

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

Dianjing Guo, Fang Chen, Kentaro Inoue,¹ Jack W. Blount, and Richard A. Dixon²

Plant Biology Division, Samuel Roberts Noble Foundation, Ardmore, Oklahoma 73401

Transgenic alfalfa plants were generated harboring caffeic acid 3-O-methyltransferase (COMT) and caffeoyl CoA 3-O-methyltransferase (CCOMT) cDNA sequences under control of the bean phenylalanine ammonia-lyase *PAL2* promoter. Strong downregulation of COMT resulted in decreased lignin content, a reduction in total guaiacyl (G) lignin units, a near total loss of syringyl (S) units in monomeric and dimeric lignin degradation products, and appearance of low levels of 5-hydroxy guaiacyl units and a novel dimer. No soluble monolignol precursors accumulated. In contrast, strong downregulation of CCOMT led to reduced lignin levels, a reduction in G units without reduction in S units, and increases in β -5 linked dimers of G units. Accumulation of soluble caffeic acid β -D-glucoside occurred only in CCOMT downregulated plants. The results suggest that CCOMT does not significantly contribute to the 3-O-methylation step in S lignin biosynthesis in alfalfa and that there is redundancy with respect to the 3-O-methylation reaction of G lignin biosynthesis. COMT is unlikely to catalyze the in vivo methylation of caffeic acid during lignin biosynthesis.

INTRODUCTION

Lignin is the major structural component of secondarily thickened plant cell walls. It is a complex polymer of hydroxylated and methoxylated phenylpropane units, linked via oxidative coupling that probably is catalyzed by both peroxidases and laccases (Boudet et al., 1995). Lignin imparts mechanical strength to stems and trunks, and hydrophobicity to water-conducting vascular elements. Dicotyledonous angiosperm lignins contain two major monomer species, termed guaiacyl (G) and syringyl (S) units. The S and G units are linked through at least five different dimer bonding patterns (Davin and Lewis, 1992). The mechanisms that determine the relative proportions of these linkage types in a particular lignin polymer are currently unknown. Furthermore, there is considerable debate whether lignin composition and structure are tightly controlled or are flexible depending on monomer availability (Lewis, 1999; Sederoff et al., 1999).

There is considerable interest in the potential for genetic manipulation of lignin levels and/or composition to help

improve digestibility of forages and pulping properties of trees (Whetten and Sederoff, 1991; Boudet and Grima-Pettenati, 1996; Dixon et al., 1996). Lignin levels increase with progressive maturity in stems of forage crops, including legumes such as alfalfa (Jung et al., 1997b) and in grasses such as tall fescue (Buxton and Redfearn, 1997). In addition, the lignin composition changes with advanced maturity toward a progressively higher S/G ratio (Buxton and Russell, 1988). Both lignin concentration (Albrecht et al., 1987; Casler, 1987; Jung et al., 1997a) and lignin methoxyl content (S/G ratio; Sewalt et al., 1996) have been negatively correlated with forage digestibility for ruminant animals. Although several studies have linked decreased forage digestibility to increased S/G ratio as a function of increased maturity (Buxton and Russell, 1988; Grabber et al., 1992), other studies have questioned the effect of lignin composition on digestibility (Grabber et al., 1997). Softwood gymnosperm lignins essentially lack S residues and therefore are highly condensed. They are less amenable to chemical pulping, a finding that contradicts the suggested effect of S/G ratio on forage digestibility.

The formation of the G and S units of lignin requires the activity of O-methyltransferase enzymes. In angiosperms, the caffeic acid 3-O-methyltransferase (COMT) of lignin biosynthesis originally was described as bifunctional, converting caffeic acid to ferulic acid and 5-hydroxyferulic acid to

¹ Current address: Plant Research Laboratory, Michigan State University, East Lansing, MI 48824.

² To whom correspondence should be addressed. E-mail radixon@noble.org; fax 580-221-7380.

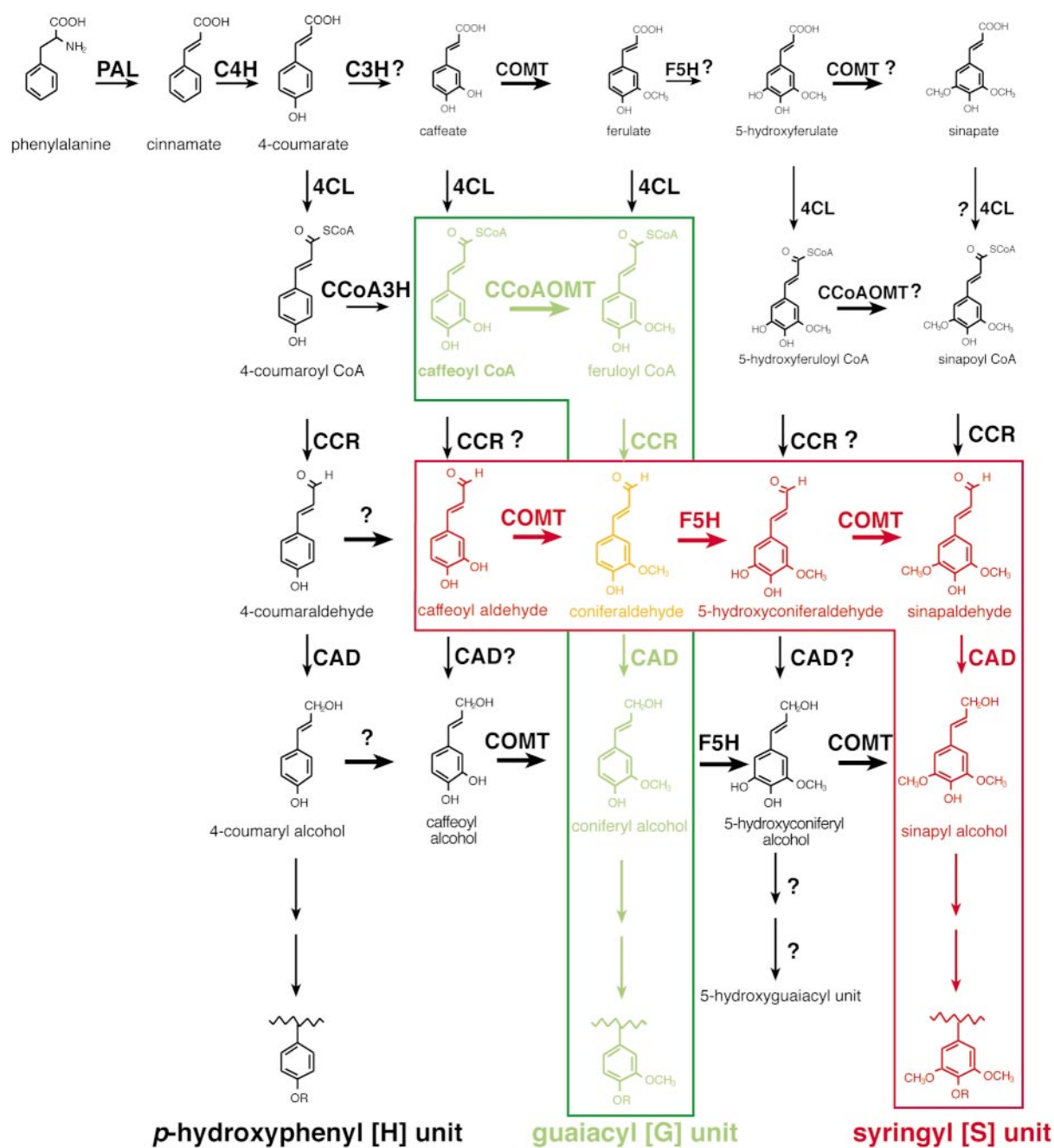


Figure 1. Pathways to Lignin Monomers.

The “metabolic grid” shown in this scheme incorporates the results of recent studies suggesting previously unexpected substrate specificities for F5H and COMT (Humphreys et al., 1999; Osakabe et al., 1999; Li et al., 2000). The pathway in green represents the most likely set of reactions leading to G lignin, and the reactions in red represent a favored pathway to S lignin. 4CL, 4-coumarate coenzyme A ligase; CAD, cinnamyl alcohol dehydrogenase; CCR, cinnamyl coenzyme A reductase.

sinapic acid (Davin and Lewis, 1992), as shown in Figure 1. Methylation of the caffeate moiety also has been shown to occur at the level of the CoA thiolester, catalyzed by caffeoyl CoA 3-O-methyltransferase (CCOMT) (Pakusch et al., 1989). The involvement of CCOMT in a parallel pathway to lignin monomer formation has been proposed (Ye et al., 1994; Zhong et al., 1998). It recently has been shown by in vivo labeling studies in *Magnolia kobus* that the methylation status of lignin monomers also can be determined at the level of the aldehyde or alcohol (Chen et al., 1999). This is supported by the observation that the enzyme designated as ferulate 5-hydroxylase (F5H) has a higher affinity for conifer-aldehyde than for ferulic acid, at least in sweet gum (Osakabe et al., 1999) and *Arabidopsis* (Humphreys et al., 1999). Furthermore, 5-hydroxyconifer-aldehyde recently has been shown to be a good substrate for COMT from various tree species (Li et al., 2000). It has been suggested that the inhibitory effect of 5-hydroxyconifer-aldehyde on methylation of caffeate by COMT might prevent COMT from performing the first methylation step in the biosynthesis of S lignin (Li et al., 2000). Thus, although studies of enzyme substrate specificity in vitro suggest that lignin monomers can be formed via the operation of a complex metabolic grid, involving O-methylation at multiple stages as shown in Figure 1, whether this occurs in vivo is now in doubt.

We are interested in the role of O-methyltransferases in lignin biosynthesis in the world's major forage legume, alfalfa (*Medicago sativa*). COMT from alfalfa expressed in *Escherichia coli* shows preference (~2:1) for 5-hydroxyferulic acid over caffeic acid, whereas CCOMT shows a similar degree of preference for caffeoyl CoA compared with 5-hydroxyferuloyl CoA (Inoue et al., 1998). This suggests, but does not prove, that COMT may be involved preferentially in the formation of S lignin in alfalfa, and CCOMT in the formation of G lignin.

Tissue print hybridization analysis indicated that both COMT and CCOMT transcripts are localized to developing xylem elements in alfalfa stems, whereas CCOMT transcripts also are found in phloem (Inoue et al., 1998). Immunolocalization studies at the light and electron microscope levels demonstrated expression of both COMT and CCOMT in the cytoplasm of alfalfa xylem parenchyma cells (Kersey et al., 1999). The presence of both enzymes in the same cells is consistent with the "metabolic grid" hypothesis for lignin monomer formation.

By independently downregulating COMT and CCOMT by gene-silencing and antisense approaches utilizing a lignin pathway gene promoter, we here assess whether these two enzymes function independently or redundantly with respect to the control of lignin content, composition, and structure (as determined by dimer linkage pattern) in alfalfa. Our results suggest, contrary to previously accepted models, that different pathways may operate for 3-O-methylation of monolignol precursors destined for G as compared with S lignin and that methylation of caffeic acid by COMT is unlikely to be a reaction in monolignol biosynthesis.

RESULTS

Cell Type Specificity of the Bean *PAL2* Promoter in Transgenic Alfalfa

Most studies on genetic modification of lignin biosynthesis in transgenic plants have utilized the cauliflower mosaic virus 35S promoter to drive expression of sense or antisense lignification-associated genes (Halpin et al., 1994; Ni et al., 1994; Atanassova et al., 1995; Doorsselaere et al., 1995; Piquemal et al., 1998; Zhong et al., 1998; Baucher et al., 1999). However, antisense downregulation of COMT and CCOMT in transgenic alfalfa is inefficient and relatively weak using 35S promoter constructs (V.J.H. Sewalt and R.A. Dixon, unpublished results). Recently, it was shown that modification of lignin composition by overexpression of F5H in transgenic *Arabidopsis* was more effective if the transgene was driven by the lignification-associated *Arabidopsis* cinnamate 4-hydroxylase promoter than by the constitutive 35S promoter (Meyer et al., 1998). We therefore decided to utilize the previously characterized lignification-associated bean phenylalanine ammonia-lyase *PAL2* promoter, which is strongly expressed in the vascular tissue of transgenic tobacco (Leyva et al., 1992; Shuffelbottom et al., 1993), to drive expression of COMT and CCOMT transgenes in alfalfa.

To confirm tissue specificity of the bean *PAL2* promoter in transgenic alfalfa, we generated several independent plants

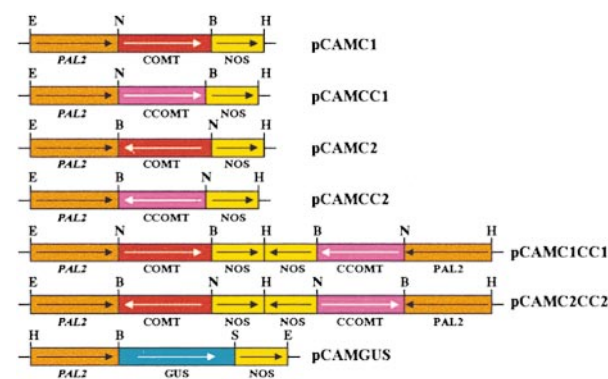


Figure 2. Binary Constructs Used for Genetic Modification of COMT and CCOMT Expression in Transgenic Alfalfa.

PAL2 is the bean phenylalanine ammonia-lyase *PAL2* promoter from -183 to -1226 bp (Liang et al., 1989), and NOS is the nopaline synthase terminator. COMT and CCOMT sequences are color-coded, and their directionality is indicated by the arrows relative to the direction of the *PAL2* promoter. Constructs containing both COMT and CCOMT in sense or antisense orientations were made by duplication of the *PAL2*/COMT/NOS and *PAL2*/CCOMT/NOS cassettes, and therefore each cDNA is under control of a separate *PAL2* promoter. All constructs are in the binary vector pCAMBIA3300. B, BamHI; E, EcoRI; H, HindIII; N, NdeI; S, SmaI.

via *Agrobacterium*-mediated transformation with a binary vector containing the β -glucuronidase (*GUS*) reporter gene under control of the full-length (182 to –1226) bean *PAL2* promoter, as illustrated in Figure 2. Figure 3 shows that staining transverse sections from these plants with the chromogenic substrate X-gluc revealed *GUS* expression in the vascular tissue of roots, stems, and petioles that was absent from similarly stained nontransgenic control tissue. Although most of the staining in stem and petiole tissue was localized to vascular parenchyma and cambial cells, there was also some staining of parenchyma cells and epidermal cells of petioles, and some sporadic staining in parenchyma cells of stems. These results were reproduced in other independent transformants. The vascular tissue staining suggested that the bean *PAL2* promoter would be suitable for directing expression of COMT and CCOMT sense and antisense transgenes for lignin modification in alfalfa.

Generation of Transgenic Alfalfa Plants with Altered Expression of COMT and CCOMT

Full-length alfalfa COMT and CCOMT cDNA sequences in the sense and antisense orientations were placed under

control of the bean *PAL2* promoter in the binary vector pCAMBIA3300, as described in Methods and summarized in Figure 2. Additional constructs contained tandem COMT and CCOMT cDNAs, in sense or antisense orientations, with each cDNA driven independently by a bean *PAL2* promoter, as shown in Figure 2, and introduction of both transgenes into a single plant was also achieved by co-transformation (Irdani et al., 1998) with single COMT and CCOMT constructs. Constructs were introduced into alfalfa by *Agrobacterium*-mediated transformation of leaf discs followed by regeneration through somatic embryogenesis. After regeneration and transfer to the greenhouse, plants first were analyzed for integration of COMT and CCOMT transgenes by polymerase chain reaction (PCR). Approximately 80% of the plants surviving selection were PCR positive.

Internode samples (sixth to ninth internodes) from stems of putative transformants were harvested from greenhouse grown plants of the same developmental age and assayed for COMT and CCOMT enzymatic activity, as shown in Figure 4. It was important to exclude younger internodes (first to fourth) from the tissue used for enzyme analysis, because these contain a second form of COMT that is not recognized by the antiserum raised against the alfalfa COMT targeted by the present transgenic strategy (Inoue et al., 2000). There

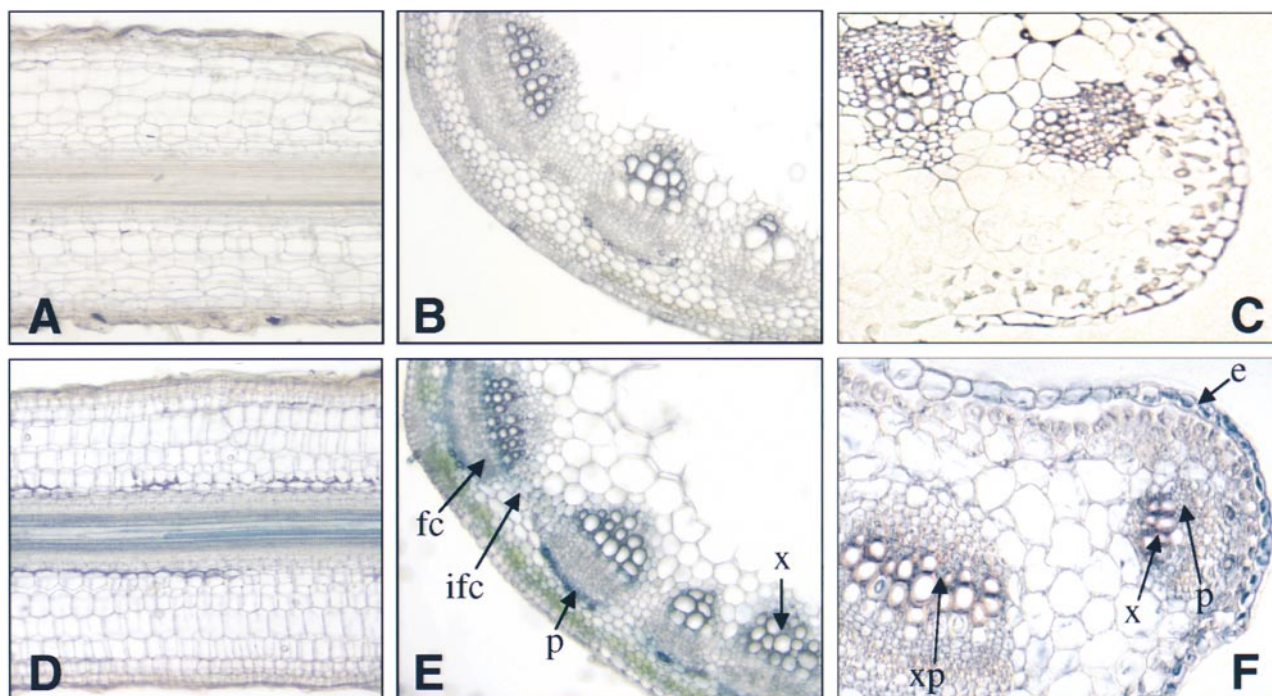


Figure 3. Tissue-Specific Expression of the Bean *PAL2* Promoter in Transgenic Alfalfa.

Cross-sections were taken of root ([A] and [D]), stem ([B] and [E]), and petiole ([C] and [F]) of transgenic alfalfa containing empty pCAMBIA3300 vector ([A] to [C]) or the *PAL2*-GUS construct pCAMGUS ([D] to [F]), and stained with X-Gluc to show *GUS* expression. e, epidermis; fc, fascicular cambium; ifc, interfascicular cambium; p, phloem; x, xylem.

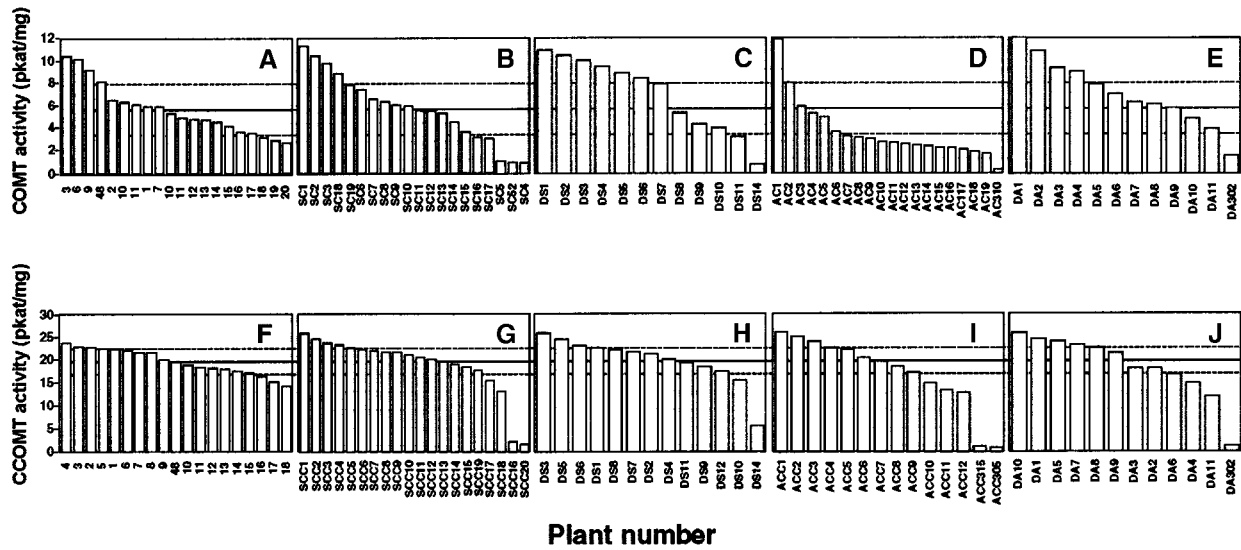


Figure 4. COMT or CCOMT Activities in Stem Tissue of Control and COMT/CCOMT Transgenic Lines.

(A) COMT activity in control plants transformed with empty pCAMBIA3300 vector.

(B) COMT activity in plants transformed with COMT in the sense orientation.

(C) COMT activity in plants transformed with a construct containing both COMT and CCOMT in the sense orientation, or by co-transformation with individual sense COMT and CCOMT constructs.

(D) COMT activity in plants transformed with COMT in the antisense orientation.

(E) COMT activity in plants transformed with a construct containing both COMT and CCOMT in the antisense orientation, or by co-transformation with individual sense COMT and CCOMT constructs.

(F) CCOMT activity in control plants transformed with empty pCAMBIA3300 vector.

(G) CCOMT activity in plants transformed with CCOMT in the sense orientation.

(H) CCOMT activity in plants transformed with a construct containing both COMT and CCOMT in the sense orientation, or by co-transformation with individual sense COMT and CCOMT constructs.

(I) CCOMT activity in plants transformed with CCOMT in the antisense orientation.

(J) CCOMT activity in plants transformed with a construct containing both COMT and CCOMT in the antisense orientation, or by co-transformation with individual sense COMT and CCOMT constructs.

The bars represent the mean and standard deviation of the control population. Enzyme activities were determined in the sixth to ninth internodes of stems of identical developmental stage.

was a wide (nearly fourfold) variation in COMT activity in a control population of 20 independent plants transformed with empty pCAMBIA3300 vector, as shown in Figure 4A. Of 20 transformants containing the single COMT sense sequence, three lines (SC4, SC5, and SC52) had strongly reduced COMT activities, whereas the remainder of the population exhibited, on average, a small increase in COMT activity (6.56 ± 2.46 pkat/mg protein, $n = 17$) compared with the average value for the control population (5.70 ± 2.43 pkat/mg protein, $n = 20$), as shown in Figure 4B. A similar situation was seen with respect to COMT activity in the double sense transformants shown in Figure 4C, with one plant (DS14) showing strongly downregulated COMT activity and the remainder of the population having a slightly elevated average COMT activity (7.54 ± 2.80 pkat/mg protein, $n = 11$) compared with the controls. In the COMT antisense population shown in Figure 4D, a single plant (AC310)

had strongly reduced COMT activity, with the remainder of the overall population showing on average a small reduction in COMT activity (3.81 ± 2.54 pkat/mg protein, $n = 19$) compared with the average value for the control population. In the double antisense lines (Figure 4E), one plant (DA302) showed strongly reduced COMT activity. It should be noted that although PCR analysis suggested genomic integration of both COMT and CCOMT sequences from the tandem constructs, the only double transformants showing enzyme downregulation arose from co-transformation with single COMT and CCOMT constructs.

There was less variation in CCOMT than in COMT activity in the empty vector control population, as seen by a comparison of Figures 4A and 4F. Otherwise, the pattern of CCOMT activities in the transformants harboring sense and antisense CCOMT constructs was very similar to that observed for COMT. CCOMT activity was strongly downregulated in

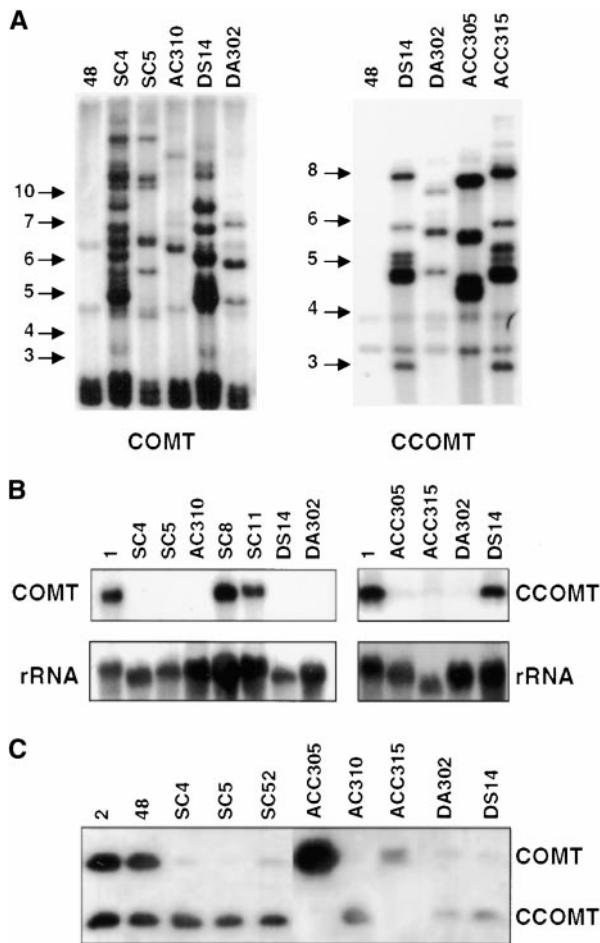


Figure 5. Molecular Characterization of Transgenic Alfalfa Harboring COMT and CCOMT Sense and Antisense Constructs.

(A) DNA gel blot analysis of selected lines. DNA was cut with HindIII, and blots were probed with full-length COMT or CCOMT cDNA sequences at high stringency. Molecular size markers (in kilobases) are shown at the left-hand sides of each panel.

(B) RNA gel blot analysis of selected lines. Blots were probed with full-length COMT or CCOMT cDNA sequences at high stringency.

(C) Protein gel blot analysis of selected lines. Protein extracts from the sixth to ninth internodes of the individual COMT and CCOMT transformants shown, and two empty vector control plants (2 and 48), were separated by SDS-PAGE, blotted to nylon membranes, and probed with monospecific polyclonal antisera raised against recombinant alfalfa COMT or CCOMT proteins.

two CCOMT sense lines shown in Figure 4G, in one double sense line (DS14; Figure 4H), in two antisense lines (ACC305 and ACC315; Figure 4I), and in one double COMT/CCOMT antisense line (DA302; Figure 4J). As with the COMT transgenic lines, we could find no sense CCOMT lines in which CCOMT activity was highly elevated.

Figure 5A shows transgene integration patterns for se-

lected control and COMT/CCOMT downregulated transgenic lines as analyzed by DNA gel blot border analysis. The results reflect multiple transgene insertions in independent transformants. The sense transformants SC4 and DS14 contained particularly high transgene copy numbers. The integration patterns in double transformants DS14 and DA302 reflected their arising from co-transformation events.

RNA gel blot analysis confirmed that the reduced COMT or CCOMT activity in the various lines resulted from a severe reduction in COMT or CCOMT transcript levels, as shown in Figure 5B. COMT transcripts were almost undetectable in the total RNA fraction from sense lines SC4, SC5, antisense line AC310, the double sense line DS14, and the double antisense line DA302. CCOMT transcripts were likewise virtually undetectable in antisense lines ACC305 and ACC315 and in the double antisense line DA302. However, CCOMT transcripts were relatively unaffected in the double sense line DS14, in which CCOMT activity is reduced to ~23% of control levels.

Comparison of COMT and CCOMT protein levels by protein gel blot analysis in Figure 5C indicated almost complete loss of COMT protein in the sense lines SC4, SC5, and SC52, in the antisense line AC310, in the double antisense line DA302, and in the double sense line DS14. CCOMT protein levels were almost undetectable in the antisense lines ACC305 and ACC315 and were strongly reduced in the double antisense line DA302 and the double sense line DS14. Interestingly, complete loss of CCOMT protein in the CCOMT antisense line ACC305 was accompanied by an increase in COMT protein level, as documented in Figure 5C, and in COMT enzymatic activity (see below). The above results indicate that expression of OMT sequences from the bean *PAL2* promoter results in downregulation of COMT and CCOMT that is similar to or greater than that obtained in previous studies in tobacco and poplar (Ni et al., 1994; Atanassova et al., 1995; Van Doorselaere et al., 1995; Zhong et al., 1998), in contrast with our previous results using 35S promoter-driven constructs in alfalfa.

Reduction of enzymatic activity resulting from reduced transcript levels in plants expressing gene constructs in the sense orientation is characteristic of epigenetic gene silencing, which may occur at the transcriptional or post-transcriptional level (Vaucheret et al., 1998). To determine the basis for the reduced COMT and CCOMT activities in some of the sense transgenic lines, nuclear run-on transcription analyses were performed with transcripts completed in vitro from nuclei isolated from empty vector and COMT or CCOMT downregulated sense lines. The transcription rates of both COMT and CCOMT were essentially the same in empty vector control and OMT downregulated lines (data not shown). However, the data in Figure 5B indicate that the steady state transcript levels in the sense COMT and CCOMT lines were only a fraction of the control levels, consistent with post-transcriptional gene silencing being responsible for reduced COMT and CCOMT expression in the sense transgene lines.

Effects of Downregulation of COMT and CCOMT on Lignin Content and Composition

Table 1 summarizes the COMT and CCOMT activities in several independent transgenic alfalfa lines selected for further study of lignin characteristics. Downregulation of COMT had no effect on the activity of CCOMT, and vice versa, with one exception. The reduction of CCOMT to <4% of wild-type activity in line ACC305 was associated with an approximate doubling of COMT activity as compared with wild-type levels, a finding consistent with the protein gel blot data in Figure 5C.

Klason lignin levels of three independent control lines averaged 17.6% of dry matter; this value was reduced to between 15.5 and 12.5% in all lines with downregulated COMT or CCOMT activity. The largest reductions in Klason lignin (down to 70% of the wild-type value) were in lines with gene-silenced COMT. However, Klason lignin also was reduced in line ACC305, which has only 3.6% of the wild-type CCOMT activity but nearly double the wild-type COMT activity, and in line AC315, with <5% wild-type CCOMT activity but normal COMT activity. Thus, reductions in either COMT or CCOMT activities can independently reduce Klason lignin levels in alfalfa. In contrast to the effects on Klason lignin, downregulation of neither OMT appeared to have any significant effect on acetyl bromide extractable lignin.

Staining of transverse stem sections with phloroglucinol-HCl was similar in control and COMT and CCOMT downregulated lines, as shown in Figure 6. The intensity of phloro-

glucinol staining often is taken as reflecting lignin content, although the reagent appears most specific for coniferaldehyde end groups in lignin (Lewis and Yamamoto, 1990). In contrast, staining with Maule reagent gave a red coloration in wild-type plants but a brown coloration in COMT downregulated lines. Such a color shift from red to brown is believed to be diagnostic for reduction of S lignin (Lewis and Yamamoto, 1990).

Thioacidolysis is the most efficient method for degrading the lignin polymer to reveal lignin monomer composition (Lapierre et al., 1985). The monomeric lignin degradation products then are determined by gas chromatography mass spectrometry (GC/MS). The data from such analyses shown in Figure 7 and Table 1 indicate that reduction in lignin levels in plants with downregulated COMT activity is associated with a much greater decrease in S units than in G units following thioacidolysis, resulting in a large decrease in S/G ratio, consistent with the results of histochemical staining with Maule reagent. In fact, thioacidolysis products of S lignin were not detected at all in the COMT antisense line AC310. In contrast, there was no reduction in S lignin in lines with reduced CCOMT activity, unless there was a corresponding decrease in COMT activity, as in the double sense and antisense lines. However, levels of G lignin were most strongly reduced in line ACC305, the line with the greatest decrease in CCOMT activity, but in which COMT was double wild-type levels. Overall, the data clearly indicate that COMT downregulation impacts both S and G lignin, with greatest effects on S lignin, whereas CCOMT downregulation only

Table 1. O-Methyltransferase Activities and Lignin Content and Composition in Transgenic Alfalfa with Modified Expression of COMT and CCOMT

Line	COMT (pkat/mg)	CCOMT (pkat/mg)	Acetyl Bromide Lignin (% Dry Wt ^a)	Klason Lignin (% Dry Wt)	S Lignin (μ mol/g Dry Wt)	G Lignin (μ mol/g Dry Wt)	5-Hydroxy G Lignin (μ mol/g Dry Wt)	S/G Ratio
Control								
1	5.98	22.35	16.07	17.21	158.8	305.4	0	0.52
2	6.55	23.77	17.79	17.91	152.8	325.2	0	0.47
48	8.19	21.13	17.52	17.64	156.2	279.4	0	0.56
COMT silenced								
SC4	1.06	20.11	16.48	12.67	10.4	227.9	7.4	0.05
SC5	1.24	22.26	16.35	12.46	8.6	246.1	8.8	0.04
SC52	1.19	22.36	16.83	14.15	17.1	223.7	1.8	0.07
COMT antisense								
AC310	0.31	22.17	16.97	15.30	0	248.2	0	0
CCOMT antisense								
ACC305	14.39	0.78	16.36	14.58	159.1	150.2	0	1.05
ACC315	8.06	1.07	15.31	15.50	164.0	243.6	0	0.69
COMT/CCOMT silenced								
DS14	0.78	5.59	16.54	14.72	54.0	223.7	0	0.23
COMT/CCOMT antisense								
DA302	0.81	1.15	16.78	15.06	14.0	303.0	0	0.05

^aWt, weight.

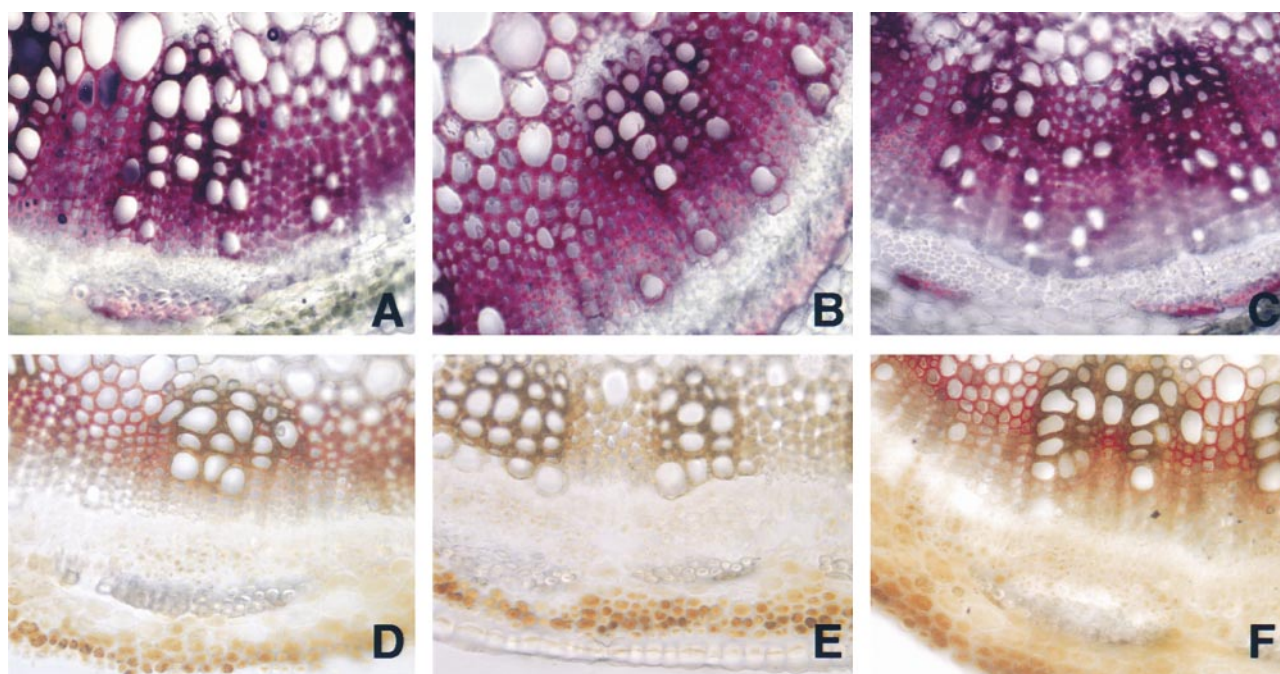


Figure 6. Histochemical Analysis of Lignin in Transverse Stem Sections (Fifth Internode) of Control and COMT or CCOMT Antisense Alfalfa. (A) to (C) Phloroglucinol staining of wild type (empty vector control) (A), antisense COMT line AC310 (B), and antisense CCOMT line ACC305 (C). (D) to (F) Maule reagent staining of wild type (empty vector control) (D), antisense COMT line AC310 (E), and antisense CCOMT line ACC305 (F).

affects extractable G lignin in alfalfa stems. Surprisingly, simultaneous reduction of both COMT and CCOMT reduced G lignin no further than independent reduction of either enzyme, as seen for lines DS14 and DA302.

Analysis of GC traces from the thioacidolysis reactions revealed new peaks in the reaction products from lignin with reduced S/G ratio, as shown in Figures 7A to 7C. These peaks were identified as originating from 5-hydroxyguaiacyl moieties that might be expected to be present if S lignin biosynthesis were being blocked primarily at the second methylation stage in COMT downregulated plants. However, the levels of 5-hydroxyguaiacyl units were always much less than the corresponding reduction in S units, as shown for three COMT downregulated lines in Table 1. Interestingly, the COMT downregulated line AC310, which completely lacked thioacidolysis recoverable S lignin, did not appear to contain 5-hydroxyguaiacyl units in its lignin.

Effects of Downregulation of COMT and CCOMT on Lignin Monomer Linkage Pattern

In the intact lignin polymer, the various monomeric units are linked to each other through covalent bonding at several different positions. Thioacidolysis releases the monomers that

are joined together by the most abundant linkage between the β position on the side chain of one monolignol and the 4-O position on a second unit. Other linkages are less easy to degrade, but their nature can be determined by analysis of dimers by GC/MS after thioacidolysis followed by Raney nickel desulfurization (Lapierre et al., 1995). These linkage types are illustrated in Figure 8. 5-5 and 4-O-5 linkages only occur between G units, whereas β - β linkages only occur between S units. β -1, β -5, and cyclic β -5 linkages can occur between two G units or between a G and an S unit. Thus, these five basic linkage types can result in nine different lignin dimers.

The levels of these various dimers were analyzed by GC/MS, as described in Methods, from the series of control and COMT or CCOMT downregulated alfalfa plants previously analyzed for lignin content and monomer composition. Representative GC chromatograms are shown in Figures 7D to 7F. The results in Figure 8 indicate that reduction of COMT activity resulted in at most a small increase in the recovery of dimers consisting of two G units (5-5, 4-O-5, β -1 [G], β -5 [G], cyclic β -5 [G]). However, there was a total loss of recovered dimers with β - β or mixed β -1 or cyclic β -5 linkages, which all involve S units, in plants with reduced COMT activity. In contrast, reduction of CCOMT activity did not lead to a reduction in dimers containing S units. Rather, CCOMT

downregulation appeared to lead to increased recovery of β -5 (G) dimers but a reduction in cyclic β -5 (G) dimers. Lignin from line ACC305, which is reduced in CCOMT but has approximately double the wild-type COMT activity, had the highest proportion of β - β linked S units.

Qualitative changes in lignin dimers resulting from OMT downregulation also were noted in this study. Thus, gas chromatograms of thioacidolysis/Raney nickel desulfurization products of lignin from five independent COMT downregulated plants exhibited a new dimer peak at 52.9 min retention time that was absent from corresponding traces from all empty vector or CCOMT downregulated plants, as shown by comparing the GC traces in Figures 7D to 7F. This compound was analyzed by mass spectrometry and shown to have a molecular ion of m/z 504, identical to that of the γ -*p*-coumarate ester of a syringyl unit, a dimer previously identified in maize lignin (Grabber et al., 1996). However, the retention time of the new dimer and its MS fragmentation pattern were very similar, but not identical, to those of an authentic sample of the S-coumarate ester. The structure of this new dimer that was only recoverable after downregulation of COMT must await NMR analysis.

Effects of Downregulation of COMT and CCOMT on Wall-Esterified Hydroxycinnamic Acids and Soluble Monolignol Precursors

To determine whether downregulation of COMT or CCOMT has quantitative and/or qualitative effects on wall-bound phenylpropanoids other than lignin, we extracted cell wall-esterified phenolic compounds from internodes 5 to 9 of the various transgenic lines analyzed previously. HPLC analysis of wall-bound hydroxycinnamic acids released by alkali hydrolysis revealed no significant differences in the levels of 4-coumaric and ferulic acids between control and OMT downregulated lines, as shown in Table 2. This suggests that neither COMT nor CCOMT is involved in the biosynthesis of wall-bound ferulic acid in lignifying alfalfa stems.

Analysis of soluble phenolic compounds in stem extracts revealed striking differences between control, COMT downregulated, and CCOMT downregulated plants, as shown in Figure 9. Several new peaks, including a major one of retention time $\sim$ 18 min (peak 2), were observed in soluble extracts from CCOMT downregulated plants. These peaks were present in extracts from all lines downregulated in CCOMT, including the double antisense line DA302 (data not shown). In contrast, the only significant peak in addition to the common peak 1 in extracts from COMT only downregulated plants appeared at retention time 41 min (peak 3) and was highest in all the COMT only downregulated lines.

Peak 2 was identified as caffeoyl β -D-glucoside by analysis of chromatographic behavior and UV light absorption spectra of the purified material before and after treatment with β -glucosidase, and by HPLC/MS, which revealed a molecular ion (negative ion mode) of m/z 341, with a second

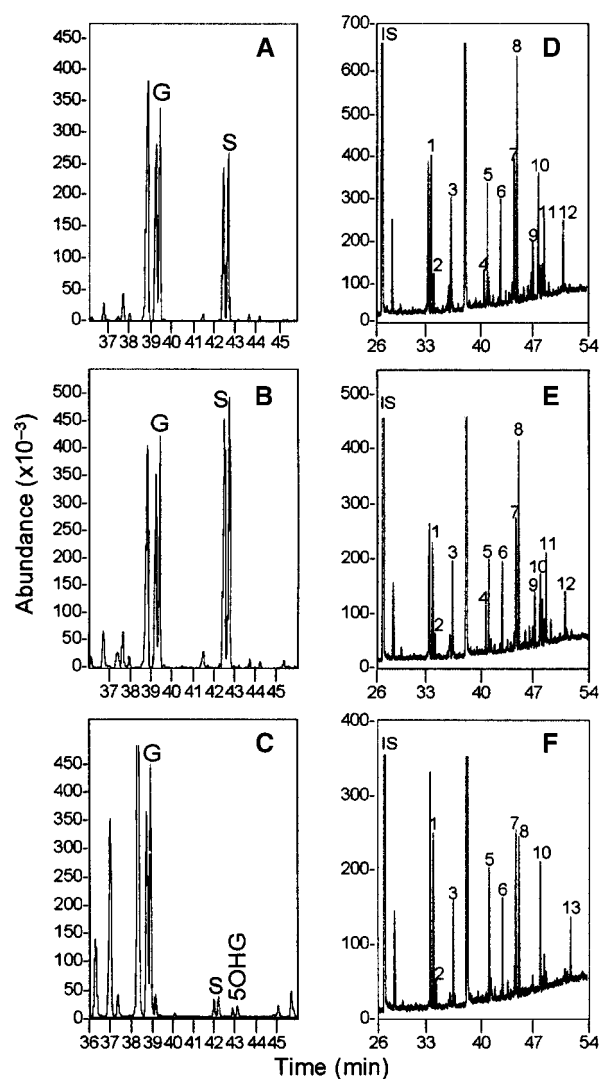
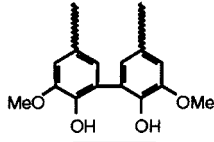


Figure 7. Typical Gas Chromatograms Showing Thioacidolysis Products from Lignin Samples of Wild-Type (Empty Vector Control; [A] and [D]), CCOMT-Suppressed Line ACC305 ([B] and [E]), and COMT-Suppressed Line SC5 ([C] and [F]) Alfalfa Plants.

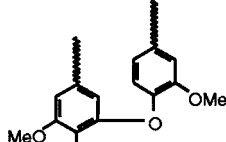
(A) to (C) Monomeric thioacidolysis products. G, S, and 5-hydroxyguaiacyl (5OHG) units are marked. The peaks appear as doublets because of the formation of *erythro* and *threo* isomers of each degradation product.

(D) to (F) Dimeric thioacidolysis products. The peaks represent the following linkage types: 1, 5-5 (G); 2, 4-O-5 (G); 3, β -1 (G); 4, β -1 (M); 5, β -5 (G); 6, β -1 (G); 7, β -5 (G); 8, β -5 (M); 9, β - β (S); 10, cyclic β -5 (G); 11, β - β (S); 12, cyclic β -5 (M); 13, unidentified dimer "x." M, mixture of S and G units.

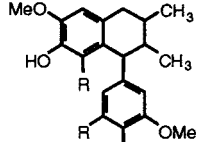
Line	5-5,G	4-O-5,G	β -1,G	β -1,M	β -5,G	β -5,M	β - β ,S	Cyclic β -5,G	Cyclic β -5,M	X
2 (control)	13.0	3.6	17.9	3.2	19.4	16.9	9.6	11.0	5.0	0.0
48 (control)	13.3	3.6	17.5	2.4	20.4	14.0	9.5	12.4	6.3	0.0
SC4	16.6	4.1	21.9	0.0	24.6	19.0	0.0	14.2	0.0	9.4
SC5	17.3	3.6	20.3	0.0	24.7	14.0	0.0	19.2	0.0	7.1
AC310	15.5	3.4	19.1	0.0	23.7	15.0	0.0	10.6	0.0	12.4
DA302	16.9	4.0	21.9	0.0	30.7	6.4	0.0	9.0	0.0	10.5
DS14	15.0	3.3	20.7	0.0	21.5	18.9	0.0	12.8	0.0	7.5
ACC305	11.2	2.9	18.6	5.2	22.2	19.5	14.8	10.1	6.6	0.0
ACC315	14.8	3.6	23.5	5.7	30.1	10.3	4.5	6.2	3.1	0.0



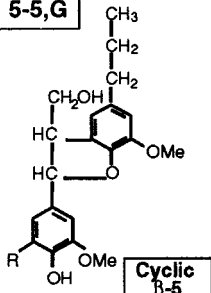
5-5,G



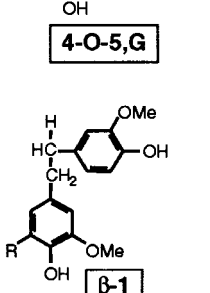
4-O-5,G



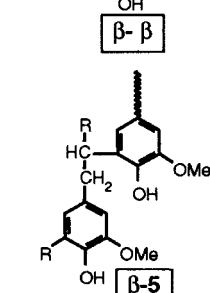
β - β



Cyclic
 β -5



β -1



β -5

Figure 8. The Dimer Bonding Patterns of Lignin Samples from Empty Vector Control, COMT-Suppressed, and CCOMT-Suppressed Alfalfa Plants.

Dimer composition was determined by thioacidolysis followed by Raney nickel desulfurization. Units for recovered dimer levels are molar percentage of total dimers. The Klason lignin levels and S/G ratios of the various lines are shown in Table 1. X is the unidentified dimer with M/z ratio of 504. The footnote shows the chemical structures of the major dimers recovered from lignin after thioacidolysis and Raney nickel desulfurization. G, guaiacyl units; S, syringyl units; M, mixture of G and S units.

major ion of m/z 179, corresponding to the caffeic acid aglycone. Peak 3 could not be identified by HPLC/MS, but on the basis of selective ion monitoring MS was not a monolignol precursor. Indeed, the only monolignol-related metabolite that increased compared with controls in selective ion monitoring analysis was caffeoyl β -D-glucoside in extracts from CCOMT downregulated plants. Thus, COMT downregulation, although having profound effects on S lignin biosynthesis, surprisingly does not appear to lead to the accumulation of any soluble precursors of S or G lignin.

Caffeoyl β -D-glucoside was purified by HPLC and tested as a substrate for O-methylation by incubation with alfalfa stem extracts and ^{14}C -S-adenosyl-L-methionine. Positive control incubations were performed with caffeoyl CoA as substrate. No methylation of caffeoyl β -D-glucoside was observed with extracts from wild-type or CCOMT downregulated plants, indicating that this compound arises by conjugation of caffeic acid following downregulation of CCOMT, rather than by virtue of its being a natural substrate for CCOMT.

DISCUSSION

The Role of COMT and CCOMT in the Biosynthesis of Wall-Bound Phenolic Compounds

Perhaps surprisingly, considering that ferulic acid is the direct product of the action of COMT on caffeic acid, and that CCOMT was first proposed to be involved in production of cell wall-esterified ferulic acid during plant defense (Pakusch et al., 1989), we could detect no significant effect of COMT or CCOMT downregulation on the levels of cell wall-esterified ferulic acid. This contrasts with a previous observation of increased wall-esterified hydroxycinnamic acids in transgenic plants in which lignin content had been reduced by downregulation of coumarate CoA ligase (Hu et al., 1999). One possible explanation is that wall-esterified ferulic acid in alfalfa is produced by the action of the COMT II enzyme with a low K_m for caffeic acid that is present only in young internodes (Inoue et al., 2000). A similar model involving an

“early” form of COMT recently has been proposed for esterified ferulic acid formation in maize, in which, as in alfalfa, multiple forms of COMT exist with different temporal developmental expression patterns (Lam et al., 1996).

Both COMT and CCOMT Impact Lignin Quantity in Alfalfa

There have been several reports on the effects of downregulation of COMT activity on lignin content and composition in transgenic plants (Ni et al., 1994; Atanassova et al., 1995; Van Doorselaere et al., 1995; Zhong et al., 1998). The results of these studies have been somewhat contradictory, possibly due to unspecified differences in tissue maturity, use of homologous versus heterologous transgenes, and use of different methods for lignin analysis. However, in cases in which COMT has been reduced to levels below ~20% of wild type by expression of a homologous transgene, a strong reduction in S/G ratio is accompanied by no apparent change in lignin content (Atanassova et al., 1995; Van Doorselaere et al., 1995). In the only report we are aware of regarding downregulation of CCOMT, there is a corresponding decrease in Klason lignin levels (Zhong et al., 1998).

The present results indicate that strong downregulation of either enzyme in alfalfa leads to up to 30% decreases in Klason lignin levels. However, levels of acetyl bromide soluble lignin were not reduced. The Klason and acetyl bromide methods measure different lignin fractions, with the Klason method determining the total residue remaining after removal of cell wall polysaccharides by sulfuric acid, and the acetyl bromide method determining lignin monomers solubilized primarily by cleavage at α -ether linkages (Dean, 1997).

Note that the values for recovery of total G and S residues by thioacidolysis imply a greater downregulation of lignin in COMT suppressed plants than observed by determination of Klason lignin. In this respect, it is important to realize that

thioacidolysis, which targets alkylaryl ether bonds, is <100% efficient and can degrade at most 50% of total Klason lignin from hardwood species (Dean, 1997). In the present work, and in all previous reports of modifications to lignin content and composition in transgenic plants utilizing “wet chemistry” techniques, a significant proportion of the lignin polymer remains recalcitrant to analysis, and this must always be kept in mind when proposing models for metabolic control of lignin biosynthesis from such studies.

Significantly greater reductions in Klason lignin levels have been obtained by downregulation of the phenylpropanoid pathway entry point enzymes PAL or cinnamate 4-hydroxylase than by downregulation of COMT or CCOMT (Sewalt et al., 1997), at least in tobacco, suggesting that there may be unsuspected redundancy for monolignol O-methylation pathways.

CCOMT Is Nonessential for S Lignin Biosynthesis in Alfalfa

Reduction of CCOMT to <5% of wild-type activity leads to reduction in G lignin with no apparent effect on S lignin in alfalfa. This contrasts with reported reductions in both G and S lignin in transgenic tobacco downregulated in CCOMT expression (Zhong et al., 1998). CCOMT therefore would appear to function in the biosynthesis of G lignin in alfalfa, as has been proposed previously in tobacco (Ye, 1997; Zhong et al., 1998), but is not essential for S lignin biosynthesis. It is possible that COMT might compensate for CCOMT in catalyzing the introduction of the 3-O-methyl group into S lignin precursors in CCOMT downregulated plants. This challenges the recent model based on in vitro studies of enzyme specificity that ascribes the first methylation reaction in S lignin biosynthesis to CCOMT, not COMT (Li et al., 2000). However, if COMT is involved in 3-O-methylation of precursors destined for S units, caffeic acid is unlikely to be a substrate in view of the lack of caffeoyl glucose accumulation

Table 2. Levels of Wall-Bound Phenolic Compounds in Empty Vector Control, COMT-Suppressed, and CCOMT-Suppressed Alfalfa Plants

Line	Coumaric Acid ^a ($\mu\text{g/g}$ Dry Wt ^b)	Ferulic Acid ^a ($\mu\text{g/g}$ Dry Wt)	Coumaric Acid (Average $\pm$ SD for Set)	Ferulic Acid (Average $\pm$ SD for Set)
Control 48	13.24	23.92		
Control 2	26.26	31.50	19.75 $\pm$ 9.20	27.71 $\pm$ 5.36
SC4	23.97	31.98		
AC310	16.50	27.35		
SC5	26.89	49.21	22.46 $\pm$ 5.36	36.18 $\pm$ 11.52
ACC305	28.53	29.82		
ACC315	21.49	24.04	25.01 $\pm$ 4.98	26.93 $\pm$ 4.09
DS14	25.20	34.11		
DA302	13.77	17.80	19.48 $\pm$ 8.08	25.95 $\pm$ 11.53

^a Phenolic compounds were extracted from cell wall material after alkaline hydrolysis and analyzed by HPLC. Differences between sets of lines were not significant ($P = 0.4$).

^b Wt, weight.

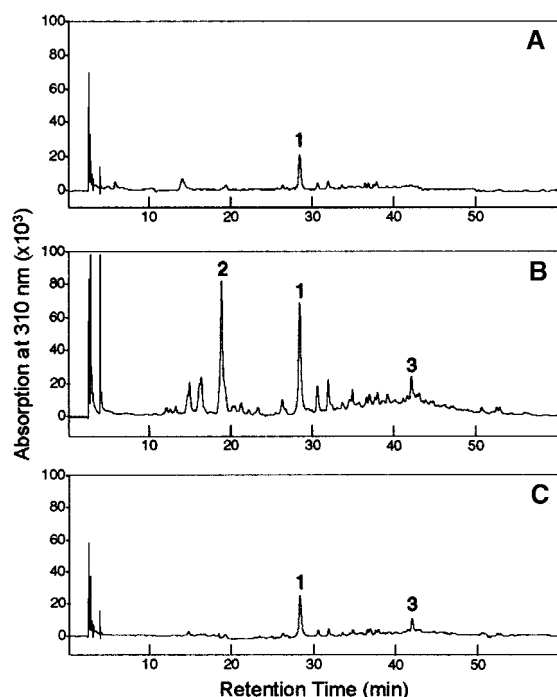


Figure 9. HPLC Analysis of Soluble Phenolic Compounds in Alfalfa Stem Extracts.

Powdered stem extracts (sixth to ninth internodes) were extracted in acetone and resuspended in methanol at 2 mL/g dry weight, and 20- μ L aliquots were analyzed by HPLC with diode array detection at 310 nm.

(A) Extract from wild-type (empty vector) plant 48.

(B) Extract from CCOMT downregulated plant ACC305.

(C) Extract from COMT downregulated plant SC5.

Qualitatively and quantitatively similar results were obtained with other independent transgenic lines harboring the same constructs. The profiles in lines DS14 and DA302 with reduction in both COMT and CCOMT levels were identical to those in (B).

in COMT downregulated plants compared with the high level of accumulation of this compound when CCOMT is downregulated.

COMT Is Essential for S Lignin Biosynthesis

Previous studies have reported that downregulation of COMT has a larger impact on S lignin than on G lignin, and that 5-hydroxyguaiacyl residues accumulate, albeit at a low level, in the lignin of COMT downregulated plants (Atanassova et al., 1995; Van Doorselaere et al., 1995). Our data extend these results to show that it is possible to completely eliminate thioacidolysis-recoverable S units from lignin by reduction of COMT to 4.5% of the control level in the forage legume alfalfa. Importantly, we analyzed not only the S units that would be released as monomers from the common uncondensed β -O-4 linked units, but also the S units in the

more condensed lignin fraction recovered as dimers. The fact that we observed a near total loss of S units in both the monomer and dimer fractions indicates that the loss of S units as a result of COMT downregulation reflects a true block in S monomer biosynthesis rather than a structural change in the lignin resulting in lower recovery of S units.

Analysis of lignin dimers indicated a complete loss of recovery of β - β linkages in lignin from COMT downregulated plants, as would be expected because these linkages only form between S units. S units are also involved in some β -1 and cyclic β -5 linkages, and these likewise were not recovered in COMT downregulated plants. Thus, the effect of COMT downregulation on S/G ratio was directly reflected in the associated dimer bonding patterns, suggesting that monomer availability might be the main factor controlling lignin linkage pattern. Somewhat similar results have been observed by NMR analysis of the lignin from the *fah1-2* mutant of *Arabidopsis*, in which complete loss of S units resulted in increased recovery of β -5 and 5-5 linkages (Marita et al., 1999). Interestingly, a new type of dimeric unit was found only in the lignin from COMT downregulated plants. Although the structure of this unit remains to be unequivocally determined, it is interesting that it was never observed in lignin from control or CCOMT downregulated lines. Clearly, there is some flexibility in the types of linkages that can occur in lignin, with the strong reduction in S units in COMT downregulated plants somehow facilitating formation of novel linkage types.

Enzymatic Redundancy for Monolignol 3-O-Methylation

Downregulation of either COMT or CCOMT leads to a similar percentage reduction in G lignin in alfalfa, suggesting that both enzymes participate in insertion of the 3-O-methyl group into a G monolignol precursor. This precursor is presumably caffeoyl CoA in the case of the CCOMT catalyzed reaction. Accumulation of large amounts of soluble caffeoyl glucose in CCOMT downregulated plants is consistent with a blockage of caffeoyl CoA methylation leading to caffeic acid formation via thioesterase activity with subsequent glucose conjugation. Caffeoyl glucose itself is not a substrate for CCOMT.

Kinetic considerations have argued against effective methylation of caffeic acid by COMT in monolignol biosynthesis. Thus, the K_m of aspen COMT for caffeic acid is some 30-fold higher than the K_m for 5-hydroxyconiferaldehyde (Li et al., 2000). The lack of accumulation of either free caffeic acid or caffeoyl glucose in COMT downregulated alfalfa is, as argued above, consistent with the noninvolvement of caffeic acid methylation in lignin biosynthesis in alfalfa. However, previous authors have failed to consider the possibility that COMT might act on either caffeoyl aldehyde or caffeoyl alcohol, and recombinant alfalfa COMT catalyzes kinetically favorable reactions (comparable to that with 5-hydroxyconiferaldehyde) with both these substrates (Parvathi et al., 2001). Flux through the putative caffeoyl aldehyde/alcohol pathway might be sufficient to

maintain S lignin biosynthesis in the absence of CCOMT expression in alfalfa. Such a model would bypass caffeoyl CoA methylation in the biosynthesis of S lignin, consistent with the lack of effect of CCOMT downregulation on S lignin levels in alfalfa. Consistent with a block in 3-O-methylation, COMT downregulation results in incorporation of far fewer 5-hydroxyguaiacyl units in lignin (and none in the soluble fraction) than would be predicted by the extent of S lignin reduction.

Redundancy in the 3-O-methylation reaction of G lignin biosynthesis may be more extensive than the above arguments suggest. Thus, simultaneous downregulation of both COMT and CCOMT in line DA302 only resulted in the same ~25% reduction in the level of G lignin as reduction in either enzyme independently. Large expressed sequence tag databases (<http://www.ncgr.org/research/mgi/>; <http://www.tigr.org/tdp/mtgi/>) are now available for *Medicago truncatula*, a model legume that is very closely related to alfalfa, and several O-methyltransferase genes in addition to COMT and CCOMT are quite strongly expressed in stem tissues. Future functional analysis of these genes may reveal the full repertoire of methylation reactions involved in monolignol biosynthesis. Clearly, the exact sequence of enzymatic events leading to the G and S monolignols still is not fully understood.

METHODS

Plant Material

Alfalfa (*Medicago sativa* cv Regen SY) plants were grown in the greenhouse under standard conditions. All transformations were performed with clonally propagated material of one selected highly regenerable line (4D).

Generation of Plasmids and Alfalfa Transformation

The bean *PAL2* promoter was obtained from the genomic clone gPAL2 (Cramer et al., 1989) and was cloned into the EcoRI-BamHI sites of pUC18. Site-directed mutagenesis was used to delete the NdeI site in pUC18 to create the plasmid pUC18-PAL. The β -glucuronidase (GUS) open reading frame was excised from the plasmid pGN100 (Reimann-Philipp and Beachy, 1993) by EcoRI-SmaI digestion, and two DNA polylinkers containing different restriction sites, EcoRI-BglII-NdeI-BamHI-SmaI and EcoRI-BglII-BamHI-NdeI-SmaI, were introduced independently between the EcoRI and SmaI sites, respectively. A BglII-PstI fragment containing the *nos* terminator sequence was inserted into the BamHI-PstI sites of pUC18-PAL to give the plasmids pPTN1 and pPTN2, which contain the bean *PAL2* promoter and *nos* terminator. To create the cassette for *gusA* gene expression, we released the bean *PAL2* promoter from the plasmid pPTN2 by digestion with EcoRI, and the ends were filled in with Klenow fragment and then digested with BamHI. The plasmid *ubi3*-GUS (Garabino and Belknap, 1994) was treated with XbaI, Klenow, and BamHI to replace the *ubi3* promoter with the isolated bean *PAL2* promoter. The *gusA* expression cassette then was cloned into HindIII-EcoRI cut pCAMBIA3300 to create the *gusA* expression construct pCAMGUS.

The alfalfa caffeic acid 3-O-methyltransferase (COMT) and caffeoyl CoA 3-O-methyltransferase (CCOMT) coding sequences were isolated from the 1107-bp full-length COMT or the 753-bp full-length CCOMT cDNAs cloned in pET vectors (Inoue et al., 1998). The COMT and CCOMT inserts were removed as NdeI-BamHI fragments and ligated into the NdeI-BamHI sites of pPTN1 and pPTN2, resulting in plasmids pPTN1-COMT and pPTN1-CCOMT for sense expression of COMT or CCOMT, respectively, and pPTN2-COMT and pPTN2-CCOMT for antisense expression of COMT or CCOMT, respectively. The chimeric genes then were cloned as EcoRI-HindIII fragments into the EcoRI-HindIII sites of the binary vector pCAMBIA3300, which has a phosphinothricin resistance gene as selectable marker. Resulting binary constructs were designated pCAMC1 (single COMT, sense), pCAMC2 (single COMT, antisense), pCAMCC1 (single CCOMT, sense), and pCAMCC2 (single CCOMT, antisense), as shown in Figure 2.

To make constructs for sense or antisense expression of tandem COMT and CCOMT genes, plasmids pPTN1-COMT and pPTN2-COMT first were cut with EcoRI, filled in with the Klenow fragment of DNA polymerase I, and then digested with HindIII. The isolated fragments were ligated into NarI-, Klenow- and HindIII-treated pPTN1 to create the shuttle vector pPTN1-D. The tandem COMT and CCOMT region together with the *PAL2* promoter and *nos* terminator was cut out with AatII, filled in with Klenow, digested with EcoRI, and finally ligated into SmaI-EcoRI cut pCAMBIA3300 to give binary expression constructs with both OMTs in the sense or antisense orientation. These were designated pCAMC1CC1 (tandem COMT sense, CCOMT sense) and pCAMC2CC2 (tandem COMT antisense, CCOMT antisense). Introduction of both COMT and CCOMT transgenes into the same plant was also achieved by co-transformation using the above single COMT and CCOMT constructs.

Constructs were introduced into *Agrobacterium tumefaciens* LBA4404 using the Gibco BRL electroporation procedure. Leaf disc transformation of alfalfa was performed based on a method described previously (Thomas et al., 1990). Phosphinothricin (5 mg/L) was added to the culture medium for selection of resistant transformants.

Polymerase Chain Reaction Analysis of Transformants

The primers used were 5'-GGGTTCACAGGTGAACTC-3' and 5'-CCTTCTTAAGAACTCCATGATG-3' for COMT and 5'-GGCAACCAACGAAGATCAAAAGC-3' and 5'-CTTGATCTACGGCAGATAGTGATTCC-3' for CCOMT, which yielded diagnostic 1.1- or 0.75-kb amplification products in COMT or CCOMT transformants, respectively. The temperature program for polymerase chain reactions (PCRs) was 4 min at 94°C, followed by 30 cycles of 1 min at 94°C, 2 min at 54°C, and 3 min at 72°C, followed by 10 min at 72°C.

DNA Gel Blot Analysis

Total DNA was isolated from leaf tissue of each alfalfa line by using a Nucleon Phytopure plant DNA extraction kit (Amersham). DNA samples (7 μ g) were digested with HindIII, electrophoretically separated, and transferred to a nylon membrane (Hybond-N, Amersham) by standard procedures (Sambrook et al., 1989). Blots were probed with ³²P-labeled 1.1-kb COMT or 0.75-kb CCOMT coding sequence probe and washed at high stringency conditions (final wash 0.1 $\times$ SSC [1 $\times$ SSC is 0.15 M NaCl, 0.015 M sodium citrate], 0.1% SDS, 65°C). The probe was labeled with an α -³²P-dCTP labeling kit (Amersham).

RNA Gel Blot Analysis

RNA was prepared from alfalfa leaves using TRIAGENT (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's suggested protocol. Total RNA samples (10 µg) were fractionated on a formaldehyde denaturing gel according to standard protocols (Sambrook et al., 1989), transferred to a Hybond-N nylon membrane, and hybridized with radiolabeled COMT and CCOMT probes as for DNA gel blots.

Protein Gel Blot Analysis

Crude proteins were extracted, separated on 8 to 12% gradient SDS-polyacrylamide gels, and electrotransferred onto nitrocellulose membranes. The membranes were incubated in blocking buffer (PBS containing 0.05% Tween 20 and 5% skim milk) for 2 hr, then incubated in blocking buffer with antibodies raised against COMT and CCOMT protein for 2 hr. The signals were detected with ECL Protein gel blotting detection reagents (Amersham) according to the manufacturer's protocol.

Enzyme Extraction and Assay

Alfalfa stems (internodes 6 to 9, counting from the first fully opened leaf at the top) were collected and homogenized in liquid nitrogen. Powdered tissue was extracted for 1 hr at 4°C in extraction buffer (100 mM Tris-HCl, pH 7.5, 10% glycerol, 2 mM DTT, 0.2 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride) and desalted on PD-10 columns (Pharmacia). Protein concentrations were determined using Bradford dye binding reagent (Bio-Rad) with BSA as standard. Enzyme activities were assayed essentially as described before (Gowri et al., 1991; Ni et al., 1996) with the following modifications. The assay mixtures contained 5 µL of [¹⁴CH₃]-S-adenosyl-L-Met (0.6 mM, 13 µCi/µmol), 5 µL of caffeic acid (1 mM) or caffeoyl CoA (1 mM), 30 µL of assay buffer (100 mM Tris-HCl, pH 7.5, 10% glycerol, 2 mM DTT, 0.2 mM MgCl₂), and 5 µL of protein extract. They were incubated at 30°C for 30 min, stopped by adding 50 µL of 0.2 M HCl (for COMT) or 10 µL of 3 M NaOH (for CCOMT), incubated at 37°C for 10 min, then (for CCOMT) acidified by adding 40 µL of 1 M HCl. Labeled ferulic acid was extracted into 200 µL of hexane:ethyl acetate (1:1, v/v), and 150 µL of the separated organic phases was transferred to scintillation vials for determination of radioactivity.

Histochemical GUS Assays

Sections of alfalfa stems, roots, and petioles were hand cut with a vibratome (Series 1000, Ted Pella Inc., Redding, CA) and incubated on ice for 30 min in 2% paraformaldehyde, 100 mM Na-phosphate, pH 7.0. They then were vacuum infiltrated in 2 mM X-gluc in 50 mM Na-phosphate, pH 7.0, 0.5% Triton X-100 for 10 sec, followed by a 2 hr incubation at 37°C. After staining, green tissues were bleached in 70% ethanol several times to allow visualization of the blue staining.

Histochemical Staining of Lignin

For Maule staining, vibratome-cut sections of alfalfa stems were immersed in 1% (w/v) potassium permanganate solution for 5 min at room temperature and then washed twice with 3% hydrochloric acid

until the color turned from black or dark brown to light brown. Phloroglucinol-HCl reagent was prepared by mixing 2 volumes of 2% (w/v) phloroglucinol in 95% ethanol with 1 volume of concentrated HCl. All photographs were taken within 30 min of staining.

Determination of Lignin Content and Composition

Klason lignin content was determined according to standard procedures (Lin and Dence, 1992). Two hundred milligrams of dried sample was used for lignin analysis, and Klason lignin content was calculated as weight percentage of the extract-free sample. Acetyl bromide lignin was determined according to the procedure of Iiyama and Wallis (1990). Thioacidolysis and the Raney nickel desulfurization method of Lapiere et al. (1985, 1995) were used for the determination of lignin composition and resistant interunit bonds. Thioacidolysis was performed using ~20 mg of extractive-free samples reacted with 15 mL of 0.2 M BF₃ etherate in an 8.75:1 dioxane/ethanethiol mixture. An aliquot of the thioacidolysis solution in CH₂Cl₂ was mixed with 1 mL Raney nickel aqueous slurry (Aldrich Chemical Company, Inc., Milwaukee, WI) for desulfurization. Lignin-derived monomers and dimers were identified by gas chromatography mass spectrometry (GC/MS) and quantified by GC, as their trimethylsilyl derivatives. The GC/MS was performed on a Hewlett Packard 5890 series II gas chromatograph with a 5971 series mass selective detector (column: HP-1, 60-m × 0.25-mm × 0.25-µm film thickness), and the mass spectra were recorded in electron impact mode (70 eV) with 60 to 650 m/z scanning range.

Determination of Soluble and Wall-Bound Phenolics

Stem tissue was ground in liquid nitrogen. Residues previously extracted for soluble phenolics (Howles et al., 1996) were washed three times with absolute ethanol, dried under N₂, and subjected to base hydrolysis for 18 hr in 10 mL of 1 N NaOH at room temperature. After centrifugation (8000g at 4°C for 15 min), 60% of the supernatant was removed, acidified to pH 1.0 to 2.0 with 2 N HCl, and extracted three times with an equal volume of ethyl acetate. The organic phases were combined, taken to dryness, and resuspended in HPLC grade methanol to a final concentration equivalent to 200 mg dry weight of original stem tissue per milliliter methanol. Twenty microliters of solution was analyzed by HPLC as described (Howles et al., 1996), monitoring at 235, 270, and 310 nm.

Soluble phenolics were analyzed by HPLC as described (Howles et al., 1996). All mass spectra were acquired using a Bruker Esquire (Billerica, MA) ion trap mass spectrometer in the negative ion mode.

ACKNOWLEDGMENTS

We thank Cuc Ly for assistance with artwork, David Huhman and Dr. Lloyd Sumner for assistance with mass spectrometry, Dr. John Ralph (U.S. Dairy Forage Research Center, Madison, WI) for an authentic sample of syringyl-γ-p-coumarate ester, and Drs. K. Parvathi and Gregory May for critical reading of the manuscript. This work was supported by AgriBiotech Inc. and The Samuel Roberts Noble Foundation.

Received August 21, 2000; accepted November 9, 2000.

REFERENCES

- Albrecht, K.A., Wedin, W.F., and Buxton, D.R. (1987). Cell-wall composition and digestibility of alfalfa stems and leaves. *Crop Sci.* **27**, 735–741.
- Atanassova, R., Favet, N., Martz, F., Chabbert, B., Tollier, M.-T., Monties, B., Fritig, B., and Legrand, M. (1995). Altered lignin composition in transgenic tobacco expressing *O*-methyltransferase sequences in sense and antisense orientation. *Plant J.* **8**, 465–477.
- Baucher, M., BernardVailhe, M.A., Chabbert, B., Besle, J.M., Opsomer, C., VanMontagu, M., and Botterman, J. (1999). Down-regulation of cinnamyl alcohol dehydrogenase in transgenic alfalfa (*Medicago sativa* L.) and the effect on lignin composition and digestibility. *Plant Mol. Biol.* **39**, 437–447.
- Boudet, A.M., and Grima-Pettenati, J. (1996). Lignin genetic engineering. *Mol. Breeding* **2**, 25–39.
- Boudet, A.M., Lapierre, C., and Grima-Pettenati, J. (1995). Tansley review No. 80. Biochemistry and molecular biology of lignification. *New Phytol.* **129**, 203–236.
- Buxton, D.R., and Redfearn, D.D. (1997). Plant limitations to fiber digestion and utilization. *J. Nutr.* **127**, S814–S818.
- Buxton, D.R., and Russell, J.R. (1988). Lignin constituents and cell-wall digestibility of grass and legume stems. *Crop Sci.* **28**, 553–558.
- Casler, M.D. (1987). In vitro digestibility of dry matter and cell wall constituents of smooth brome grass forage. *Crop Sci.* **27**, 931–934.
- Chen, F., Yasuda, S., and 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.
- Cramer, C.L., Edwards, K., Dron, M., Liang, X., Dildine, S.L., Bolwell, G.P., Dixon, R.A., Lamb, C.J., and Schuch, W. (1989). Phenylalanine ammonia-lyase gene organization and structure. *Plant Mol. Biol.* **12**, 367–383.
- Davin, L.B., and Lewis, N.G. (1992). Phenylpropanoid metabolism: Biosynthesis of monolignols, lignans and neolignans, lignins and suberins. *Rec. Adv. Phytochem.* **26**, 325–375.
- Dean, J.F.D. (1997). Lignin analysis. In: *Methods in Plant Biochemistry and Molecular Biology*, W.V. Dashek, eds (Boca Raton, FL: CRC Press), pp. 199–215.
- Dixon, R.A., Lamb, C.J., Masoud, S., Sewalt, V.J.H., and Paiva, N.L. (1996). Metabolic engineering: Prospects for crop improvement through the genetic manipulation of phenylpropanoid biosynthesis and defense responses—A review. *Gene* **179**, 61–71.
- Garabino, J.E., and Belknap, W.R. (1994). Isolation of a ubiquitin-ribosomal protein gene (*ubi3*) from potato and expression of its promoter in transgenic plants. *Plant Mol. Biol.* **24**, 119–127.
- Gowri, G., Bugos, R.C., Campbell, W.H., Maxwell, C.A., and Dixon, R.A. (1991). Stress responses in alfalfa (*Medicago sativa* L.) X. Molecular cloning and expression of S-adenosyl-L-methionine: Caffeic acid 3-*O*-methyltransferase, a key enzyme of lignin biosynthesis. *Plant Physiol.* **97**, 7–14.
- Grabber, J.H., Jung, G.A., Abrams, S.M., and Howard, D.B. (1992). Digestion kinetics of parenchyma and sclerenchyma cell walls isolated from orchardgrass and switchgrass. *Crop Sci.* **32**, 806–810.
- Grabber, J.H., Quideau, S., and Ralph, J. (1996). *p*-Coumaroylated syringyl units in maize lignin: implications for β -ether cleavage by thioacidolysis. *Phytochemistry* **43**, 1189–1194.
- Grabber, J.H., Ralph, J., Hatfield, R.D., and Quideau, S. (1997). *p*-Hydroxyphenyl, guaiacyl, and syringyl lignins have similar inhibitory effects on wall degradability. *J. Agric. Food Chem.* **45**, 2530–2532.
- Halpin, C., Knight, M.E., Foxon, G.A., Campbell, M.M., Boudet, A.M., Boon, J.A., Chabbert, B., Tollier, M.-T., and Schuch, W. (1994). Manipulation of lignin quality by down-regulation of cinnamyl alcohol dehydrogenase. *Plant J.* **6**, 339–350.
- Howles, P.A., Paiva, N.L., Sewalt, V.J.H., Elkind, N.L., Bate, Y., Lamb, C.J., and Dixon, R.A. (1996). Overexpression of L-phenylalanine ammonia-lyase in transgenic tobacco plants reveals control points for flux into phenylpropanoid biosynthesis. *Plant Physiol.* **112**, 1617–1624.
- Hu, W.-J., Harding, S.A., Lung, J., Popko, J.L., Ralph, J., Stokke, D.D., Tsai, C.-J., and Chiang, V.L. (1999). Repression of lignin biosynthesis promotes cellulose accumulation and growth of transgenic trees. *Nat. Biotech.* **17**, 808–812.
- Humphreys, J.M., Hemm, M.R., and Chapple, C. (1999). New routes for lignin biosynthesis defined by biochemical characterization of recombinant ferulate 5-hydroxylase, a multifunctional cytochrome P450-dependent monooxygenase. *Proc. Natl. Acad. Sci. USA* **96**, 10045–10050.
- Iiyama, K., and Wallis, A.F.A. (1990). Determination of lignin in herbaceous plants by an improved acetyl bromide procedure. *J. Sci. Food Agric.* **51**, 145–161.
- Inoue, K., Sewalt, V.J.H., Ballance, G.M., Ni, W., Stürzer, C., and Dixon, R.A. (1998). Developmental expression and substrate specificities of alfalfa caffeic acid 3-*O*-methyltransferase and caffeoyl CoA 3-*O*-methyltransferase in relation to lignification. *Plant Physiol.* **117**, 761–770.
- Inoue, K., Parvathi, K., and Dixon, R.A. (2000). Substrate preferences of caffeic acid/5-hydroxyferulic acid 3-*O*-methyltransferases in developing stems of alfalfa (*Medicago sativa* L.). *Arch. Biochem. Biophys.* **375**, 175–182.
- Irdani, T., Bogani, P., Mengoni, A., Mastromei, G., and Buiatti, M. (1998). Construction of a new vector conferring methotrexate resistance in *Nicotiana tabacum* plants. *Plant Mol. Biol.* **37**, 1079–1086.
- Jung, H.G., Mertens, D.R., and Payne, A.J. (1997a). Correlation of acid detergent lignin and Klason lignin with digestibility of forage dry matter and neutral detergent fiber. *J. Dairy Sci.* **80**, 1622–1628.
- Jung, H.G., Sheaffer, C.C., Barnes, D.K., and Halgerson, J.L. (1997b). Forage quality variation in the US alfalfa core collection. *Crop Sci.* **37**, 1361–1366.
- Kersey, R., Inoue, K., Schubert, K.R., and Dixon, R.A. (1999). Immunolocalization of two lignin *O*-methyltransferases in stems of alfalfa (*Medicago sativa* L.). *Protoplasma* **209**, 46–57.
- Lam, T.B.T., Iiyama, K., and Stone, B.A. (1996). Caffeic acid: *O*-Methyltransferases and the biosynthesis of ferulic acid in primary cell walls of wheat seedlings. *Phytochemistry* **41**, 1507–1510.
- Lapierre, C., Monties, B., and Rolando, C. (1985). Thioacidolysis of lignin: Comparison with acidolysis. *J. Wood Chem. Technol.* **5**, 277–292.

- Lapierre, C., Pollet, B., and Rolando, C. (1995). New insight into the molecular architecture of hardwood lignins by chemical degradative method. *Res. Chem. Intermed.* **21**, 397–412.
- Lewis, N.G. (1999). A 20th century roller coaster ride: A short account of lignification. *Curr. Opin. Plant Biol.* **2**, 153–162.
- Lewis, N.G., and Yamamoto, E. (1990). Lignin: Occurrence, biogenesis and biodegradation. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **41**, 455–496.
- Leyva, A., Liang, X., Pintor-Toro, J.A., Dixon, R.A., and Lamb, C.J. (1992). *Cis*-element combinations determine phenylalanine ammonia-lyase gene tissue specific expression patterns. *Plant Cell* **4**, 263–271.
- Li, L., Popko, J.L., Umezawa, T., and Chiang, V.L. (2000). 5-Hydroxyconiferyl aldehyde modulates enzymatic methylation for syringyl monolignol formation, a new view of monolignol biosynthesis in angiosperms. *J. Biol. Chem.* **275**, 6537–6545.
- Liang, X., Dron, M., Schmid, J., Dixon, R.A., and Lamb, C.J. (1989). Developmental and environmental regulation of a phenylalanine ammonia-lyase β -glucuronidase gene fusion in transgenic tobacco plants. *Proc. Natl. Acad. Sci. USA* **86**, 9284–9288.
- Lin, S.Y., and Dence, C.W. (1992). *Methods in Lignin Chemistry*. Springer Series in Wood Science. (Berlin, Heidelberg, Springer-Verlag).
- Marita, J.M., Ralph, J., Hatfield, R.D., and Chapple, C. (1999). NMR characterization of lignins in *Arabidopsis* altered in the activity of ferulate 5-hydroxylase. *Proc. Natl. Acad. Sci. USA* **96**, 12328–12332.
- Meyer, K., Shirley, A.M., Cusumano, J.C., Bell-Lelong, D.A., and Chapple, C. (1998). Lignin monomer composition is determined by the expression of a cytochrome P450-dependent monooxygenase in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* **95**, 6619–6623.
- Ni, W., Paiva, N.L., and Dixon, R.A. (1994). Reduced lignin in transgenic plants containing an engineered caffeic acid *O*-methyltransferase antisense gene. *Transgenic Res.* **3**, 120–126.
- Ni, W., Sewalt, V.J.H., Korth, K.L., Blount, J.W., Ballance, G.M., and Dixon, R.A. (1996). Stress responses in alfalfa (*Medicago sativa* L.). XXI. Activation of caffeic acid 3-*O*-methyltransferase and caffeoyl CoA 3-*O*-methyltransferase genes does not contribute to changes in metabolite accumulation in elicitor-treated cell suspension cultures. *Plant Physiol.* **112**, 717–726.
- Osakabe, K., Tsao, C.C., Li, L., Popko, J.L., Umezawa, T., Carraway, D.T., Smeltzer, R.H., Joshi, C.P., and Chiang, V.L. (1999). Coniferyl aldehyde 5-hydroxylation and methylation direct syringyl lignin biosynthesis in angiosperms. *Proc. Natl. Acad. Sci. USA* **96**, 8955–8960.
- Pakusch, A.E., Kneusel, R.E., and Matern, U. (1989). S-adenosyl-L-methionine:trans-caffeoyl-coenzyme A 3-*O*-methyltransferase from elicitor-treated parsley cell suspension cultures. *Arch. Biochem. Biophys.* **271**, 488–494.
- Parvathi, K., Chen, F., Guo, D., Blount, J.W., and Dixon, R.A. (2001). Substrate preferences of *O*-methyltransferases in alfalfa suggest new pathways for 3-*O*-methylation of monolignols. *Plant J.*, in press.
- Piquemal, J., Lapierre, C., Myton, K., O'Connell, A., Schuch, W., Grima-Pettenati, J., and Boudet, A.M. (1998). Down-regulation of cinnamoyl-CoA reductase induces significant changes of lignin profiles in transgenic tobacco plants. *Plant J.* **13**, 71–83.
- Reimann-Philipp, R., and Beachy, R.N. (1993). Coat protein-mediated resistance in transgenic tobacco expressing the tobacco mosaic virus coat protein from tissue-specific promoters. *Mol. Plant-Microbe Interact.* **6**, 323–330.
- Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).
- Sederoff, R.R., MacKay, J.J., Ralph, J., and Hatfield, R.D. (1999). Unexpected variation in lignin. *Curr. Opin. Plant Biol.* **2**, 145–152.
- Sewalt, V.J.H., Glasser, W.G., Fontenot, J.P., and Allen, V.G. (1996). Lignin impact on fiber degradation. 1. Quinone methide intermediates formed from lignin during *in vitro* fermentation of corn stover. *J. Sci. Food Agric.* **71**, 195–203.
- Sewalt, V.J.H., Ni, W., Blount, J.W., Jung, H.G., Howles, P.A., Masoud, S.A., Lamb, C., and Dixon, R.A. (1997). Reduced lignin content and altered lignin composition in transgenic tobacco down-regulated in expression of phenylalanine ammonia-lyase or cinnamate 4-hydroxylase. *Plant Physiol.* **115**, 41–50.
- Shufflebottom, D., Edwards, K., Schuch, W., and Bevan, M. (1993). Transcription of two members of a gene family encoding phenylalanine ammonia-lyase leads to remarkably different cell specificities and induction patterns. *Plant J.* **3**, 835–845.
- Thomas, M.R., Johnson, L.B., and White, F.F. (1990). Selection of interspecific somatic hybrids of *Medicago* by using *Agrabacterium*-transformed tissue. *Plant Sci.* **69**, 189–198.
- Van Doorselaere, J., et al. (1995). A novel lignin in poplar trees with a reduced caffeic acid/5-hydroxyferulic acid *O*-methyltransferase activity. *Plant J.* **8**, 855–864.
- Vaucheret, H., Béclin, C., Elmayan, T., Feuerbach, F., Godon, C., Morel, J.B., Mourrain, P., Palauqui, J.C., and Vernhettes, S. (1998). Transgene-induced gene silencing in plants. *Plant J.* **16**, 651–659.
- Whetten, R., and Sederoff, R. (1991). Genetic engineering of wood. *For. Ecol. Manage.* **43**, 301–316.
- Ye, Z.-H. (1997). Association of caffeoyl CoA 3-*O*-methyltransferase expression with lignifying tissues in several dicot plants. *Plant Physiol.* **115**, 1341–1350.
- Ye, Z.H., Kneusel, R.E., Matern, U., and Varner, J.E. (1994). An alternative methylation pathway in lignin biosynthesis in *Zinnia*. *Plant Cell* **6**, 1427–1439.
- Zhong, R., Morrison III, W.H., Negrel, J., and Ye, Z.-H. (1998). Dual methylation pathways in lignin biosynthesis. *Plant Cell* **10**, 2033–2046.