Lignin Impact on Fiber Degradation: Increased Enzymatic Digestibility of Genetically Engineered Tobacco (*Nicotiana tabacum*) Stems Reduced in Lignin Content

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Cell wall digestibility, lignin content, and lignin composition were analyzed in transgenic tobacco altered in the expression of the phenylpropanoid biosynthetic enzymes caffeic acid 3-*O*-methyltransferase (COMT) and L-phenylalanine ammonia-lyase (PAL). Reduction of COMT activity by antisense technology resulted in reduced lignin content accompanied by an increased syringyl (S)/guaiacyl (G) monomer ratio, as determined by pyrolysis/GC/MS and measurement of lignin methoxyl content by wet chemistry. These results resemble those obtained by reduction of flux of lignin precursors into the phenylpropanoid pathway by PAL suppression, which results in drastically reduced lignin with sharply increased methoxyl content. Enzymatic digestibility of cell walls from stem internodes was improved in the transgenic lines and was highly negatively correlated with lignin concentration ($r = -0.97$). Although lignin composition was also affected, lignin concentration was the overriding factor influencing cell wall digestibility. The results provide a basis for new strategies for lignin modification to improve digestibility of forages.

Keywords: *Lignin; genetic engineering; digestibility; tobacco*

INTRODUCTION

Lignin, a structural phenylpropanoid polymer in plant cell walls, imposes limitations to forage digestibility (Jung, 1989) and the efficiency of paper pulping (Glasser and Kelley, 1987). The inhibitory effects of lignin depend on its monomer composition (Reeves, 1985; Buxton and Russell, 1988; Sewalt et al., 1996a) , functional groups (Sewalt et al., 1996b, 1997a), and the extent of cross-linking to wall polysaccharides. The ratio of indigestible residue to lignin content has been reported to increase with maturity [e.g., Quicke and Bentley (1959), Grabber et al. (1992)], which may be caused by a different type of lignin (with respect to monomer composition) being deposited in the cell wall at a later stage of maturity. Increased lignification with advancing maturity depresses digestibility of grasses more than of legumes, which is associated with a more rapid increase in syringyl-rich lignin in grasses (Buxton and Russell, 1988).

Unfortunately, reduction in digestibility with increasing maturity cannot be conclusively attributed to changes in lignin content, lignin composition, or lignin structure or to any other single cell wall component, due to many associated changes in chemical constituents and physical structure occurring during plant maturation (Titgemeyer et al., 1996). An alternative approach to elucidation of inhibitory factors toward digestibility has been by chemical or biological delignification [e.g., Jung et al. (1992), Sewalt et al. (1997b)]. However, such delignification strategies, although effective in enhancing fiber digestibility, never exclusively reduce lignin content, as hemicellulose components, hydroxycinnamic acids, lignin composition, and physical structure (pore size) are also altered. Although *in vitro* studies employing extraneously added industrial lignins have pointed to the crucial role of the phenolic hydroxyl group in inhibiting cellulase activity (Sewalt et al., 1997a) and microbial fiber digestion (Sewalt et al., 1996b), the relative impact of lignin content and lignin composition or structure on digestibility of plant cell walls has not been adequately addressed.

To avoid confounding effects associated with comparisons of maturity stages, species or cultivars, plant morphological parts, and pretreatments, the use of near-isogenic lines altered in lignin biosynthesis may prove useful. Although natural brown midrib lignin mutants with improved digestibility are available (Kuc and Nelson, 1964; Hartley and Jones, 1978, Sommerfeldt et al., 1979), genetically engineered plants with targeted alterations in expression of single lignin biosynthetic genes potentially provide a cleaner system to study the relative impact of lignin content and composition on forage digestibility. In addition, interest in genetic modification of lignin content and/or composition as a means to improve the efficiency of paper pulping or to increase digestibility of forages is intensifying (Campbell and Sederoff, 1996; Boudet and Grima-Pettenati, 1996).

Recent approaches in lignin genetic engineering involve down-regulation of the enzymes involved in lignin monomer synthesis by expression of homologous or heterologous antisense genes in transgenic plants (Dwivedi et al., 1994; Halpin et al., 1994; Ni et al., 1994; Atanassova et al., 1995; Van Doorsselaere et al., 1995). Most reports to date suggest that reduced expression of late enzymes of lignin monomer synthesis [caffeic acid 3-*O*-methyltransferase (COMT) and cinnamyl alcohol dehydrogenase (CAD)] affect lignin composition without affecting content (Halpin et al., 1994; Atanassova et al., 1995; Van Doorsselaere et al., 1995), although one study (Ni et al., 1994) revealed reduced lignin as an effect of COMT suppression. In contrast, reduction of the flux

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into the lignin pathway at an earlier stage results in reduced lignin content (Elkind et al., 1990; Bate et al., 1994; Yao et al., 1995). Whether the differential effects of suppression of early and late pathway enzymes are universal remains to be verified.

A recent publication has reported that a decrease in syringyl/guaiacyl (S/G) ratio of lignin in the apparent absence of changes in lignin content can lead to a small improvement in enzymatic digestibility of tobacco stems (Bernard Vailhé et al., 1996). The purpose of the present work was twofold: to examine more rigorously the lignin content and composition of our previously described transgenic COMT antisense tobacco (Ni et al., 1994) in comparison with L-phenylalanine ammonia-lyase (PAL)-suppressed tobacco in which the flux into the phenylpropanoid pathway is severely reduced (Elkind et al., 1990; Bate et al., 1994); and to determine the relative importance of lignin content and lignin composition toward *in vitro* digestibility in near-isogenic lines modified in phenylpropanoid pathway enzyme expression.

MATERIALS AND METHODS

Plant Material. Transgenic tobacco (*Nicotiana tabacum* L. cv. Xanthi) plants originated from two internally controlled experiments in which expression levels of either COMT or PAL were altered. Within each experiment, all plants were grown simultaneously under identical conditions. COMT-suppressed tobacco was obtained by expression of an alfalfa antisense COMT cDNA fragment (Ni et al., 1994). Plants evaluated were 5-week regrowth of three independent untransformed control lines, three primary COMT transformants, and six T_1-progeny plants of the most severely COMT-downregulated primary transformant.

Tobacco lines altered in PAL expression were produced by introduction of the bean *PAL2* transgene in the sense orientation (Elkind et al., 1990; Bate et al., 1994). Selected lines analyzed represent severely PAL-suppressed, recovery (after five generations of self-fertilization), wild-type (after loss of transgene), and PAL-overexpressing phenotypes. The PAL lines were grown from seed and harvested after 5 weeks. PAL-suppressed plants are reduced in lignin content in stems and chlorogenic acid in leaves (Elkind et al., 1990; Bate et al., 1994). PAL overexpressors are increased in leaf chlorogenic acid levels (Bate et al., 1994; Howles et al., 1996). For both COMT antisense plants and plants altered in PAL expression, middle stem sections (internodes 10 and 11, counting from the first fully opened leaf at the top) were collected and ground under liquid N_2. Powdered tissue was divided in two tubes (one for assay of enzyme activities, the other for lignin analysis) and stored at −70 °C.

Enzyme Extraction and Assays. Powdered tissue for assay of COMT activity was extracted for 20 min at 4 °C in extraction buffer [100 mM Tris-HCl, 0.2 mM $MgCl_2$, 2.0 mM DTT, 10% (v/v) glycerol, pH 7.2]. After centrifugation (12000*g*, 4 °C, 10 min), extracts were desalted on a PD-10 column. Soluble protein in the enzyme extracts was determined using the Bradford dye binding reagent (Bio-Rad) with BSA as standard (Bradford, 1976). Enzyme activity was assayed as described previously for COMT (Gowri et al., 1991) and PAL (Edwards and Kessmann, 1992).

Lignin Analysis. Powdered stem samples were freeze-dried and ground in a cyclone mill to pass a 1-mm sieve. Neutral detergent fiber (NDF) was prepared according to the method of Van Soest et al. (1991) using a batch fiber analysis apparatus (ANKOM Co., Fairport, NY) and was subjected to two-stage acid hydrolysis to determine Klason lignin (Sewalt et al., 1996a). The lignin residue was used to determine lignin methoxyl groups (TAPPI, 1972). Nonextracted dried stem material was subjected to pyrolysis/GC/MS to determine the lignin S/G ratio (Ralph and Hatfield, 1991).

***In Vitro* Cell Wall Digestibility.** Cell wall digestibility was estimated by enzymatic hydrolysis of NDF for 18 h at 40 °C using commercial cellulase (CEP cellulase, Biovance Technologies Inc., Omaha, NE) in 0.1 N sodium acetate buffer, pH 4.8, at 80 FPU/g NDF and measurement of reducing sugars using dinitrosalicylic acid (Miller, 1959). In this notation, 1 FPU (filter paper unit) is the activity required to release 1 μmol of glucose/min from milled Whatman No. 1 filter paper at pH 4.8, 50 °C.

Table 1. COMT Activity, NDF, Klason Lignin, and Lignin Methoxyl Content in Control and COMT Antisense Tobacco Plants[a]

	control[b] ($n = 3$)	primary transformants[b] ($n = 3$)	T_1 progeny of 105p ($n = 6$)	P value ($n = 12$)
COMT activity (% of control)	100^{x} (19.2)[c]	71.9^{xy} (23.1)	65.2^{y} (11.6)	0.045
NDF (mg/g of DM)	469^{x} (23.0)	416^{x} (14.8)	446^{x} (25.7)	0.093
Klason lignin (mg/g of NDF)	168^{x} (5.4)	142^{y} (13.1)	141^{y} (16.9)	0.064
OCH_3 (mg/g of lignin)	161^{x} (5.6)	179^{y} (3.8)	185^{y} (13.0)	0.049
S/G ratio	1.06^{x} (0.02)	1.27^{xy} (0.26)	1.51^{y} (0.29)	0.23

[a] Dissimilar superscripts (x−z) indicate significant ($P < 0.05$) treatment differences. [b] Control plants (101c, 103c, and 105c) were used as parent material to generate the respective primary transformants (101p, 103p, and 105p). [c] Values in parentheses represent standard deviations.

Table 2. PAL Activity, NDF, Klason Lignin, and Lignin Methoxyl Content in Control and PAL-Modified Tobacco Plants[a]

	control[b]	sense suppressed	recovering	P value
PAL activity (% of control)	100^{yz} (49)[c]	17^{x} (8.2)	49^{y} (7.3)	0.001
NDF (mg/g of DM)	340^{x} (25)	296^{x} (71)	329^{x} (55)	0.55
Klason lignin (mg/g of NDF)	110^{y} (2.0)	56.8^{x} (23.1)	90.2^{xy} (16.5)	0.03
OCH_3 (mg/g of lignin)	155^{x} (15)	222^{y} (58)	154^{x} (30)	0.049

[a] Dissimilar superscripts (x−z) indicate significant ($P < 0.05$) treatment differences. [b] Control and PAL-suppressed groups comprise three replicates each. PAL overexpressing plants, expressing PAL at 200% of control, were not different from the control in NDF, Klason lignin, and methoxyl contents. [c] Values in parentheses represent standard deviations.

Histochemical Analysis. The 10th internode from control and selected COMT antisense tobacco plants was collected in a second sampling from regrown plants. Free-hand sections were stained for lignin using phloroglucinol hydrochloride and Mäule's reagent according to the procedure of Nakano and Meshitsuka (1992). Phloroglucinol-stained sections were photographed within 30 min. In addition, sections were stained with safranin-O and astra-blue using a procedure modified from that of Srebotnik and Messner (1994). Sections were stained for 3 min with 0.1% aqueous safranin-O (color index no. 50240; Sigma catalog no. S-2255), washed with distilled water, and subsequently stained for 3 min with 1% aqueous astra-blue (Sigma catalog no. A-2077).

Statistical Analysis. Differences in enzymatic activity and lignin characteristics between groups of control and genetically modified plants were examined by one-way analysis of variance (Snedecor and Cochran, 1989). Seedlings or vegetatively propagated progeny of independent transformants and control lines were used as replicates (see Tables 1 and 2).

RESULTS AND DISCUSSION

Lignin Content and Composition in COMT Antisense Tobacco. One of the first papers on genetic modification of lignin (Ni et al., 1994) demonstrated that expression of an alfalfa COMT cDNA in the antisense orientation results in reduced levels of thioglycollic acid (TGA)-extractable lignin in transgenic tobacco, ac-

companied by a reduction of phloroglucinol staining of stem vascular tissue. Alkaline nitrobenzene oxidation (NBO) analysis indicated a small increase in syringyl (S) residues and a corresponding small decrease in guaiacyl (G) residues which was, at the time, not considered significant. That study relied on the determination of TGA-extractable lignin (Doster and Bostock, 1988) and NBO (Chen, 1992), which are not necessarily representative of the whole lignin polymer. Therefore, we decided to re-evaluate the lignin content and composition of the COMT antisense plants generated by Ni et al. (1994) using more rigorous methods of analysis.

Extractable COMT activity and concentrations of NDF, Klason lignin, and lignin methoxyl groups are summarized in Table 1 for three independent untransformed tobacco lines, three independent antisense COMT primary transformants, and six progeny of primary transformant 105 retaining the alfalfa COMT antisense transgene. On average, the plants containing the antisense COMT construct had a 32% reduction in COMT activity, which resulted in a reduction in lignin level in the cell wall of 15%, consistent with the previous analysis of TGA lignin in these plants (Ni et al., 1994). However, on a total dry matter (DM) basis, the reduction in lignin content was 29%, resulting from the reduced level of NDF in the transgenics. In the most severely suppressed primary transformant (105p), COMT activity was reduced to 40% of wild-type, resulting in a 35% decrease in lignin on a DM basis. Concentration of NDF was positively correlated with lignin content, on both DM and NDF bases (r = 0.87 and 0.67, respectively). Whether the reduction in NDF is due to reduced cell wall deposition or to enhanced solubility of the cell wall in neutral detergent awaits further investigation.

The lignin of primary transformant 105p and its progeny plants was significantly altered in composition from the control (Table 1). Lignin methoxyl content, as determined by titration after reaction with hydriodic acid, was increased by approximately 15%. Pyrolysis/GC/MS, which provides detailed information on lignin monomer composition (Ralph and Hatfield, 1991), confirmed the wet chemistry results. The S/G ratio of the lignin of the T_1 progeny of the most down-regulated primary transformant (105p) averaged 1.5, significantly (P = 0.049) increased above the control value (1.06). The level of S-derived pyrolysis products relative to the G-derived products was greater in COMT antisense lines than in the control line, due to a larger decrease in G units than in S units. The S/G ratio of the other two primary transformants (101p and 103p) was not affected, which caused the overall difference between control and COMT antisense plants according to the one-way ANOVA to be nonsignificant (see Table 1).

The effect of reduced COMT activity on overall lignin content (expressed on DM basis rather than NDF) and S/G ratio in the 12 independent lines listed in Table 1 is shown in Figure 1. As expected, the lignin concentration declined gradually from about 80 to 50 mg/g DM as COMT activity decreased to 50% of the control. The negative relationship between COMT activity and S/G ratio was very strong; as the level of COMT suppression approached 50%, the lignin composition (S/G ratio) changed drastically from around 1.1 to 1.7. Interestingly, lignin methoxyl content and S/G ratio were both negatively correlated with lignin content (r = −0.57 and −0.52, respectively), whereas across maturity stages

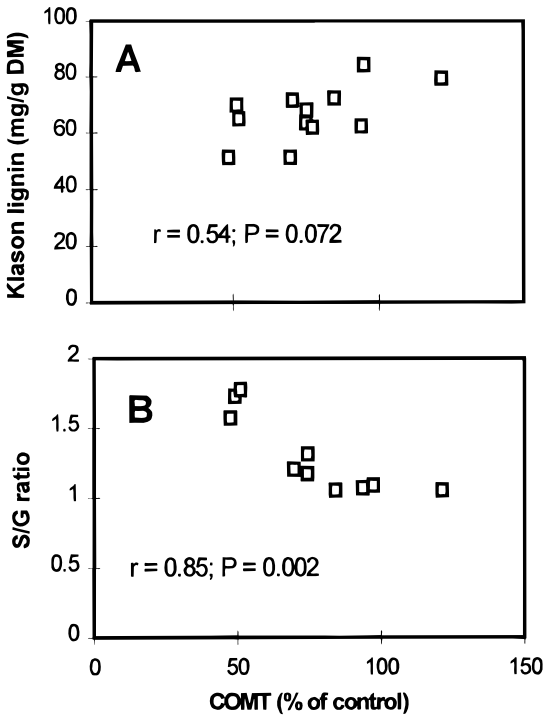

Figure 1. Relations between COMT activity and lignin content and composition of stems of a range of control and transgenic tobacco plants harboring an alfalfa COMT antisense construct: (A) Klason lignin (mg/g of DM); (B) syringyl/guaiacyl (S/G) ratio.

they are normally positively correlated with lignin content [e.g., Sewalt et al. (1996a)].

In confirmation of our earlier paper (Ni et al., 1994), COMT antisense plants displayed reduced phloroglucinol staining of their vascular tissue compared to the respective control lines (Figure 2), consistent with a reduced level of lignin. In addition, sections were stained with safranin-O, a basic dye, which stains lignin red, and astra-blue, a phthalocyanin dye, which is incorporated into cellulose fibers and stains them blue in the absence of lignin. Double-staining with safranin-O and astra-blue confirmed the results obtained with phloroglucinol; shifts from red to purple or blue in the vascular ring in plants with reduced COMT activity were observed, which are indicative of reduced lignin level and increased accessibility of cellulose to the stain (Figure 2). Finally, reduced COMT activity did not cause a change in the staining by Mäule's reagent, consistent with the fact that both wild-type and COMT antisense plants contained S and G residues (data not shown).

In contrast to our findings of the reduced lignin level and increased S/G of the remaining lignin in COMT antisense tobacco, other groups have shown that strong down-regulation of COMT in tobacco or poplar leads to a drastic reduction in S units, with corresponding incorporation of 5-hydroxyguaiacyl units into lignin, the overall level of which is not reduced (Atanassova et al., 1995; Van Doorsselaere et al., 1995). Parallel pathways for monolignol *O*-methylation occur at the level of the free acids and their respective CoA thioesters (Ye et al., 1994) and possibly even at the level of the aldehydes formed after the first reduction of the CoA thioesters (Matsui et al., 1994) (see Figure 2). These pathways may be under the control of environmental and developmental factors that might differentially affect the relative activities of the COMT and potentially competing OMT pathways, which may explain the variable

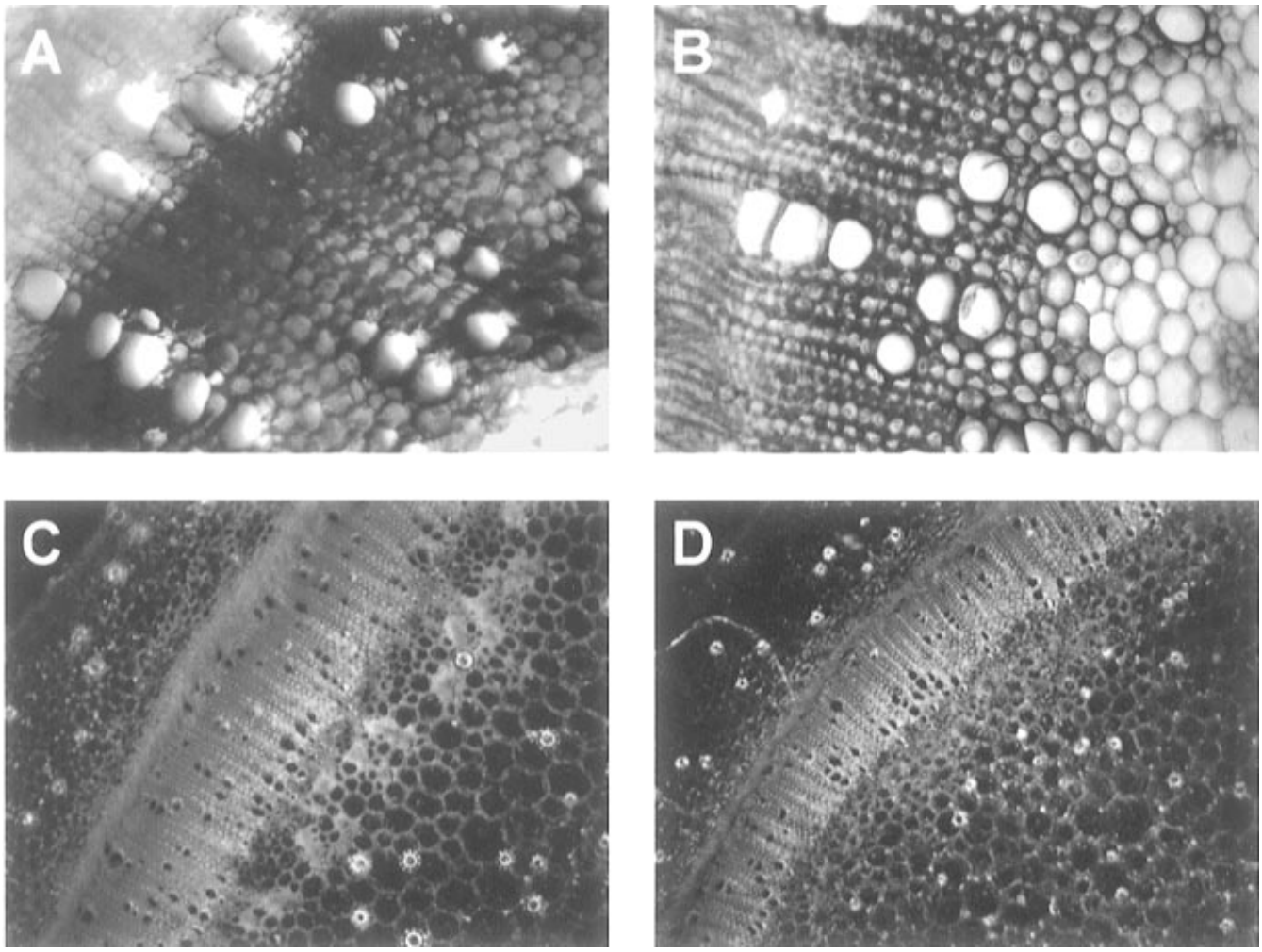

Figure 2. Histochemical staining of lignin in transgenic tobacco: cross sections of stems (10th internode from top) stained with (A, B) phloroglucinol hydrochloride or (C, D) safranin-O and astra-blue; (A and C) untransformed control (105c); (B) COMT antisense primary transformant (105p); (D) COMT antisense T_1 progeny plant (105−22).

effects of reduction in COMT activity on lignin biosynthesis. Alternatively, the variability could be due to the use of different tobacco cultivars or the use of heterologous versus homologous antisense sequences that might differentially down-regulate different OMT isoforms.

The variation in results of genetic manipulation of OMT levels is, however, not unlike the contradictory data for the naturally occurring brown midrib phenotype in corn and other cereals. The corn *bm3* mutation was recently established to be in the gene encoding COMT (Vignols et al., 1995). The most common phenotype ascribed to the brown midrib mutation, including the *bm3* mutation in corn, is a reduced S/G ratio (Gaudillere and Monties, 1989; Lam et al., 1996) and the appearance of the new 5-hydroxyguaiacyl lignin subunit (Chabbert et al., 1994), which is derived from 5-hydroxyferulic acid, the intermediate product between ferulic acid (monomethoxylated) and sinapic acid (dimethoxylated). However, the effect on lignin content has varied from no change in acid insoluble lignin (Sommerfeldt et al., 1979) or acetyl bromide lignin (Lam et al., 1996) to substantial reductions in acid insoluble lignin (Gaudillere and Monties, 1989; Lam et al., 1996) and acetyl bromide lignin (Gaudillere and Monties, 1989). The *bm2* mutation in corn, for which the gene has not yet been identified, causes reduced lignin levels with an increase in S/G ratio (Chabbert et al., 1994), opposite to the reduced S/G ratio in the *bm3* mutant. Clearly, the complexity of the lignin biosynthetic pathway makes it difficult to predict the effect of down-regulating single enzymes of the pathway. Although several reports of targeted down-regulation of late enzymes of lignin biosynthesis have failed to demonstrate reductions in lignin content (Dwivedi et al., 1994; Halpin et al., 1994; Atanassova et al., 1995; Van Doorsselaere et al., 1995), the present results imply the possibility of moderate reduction in lignin content and increased S/G ratio by targeting COMT.

Strategies to engineer plants with agronomically useful lignin-related traits need to flexibly and predictably yield reductions in lignin content and/or changes in lignin monomer composition. Drastically reduced levels of lignin can be obtained by decreasing the phenylalanine pool size (Yao et al., 1995) or reducing the activity of PAL (Elkind et al., 1990; Bate et al., 1994) or cinnamate 4-hydroxylase (C4H) (unpublished results), the entry enzymes into the phenylpropanoid pathway.

Extractable PAL activity and cell wall composition of control and PAL-suppressed tobacco are summarized in Table 2. Tobacco plants suppressed in PAL accumulate sharply reduced levels of lignin in their stems, concomitant with very large increases in methoxyl content (Table 2). The change in lignin composition following PAL suppression suggests that inhibition of the precursor flux into the phenylpropanoid pathway has qualitative as well as quantitative effects on lignin synthesis. Those results, although more magnified, are similar to

those obtained by COMT suppression. As with COMT suppression, NDF content is reduced by PAL suppression, and NDF levels are even more strongly correlated with lignin content, on both DM and NDF bases (r = 0.90 and 0.80, respectively). Staining results with phloroglucinol, Mäule's reagent, and safanin-O/astrablue are also very similar to those reported here for the COMT antisense tobacco (Sewalt and Dixon, unpublished data).

Lignin−Digestibility Relationship. The presence of lignin and other phenolics in plant cell walls reduces the rate and extent of polysaccharide degradation by rumen microorganisms, resulting in an inverse relationship between lignin content and digestibility of forages (Jung and Vogel, 1986). In addition to lignin content, the composition of lignin may affect its inhibition of cell wall degradation (Reeves, 1985; Sewalt et al., 1996a). Lignin composition changes with maturity toward a more syringyl-rich lignin (Buxton and Russell, 1988), which could change the type and extent of interactions between lignin and polysaccharides. Although cell walls of the more digestible brown midrib mutant (bm_3) of corn often contain less lignin [e.g., Hartley and Jones (1978)], digestibility is higher for the mutant even at lignin content similar to that of normal corn varieties (Sommerfeldt et al., 1979).

In contrast to most studies relying on maturity-associated or treatment-induced changes in lignin, we can now demonstrate a clear relation between cell wall digestibility and lignin content without the confounding effects usually present in maturity studies or pretreatment experiments. NDF preparations were made from dried and ground stem samples selected from various near-isogenic control and COMT or PAL transgenic plants covering a wide range of Klason lignin and methoxyl group values, and *in vitro* enzymatic digestibility was determined by measuring reducing sugar production following incubation with cellulase. Digestibility of tobacco stem internodes increased significantly with reduced lignin content and with increased lignin methoxyl group content (Figure 3). Although both lignin content and lignin methoxyl content were significantly correlated with enzymatic NDF digestibility, lignin content seemed to be the overriding factor as judged by the magnitude of its negative correlation with digestibility.

Lignin content has been previously related to digestibility by curvilinear models (Jung and Vogel, 1986), but the data of the present experiment suggest linear trends according to the following regression equations:

$$\text{DM digestibility (mg/g)} = 894 - 3.44 \times \text{Klason lignin (mg/g of DM)} \quad (r^2 = 0.97,\ n = 14)$$

$$\text{NDF digestibility (mg/g)} = 723 - 2.74 \times \text{Klason lignin (mg/g of NDF)} \quad (r^2 = 0.95,\ n = 14)$$

The second equation predicts a noticeable indigestible NDF residue even at lignin levels approaching zero, which is consistent with observations that additional factors, such as the presence of wall-bound hydroxycinnamic acids, polysaccharide structure, or physical structure, are potentially limiting to cell wall degradation. Notwithstanding, the improvements in cell wall digestibility by reducing lignin content are substantial: in PAL-suppressed tobacco, a reduction in lignin content by approximately 50% caused an increase in cell wall digestibility by 35% (147 mg/g). Similarly, in the case of primary COMT antisense transformant 105p, a reduction in lignin content from 164 to 127 mg/g of NDF (a 22% reduction) resulted in an improvement in cell wall digestibility of 48% (113 mg/g). Clearly, these improvements in DM and cell wall digestibility obtained by reducing lignin levels are larger than those resulting from altered lignin composition (reduced S/G ratio).

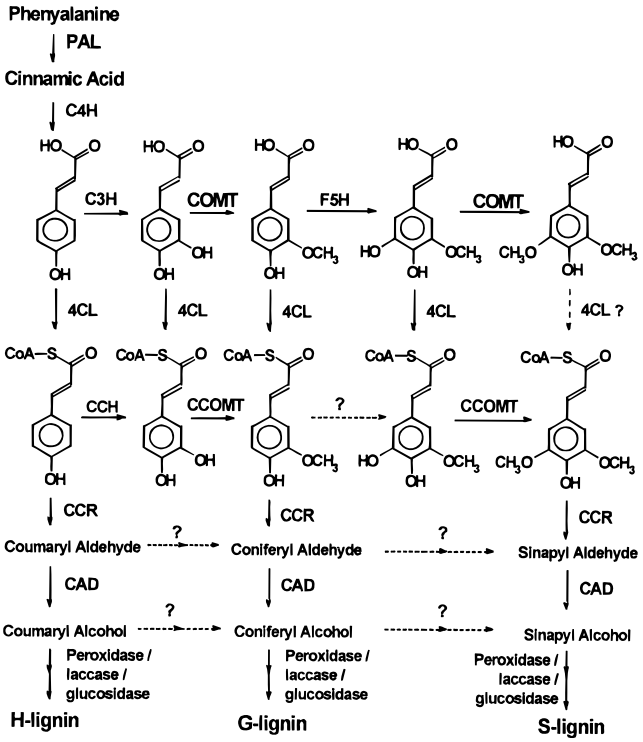

Figure 3. Biosynthetic pathways for the formation of lignin monomers. The enzymes are PAL, L-phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; C3H, 4-coumarate hydroxylase; COMT, bifunctional caffeic/5-hydroxyferulic acid *O*-methyltransferase; F5H, ferulate 5-hydroxylase; 4CL, coumarate:CoA ligase; CCH, coumaroyl CoA hydroxylase; CCOMT, caffeoyl CoA 3-*O*-methyltransferase; CCR, cinnamoyl CoA reductase; CAD, cinnamyl alcohol dehydrogenase. Dotted arrows indicate reactions that are still uncertain in the context of lignin biosynthesis.

The importance of lignin composition *per se* toward its inhibitory effect on digestibility was recently demonstrated utilizing transgenic tobacco with altered lignin composition (S/G ratio) but equal lignin concentration (Bernard Vailhé et al., 1996). In those plants, a 2-fold reduction in S/G ratio in COMT antisense tobacco caused a 6% (3.5 percentage units) increase in DM digestibility, whereas a 10-fold decrease in S/G ratio in COMT sense-suppressed tobacco resulted in a 9% (5.6 percentage units) increase. In contrast, in an *Arabidopsis* mutant that produces no syringyl-type lignin due to a mutation in the gene encoding ferulate 5-hydroxylase, the change from the wild-type mixed syringyl/guaiacyl lignin to the mutant guaiacyl lignin did not alter cell wall degradability (Jung and Chapple, 1996).

In conclusion, the present results indicate the feasibility of genetic engineering of lignin for forage improvement by manipulating either early or late lignin biosynthetic pathway enzymes. Although the potential complications of a metabolic grid associated with the ring substitution reactions in the later stages of monolignol synthesis might, at first sight, make the simple approach of reducing lignin by limiting flux at an earlier stage in the pathway more attractive, such modifications would ultimately need to be engineered under

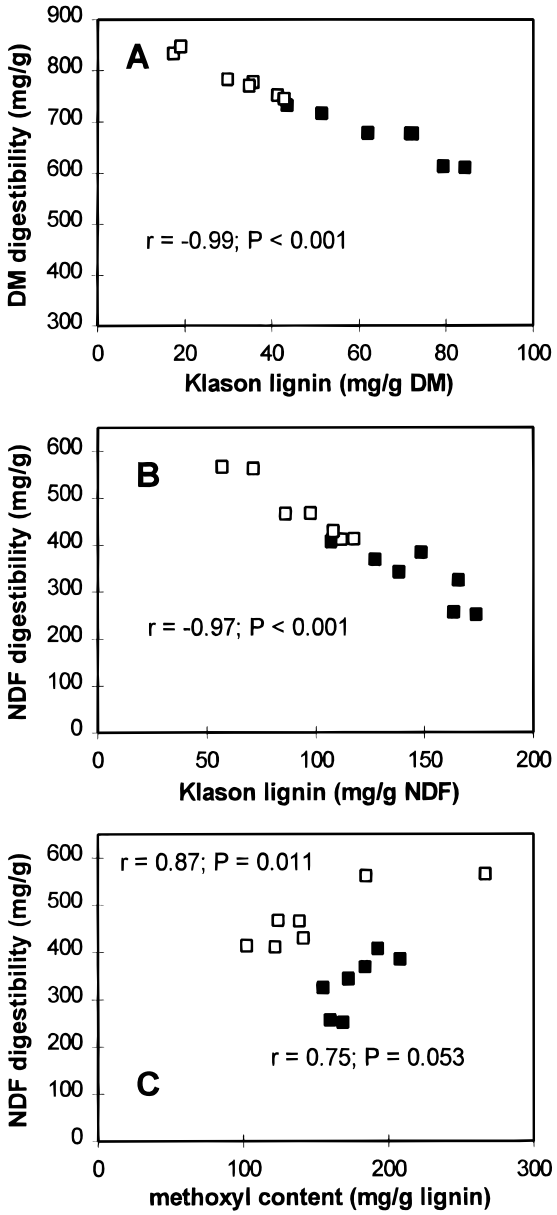

Figure 4. Relations between lignin content and composition with *in vitro* digestibility of NDF or DM from a range of transgenic tobacco lines. The individual transformants included control and PAL-modified plants (Bate et al., 1994; open symbols) and control and COMT antisense plants (Ni et al., 1994; solid symbols) from two independent experiments: (A) DM digestibility and Klason lignin (mg/g of DM; DM digestibility was calculated assuming 95% digestion of neutral detergent solubles); (B) NDF digestibility and Klason lignin (mg/g of NDF); (C) NDF digestibility and lignin methoxyl content (mg/g of Klason lignin).

tight developmental control (by choice of an appropriate gene promoter) to avoid pleiotropic effects, such as increases in disease susceptibility (Maher et al., 1994; Pallas et al., 1996) and lodging. Using a near-isogenic system, we also demonstrate that a reduced lignin content overrules possible negative effects of altered lignin composition on dry matter and cell wall digestibility.

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LITERATURE CITED

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

Bate, N. J.; Orr, J.; Ni, W.; Meroni, A.; Nadler-Hassar, T.; Doerner, P. W.; Dixon, R. A.; Lamb, C. J.; Elkind, Y. Quantitative relationship between phenylalanine ammonia-lyase levels and phenylpropanoid accumulation in transgenic tobacco identifies a rate determining step in natural product synthesis. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 7608−7612.

Bernard Vailhé, M. A.; Migné, C.; Cornu, A.; Maillot, M. P.; Grenet, E.; Besle, J. M.; Atanassova, R.; Martz, F.; Legrand, M. Effect of modification of the *O*-methyltransferase activity on cell wall composition, ultrastructure and degradability of transgenic tobacco. *J. Sci. Food Agric.* **1996**, *72*, 385−391.

Boudet, A. M.; Grima-Pettenati, J. Lignin genetic engineering. *Mol. Breed.* **1996**, *2*, 25−39.

Bradford, M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

Buxton, D. R.; Russell, J. R. Lignin constituents and cell-wall digestibility of grass and legume stems. *Crop Sci.* **1988**, *28*, 553−558.

Campbell, M. M.; Sederoff, R. R. Variation in lignin content and composition. Mechanisms of control and implications for the genetic improvement of plants. *Plant Physiol.* **1996**, *110*, 3−13.

Chabbert, B.; Tollier, M. T.; Monties, B.; Barrière, Y.; Argillier, O. Biological variability in lignification of maize: expression of the brown midrib bm2 mutation. *J. Sci. Food Agric.* **1994**, *64*, 455−460.

Chen, C.-L. Nitrobenzene and cupric oxide oxidations. In *Methods in Lignin Chemistry*; Lin, S. Y., Dence, C. W., Eds.; Springer-Verlag: Berlin, 1992; pp 301−321.

Doster, M. A.; Bostock, R. M. Quantification of lignin formation in almond bark in response to wounding and infection by *Phytophthora* species. *Phytopathology* **1988**, *78*, 473−477.

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

Edwards, R.; Kessmann, H. Isoflavanoid phytoalexins and their biosynthetic enzymes. In *Molecular Plant Pathology—A Practical Approach*; Gurr, S. J., McPherson, M. J., Bowles, D. J., Eds.; IRL Press: Oxford, U.K., 1992; Vol. 2, pp 45−62.

Elkind, Y.; Edwards, R.; Mavandad, M.; Hedrick, S. A.; Ribak, O.; Dixon, R. A.; Lamb, C. J. Abnormal plant development and down regulation of phenylpropanoid biosynthesis in transgenic tobacco containing a heterologous phenylalanine ammonia-lyase gene. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 9057−9061.

Gaudillere, M.; Monties, B. Biochemical and biosynthetitc studies on lignification of graminea. In *Plant Cell Wall Polymers. Biogenesis and Biodegradation*; ACS Symposium Series 399; Americal Chemical Society: Washington, DC, 1989; pp 182−192.

Glasser, W. G.; Kelley, S. S. Lignin. In *Encyclopedia of Polymer Science and Engineering*, 2nd ed.; Wiley: New York, 1987; Vol. 8, pp 795−852.

Gowri, G.; Bugos, R. C.; Campbell, W. H.; Maxwell, C. A.; Dixon, R. A. 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.* **1991**, *97*, 7−14.

Grabber, J. H.; Jung, G. A.; Abrams, S. M.; Howard, D. B. Digestion kinetics of parenchyma and sclerenchyma cell

walls isolated from orchardgrass and switchgrass. *Crop Sci.* **1992**, *32*, 806−810.

Halpin, C.; Knight, M. E.; Foxon, G. A.; Campbell, M. M.; Boudet, A. M.; Boon, J. A.; Chabbert, B.; Tollier, M.-T.; Schuch, W. Manipulation of lignin quality by down-regulation of cinnamyl alcohol dehydrogenase. *Plant J.* **1994**, *6*, 339−350.

Hartley, R. D.; Jones, E. C. Phenolic components and degradability of the cell walls of the brown midrib mutant, bm_3, of *Zea mays*. *J. Sci. Food Agric.* **1978**, *29*, 777−789.

Howles, P. A.; Sewalt, V. J. H.; Paiva, N. L.; Lamb, C. J.; Elkind, Y.; Bate, N. J.; Dixon, R. A. Overexpression of L-phenylalanine ammonia-lyase in transgenic tobacco plants reveals control points for flux into phenylpropanoid synthesis. *Plant Physiol.* **1996**, *112*, 1617−1624.

Jung, H. G. Forage lignins and their effects on fiber digestibility. *Agron. J.* **1989**, *81*, 33−38.

Jung, H. G.; Chapple, C. C. S. Does lignin composition impact cell-wall degradability? *J. Dairy Sci.* **1996**, *79* (Suppl. 1), 151.

Jung, H. G.; Vogel, K. P. Influence of lignin on digestibility of forage cell wall material. *J. Anim. Sci.* **1986**, *62*, 1703−1712.

Jung, H. G.; Valdez, F. R.; Hartfield, R. D.; Blanchette, R. A. Cell wall composition and degradability of forage stems following chemical and biological delignification. *J. Sci. Food Agric.* **1992**, *58*, 347−355.

Kuc, J.; Nelson, O. E. The abnormal lignins produced by the brow-midrib mutants of maize, I. The brown-midrib-1 mutant. *Arch. Biochem. Biophys.* **1964**, *105*, 103−113.

Lam, T. B. T.; Iiyama, K.; Stone, B. A. Lignin and hydroxycinnamic acids in walls of brown midrid mutants of sorghum, pearl millet and maize stems. *J. Sci. Food Agric.* **1996**, *71*, 174−178.

Maher, E. A.; Bate, N. J.; Ni, W.; Elkind, Y.; Dixon, R. A.; Lamb, C. J. Increased disease susceptibility of transgenic tobacco plants with suppressed levels of preformed phenylpropanoid products. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 7802−7806.

Matsui, N.; Fukushima, K.; Yasuda, S.; Terashima, N. On the behavior of monolignol glucosides in lignin biosynthesis. *Holzforschung* **1994**, *48*, 375−380.

Miller, G. L. Use of dinitrosalicylic reagent for determination of reducing sugar. *Anal. Chem.* **1959**, *31*, 426−428.

Nakano, J.; Meshitsuka, G. The detection of lignin. In *Methods in Lignin Chemistry*; Lin, S. Y., Dence, C. W., Eds.; Springer-Verlag: Berlin, 1992; pp 23−32.

Ni, W.; Paiva, N. L.; Dixon, R. A. Reduced lignin in transgenic plants containing an engineered caffeic acid O-methyltransferase antisense gene. *Transgenic Res.* **1994**, *3*, 120−126.

Pallas, J. A.; Paiva, N. L.; Lamb, C. J.; Dixon, R. A. Tobacco plants epigenetically suppressed in phenylalanine ammonia-lyase expression do not develop systemic acquired resistance in response to infection by tobacco mosaic virus. *Plant J.* **1996**, *10*, 282−294.

Quicke, G. V.; Bentley, O. G. Lignin and methoxyl groups as related to the decreased digestibility of mature forages. *J. Anim. Sci.* **1959**, *18*, 356−373.

Ralph, J.; Hatfield, R. D. Pyrolysis-GC-MS characterization of forage materials. *J. Food Agric. Chem.* **1991**, *39*, 1426−1437.

Reeves, III, J. B. Lignin composition and in vitro digestibility of feeds. *J. Anim. Sci.* **1985**, *60*, 316−322.

Sewalt, V. J. H.; Glasser, W. G.; Fontenot, J. P.; Allen, V. G. Lignin impact on fiber degradation. 1. Quinone methide intermediates formed from lignin during *in vitro* fermentation of corn stover. *J. Sci. Food Agric.* **1996a**, *71*, 195−203.

Sewalt, V. J. H.; De Olivera, W.; Glasser, W. G.; Fontenot, J. P. Lignin impact on fiber degradation. 2. A model study using cellulose hydrogels. *J. Sci. Food Agric.* **1996b**, *71*, 204−208.

Sewalt, V. J. H.; Glasser, W. G.; Beauchemin, K. A. Lignin impact on fiber degradation. 3. Reversal of inhibition of enzymatic hydrolysis by chemical modification of lignin and by additives. *J. Agric. Food Chem.* **1997a**, *45*, 1823−1828.

Sewalt, V. J. H.; Beauchemin, K. A.; Rode, L. M.; Acharya, S.; Baron, V. S. Lignin impact on fiber degradation. IV. Enzymatic saccharification and *in vitro* digestibility of alfalfa and grasses following selective solvent delignification. *Bioresour. Technol.* **1997b** (in press).

Snedecor, G. W.; Cochran, W. G. *Statistical Methods*, 8th ed.; Iowa State University Press: Ames, IA, 1989.

Sommerfeldt, J. L.; Schingoethe, D. J.; Muller, L. D. Brown-midrib corn silage for lactating dairy cows. *J. Dairy Sci.* **1979**, *62*, 1611−1618.

Srebotnik, E.; Messner, K. A simple method that uses differential staining and light microscopy to assess the selectivity of wood delignification by white rot fungi. *Appl. Environ. Microbiol.* **1994**, *60*, 1383−1386.

TAPPI. *Methoxyl Content of Pulp and Wood*; TAPPI standard method 209 su-72; Technical Association of the Pulp and Paper Industry (TAPPI): Atlanta, GA, 1972.

Titgemeyer, E. C.; Cochran, R. C.; Towne, E. G.; Armendariz, C. K.; Olson, K. C. Elucidation of factors associated with the maturity-related decline in degradability of big bluestem cell wall. *J. Anim. Sci.* **1996**, *74*, 648−657.

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. A novel lignin in poplar trees with a reduced caffeic acid/5-hydroxyferulic acid O-methyltransferase activity. *Plant J.* **1995**, *8*, 855−864.

Van Soest, P. J.; Robertson, J. B.; Lewis, B. A. Methods for dietary fiber, neutral detergent fiber, and non-starch polysaccharides in relation to animal nutrition. *J. Dairy Sci.* **1991**, *74*, 3583−3597.

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

Yao, K. N.; Deluca, V.; Brisson, N. Creation of a metabolic sink for tryptophan alters the phenylpropanoid pathway and the susceptibility of potato to *Phytophthora infestans*. *Plant Cell* **1995**, *7*, 1787−1799.

Ye, Z. H.; Kneusel, R. E.; Matern, U.; Varner, J. E. An alternative methylation pathway in lignin biosynthesis in *Zinnia*. *Plant Cell* **1994**, *6*, 1427−1439.

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