifunctional *O*-methyltransferase implicated in a dual methylation pathway associated with lignin biosynthesis in loblolly pine. Proceedings of the National Academy of Sciences of the United States of America 94, 5461–5466.

Liang, X., Dron, M., Cramer, C.L., Dixon, R.A., Lamb, C.J., 1989. Differential regulation of phenylalanine ammonia-lyase genes during plant development and by environmental cues. The Journal of Biological Chemistry 264, 14486–14492.

Lindermayr, C., Möllers, B., Fliegmann, J., Uhlmann, A., Lottspeich, F., Meimberg, H., Ebel, J., 2002. Divergent members of a soybean (*Glycine max* L.) 4-coumarate:coenzyme A ligase gene family: primary structure, catalytic properties and differential expression. European Journal of Biochemistry 296, 1304–1315.

Lorenz, D., Morris, D., 1995. How much energy does it take to make a gallon of ethanol? http://www.carbohydrateeconomy.org.

Lüderitz, T., Grisebach, H., 1981. Enzymic synthesis of lignin precursors: comparison of cinnamoyl-CoA reductase and cinnamyl alcohol:NADP$^+$ dehydrogenase from spruce (*Picea abies* L.) and soybean (*Glycine max* L.). European Journal of Biochemistry 119, 115–124.

Luthe, C.E., Lewis, N.G., 1986. Identification and characterization of paucidisperse lignosulphonates. Holzforschung 40 (Suppl.), 153–157.

Mansell, R.L., Babbel, G.R., Zenk, M.H., 1976. Multiple forms and specificity of coniferyl alcohol dehydrogenase from cambial regions of higher plants. Phytochemistry 15, 1849–4853.

Marita, J.M., Ralph, J., Hatfield, R.D., Chapple, C., 1999. NMR characterization of lignins in *Arabidopsis* altered in the activity of ferulate 5-hydroxylase. Proceedings of the National Academy of Sciences of the United States of America 96, 12328–12332.

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

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

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

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

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 of the United States of America 95, 6619–6623.

Meyermans, H., Morreel, K., Lapierre, C., Pollet, B., De Bruyn, A., Busson, R., Herdewijn, P., Devreese, B., Van Beeumen, J., Marita, J.M., Ralph, J., Chen, C., Burggraeve, B., Van Montagu, M., Messens, E., Boerjan, W., 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. The Journal of Biological Chemistry 275, 36899–36909.

Muller, L.D., Barnes, R.F., Bauman, L.F., Colenbrander, V.F., 1971. Variations in lignin and other structural components of brown midrib mutants of maize. Crop Science 11, 413–415.

Nedelkina, S., Jupe, S.C., Blee, K.A., Schalk, M., Werck-Reichhart, D., Bolwell, G.P., 1999. Novel characteristics and regulation of a divergent cinnamate 4-hydroxylase (CYP73A15) from French bean: engineering expression in yeast. Plant Molecular Biology 39, 1079–1090.

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.

Nose, M., Bernards, M.A., Furlan, M., Zajicek, J., Eberhardt, T.L., Lewis, N.G., 1995. Towards the specification of consecutive steps in macromolecular lignin assembly. Phytochemistry 39, 71–79.

Ohashi, H., Yamamoto, E., Lewis, N.G., Towers, G.H.N., 1987. 5-Hydroxyferulic acid in *Zea mays* and *Hordeum vulgare* cell walls. Phytochemistry 26, 1915–1916.

O'Malley, D., Porter, S., Sederoff, R., 1992. Purification, characterization, and cloning of cinnamyl alcohol dehydrogenase in loblolly pine (*Pinus taeda* L.). Plant Physiology 98, 1364–1371.

Osakabe, K., Tsao, C.C., Li, L., Popko, J.L., Umezawa, T., Carraway, D.T., Smeltzer, R.H., Joshi, C.P., Chiang, V.L., 1999. Coniferyl aldehyde 5-hydroxylation and methylation direct syringyl lignin biosynthesis in angiosperms. Proceedings of the National Academy of Sciences of the United States of America 96, 8955–8960.

Padmapriya, A.A., Just, G., Lewis, N.G., 1985. A new method for the esterification of sulphonic acids. Synthetic Communications 15, 1057–1062.

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. The Plant Journal 25, 193–202.

Pearce, G., Marchand, P.A., Griswold, J., Lewis, N.G., Ryan, C.A., 1998. Accumulation of feruloyltyramine and *p*-coumaroyltyramine in tomato leaves in response to wounding. Phytochemistry 47, 659–664.

Pichon, M., Courbou, I., Beckert, M., Boudet, A.-M., Grima-Pettenati, J., 1998. Cloning and characterization of two maize cDNAs encoding cinnamoyl-CoA reductase (CCR) and differential expression of the corresponding genes. Plant Molecular Biology 38, 671–676.

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

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

Provan, G.J., Scobbie, L., Chesson, A., 1997. Characterisation of lignin from CAD and OMT deficient *Bm* mutants of maize. Journal of the Science of Food and Agriculture 73, 133–142.

Ralph, J., 1997. Recent advances in characterizing 'non-traditional' lignins. 9th International symposium on wood and pulping chemistry 1, PL2/1–PL2/7.

Ralph, J., Hatfield, R.D., 1991. Pyrolysis-GC-MS characterization of forage materials. Journal of Agricultural and Food Chemistry 39, 1426–1437.

Ralph, J., Hatfield, R.D., Piquemal, J., Yahiaoui, N., Pean, M., Lapierre, C., Boudet, A.M., 1998. NMR characterization of altered lignins extracted from tobacco plants down-regulated for lignification enzymes cinnamyl-alcohol dehydrogenase and cinnamoyl-CoA reductase. Proceedings of the National Academy of Sciences of the United States of America 95, 12803–12808.

Ralph, J., Lapierre, C., Lu, F., Marita, J.M., Pilate, G., Doorsselaere, J.V., Boerjan, W., Jouanin, L., 2001. NMR evidence for benzodioxane structures resulting from incorporation of 5-hydroxyconiferyl alcohol into lignins of *O*-methyltransferase-deficient poplars. Journal of Agricultural and Food Chemistry 49, 86–91.

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.

Rubery, P.H., Fosket, D.E., 1969. Changes in phenylalanine ammonia lyase activity during xylem differentiation in *Coleus* and soybean. Planta 87, 54–62.

Sakakibara, A., Sasaya, T., Miki, K., Takahashi, H., 1987. Lignans and Brauns' lignins from softwoods. Holzforschung 41, 1–11.

Sarkanen, K.V., Ludwig, C.H., 1971. Definition and nomenclature. In: Sarkanen, K.V., Ludwig, C.H. (Eds.), Lignins. Occurrence, Formation, Structure and Reactions. Wiley Interscience, New York, pp. 1–41.

Sarkanen, S., 1998. Template polymerization in lignin biosynthesis. In: Lewis, N.G., Sarkanen, S. (Eds.), Lignin and Lignan Biosynthesis, Vol. 697. ACS Symposium Series, Washington, DC, pp. 194–208.

Schmitt, D., Pakusch, A.-E., Matern, U., 1991. Molecular cloning, induction, and taxonomic distribution of caffeoyl-CoA 3-*O*-methyltransferase, an enzyme involved in disease resistance. The Journal of Biological Chemistry 266, 17416–17423.

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

Sewalt, V.J.H., Ni, W., Blount, J.W., Jung, H.G., Masoud, S.A., Howles, P.A., Lamb, C., Dixon, R.A., 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.H., 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.

Smart, C.C., Amrhein, N., 1985. The influence of lignification on the development of vascular tissue in *Vigna radiata* L.. Protoplasma 124, 87–95.

Smith, D.C.C., 1955. *p*-Hydroxybenzoate groups in the lignin of aspen (*Populus tremula*). Journal of the Chemical Society 2347–2351.

Tamagnone, L., Merida, A., Parr, A., MacKay, S., Culianez-Macia, F.A., Roberts, K., Martin, C., 1998. The AmMYB308 and AmMYB330 transcription factors from antirrhinum regulate phenylpropanoid and lignin biosynthesis in transgenic tobacco. The Plant Cell 10, 135–154.

Tanaka, M., Kojima, M., 1991. Purification and characterization of *p*-coumaroyl-D-glucose hydroxylase of sweet potato (*Ipomoea batatas*) roots. Archives of Biochemistry and Biophysics 284, 151–157.

The Arabidopsis Genome Initiative, 2000. Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature 408, 796–815.

Timell, T.E., 1986. Compression Wood in Gymnosperms. Springer-Verlag, Berlin.

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.

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

Van Doorsselaere, J., Baucher, M., Feuillet, C., Boudet, A.M., Van Montagu, M., Inze, D., 1995b. Isolation of cinnamyl alcohol dehydrogenase cDNAs from two important economic species: alfalfa and poplar. Demonstration of a high homology of the gene within angiosperms. Plant Physiology and Biochemistry 33, 105–109.

van Heerden, P.S., Towers, G.H.N., Lewis, N.G., 1996. Nitrogen metabolism in lignifying *Pinus taeda* cell cultures. The Journal of Biological Chemistry 271, 12350–12355.

van Rensburg, H., Anterola, A.M., Levine, L.H., Davin, L.B., Lewis, N.G., 2000. Monolignol compositional determinants in loblolly pine: aromatic amino acid metabolism and associated rate-limiting steps. In: Glasser, W.G., Northey, R.A., Schultz, T.P. (Eds.), Lignin: Historical, Biological, and Materials Perspectives, Vol. 742. ACS Symposium Series, Washington, DC, pp. 118–144.

Van Soest, P.J., Robertson, J.B., Lewis, B.A., 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. Journal of Dairy Science 74, 3583–3597.

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

Wang, Z.-X., Li, S.-M., Löscher, R., Heide, L., 1997. 4-Coumaroyl coenzyme A 3-hydroxylase activity from cell cultures of *Lithospermum erythrorhizon* and its relationship to polyphenol oxidase. Archives of Biochemistry and Biophysics 347, 249–255.

Weller, R.F., Phipps, R.H., Cooper, A., 1985. The effect of the brown midrib-3 gene on the maturity and yield of forage maize. Grass and Forage Science 40, 335–339.

Westing, A.H., 1965. Formation and function of compression wood in gymnosperms. Botanical Reviews 31, 381–480.

Whiting, P., Goring, D.A.I., 1982. Chemical characterization of tissue fractions from the middle lamella and secondary wall of black spruce tracheids. Wood Science and Technology 16, 261–267.

Wyrambik, D., Grisebach, H., 1975. Purification and properties of isoenzymes of cinnamyl-alcohol dehydrogenase from soybean-cell-suspension cultures. European Journal of Biochemistry 59, 9–15.

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

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

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

Zhang, X.H., Dickson, E.E., Chinnappa, C.C., 1995. Nucleotide sequence of a cDNA clone encoding caffeoyl-coenzyme A 3-*O*-methyltransferase of *Stellaria longipes* (Caryophyllaceae). Plant Physiology 108, 429–430.

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

Zhong, R., Morrison III, W.H., Negrel, J., Ye, Z.-H., 1998. Dual methylation pathways in lignin biosynthesis. The Plant Cell 10, 2033–2045.

Zou, J., Taylor, D.C., 1994. Isolation of an *Arabidopsis thaliana* cDNA homologous to parsley (*Petroselinum crispum*) *S*-adenosyl-L-methionine:*trans*-caffeoyl-coenzyme A 3-*O*-methyltransferase, an enzyme involved in disease resistance. Plant Physiology and Biochemistry 32, 423–427.

Zuber, M.S., Colbert, T.R., Bauman, L.F., 1977. Effect of brown-midrib-3 mutant in maize (*Zea mays* L.) on stalk strength. Zeitschrift für Pflanzenzüchtung 79, 310–314.

Aldwin M. Anterola holds a PhD (2001) degree in Plant Physiology from Washington State University. He graduated cum laude from the University of the Philippines, with a BS Degree in Agricultural Chemistry (1994). He worked for a year in the same University, as a junior faculty teaching laboratory classes in general chemistry, organic chemistry and biochemistry. As a graduate student, he received the Helen and Loyal H. Davis Fellowship, a Student Award for Best Paper from the Phytochemical Society of North America, and an American Chemical Society (Cellulose and Paper Division) Graduate Student Award for his research. He currently works as a Scientific Editor for Phytochemistry, and as a part-time Postdoctoral Research Associate in the co-author's laboratory.

Norman G. Lewis is the Director of the Institute of Biological Chemistry and the Arthur M. and Katie Eisig-Tode Distinguished Professor at Washington State University. He was initially trained in Chemistry (BSc Honors, 1973, University of Strathclyde) before completing a PhD (1977) in organic chemistry (alkaloid biosynthesis) at the University of British Columbia (Vancouver, BC). Postdoctoral training was in alkaloid/Vitamin B12 biosynthesis with Sir Alan R. Battersby at Cambridge University. Professor Lewis' current research interests are mainly associated with biosynthesis of plant phenolics, including how their ordered deposition into various cell wall types is achieved. He is a Regional Editor of Phytochemistry, as well as serving on other editorial boards.