In Search of a Maize Ideotype for Cell Wall Enzymatic Degradability Using Histological and Biochemical Lignin Characterization

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Grass cell wall degradability is conventionally related to the lignin content and to the ferulic-mediated cross-linking of lignins to polysaccharides. To better understand the variations in degradability, 22 maize inbred lines were subjected to image analyses of Fasga- and Mäule-stained stem sections and to chemical analyses of lignins and *p*-hydroxycinnamic acids. For the first time, the nearness of biochemical and histological estimates of lignin levels was established. Combination of histological and biochemical traits could explain 89% of the variations for cell wall degradability and define a maize ideotype for cell wall degradability. In addition to a reduced lignin level, such an ideotype would contain lignins richer in syringyl than in guaiacyl units and preferentially localized in the cortical region rather than in the pith. Such enrichment in syringyl units would favor wall degradability in grasses, contrary to dicots, and could be related to the fact that grass syringyl units are noticeably *p*-coumaroylated. This might affect the interaction capabilities of lignins and polysaccharides.

INTRODUCTION

Most studies related to the biochemistry and genetics of lignification have been performed in perennial or annual dicots and in woody gymnosperm species, whereas most forage plants are grasses. This focus is due to the major interest in reducing the cost and environmental impact of removing lignin from woody plants during pulping for paper production. Among grasses, maize has probably been the most studied species and especially in approaches devoted to cell wall lignification and digestibility. Grass cell walls are complex materials comprising polysaccharidic (cellulose and hemicelluloses) and phenolic components (lignins and *p*-hydroxycinnamic acids). Lignins are polymers essentially made of syringyl (S) and guaiacyl (G) units interconnected by labile ether bonds (β-*O*-4 linkages) and/or resistant carbon–carbon bonds. *p*-Hydroxycinnamic acids, namely, ferulic (FA) and *p*-coumaric (pCA) acids, are able to create cross-links between grass cell wall polymers. Although *p*-coumaric acid is known to be ester-linked to the γ-position

of the side chains of S lignin units (*1*), ferulic acid may be associated both with polysaccharides through ester linkages and with lignins via radical coupling mechanisms, which leads to a variety of bonding patterns (ether and carbon–carbon types) (*2–4*). Both lignins and *p*-hydroxycinnamic acids have been reported to limit the digestibility of forage cell walls by increasing their resistance to enzymatic or microbial degradation (*5, 6*).

Studies to identify the chemical mechanisms that limit grass cell wall digestibility are often restricted to correlative studies using maturation as a way to control the changes in cell wall composition or using brown midrib (bm) mutants in comparison to their normal counterparts (*6–9*). Nevertheless, although both the bm3 mutation and maturity stage drastically affect cell wall composition and digestibility, the evaluation of normal maize harvested at the standard ensiling stage has revealed that large genetic variations in digestibility could be observed in normal lines as well (*10*). Lignin concentration is generally recognized as the first factor negatively correlated with cell wall digestibility. However, this correlation is greater when samples of different maturity stages are considered, in contrast to samples of similar maturity (*8, 11*). In the latter case, it seems that wall composition cannot entirely account for the observed variations in cell wall digestibility (*12*). Wilson and Mertens (*13*) made

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the hypothesis that the anatomical structures of grass cells and tissues are to be considered to better understand the cell wall digestibility, a hypothesis then confirmed by Wilson and Hatfield (*14*).

We thus wondered whether differences in the cell wall degradability of maize stems could be more efficiently explained by a combination of histological factors together with more conventional compositional parameters. With this objective, we studied the biochemical and histological parameters of 22 maize inbred lines comprising 4 bm3 mutants and their corresponding normal counterparts. The maize stems were collected at the same developmental stage but, nevertheless, displayed distinct cell wall degradabilities (*10*). In addition to conventional wall chemistry parameters previously reported (*12*), we studied the various inbred lines on the basis of histological parameters. Among the various candidate parameters, we selected the cortical area (A_C), which includes the thick-walled sclerenchyma tissue, the thickness (T_{CS}), and the number of cell layers (N_{CS}) of this cortical sclerenchyma. In addition, we chose the Fasga staining method to evaluate the lignified tissue. In this method, the safranin and alcian blue dies stain lignified cell walls in red and nonlignified (or only weakly lignified) cell walls in blue, which allowed us to run image analyses to measure the red (A_R) and blue (A_B) areas as indicators of lignified and nonlignified tissues, respectively. We also tentatively examined the distribution of S and G lignin units using the Mäule staining method. In this study, we examined the genetic variation for all of these parameters among the normal and bm3 inbred lines. Then we studied the relationships of biochemical and histological parameters to the in vitro cell wall digestibility (IVNDFD) of the stem harvested at ensiling stage. This extensive study is aimed at further deciphering the relationship of biochemical and histological parameters to the enzymatic degradability of silage-stage maize stems.

MATERIALS AND METHODS

Plant Material. Twenty-two maize inbred lines, including 4 bm3 genotypes and their normal isogenic counterparts (only quasi-isogenic normal counterpart F7025 for F7026bm3), were selected from preliminary experiments (*15*; unpublished data). The field experiments were run at Lusignan (INRA, Unité de Génétique et d'Amélioration des Plantes Fourragères, France) during three successive years. The trials were randomized block designs with three replicates of two 4 m long rows. Row spacing was 0.75 m, and the density was 95 000 plants per hectare. The plots were irrigated at 90−120 mm per year to prevent any summer water stress.

In 1996 and 1997, in each plot, 20 stem fractions, from the bottom to the ear, were collected at ensiling stage, carefully mixed, and then chopped. Three sampling dates were chosen according to the earliness of the inbred lines in order to harvest at the same maturity stage (∼30% of dry matter content). The samples were oven-dried (70 °C) and ground with a hammer mill, first to pass through a 5 mm screen and then through a 1 mm screen. Sampling was done from the bottom to the ear of the stem because this major and highly lignified fraction of the plant may provide the greatest opportunity to discriminate maize genotypes on the basis of cell wall composition and digestibility.

In 1998, at ensiling stage (always with respect to three sample dates in order to harvest at the same maturity stage), a 1 cm long segment was sampled in the middle part of the internode below the node from which the ear arises on one plant for each inbred line. The 1 cm long fractions were preserved in a 2% paraformaldehyde and 25% glutaraldehyde fixative solution for histological analysis.

Histological Analyses. For each of the 22 maize inbred lines, 15 serial stem sections of 70 μm thickness were made with a Vibratome. Four randomly chosen slides were stained using the Mäule reaction (*16*). This staining was originally used to distinguish angiospermous and gymnospermous woods. A red color is produced only for woods
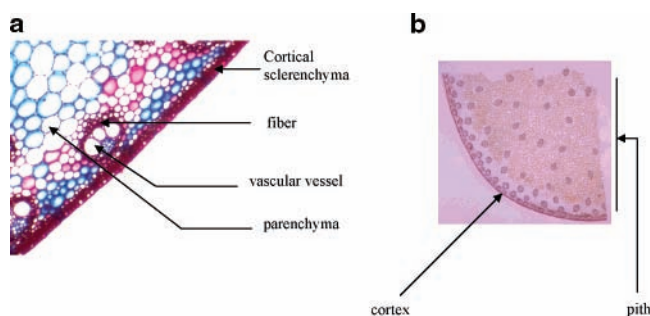
Figure 1. Fasga staining (×4, **a**) and Mäule staining (×0.8, **b**) of two different stems cross sections.

that contain syringyl units. Thus, angiosperms produce an intense purple-red color, whereas gymnosperms yield an indefinite brownish shade. No reaction was obtained for pine, but work has shown that the Mäule reaction is good for slightly lignified cell walls, until they become more lignified and reach a stage where the phloroglucinol reaction works better (*17*). We thus stained four other randomly chosen slides using a solution of phloroglucinol (*18*). The phloroglucinol reaction has been attributed to the formation of a condensation product between phloroglucinol and aldehyde grouping (*19*). Finally, we performed a third staining, the Fasga staining, on four randomly chosen slides. The reagent used contained safranin in combination with alcian blue. Safranin is a red, basic, cationic dye, and alcian blue is an acidic anionic dye. Because lignin is acidic (due to its phenolic hydroxyl groups), lignified tissues are stained in red even if this stain is certainly not specific for only lignin. Thus, the Fasga staining presents lignified tissues in red, whereas nonlignified or poorly lignified tissues are in blue.

After staining, all sections were mounted on slides in distilled water and examined with a magnifying glass (×0.8) or a microscope with a ×4 or ×10 magnification. Color image analysis of stained stem cross section was carried out with a video camera installed on the magnifying glass or on the microscope. A semiautomated image analysis was developed using the 6.1 version of Optimas (*20*).

A first group of characters was estimated using the Fasga staining (**Figure 1a**). The whole histological stem cross-section area (in this paper, all areas include cell lumen) was first automatically delimited on the Fasga-stained section image (×0.8 magnification) and estimated using Optimas software. The lignified area (red area, A_R) and the cellulosic area (blue area, A_B) were measured with parametrizing Optimas software to distinguish, delimit, and calculate both blue and red areas. Many lines were then manually drawn across the sclerenchyma cell layers or cell walls on the 4× magnified Fasga-stained section, and the means of these line lengths were automatically calculated to estimate the thickness of cell layers in the cortical sclerenchyma (T_{CS}) and the thickness of the cell wall in the cortical sclerenchyma. Finally, a 10× magnification was sufficient to count easily the number of cell layers (N_{CS}) in the cortical sclerenchyma.

A second group of characters was estimated using the Mäule staining (**Figure 1b**). The cortical area (A_C) was defined as the red-purple color located under the epidermal cells of the section and thus automatically calculated with Optimas software. In a similar manner, red-purple (A_S) and brownish areas (A_G), attributed, respectively, to S-rich and G-rich lignins, were determined on Mäule-stained sections (×0.8 magnification). The total lignified area on the Mäule-stained section ($A_S + A_G$) was defined as the red-purple area plus the brownish area.

Chemical Analyses. The neutral detergent fiber (NDF) of dried stem samples was measured according to the method of Goering and Van Soest (*21*) and expressed as weight percentage of dry matter (DM). The lignin content was determined according to the Klason procedure (*22*) and expressed as the weight percentage of Klason lignin (KL) in NDF.

In vitro dry matter digestibility (IVDMD) of the stem samples was determined according to an adapted Libramont−Limagrain protocol (*23*). The dried samples were placed in a nylon bag and subjected to a first enzymatic degradation with pepsin in acidic conditions. A second attack was then performed using a cellulases/hemicellulases cocktail.

IVDMD is simply the amount of sample that enters the system minus the amount of sample that exits the system. In vitro NDF digestibility (IVNDFD) was estimated by measuring the NDF content of the residue (NDFres) recovered after enzymatic hydrolysis (*10*). The final result was calculated as follows:

$$IVNDFD = 100 - \frac{NDFres\% \times (100 - IVDMD)}{NDF\%}$$

According to previous results (*10*), 2 field replicates and 16 (including 3 bm3 mutants and their normal counterparts) of the 22 maize inbred lines presented above were retained for *p*-hydroxycinnamic acid content and lignin structure studies (*12*). The *p*-hydroxycinnamic acid content of NDF was measured according to a previously published procedure (*24*), which involves two alkaline treatments differing in their severity. Esterified *p*-hydroxycinnamic acids were released by subjecting NDF samples (100 mg) to a mild alkaline hydrolysis (5 mL of 2 M NaOH, room temperature, 20 h, under N_2 and with mechanical shaking). Other NDF samples (100 mg) were placed in a Teflon screw-cap vial containing 5 mL of 4 M NaOH and purged with nitrogen. This vial was then sealed in a stainless steel reaction vessel, which was placed at 170 °C (oven) for 2 h. This severe alkaline treatment allows release of both the esterified and the etherified *p*-hydroxycinnamic acids. The samples recovered from mild and severe alkaline hydrolyses were subjected to the same extraction procedure, as follows. An internal standard (*p*-anisic acid) was added to the reaction medium prior to centrifugation (380*g* for 10 min). The supernatants were acidified to pH 2 with concentrated HCl and kept at 4 °C overnight before another centrifugation step. *p*-Hydroxycinnamic acids were extracted from the aqueous supernatant with ethyl acetate and analyzed by high-performance liquid chromatography (HPLC), according to the method of Chabbert et al. (*25*). The concentration of etherified ferulic acid was calculated as the difference between ferulic acid amounts released by the severe and mild alkaline treatments.

Lignin structure was studied by thioacidolysis performed on 10 mg of NDF placed in a screw-cap glass tube together with 8 mL of dioxane/ethanethiol mixture (9:1, v/v) containing 0.2 M boron trifluoride etherate, for 4 h, at 100 °C (oil bath). After extraction of the lignin-derived monomers, the analysis of their trimethylsilyl (TMS) derivatives was run by gas chromatography (GC). The molar yield in G and S lignin-derived monomers released upon thioacidolysis of the NDF sample was calculated on the basis of its Klason lignin content.

Most of the biochemical analyses were performed in triplicate except thioacidolysis, which was run in duplicate for the samples harvested in one field replicate and in single assays for the samples issued from the second field replicate.

Statistical Analyses. For biochemical parameters, variance analysis was carried out following standard procedures of the fixed following model:

$$Y_{ijkl} = \mu + A_j + B_k/A_j + G_i + G_i \times A_j + G_i \times B_k/A_j + R_{ijkl}$$

where Y_{ijkl} = the value of genotype *i* in year *j*, for plot replicate *k* and analysis replicate *l*; μ = the overall mean; A_j = the main effect of year *j*; B_k/A_j = the main effect of replicate *k* nested in year *j*; G_i = the effect of genotype *i*; $G_i \times A_j$ = the interaction effect between genotype *i* and year *j*; $G_i \times B_k/A_j$ = the interaction effect between genotype *i* and replicate *k* nested in year *j*; and R_{ijkl} = the random residual term.

For histological parameters, variance analysis was carried out following standard procedures of the fixed following model:

$$Y_{il} = \mu + G_i + R_{il}$$

where Y_{il} = the value of genotype *i* in the analytical repetition *l*, μ = the overall mean, G_i = the effect of genotype *i*, and R_{il} = the random residual term.

Data for inbred lines were combined over year, field, and analysis replicates before determination of correlation coefficients and multiple-regression analysis using a backward elimination procedure. The dataset employed to perform the simple correlation study and the multiple regression analysis was composed of the 13 normal inbred lines characterized for all histological and biochemical parameters. All of the statistical analyses were performed with the Splus package (*26*).

RESULTS

Genetic Variation for Histological and Biochemical Traits within Normal and bm3 Inbred lines. The histological traits studied herein were measured from Fasga- and Mäule-stained sections collected in the middle part of the internode below the node from which the ear arises. Some attempts to use sections stained by the Wiesner (phloroglucinol−HCl) reagent failed as the samples were not contrasted enough for automatic image analysis. The histological characters considered in this study were related to lignification and to cortex specificities. Fasga-stained slides were used to measure the stem cross-section area, the red and lignified wall area A_R, the nonlignified wall blue area A_B, and the thickness and number of cell layers in the cortical sclerenchyma (T_{CS} and N_{CS}, respectively). The red area A_R could be mainly associated with the pith lignification, as shown in **Figure 2**. The Mäule-stained sections were employed to measure the cortical area A_C and to try to discriminate the lignified areas on the basis of their content in G and S units. Whereas tissues with G-rich lignins correspond to the orange-brown area A_G, the red-purple area A_S was attributed to lignins containing enough S units to provide the red-purple 3-methoxy *o*-quinone structures upon Mäule treatment. The total lignified area was defined as the sum of A_S and A_G.

As shown in **Tables 1** and **2**, the main histological parameters determined for the 22 inbred lines displayed substantial variations. The A_R, A_B, and $A_R/(A_R + A_B)$ (lignified surface related to the total Fasga-stained surface) measures varied over an important range, but nevertheless allowed discrimination of three bm3 lines (excepted F2bm3) and two normal inbred lines (F4 and CM484) from all other normal lines (**Table 1**). Contrary to the three other bm3 mutants, F2bm3 had the same A_B area as its normal isogenic line F2, and both lines presented the smallest A_B. The cortical sclerenchyma thickness, T_{CS}, varied from 0.029 to 0.069 mm (**Table 2**) and did not allow separation of normal from bm3 lines. The maximal and minimal cortical areas (A_C) were occupied by two normal inbred lines (LAN496 and F4) and did not discriminate normal from bm3 lines.

The tentative estimation of the distribution of S and G lignin units, based on the red-purple A_S and orange-brown A_G areas of the Mäule-stained sections, was given up because of a poor intensity in our Mäule staining. With a few exceptions (**Table 1**), it can be seen that the total lignified area A_R, estimated from the Fasga staining, and the total Mäule-stained areas ($A_S + A_G$) correspond to close values and are nicely correlated (**Table 3**).

Similar to the histological traits, the biochemical traits studied in this work displayed a large genetic variability within the 22 maize inbred lines, as previously reported (*12*). The IVNDFD varied over a range of 20 points within the 22 inbred lines (**Table 2**). Not unexpectedly, the highest IVNDFD was observed for the bm3 mutant lines. However, if we look at its variations within normal inbred lines, we also found important differences with a range of >14 points (from 24 to 38%). The cell wall content (estimated by NDF measurement) also varied over a wide range (>14 points) (**Table 2**). This amplitude of variation was the same when only normal inbred lines or both normal and bm3 lines were considered. The Klason lignin content in the cell wall [KL(%NDF)] was found to be lower in bm3 inbred lines than in normal lines (**Table 2**). Whereas the KL(%NDF) of the normal inbred lines varied from 14.74 to 20.32%, the lowest level was found to be quite close to the values of bm3 lines. The content in esterified pCA displayed a large variability
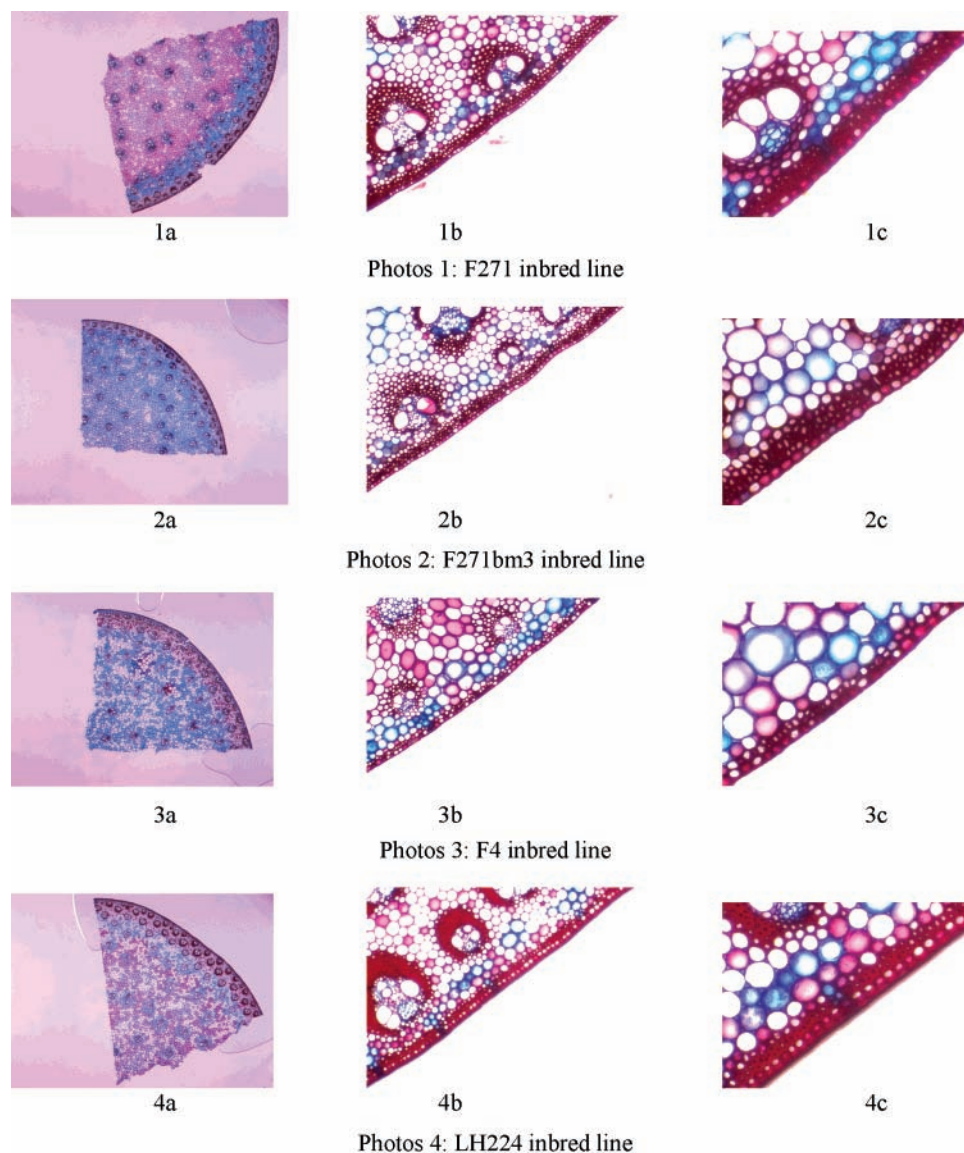
1a   1b   1c

Photos 1: F271 inbred line

2a   2b   2c

Photos 2: F271bm3 inbred line

3a   3b   3c

Photos 3: F4 inbred line

4a   4b   4c

Photos 4: LH224 inbred line

Figure 2. Fasga-stained sections, ×0.8 (1a–4a), ×4 (1b–4b), and ×10 (1c–4c).

within the maize inbred lines and allowed discrimination of normal and bm3 lines, which was not the case of etherified FA. The evaluation of the frequency of S units in lignin by thioacidolysis very clearly distinguished normal and bm3 lines (**Table 2**). The S/G thioacidolysis ratio was found to be dramatically reduced in the bm3 lines. This ratio also displayed substantial variations within normal inbred lines (**Table 2**). The total yield in thioacidolysis monomers (S + G) (**Table 2**), calculated on the basis of the KL content in the NDF, provides an estimate of the proportion of lignin units involved in only labile β-O-4 bonds. This total yield presented a large range of variation of >500 μmol/g of KL. Despite a severe reduction in S units, the (S + G) thioacidolysis yields were not systematically lower in the bm3 lines than in their normal counterparts.

Simple Correlative Studies between Histological Traits, Biochemical Parameters, and IVNDFD in Normal and bm3 Maize Stems. A simple correlation study was performed to further understand the relationships between histological and biochemical traits, with a special focus at lignin-related traits (**Table 3**). The employed dataset was composed by the 13 normal inbred lines characterized for all biochemical and histological parameters. A significant and positive correlation between KL(%NDF) and A_R or $A_R/(A_R + A_B)$ could be

evidenced herein for the first time. Consistently enough, an inverse correlation also existed between KL(%NDF) and A_B. Not unexpectedly, the KL content in the cell wall, the Fasga A_R, and the Mäule ($A_S + A_G$) were also significantly and positively correlated with the number of cell layers in the sclerenchyma, which is a thick-walled and lignified tissue. Within the biochemical parameters, it was worth noting a negative tendency between the S/G thioacidolysis ratio and the lignin content in the cell wall (**Table 3**).

Among the chemical factors, a simple correlative study between compositional features and IVNDFD confirmed that the lignin content KL(%NDF) and the NDF content were significantly and negatively correlated to IVNDFD (**Table 4**). In addition, this simple correlative study revealed that the histological factor A_R, which is a measure of the area of lignified tissues in Fasga-stained stem cross sections, displayed a negative correlation with IVNDFD. When expressed on a relative basis, the relative importance of lignified cell walls, $A_R/(A_R + A_B)$ as judged by the Fasga stain reaction, was significantly and negatively correlated to IVNDFD. Other biochemical and histological traits presented nonsignificant negative correlations with IVNDFD (except the S/G thioacidolysis ratio, which presented a nonsignificant positive relation with IVNDFD)

Table 1. Mean Values for in Vitro Cell Wall Digestibility, Klason Lignin Content, and Histological Parameters[a] for the 22 Inbred Lines (Including 4 bm3 Inbred Lines and Their Normal Isogenic Counterparts)

genotype	IVNDFD	KL(%NDF)	A_R (mm²)	A_B (mm²)	$A_R/A_R + A_B$	T_{CS} (mm)	N_{CS}	$A_S + A_G$ (mm²)
F271	25.66	16.64	29.97	10.97	0.73	0.052	3.5	26.2
F271bm3	41.50	12.14	3.41	22.39	0.13	0.048	3.5	4.77
W117	26.19	17.29	30.70	15.54	0.66	0.051	4.0	24.79
W117bm3	40.48	12.27	9.18	29.24	0.24	0.045	3.5	7.09
F2	29.11	17.45	22.9	6.40	0.78	0.039	3.5	21.1
F2bm3	37.99	13.38	12.69	6.7	0.65	0.036	3	11.73
F7025	30.67	18.24	30.31	16.75	0.64	0.043	4.0	28.39
F7026bm3	41.74	13.21	14.13	27.92	0.34	0.069	3.5	10.5
F292	25.74	16.57	16.74	7.5	0.69	0.039	2.5	17.2
F283	26.29	17.95	16.1	7.81	0.67	0.035	2.5	11.96
F311	27.82	18.68	26.31	13.75	0.66	0.041	2.0	22.44
F7019	28.09	17.59	18.94	17.52	0.52	0.048	4.0	22.01
F288	28.78	18.53	19.3	14.64	0.57	0.067	4.0	16.42
LAN496	28.83	19.31	19.39	10.6	0.65	0.043	3	19.36
LH224	23.95	20.32	22.79	10.31	0.69	0.047	3	21.3
F7027	25.36	17.16	20.99	14.69	0.59	0.057	4.0	20.04
F7012	29.47	16.79	12.29	8.93	0.58	0.029	2.5	8.53
Co316	31.23	17.02	17.73	17.98	0.5	0.04	3	15.5
LH145	32.28	17.67	15.17	10.58	0.59	0.038	3.5	13.38
F4	35.91	14.74	9.11	25.5	0.26	0.034	2.0	11.75
Cm484	36.52	14.80	6.34	35.52	0.15	0.038	2.0	7.94
F251	38.02	17.40	17.01	6.97	0.71	0.041	3.5	17.44

[a] IVNDFD, in vitro NDF digestibility; KL, Klason lignin; A_R, red (lignified) area on the Fasga-stained section; A_B, blue (nonlignified) area on the Fasga-stained section; T_{CS}, thickness of cell layers of the cortical sclerenchyma; N_{CS}, number of cell layers in the cortical sclerenchyma; $A_S + A_G$, total stained area on the Mäule-stained section, attributed to lignified tissues.

Table 2. Minimum and Maximum Values for Digestibilities, Cell Wall Composition, and Histological Parameters[a] within both Normal (Min N and Max N) and bm3 (Min bm3 and Max bm3) Inbred Lines

	IVNDFD	NDF (% DM)	KL(%NDF)	IVDMD	estpCA (mg/g of NDF)	ethFA (mg/g of NDF)	G (µmol/g of KL)	S (µmol/g of KL)	S + G (µmol/g of KL)	S/G
min N	23.95	45.76	14.74	49.48	17.3	1.41	301	430	765	1.03
max N	38.02	59.71	20.32	65.24	27.8	2.32	591	632	1223	1.79
min bm3	37.99	45.23	12.14	59.20	13.21	1.61	541	156	697	0.25
max bm3	41.74	56.89	13.38	69.28	13.38	2.06	653	207	860	0.32
mean[b]	31.44	52.81	16.60	58.29	19.27	1.86	424	451	875	1.19
cl[c]	0.94	0.26	0.44	0.49	0.39	0.23	32	26	54	0.03

	A_B (mm²)	A_R (mm²)	$A_R/A_R + A_B$	A_c (mm²)	T_{CS} (mm)	N_{CS}	$A_S + A_G$ (mm²)
min N	6.40	9.11	0.15	2.57	0.029	2	7.94
max N	35.52	30.70	0.78	8.39	0.067	4	28.39
min bm3	6.70	3.41	0.13	3.37	0.036	3	4.77
max bm3	29.24	14.13	0.65	7.65	0.069	3.5	11.73
mean[b]	14.81	17.87	0.55	5.21	0.04	3.14	16.33
cl[c]	1.93	2.41			0.004		2.78

[a] IVNDFD, in vitro NDF digestibility; NDF, neutral detergent fiber; KL, Klason lignin; IVDMD, in vitro dry matter digestibility; estpCA, esterified *p*-coumaric acid; ethFA, etherified ferulic acid; G, guaiacyl thioacidolysis monomers; S, syringyl thioacidolysis monomers; S + G, total yield of syringyl and guaiacyl thioacidolysis monomers; S/G, syringyl/guaiacyl ratio; A_B, blue (nonlignified) area on the Fasga-stained section; A_R, red (lignified) area on the Fasga-stained section; A_C, cortical area on the Mäule-stained section; T_{CS}, thickness of cell layers of the cortical sclerenchyma; N_{CS}, number of cell layers in the cortical sclerenchyma; $A_S + A_G$, total stained area on the Mäule-stained section, attributed to lignified tissues. [b] The mean value was performed within the 22 inbred lines combining both normal and bm3 lines. [c] Confidence limit at $p < 0.05$.

(**Table 4**). Thus, when only one variable was considered, the histological parameter A_R, measured from the image analysis of Fasga-stained stem cross sections of the maize ear internode, was the best explanatory trait to account for the variations in cell wall digestibility. This parameter allowed us to account for 40% ($r = -0.63$) (**Table 4**) of the observed variations for cell wall digestibility.

Relationship of the in Vitro Cell Wall Digestibility Variations to both Biochemical and Histological Traits. A multiple-regression analysis was attempted to more comprehensively explain the IVNDFD variations observed among the 13 normal inbred lines characterized for all biochemical and histological parameters. When using only biochemical traits as explanatory variates and in agreement with a previous study (*12*), the best-fitting regression equation included pCA esters and S/G thioacidolysis ratio according to the following equation ($p < 0.05$, residual standard error RSE = 3.2):

$$IVNDFD = 39.14 - 1.24(estpCA) + 12.67(S/G)$$

This two-variates-based equation accounted for 57% of the observed IVNDFD variations. When using only histological traits, no model with more than one explanatory variate (and in this case, the best variate was A_R) could be detected to significantly explain IVNDFD. Another regression model based on two factors could be evidenced that combined an histological factor A_R and a biochemical factor S/G according to the following equation ($p < 0.10$, RSE = 3.2):

$$IVNDFD = 28.01 - 0.37A_R + 6.66(S/G)$$

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Table 3. Correlation Coefficients between Biochemical and Histological Traits[a] for the 13 Normal Inbred Lines

	Klason	Fasga-stained sections				Mäule-stained sections	thioacidolysis			
	KL(%NDF)	A_B (mm²)	A_R (mm²)	$A_R/(A_R + A_B)$	N_{CS}	$A_S + A_G$ (mm²)	S (µmol/ g of KL)	G (µmol/ g of KL)	S + G (µmol/ g of KL)	S/G
KL (% NDF)	−0.59*	0.56*	0.67*	0.64*	0.51	0.20	0.62*	0.50	−0.54	
A_B (mm²)		−0.44	−0.93**	−0.47	−0.38	0.21	−0.30	−0.09	0.52	
A_R (mm²)			0.72**	0.80**	0.96**	0.15	0.06	0.11	0.03	
$A_R/(A_R + A_B)$				0.67*	0.68*	−0.15	0.24	0.08	−0.40	
N_{CS}					0.73**	−0.05	0.08	0.02	−0.12	
$A_S + A_G$ (mm²)						0.25	0.05	0.16	0.12	
S (µmol/g of KL)							0.49	0.83**	0.23	
G (µmol/g of KL)								0.90**	−0.73**	
S + G (µmol/g of KL)									−0.35	
S/G										

[a] KL, Klason lignin; A_B, blue (nonlignified) area on the Fasga-stained section; A_R, red (lignified) area on the Fasga-stained section; N_{CS}, number of cell layers in the cortical sclerenchyma; $A_S + A_G$, total stained area on the Mäule-stained section, attributed to lignified tissues; S, syringyl thioacidolysis monomers; G, guaiacyl thioacidolysis monomers; S + G, total yield of syringyl and guaiacyl thioacidolysis monomers; S/G, syringyl/guaiacyl ratio. *, $p \leq 0.05$; **, $p \leq 0.01$.

Table 4. Correlation Coefficients between in Vitro Cell Wall Digestibility and the Main Biochemical and Histological Parameters for the 13 Normal Inbred Lines

trait	IVNDFD	trait	IVNDFD
NDF (% DM)	−0.55*	A_B (mm²)	0.48
KL (% DM)	−0.61*	A_R (mm²)	−0.63*
KL(%NDF)	−0.55*	$A_R/(A_R + A_B)$	−0.60*
estpCA (mg/g of NDF)	−0.41	A_C (mm²)	−0.18
ethFA (mg/g of NDF)	−0.32	N_{CS}	−0.32
S (µmol/g of KL)	0.01	T_{CS} (mm)	−0.41
G (µmol/g of KL)	−0.30		
S + G (µmol/g of KL)	−0.19		
S/G	0.36		

[a] IVNDFD, in vitro NDF digestibility; NDF, neutral detergent fiber; KL, Klason lignin; estpCA, esterified p-coumaric acid; ethFA, etherified ferulic acid; S, syringyl thioacidolysis monomers; G, guaiacyl thioacidolysis monomers; S + G, total yield of syringyl and guaiacyl thioacidolysis monomers; S/G, syringyl/guaiacyl ratio; A_B, blue (nonlignified) area on the Fasga-stained section; A_R, red (lignified) area on the Fasga-stained section; A_C, cortical area on the Mäule-stained section; N_{CS}, number of cell layers in the cortical sclerenchyma; T_{CS}, thickness of cell layers of the cortical sclerenchyma. *, $p \leq 0.05$; **, $p \leq 0.01$.

Similar to the previous one, this equation allowed us to significantly explain 54% of the observed IVNDFD variations. When we attempted to include other variates to further explain the observed cell wall digestibility variations within the 13 inbred lines, the best-fitting regression equation that we could evidence accounted for as much as 89% of the observed IVNDFD variations. This equation combined two biochemical variates, esterified pCA and S/G thioacidolysis ratio, together with two histological variates, A_R and the number of cell layers in the cortical sclerenchyma N_{CS}, according to ($p < 0.02$, RSE = 1.80)

$$IVNDFD = 26.81 - 0.97 estpCA + 13.66(S/G) - 0.57A_R + 5.12N_{CS}$$

The efficiency of this multiple-regression model, based on both biochemical and histological factors, is further outlined in **Figure 3**. Not unexpectedly, the bm3 mutant lines, which were not included in the dataset employed to determine this model, did not fit to it. The estimated IVNDFD values of these bm3 mutants were systematically and substantially lower than the observed IVNDFD (**Table 5**).
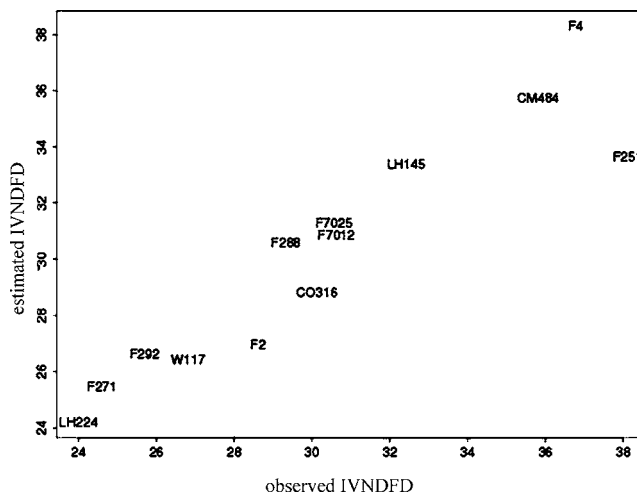
Figure 3. Observed in vitro cell wall digestibility (observed IVNDFD) versus estimated in vitro cell wall digestibility (estimated IVNDFD) for 13 genotypes by applying the regression model using two biochemical variates and two histological variates, according to the following equation: IVNDFD = 26.81 − 0.97 estPCA + 13.66(S/G) − 0.57A_R + 5.12N_{CS}.

Table 5. Comparison between the Observed in Vitro Cell Wall Digestibility and the Estimated in Vitro Cell Wall Digestibility for Brown Midrib 3 Mutants

genotype	observed IVNDFD	estimated IVNDFD
F2bm3	37.99	28.93
F271bm3	41.5	36.66
F7026bm3	41.74	31.06

DISCUSSION

Numerous studies have been carried out on the relationships of cell wall phenolics and maize digestibility. Since the pioneering studies on bm mutants in the 1960s, these mutants have been repeatedly used (7, 27, 28) as important models in digestibility studies. The genetic variation for lignin traits and for digestibility has also been studied in normal maize inbred lines and hybrids (8, 10, 12, 29−31). In the present work and for the first time, the genetic variation for histological factors was examined, and these factors were tentatively combined with biochemical factors in order to better account for the enzymatic degradability of normal and bm3 maize stems collected at the silage stage.

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Image Analysis of Fasga- and Mäule-Stained Stem Sections and Their Relationships to Lignin Content. The difficulty in providing definite relationships of wall chemistry to wall degradability has been assigned to the importance of the anatomical structure of cells and tissues (*14*). On this basis, we searched for histological factors that could be easily quantified by image analysis of maize stem sections. In addition to the unspecific measurements of the mean thickness of cell walls or of tissues carried out on the whole stem section or on the thick-walled cortical sclerenchyma, we selected lignin-specific histological factors from Fasga- and Mäule-stained sections. The Fasga staining method was chosen as the red and the blue areas could be easily discriminated by automatic image analysis of the section. However, this image analysis could obviously not be performed all along the maize stem. In addition, the red safranin stain has been reported to be not strictly specific to lignins (*32, 33*). Accordingly, the first step of our investigation was to determine to what extent the red area of the section sampled in the middle part of the ear internode and the lignin content of the whole stem are correlated. Indeed, the absolute A_R or relative $[A_R/(A_R + A_B)]$ red area of such a Fasga-stained cross section was found to be significantly and positively correlated to the Klason lignin content of the cell walls of the entire stem [KL(%NDF)], which establishes for the first time the nearness of these two biochemical and histological estimates of lignin levels. However, this correlation was not very high, which may indicate that the lignin level of stem section sampled at a specific height does not strictly mirror that of the whole stem. It may also mean that a lignin fraction is not considered either in the red-stained areas or in the Klason lignin. In normal inbred lines, the positive and high correlation between A_R or KL(%NDF) and the number of cell layers in the cortical sclerenchyma N_{CS} is consistent with the importance of lignification in the thick-walled sclerenchyma. These results further validate the A_R histological factor as a measure of lignin level in the maize stem section.

A further support of the validity of the Fasga-stained red area A_R as a measure of the lignified cell walls in maize stem sections was provided by the closeness between this red area and the Mäule-stained total area $(A_S + A_G)$.

Histological and Biochemical Specificities of Maize Lignins in bm3 Mutants and in Normal Inbred Lines. In agreement with much past literature data (*6, 7, 12, 25, 34−38*), the bm3 lines studied herein displayed major compositional and structural changes relative to their normal counterpart. Their stems collected at the silage stage were typified by substantially lower levels in lignins and in pCA esters. In addition, lignin structure was dramatically changed, as revealed by the lower S/G thioacidolysis ratio and the appearance of substantial amount of 5-hydroxyguaiacyl units in lignins (data not shown). The lower frequency in S lignin units is consistent with the decrease in pCA esters, which are mainly linked to the S units of grass lignins (*1*). In contrast to various COMT-deficient dicots, the total yield in lignin-derived monomers, when expressed on a Klason lignin basis, was similarly low in the bm3 and in the normal lines. The possibility that *p*-coumarate substituents on S lignin units could substantially obstruct the depolymerization process (*1*) has been ruled out by the fact that a mild alkaline hydrolysis of pCA esters, performed without any lignin loss, does not improve the recovery of thioacidolysis monomers (C. Lapierre, unpublished results). That maize lignins systematically provide low thioacidolysis yields is most likely indicative of a high content in condensed bonds, whatever their frequency in S units. Although sinapyl alcohol has a pronounced tendency

to be involved in β-*O*-4 endwise-type coupling upon peroxidasic polymerization in the cell walls of S-rich dicots (*39*), its *p*-coumaroylation at Cγ might change the course of the reaction toward the formation of condensed bonds, such as the β-β ones. Such a discrepancy between grass cell walls and non-grass cell walls might originate from the structural peculiarities of grass lignins, including the *p*-coumaroylation of S units, an hypothesis which underscores that results obtained from the cell walls of non-grass plants, such as *Arabidopsis*, may not be extrapolated to the case of grass cell walls. Contrary to pCA esters and despite their lower lignin level, bm3 lines displayed similar yield in measurable FA ethers (released by hot alkaline hydrolysis) as normal lines. As the release of cell wall-linked ferulic units is precluded by a higher lignin level due to their incorporation in resistant bonding pattern (*37*), this result may suggest that bm3 lines display a lower ferulate-mediated cross-linking of the lignins to polysaccharides. All of the specific biochemical traits of bm3 lines were associated with a higher cell wall digestibility, which typifies these maize mutants. In agreement with the lower lignin level, three of four bm3 lines displayed a reduced absolute A_R and relative $A_R/(A_R + A_B)$ red area on the Fasga-stained stem sections. Contrary to a few reports on the occurrence of thinner cell walls in bm3 mutants (*36, 40, 41*), we could not discriminate bm3 and normal lines from other histological parameters, such as the area and characteristics of the thick-walled cortical sclerenchyma. It was noteworthy that the F2bm3 mutant presented a specific behavior compared to the three other bm3 mutants. Indeed, this mutant displayed a Klason lignin level substantially lower than its normal counterpart, but the relative importance of the Fasga-stained red area $A_R/(A_R + A_B)$ was similar in the F2bm3 and F2 samples. This unexpected result suggests that the bm3 mutation is not a simple translation from the normal inbred line to the bm3 counterparts. As described by Barrière et al. (*42*) for biological traits, it seems that the impact of the maize bm3 gene on histological traits may vary according to the genetic background.

Remarkably enough, we could evidence three normal inbred lines, F4, Cm484, and F251, provided with a cell wall digestibility similar to that of bm3 lines. Obviously, this high performance could be assigned to a low lignin level (as detected from both the compositional and histological parameters) for F4 and CM484, which further emphasizes the major impact of lignin content on cell wall digestibility. In contrast, the abnormally high digestibility of the F251 sample could be assigned neither to a low lignin level nor to another change in lignin descriptors (thioacidolysis yield or S/G ratio) or in measurable pCA or FA units. The origin of such a good digestibility therefore remains to be addressed. Contrary to the very low S/G ratio of bm3 lines, we highlighted quite a high S/G ratio for those three normal inbred lines of high cell wall digestibility. This further emphasized the different processes involved to explain the good cell wall digestibility in bm3 lines and in normal inbred lines.

From the Relationships between Cell Wall Digestibility and Biochemical and Histological Traits to the Definition of a Maize Ideotype for Cell Wall Digestibility. The main goal of this work was to better appreciate the relationships between degradability and both histological and biochemical characteristics of maize inbred lines.

The simple correlation study performed in the present work allowed us to complete the study begun in 1997 on biochemical traits (*10, 12*). We confirmed the well-known negative relationship between cell wall digestibility and lignin content. As recently suggested by Grabber et al. (*28*), we particularly paid

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attention to the relationships between cell wall digestibility and the ratio of pCA esters to FA ethers. Indeed, Grabber et al. (*28*) suggested the potential implication of this ratio as a selection criterion, with *p*-coumarate being an indicator of lignin deposition and ferulate being an indicator of lignin distribution among plant tissues. However, in the present study, we detected only a tendency between cell wall digestibility variations and the ratio of pCA esters to FA ethers ($r = -0.51$, data not shown). Thus, it seems that this parameter needs to be more precisely examined before it is used as a selection criterion in plant-breeding programs. The histological study carried out here for the first time revealed that a highly digestible maize may present a small surface of lignified material, a low number of cell layers in the cortical sclerenchyma, a low cortex and vessel surfaces, and finally thin cortical cell walls (**Table 4**).

When considering only one explanatory histological or biochemical variable, we accounted for 40% of the observed IVNDFD variations; the combined use of histological and biochemical traits to further understand the IVNDFD variations was therefore attempted. We first observed that the introduction of histological measures with biochemical ones did not allow us to define a more efficient model with two regression factors. Whatever the two-variates-based models, the lignin content was retained as an explanatory variate. In the first one, lignin content was represented through its link with pCA esters. In the second one the surface of lignified tissue (A_R) was charged with this role. In the two models, the second retained variate was the S/G thioacidolysis ratio. Thus, the two more important points in the consideration of both histological and biochemical traits were the lignin content in combination with the structure of the uncondensed part of lignin.

The most promising result produced a regression model using a combination of two histological and two biochemical variates to significantly account for 89% of the IVNDFD variations. Even if this model should be tested using samples that were not part of the model development, it allowed us to have quite a good illustration of what could be a maize of good cell wall digestibility. The four retained factors were the esterified pCA, the S/G thioacidolysis ratio, the number of cell layers in the cortical sclerenchyma, and the A_R area. A_R could be associated mainly with the pith lignification, whereas the number of cell layers in the cortical sclerenchyma may be related to the lignification of the cortical region. Consequently, an ideotype of maize silage for cell wall digestibility, as it appeared in our study, may contain a reduced quantity of lignin and its lignin may be richer in S than in G units and preferentially localized in the cortical region than in the pith.

The negative impact of lignin content on cell wall digestibility was not surprising at all. On the other hand, the preferential repartition of lignin in the cortical part of the stem rather than in the pith was a novel trait to be considered. Chesson (*43*) has suggested that the process of cell wall degradation in lignified tissues consists of the removal of wall polysaccharides from the lumen side of the wall and that this cell wall degradation comes to an end when a continuous layer of lignin incrustation is reached. More recently, Jung et al. (*44*) showed that particle size reduction in maize and lucerne increased access to the luminal side of cell walls. This increase was due to an enlarged surface area that can be attacked by rumen microbes. In the same way, our results suggested that the lesser lignification of the pith compared to the cortical zone would allow a better digestibility by increasing the accessible and potentially degradable area. Thus, cell wall digestibility will be more favored by a weakly lignified pith than by a weakly lignified cortical zone.

Concerning the involvement of lignin structure in cell wall degradability, we highlighted one more time the controversial implication of this S/G thioacidolysis ratio. In some studies, the digestibility was found to be associated with this S/G ratio (*11*, *12*). In contrast, and at a constant lignin level, results obtained on tobacco by Bernard-Vailhé et al. (*45*) and Grand et al. (*46*), on alfafa by Baucher et al. (*47*), and on *Arabidopsis* by Goujon et al. (*48*) suggest that a better digestibility is associated with a decrease of the S/G ratio. Finally, results obtained on an F5H mutant of *Arabidopsis thaliana* by Jung et al. (*49*) or on artificially lignified maize cell walls by Grabber et al. (*50*, *51*) suggest that varying ratios of S and G units have no influence on cell wall digestibility. Therefore, it is quite difficult to reach any definite conclusions on the implication of the S/G ratio in the cell wall digestibility variations. In the present study, we shed light on a positive relationship between the S/G ratio and the variations of cell wall digestibility. However, we think that the key idea is elsewhere than in the simple monomeric composition of the uncondensed part of lignins. Indeed, a major point needs to be considered when one is working on maize: the *p*-coumaroylation of lignin units. Although a small amount of pCA is esterified to hemicelluloses in immature tissues, most *p*-coumarate accumulation occurs during lignification (*28*). Lu and Ralph (*52*) showed that sinapyl alcohol is preacylated prior to its incorporation into lignins. The impact of *p*-coumaroylation on the incorporation mode of sinapyl alcohol in lignins is not clearly understood. In our study, mature normal maize cell walls contain on average 2% of *p*-coumarate esters mainly associated with the lignin moiety, the level of which is on average close to 17% of the extractive-free stem. Keeping in mind that most pCA is linked to S units, which account for ~50% of maize lignin units, these data indicate that almost 25% of S units in maize lignins may be acylated by pCA, a very high figure disregarded in most studies devoted to maize lignins.

Such a discrepancy between grass cell walls and non-grass cell walls might originate from the structural peculiarities of grass lignins, including the *p*-coumaroylation of S units, a hypothesis which underscores that results obtained from the cell walls of non-grass plants, such as *Arabidopsis*, may not be extrapolated to the case of grass cell walls. In this way, we think it is fundamental to now consider the question of the implication of the lignin structure on cell wall digestibility in maize quite differently than in nongrass species. It is therefore particularly crucial to address now the impact of the pronounced *p*-coumaroylation of S maize units on the interaction capabilities of maize lignins with polysaccharides and, therefore, on cell wall degradability.

ABBREVIATIONS USED

A_B, blue (nonlignified) area on the Fasga-stained section; A_C, cortical area on the Mäule-stained section; A_G, orange-brown area on the Mäule-stained section attributed to guaiacyl-rich lignins; A_R, red (lignified) area on the Fasga-stained section; A_S, red-purple area on the Mäule-stained section attributed to S-rich lignins; bm, brown midrib; DM, dry matter; estpCA, esterified *p*-coumaric acid; ethFA, etherified ferulic acid; FA, ferulic acid; G, guaiacyl units; IVNDFD, in vitro NDF digestibility; KL, Klason lignin; N_{CS}, number of cell layers in the cortical sclerenchyma; NDF, neutral detergent fiber (estimation of the cell wall content); pCA, *p*-coumaric acid; S, syringyl units; T_{CS}, thickness of cell layers of the cortical sclerenchyma.

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