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Lignin Deposition and Associated Changes in Anatomy, Enzyme Activity, Gene Expression, and Ruminal Degradability in Stems of Tall Fescue at Different Developmental Stages

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Stem tissues of tall fescue (*Festuca arundinacea* Schreb.) were sampled at three elongation stages and three reproductive stages. Anatomical analysis showed the deposition of guaiacyl (G) and syringyl (S) lignin during plant development and the formation of a lignified sclerenchyma ring. A dramatic increase in Klason lignin content was found from elongation stage to reproductive stage. Lignin composition analyzed by gas chromatography−mass spectrometry revealed that S lignin content and S/G ratio increased with stem development, but contents of *p*-hydroxyphenyl (H) and G lignins decreased during the same period. S lignin content and S/G ratio also increased from the younger upper internode down to the older basal internode of the stem, but G and H lignin decreased in parallel. Relative *O*-methyltransferase activities increased during stem development and in parallel with the lignification process of stem. The pattern of enzyme activity during development varied with the choice of substrate, with highest activities seen when substrates were caffeoylaldehyde and 5-hydroxyferulic acid, and lowest activities were seen when caffeic acid and 5-hydroxyconiferyl alcohol were used as substrates. The expression of caffeic acid *O*-methyltransferase and cinnamyl alcohol dehydrogenase genes increased during the stem elongation stage and remained at high levels during the reproductive stages. The changes at anatomical, metabolic, and molecular levels during plant development were closely associated with lignification and degradability. This study provides an integrated picture of the molecular and chemical events that accompany changes in lignin deposition and ruminal degradability.

KEYWORDS: Tall fescue; lignin; stem; internode; ruminal degradability; *O*-methyltransferase; cinnamyl alcohol dehydrogenase

INTRODUCTION

Forages represent the major feed source for cattle production systems, and improvement in cell wall degradability is an important goal of many plant-ruminant animal research programs (*1*). Lignin is an important chemical component of forage cell walls; however, it is essentially undigestible and inhibits rumen fermentation of forage cell wall polysaccharides (*2*). Lignification of cell walls during plant development has been identified as the major factor limiting digestibility/degradability of forage crops (*3*, *4*).

Lignin in forage grasses is composed of guaiacyl (G) units derived from coniferyl alcohol, syringyl (S) units derived from sinapyl alcohol, and *p*-hydroxyphenyl (H) units derived from *p*-coumaryl alcohol (*5*). In addition to lignin content (or concentration), the composition of lignin is an important factor that influences cell wall degradability of forages (*3*, *4*, *6*). It has also been suggested that the anatomical structure of cells and tissues in grasses may be more important than wall chemistry in determining the rate and extent of fiber digestion in vivo, because anatomical structure significantly influences wall accessibility to rumen microorganisms (*7*, *8*).

Lignin biosynthesis comprises a highly coordinated and regulated set of metabolic events, and many enzymes are involved in the pathway (*9*−*12*). Caffeic acid *O*-methyltransferase (COMT) and cinnamyl alcohol dehydrogenase (CAD) have been shown to play important roles in lignin biosynthesis (*13*−*15*). COMT is a multispecific enzyme that not only methylates caffeic acid to ferulic acid and 5-hydroxyferulic acid to sinapic acid but also is involved in the 3-*O*-methylation of monolignol precursors at the aldehyde or alcohol levels (*9*, *10*, *16*, *17*). CAD catalyzes the last step in the biosynthesis of lignin precursors, which is the reduction of cinnamaldehydes to cinnamyl alcohols (*9*).

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There have been many studies on lignin and degradability in forage grasses; however, the research efforts were scattered in many species with emphasis on different aspects. They include studies on the influence of lignin on degradability in bluestems, orchardgrass, smooth bromegrass, and switchgrass (*3*, *5*, *6*, *18*, *19*), comparison of lignin content and/or composition between different cultivars and genotypes and the effect of selection in smooth bromegrass, orchardgrass, perennial ryegrass, switchgrass, and tall fescue (*20*−*24*), maturation effect on degradability with forage cuttings at different timepoints in big bluestem, creeping foxtail, Kentucky bluegrass, perennial ryegrass, reed canarygrass, switchgrass, tall fescue and timothy (*25*−*31*), effect of phenolic compounds on cell wall degradation in bermudagrass, orchardgrass, tall fescue, and timothy (*2*, *32*, *33*), and digestion kinetics of different cell types in Italian ryegrass, orchardgrass, perennial ryegrass, and switchgrass (*34*, *35*).

Lignin formation and deposition in cell walls is developmentally controlled and reflects changes in gene expression. Despite the diverse studies on lignin and degradability in different grass species, detailed systematic studies addressing cell wall anatomy, chemical composition, enzyme activity, gene expression, and degradability have not, to our knowledge, been reported. We have been interested in using tall fescue as a model system for such studies, because this information will help to design better strategies to improve forage digestibility by genetic engineering of lignin biosynthetic pathway.

Tall fescue is the predominant cool-season perennial forage species grown on an estimated 35 million acres in the United States (*36*). It has been used extensively as forage basis for cattle production worldwide and also serves as turf for sports grounds and soil conservation (*37*). In addition, tall fescue is being used as a model system for large-scale expressed sequence tags (EST) sequencing, gene discovery, genetic transformation, and lignin modification (*38*).

We here describe a systematic study of lignin deposition in tall fescue stems at defined developmental stages. Anatomical comparisons, cell wall chemistry, activity and substrate preference of lignin biosynthetic enzymes, expression levels of lignin-related genes, and ruminal degradability are reported for the same biological samples to provide an integrated picture of the molecular events that accompany changes in ruminal degradability in this important forage species.

MATERIALS AND METHODS

Plant Material. Tall fescue (*Festuca arundinacea* Schreb.) plants grown in the Headquarters farm of the Noble Foundation (Ardmore, OK) were sampled from April to June of 2000 and 2001. Mean annual temperature and rainfall of the experimental site were 17.7 °C and 912 mm, respectively. Stem tissues were excised by removing leaf blades and leaf sheaths from plants harvested at different developmental stages. The development of tall fescue stem was divided into three elongation stages (E1, E2, and E3) and three reproductive stages (R1, R2, and R3) according to the guidelines of Moore et al. (*39*). The collected stems were further dissected into different internodes ranging from I1 (basal, oldest section) to I4 (upper, youngest section) at the R1−R3 stage. The samples were immediately frozen in liquid N_2 and stored at −80 °C until used.

Histochemical Staining of Lignin. Two methods were used for histochemical staining of lignin.

(1) Maule Staining (40). Sections of tall fescue internodes were hand cut with a vibratome (series 1000, Ted Pella Inc., Redding, CA) and immersed in 1% neutral potassium permanganate solution for 3 min at room temperature. The sections were rinsed in distilled water, decolorized with 12% HCl for 2 min, washed thoroughly in water, and treated with a few drops of 1.5% sodium bicarbonate solution. Photographs were taken using an Olympus SZX stereomicroscope system with a SPOT RT color camera.

(2) Staining with Safranin-O (41). A 50 mm length segment in the middle of each internode was fixed in formalin/acetic acid/ethanol/water (5:5:60:30) and embedded in paraffin until processing for optical microscopy. Twenty serial sections of 12.5 μm thickness were cut with a sledge microtome from the embedded internodes. Paraffin was removed by immersion in xylene. The sections were rehydrated and stained with 5% Safranin-O in 30% EtOH at room temperature for 4 h. Photographs were taken using an Olympus SZX stereomicroscope system with a SPOT RT color camera.

Determination of Lignin Content and Composition. The content and composition of lignin were determined according to the procedures described by Guo et al. (*14*). Two hundred milligrams of dried cell walls was used for lignin analysis, and Klason lignin content was calculated as weight percentage of the extract-free sample (*40*). Lignin composition was determined after thioacidolysis and Raney nickel desulfurization (*42*, *43*). Thioacidolysis was performed using 15 mg of extractive-free cell wall samples reacted with 15 mL of 0.2 M BF_3 etherate in an 8.75:1 dioxane/ethanethiol mixture. An aliquot of the thioacidolysis solution in CH_2Cl_2 was mixed with 1 mM Raney nickel aqueous slurry (Aldrich Chemical Co., Inc., Milwaukee, WI) for desulfurization. The above thioacidolysis procedure was done under a fume hood. Lignin-derived monomers and dimers were identified by gas chromatography (GC)−mass spectrometry (MS) and quantified by GC. 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 *m*/*z* 60−650 scanning range (*14*).

Fractionation of Cell Wall Polysaccharides. The excised stem and internode segments were boiled for 15 min in methanol at 80 °C and then washed and stored in methanol until use. Cell wall polysaccharides were fractionated according to the method of Kamisaka et al. (*44*). Rehydrated segments were homogenized in ice-cold water with a mortar and pestle. The homogenate was centrifuged for 10 min at 1000*g*, and the cell wall residue was washed sequentially with ice-cold water, acetone, and a methanol/chloroform mixture (1:1, v/v). To remove the starch, the washed residues were treated at 37 °C for 3 h with 5 mL of 2 units mL^{-1} pancreatic α-amylase (type I-A, Sigma Chemical Co., St. Louis, MO) in 0.1 M sodium acetate buffer (pH 6.5). The treated material was extracted three times with 20 mM ammonium oxalate (pH 4.0) at 70 °C for 1 h to obtain the pectin fraction. The residues were further extracted with 0.1 M NaOH at room temperature under nitrogen atmosphere for 24 h in the dark to obtain hemicellulose extraction 1. The insoluble residues were extracted three times with 17.5% (w/v) NaOH solution for 8 h under nitrogen atmosphere to obtain the hemicellulose extraction 2. The total hemicellulose fraction was obtained by combining extractions 1 and 2. The alkali-insoluble fraction was sequentially washed with distilled water, 1 mM acetic acid, and ethanol and then dried at 37 °C. This fraction was dissolved in 72% H_2SO_4 for 1 h at room temperature and then diluted 30-fold with distilled water to obtain the cellulose fraction. Total sugar contents in each fraction were determined according to the phenol−sulfuric acid method (*45*) using glucose as the standard.

Enzyme Extraction and Assay. Tall fescue stems and internodes were collected and homogenized in liquid nitrogen. Powdered tissue was extracted for 1 h at 4 °C in extraction buffer (100 mM Tris-HCl, pH 7.5, 10% glycerol, 2 mM DTT, 0.2 mM $MgCl_2$, 1 mM phenylmethanesulfonyl fluoride) and desalted on PD-10 columns (Amersham Pharmacia Biotech, Uppsala, Sweden). Protein concentrations were determined using Bio-Rad Dc protein assay reagent (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as standard. The COMT activities were assayed essentially as described by Ni et al. (*46*). Six potential substrates were used: caffeic acid, 5-hydroxyferulic acid, caffeoylaldehyde, caffeoyl alcohol, 5-hydroxyconiferaldehyde, and 5-hydroxyconiferyl alcohol. The assay mixtures contained 5 μL of [$^{14}CH_3$]-*S*-adenosyl-L-Met (0.6 mM, 13 μCi/μmol), 5 μL of phenolic substrates (1 mM), 30 μL of assay buffer (100 mM Tris-HCl, pH 7.5, 10% glycerol, 2 mM DTT, 0.2 mM $MgCl_2$), and 5 μL of protein extract. They were incubated at 30 °C for 30 min, stopped by adding 50 μL of

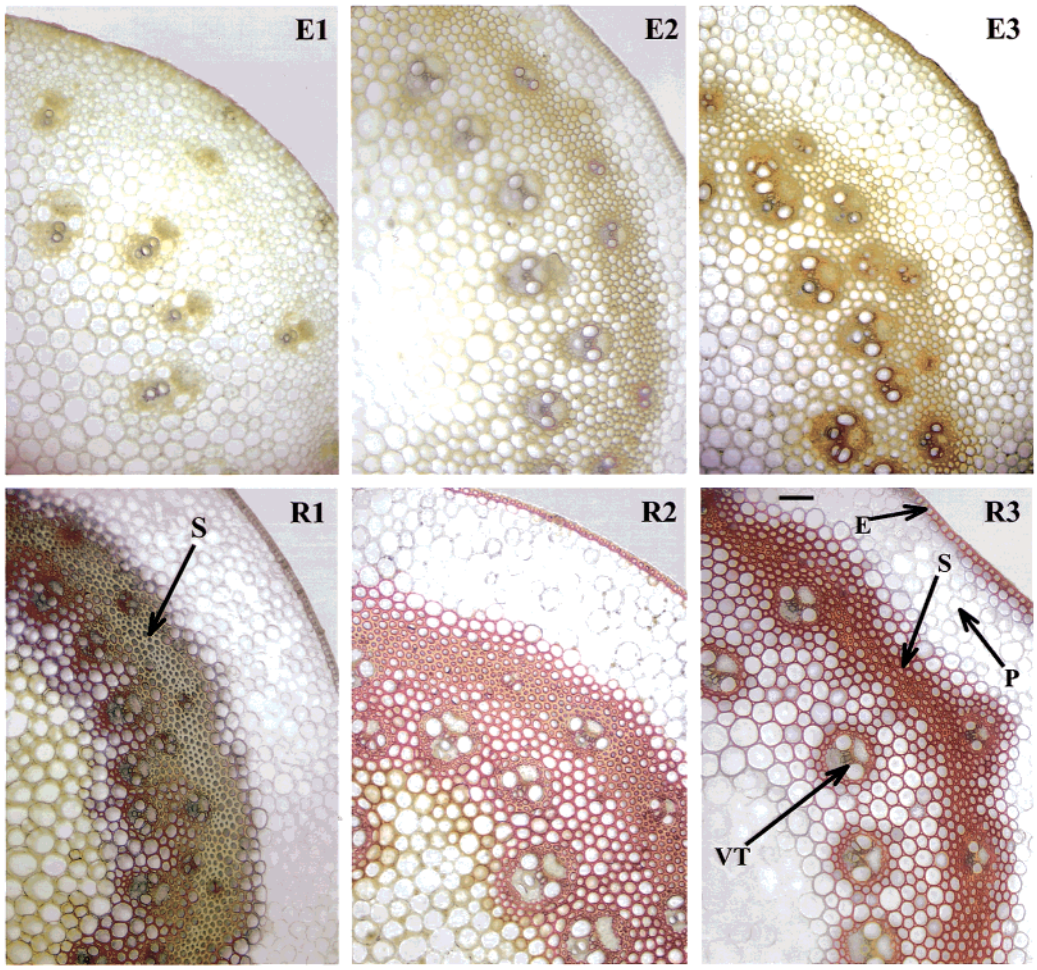

Figure 1. Cross-sections of base internodes of tall fescue stem from elongation (E1) to reproductive (R3) stage: E1, first node palpable; E2, second node palpable; E3, third node palpable/visible; R1, first spikelet visible; R2, spikelets fully emerged; R3, anthesis. Color shift from yellow to red indicated the increase of syringyl (S) lignin. VT, vascular tissue; P, parenchyma; E, epidermis; S, sclerenchyma. Bar length is 50 μm.

0.2 mM HCl, and incubated for 10 min at 37 °C. Labeled ferulic acid was extracted into 200 μL of hexane/ethyl acetate (1:1, v/v), and 150 μL of separated organic phases was transferred to scintillation vials for determination of radioactivity.

Northern Hybridization Analysis. Total RNA was isolated from whole stem and internode segments at different growth stages with TRI reagent (Molecular Research Center, Inc., Cincinnati, OH). After denaturation with glyoxal at 65 °C for 15 min, 10 μg samples of RNA were fractionated by electrophoresis on a 1% formaldehyde denaturing gel according to standard protocols (*47*). Fractionated RNAs were transferred to a Duralose-UV membrane (Stratagene, La Jolla, CA) and fixed by baking at 80 °C for 2 h after UV cross-linking. Hybridization was carried out with COMT and CAD cDNA probes using a hybridization kit (Molecular Research Center, Inc.). The probes were labeled with an α^{32}P-dCTP labeling kit (Life Technologies, Gaithersburg, MD).

In Situ Degradability Assay in Fistulated Steers. Tall fescue stem tissues harvested at different growth stages were dried at 50 °C and ground into 1 mm powder. About 5 g of ground material was put into each preweighed Ankom rumen in situ filter bag (10 × 20 cm, pore size = 50 μm; Ankom Technology, Fairport, NY). These bags were put into larger (40 × 80 cm) bags and then placed into the rumens of fistulated steers for 36 h of digestion. Steers were placed on ad libitum tall fescue hay 2 weeks prior to the trials. During the trials, they were fed only tall fescue. After digestion, bags were removed from the rumen, washed in a commercial washing machine, and vacuum-dried in a freeze-drier. Degradability was calculated on the basis of sample weight loss before and after digestion. The amount of degradation was expressed as the means of triplicate samples in the experiment.

Statistical Analysis. All of the quantitative data have at least three replications. Data from each trait (chemical component) were subjected to one-way analysis of variance. Significance of treatments were tested at the $P = 0.05$ level. Standard errors are provided in all of the tables and figures as appropriate. Correlation and linear regression analyses were performed to determine the relationship between ruminal degradability and related chemical components.

RESULTS

Lignin Deposition in Developing Tall Fescue Stems and Internodes. The stages of growth and development of tall fescue plants were identified using a system developed by Moore et al. (*39*) with minor modifications. Stem tissues were collected at three elongation stages (E) and three reproductive stages (R) according to the following criteria: E1, first node palpable; E2, second node palpable; E3, third node palpable/visible; R1, first spikelet visible; R2, spikelets fully emerged; and R3, anthesis. At the reproductive stages (R1, R2, and R3), stems were further dissected into internodes, from basal internode I1 to upper internode I4.

Lignin deposition was anatomically examined by histochemical analyses of the collected samples using the Maule (*40*) and Safranin-O staining (*41*) methods. Essentially the same lignification pattern was obtained by both methods. Because Maule staining provides a visual indication of color reactions with syringyl groups, **Figure 1** shows the results of Maule staining

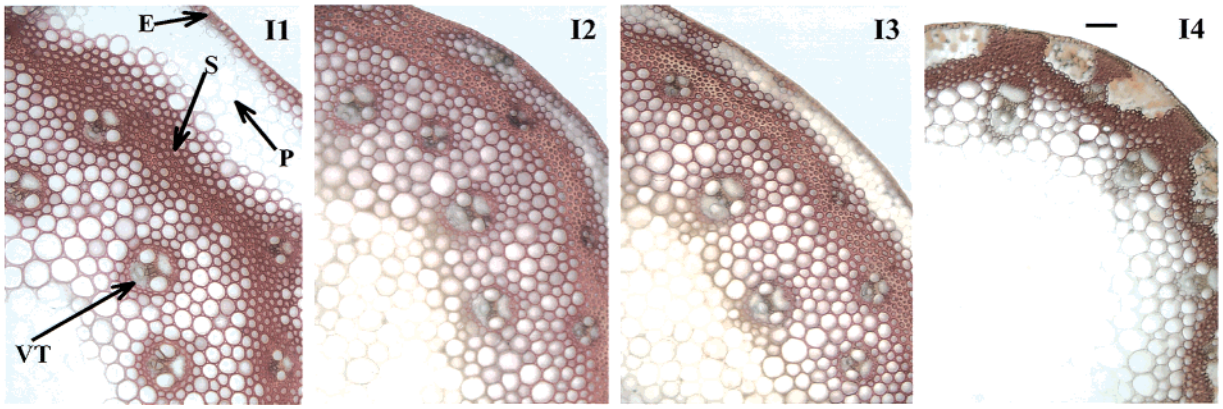

Figure 2. Cross-sections of tall fescue internodes from basal internode I1 to apical internode I4 at the anthesis (R3) stage. VT, vascular tissue; P, parenchyma; E, epidermis; S, sclerenchyma. Bar length is 50 μm.

of basal internodes at each growth stage. A color shift from yellow to red indicated that S lignin increased with progressive maturity of stems from E1 to R3, but the relative content of G lignin decreased during the same period when analyzed together with the results from Safranin-O staining. The deposition of G lignin was mainly in the intercalary meristematic region, where vascular elements were later differentiated from both inner and outer sides of the region and S lignin formed. The parenchyma cells (P) were large in diameter and comprised a substantial proportion of the tissues. These cells appeared to be thin walled at the E1 and E2 stages but became increasingly differentiated and more densely stained from E3 to R3. This observation is more obvious when the tissues were stained with Safranin-O. The thickness of parenchyma cell walls increased from 120 nm at E1 to 1500 nm at the R3 stage. The vascular tissue (VT), consisting of thick-walled and heavily lignified cells, occurred as isolated vascular bundles at the E1 stage and partially linked by sclerenchyma cells (S) at the later stages. One of the most obvious changes during stem lignification is the formation of a sclerenchyma ring. The ring became visible in the E2 stage and continued to increase in size and proportion of the cross-sectioned area through the later stages. Wall thickness of sclerenchyma cells increased from 650 nm at E2 to 4500 nm at the R3 stage. The walls of sclerenchyma cells were 3−5 times thicker than those of parenchyma cells.

Histochemical staining of different internodes at the R3 stage showed a decrease in sclerenchyma area and wall thickness from I1 to I4 (**Figure 2**). The sclerenchyma ring was closer to the epidermal cell layer in upper internodes than that in the basal internode. There were some island-like sclerenchyma tissues near and connected to the epidermises in the upper internodes (**Figure 2**). In the apical I4 internode, the central pitch cells were resorbed and a hollow was formed (**Figure 2**).

Lignin Content and Composition of Stems and Internodes at Different Developmental Stages. Klason lignin content in stem tissues increased moderately during the elongation stage (E1−E3), but the major increase in lignin content occurred when plants changed from the elongation stage to the reproductive stage (**Figure 3A**). Lignin content at the reproductive stage was always much higher than that at the elongation stage, with 10 times more lignin deposited in the cell walls at the R3 stage than at the E1 stage.

In the same stem, lignin content showed a clear trend of decreasing from the basal internodes I1 to upper internodes I4, especially at early reproductive stages R1and R2 (**Figure 3B**). At the R3 stage, lignin content declined from I1 to I3 internodes, but there was little change between I3 and I4 internodes (**Figure 3B**; **Table 1**).

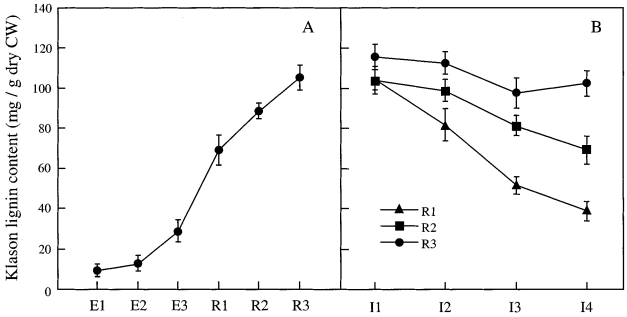

Figure 3. Klason lignin content in tall fescue stems (A) and internodes (B) at different developmental stages: E1, first node palpable; E2, second node palpable; E3, third node palpable/visible; R1, first spikelet visible; R2, spikelets fully emerged; R3, anthesis; I1–I4, basal internode I1 to apical internode I4. Values are means ± SE (n = 3).

Lignin monomer composition was revealed by GC-MS after thioacidolysis. S lignin content in total lignin and S/G ratio increased with progressive maturity of stems from E1 to R3, but the contents of H and G lignin decreased during the same period (**Table 1**).

S lignin and S/G ratio decreased from the basal internode I1 to the upper internode I4 at the R3 stage, but the H and G lignin components increased from I1 to I4 (**Table 1**).

Activities and Substrate Preference of *O*-Methyltransferase (OMT) in Stems and Internodes at Different Developmental Stages. Relative OMT activities were measured using six potential substrates in the monolignol biosynthetic pathway. Although all of the tested substrates could be methylated by OMT, highest enzyme activities were seen when substrates were caffeoylaldehyde and 5-hydroxyferulic acid (**Figure 4**). Caffeic acid and 5-hydroxyconiferyl alcohol were the poorest substrates, and the OMT measured with these two compounds remained at almost the same level irrespective of the growth stage of the stem tissues (**Figure 4A**). When caffeoylaldehyde, 5-hydroxyferulic acid, 5-hydroxyconiferaldehyde, and caffeoyl alcohol were used as substrates, significantly higher OMT activities were observed with progressive maturity of stems (**Figure 4A**). Highest activities against all substrates were recorded at the R2 or R3 stage (**Figure 4A**).

The levels of OMT activity increased moderately from the basal to the upper internodes (**Figure 4B**). Although I1 is the oldest internode structurally, its COMT activity was lower than that of I4 when caffeoylaldehyde and 5-hydroxyconiferaldehyde were used as substrates (**Figure 4B**). No significant difference was found between the internodes when OMT activity was

Table 1. Lignin Content and Composition in Stems and Internodes of Tall Fescue at Different Developmental Stages[a]

		lignin (% dry CW)	S lignin (% lignin)	G lignin (% lignin)	H lignin (% lignin)	S/G ratio
stems	E1	0.91 ± 0.29	34.34 ± 2.54	61.01 ± 2.55	4.65 ± 0.39	0.56
	E2	1.24 ± 0.37	36.90 ± 3.37	60.03 ± 3.08	3.07 ± 0.19	0.61
	E3	2.85 ± 0.59	36.72 ± 2.25	60.65 ± 3.20	2.63 ± 0.17	0.61
	R1	6.90 ± 0.78	38.85 ± 3.07	58.63 ± 1.66	2.52 ± 0.32	0.66
	R2	8.84 ± 0.37	45.54 ± 2.11	52.28 ± 3.09	2.48 ± 0.57	0.87
	R3	10.51 ± 0.55	48.52 ± 2.76	49.58 ± 3.11	1.90 ± 0.48	0.98
internodes (R3 stage)	I1	11.58 ± 0.71	50.68 ± 2.58	48.12 ± 3.09	1.20 ± 0.32	1.05
	I2	11.30 ± 0.62	50.92 ± 3.69	47.55 ± 2.26	1.53 ± 0.28	1.07
	I3	9.79 ± 0.84	47.38 ± 1.79	50.59 ± 3.62	2.03 ± 0.24	0.94
	I4	10.25 ± 0.63	44.65 ± 2.92	52.57 ± 1.87	2.78 ± 0.39	0.85

[a] CW, cell wall; S, syringyl; G, guaiacyl; H, *p*-hydroxyphenyl. Values are means ± SE.

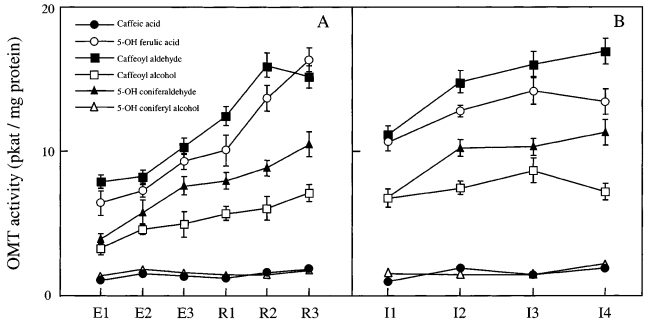

Figure 4. Developmental changes of *O*-methyltransferase (OMT) activities in tall fescue stems (A) and internodes (B): E1, first node palpable; E2, second node palpable; E3, third node palpable/visible; R1, first spikelet visible; R2, spikelets fully emerged; R3, anthesis; I1–I4, basal internode I1 to apical internode I4. Enzyme activities with the indicated substrates (final concentration = 50 μM) and [^{14}C]-*S*-adenosyl-L-Met were determined in crude extracts from stems and internodes. Values are means ± SE (n = 3).

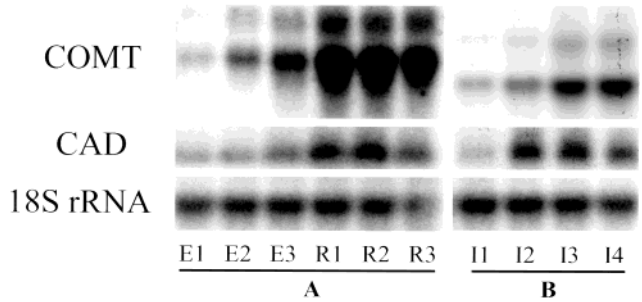

Figure 5. RNA gel blot analysis of tall fescue stems at different developmental stages (A) and basal to upper internodes at the R3 stage (B): E1, first node palpable; E2, second node palpable; E3, third node palpable/visible; R1, first spikelet visible; R2, spikelets fully emerged; R3, anthesis; I1–I4, basal internode I1 to apical internode I4. Blots were probed with tall fescue caffeic acid *O*-methyltransferase (COMT) and cinnamyl alcohol dehydrogenase (CAD) cDNA sequences.

measured against the substrates caffeoyl alcohol, 5-hydroxyconiferyl alcohol, and caffeic acid.

Gene Expression Levels of COMT and CAD in Developing Stems and Internodes. RNA gel blot analyses showed that the accumulation of *COMT* and *CAD* transcripts increased from the E1 to the R1 stage, with little change from the R1 to the R3 stage (**Figure 5A**). The basal internode I1 had the lowest expression levels for both *COMT* and *CAD* (**Figure 5B**). *COMT* expression increased from I1 to I3 internode, but no obvious differences were seen between I3 and I4 internodes. Likewise, *CAD* expression increased from I1 to I2 internode, but no differences were observed from I2 to I4 internodes (**Figure 5B**).

Table 2. Cell Wall Polysaccharides and Ruminal Degradability of Stems and Internodes of Tall Fescue at Different Developmental Stages[a]

		hemicelluloses (g kg^{-1} of dry CW)	cellulose (g kg^{-1} of dry CW)	pectin (g kg^{-1} of dry CW)	degradability (%)
stems	E1	452.2 ± 6.3	466.5 ± 4.3	78.5 ± 2.2	82.5 ± 2.6
	E2	458.9 ± 1.1	480.9 ± 5.7	56.9 ± 1.1	60.4 ± 1.4
	E3	437.4 ± 4.5	500.2 ± 3.4	57.4 ± 3.3	55.7 ± 0.9
	R1	421.3 ± 4.7	529.7 ± 2.1	42.4 ± 2.2	46.6 ± 2.6
	R2	421.9 ± 5.7	549.9 ± 4.8	20.2 ± 1.7	42.5 ± 1.3
	R3	391.7 ± 3.1	584.6 ± 5.6	20.3 ± 2.2	33.1 ± 2.8
internodes (R3 stage)	I1	377.7 ± 1.6	594.4 ± 3.1	20.3 ± 1.3	30.3 ± 1.1
	I2	372.6 ± 4.9	598.1 ± 4.5	22.3 ± 0.7	30.6 ± 2.5
	I3	410.2 ± 3.6	562.7 ± 3.6	19.1 ± 1.7	35.0 ± 1.8
	I4	405.7 ± 2.3	575.4 ± 2.6	18.9 ± 2.2	34.1 ± 1.2

[a] CW, cell wall. Values are means ± SE.

Cell Wall Polysaccharide Content and Ruminal Degradability. Hemicellulose, cellulose, and pectin fractions of cell wall polysaccharides were prepared from different stage stems and internodes. Contents of these polysaccharides were evaluated on the basis of dry cell wall mass and are shown in **Table 2**. More hemicellulose and pectin were deposited in the younger stems at elongation stage, whereas more cellulose was present in the older stems at the reproductive stage. Younger internodes (I3 and I4) also had more hemicellulose and less cellulose compared with older internodes (I1 and I2) (**Table 2**).

Cell wall degradability of stems and internodes decreased with increasing maturity (**Table 2**). The decline of ruminal degradability of stem tissue was drastic, from 82.51% at the E1 stage to 33.07% at the R3 stage. Correlations between ruminal degradability and total lignin, S, G, and H lignins, and S/G ratio as well as polysaccharides were calculated on the basis of the data in **Tables 1** and **2**. As shown in **Figure 6**, strong negative correlations were found between ruminal degradability and total lignin content ($r = -0.94$), S lignin content ($r = -0.91$), and S/G ratio ($r = -0.88$). Because G lignin content decreased at later developmental stages, positive correlations were found between ruminal degradability and G lignin content ($r = 0.88$) and H lignin content ($r = 0.92$). The content of hemicellulose had a positive correlation with ruminal degradability ($r = 0.87$), whereas cellulose content was negatively correlated with degradability ($r = -0.94$).

DISCUSSION

Stem tissue has been considered as the preferred material for studying lignin and degradability in forage grasses and legumes (*3*, *32*, *48*). Anatomical comparisons of lignin deposition showed that the structural changes in stems during development were associated with the increase in lignin content and the decrease

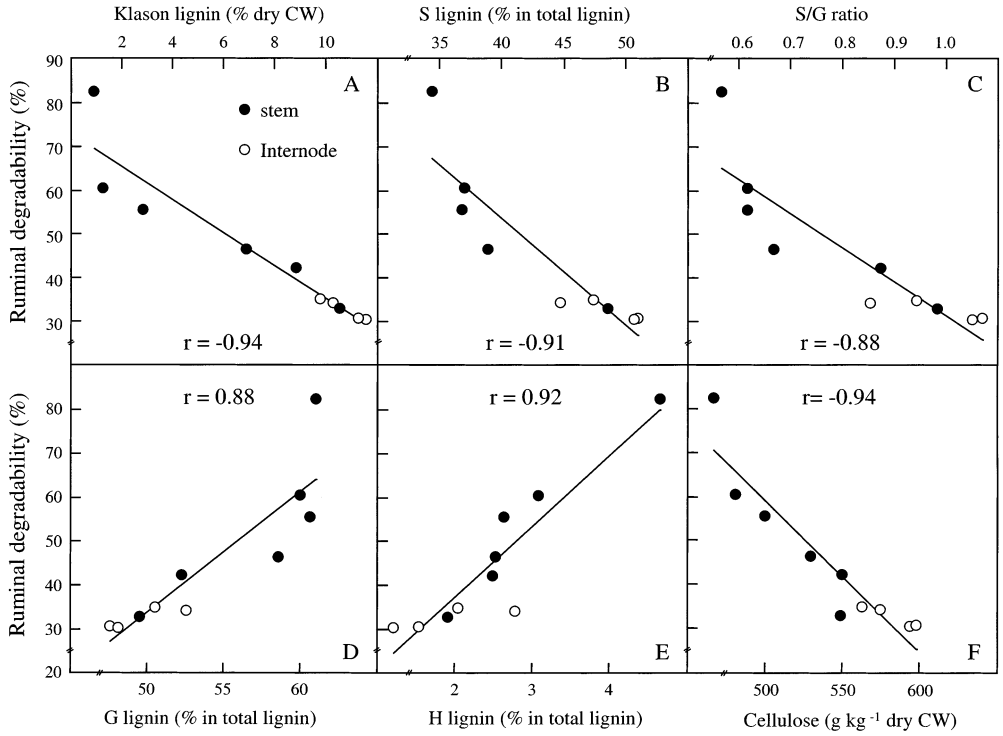

Figure 6. Correlations between ruminal degradability and Klason lignin content (A), syringyl (S) lignin content (B), S/guaiacyl (G) ratio (C), G lignin content (D), *p*-hydroxyphenyl (H) lignin content (E), and cellulose content (F).

of degradability. Because no new cells were created at later stages of stem development, the increase in structural mass of the sclerenchyma ring around the vascular tissues arose from the conversion of thin-walled parenchyma cells to thick-walled sclerenchyma-like cells. The highly lignified cells comprised only a small portion of the cross sections; however, because walls of these cells are several times thicker than those of parenchyma, their cell wall dry mass constitutes a significant part of the stems. Physical separation of cell types from sorghum showed that the wall content of the thick-wall tissues comprised ~80% of an internode on weight basis (*49*). Anatomical limitations to wall digestion have been considered as an important issue for maturing grass stems (*7*). Grabber et al. (*35*) suggested that the low degradability of lignified sclerenchyma walls was mainly due to the anatomical configuration of this cell type. The formation and development of a sclerenchyma ring with progressive maturity of tall fescue stems were clearly observed in this study. Therefore, sclerenchyma tissue can be an important target for genetic manipulation to improve forage digestibility. With the development of molecular cloning techniques, it is possible to identify tissue-specific genes and isolate tissue- or cell-specific promoters; this will provide an effective means to down-regulate lignin biosynthesis in specific tissues or cell types.

The effects of plant maturity on lignin and forage digestibility have been reported in different grass species (*5*, *50*). A dramatic increase in Klason lignin content in developing tall fescue stems was observed in this study, and this change was closely related to the decrease in degradability. Research on lignin structures in grasses has been largely on the effects of ferulic acid and cross-linking (*20*, *50*, *51*). Little information is available on developmental changes of H, G, and S lignin components in forage grasses. Chemical analysis of developing tall fescue stems revealed that H lignin remained at low levels at different developmental stages; thus, it probably does not have much effect on degradability. S lignin and S/G ratio were negatively correlated with degradability. S lignin content and S/G ratio increased when plants matured, and G lignin content decreased when plants matured and showed a positive correlation with degradability. However, this does not necessarily mean that G lignin is more digestible than S lignin. Grabber et al. (*52*) showed that H, G, and S lignin have similar inhibitory effects on wall degradability in maize. When the data of chemical analyses were combined with anatomical comparisons of the developing stems, it was concluded that G lignin was deposited at the early stage of plant growth and S lignin was preferentially deposited at the later developmental stage. Because forage quality is mainly influenced by lignification at the later developmental stage, it would be wise to design strategies to reduce S lignin biosynthesis to improve forage quality.

The lignin biosynthetic pathway is complicated, and many enzymes are involved in the process (*10*−*12*). Our analysis showed that OMT activity increased with plant development and lignification. The pattern of enzyme activity changed during plant development and varied with the choice of substrate. Caffeic acid has in the past been believed to be a major substrate for COMT (*9*); however, relative enzyme activity was low and remained almost unchanged at different developmental stages in tall fescue. Thus, COMT in tall fescue is unlikely to methylate caffeic acid during lignin biosynthesis in vivo. Recent studies in the dicot species alfalfa showed that the highest OMT activity was obtained with caffeoyl CoA and caffeoyl alcohol (*16*). It has also been proposed that 5-hydroxyconiferaldehyde is the true in vivo substrate of COMT in the biosynthesis of S lignin in aspen (*53*). In tall fescue, highest OMT activities were obtained with caffeoylaldehyde and 5-hydroxyferulic acid at different developmental stages, indicating a difference between plants species on substrate preference during lignin biosynthesis. Relatively high enzyme activity at the level of caffeoylaldehyde indicates alternative pathways to monolignols, and the new lignin biosynthetic pathway outlined for alfalfa (*11*, *14*) may be applicable to monocot grasses.

Characterization of *COMT* gene expression was reported in perennial ryegrass (*54*, *55*). However, in some cases (*55*), the stem tissue used was actually "pseudo" stem, which consisted of leaf sheath and undeveloped leaf blade. This is mainly due to the fact that perennial ryegrass, like tall fescue, requires vernalization to develop true stem. So far there is no report on the expression of important lignin genes (e.g., *COMT* and *CAD*) in stems of forage grasses at different developmental stages. This study revealed that the expression of *COMT* and *CAD* genes increased with stem elongation and remained at similar high levels at the later reproductive stages. This trend is in parallel with changes in enzyme activity and lignification during stem development. Within the stem, internode I1 had the lowest OMT activity and lowest levels of RNA transcripts. This is mainly because I1 was the oldest internode and already highly lignified. The developing internodes (I2−I4), where lignification is still actively taking place, had higher enzyme activities and higher levels of *COMT* and *CAD* gene expression. Thus, *COMT* and *CAD* are good candidate genes for genetic modification of lignin biosynthesis for forage grasses. By using sense and antisense *COMT* and *CAD* gene constructs, we have generated transgenic tall fescue plants with altered lignin (L. Chen et al., 2002, unpublished results). Because lignin content/composition and degradability change with plant development, it is important to have a uniform criteria and to sample plant material at defined developmental stages. This study also provides useful information in this aspect.

Besides lignin, another major organic constituent of forage cell walls, polysaccharides, plays an important role in forage degradability. This study showed that more hemicellulose was deposited at earlier developmental stages and in younger internodes, and it was positively correlated with ruminal degradability. More cellulose was present at the reproductive stages and in older internodes, and it had a strong negative correlation with degradability. Although most of the lignin in cell walls of grasses is linked covalently with polysaccharides (*56*), the associations between lignin and polysaccharides are not fully understood. Increasing evidence has shown that cross-linking of cell wall components by ferulic acid ester−ether bridges contributes to the resistance of lignified walls of grasses to rumen digestion (*51*, *57*).

In summary, by using the tall fescue stem as a model system, a number of degradability-related developmental changes and metabolic events were studied, including anatomical structure, lignin content and composition, polysaccharide content, enzyme activities, and expression of lignin-related genes. The changes at anatomical, metabolic, and molecular levels during development were closely associated and finally led to the changes of degradability. This is the first report on an integrated study of lignin deposition using chemical and molecular tools in the major cool-season forage grass species tall fescue. The information obtained could be useful for other important forage species such as perennial ryegrass.

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