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# Impact of Lignin Structure and Cell Wall Reticulation on Maize Cell Wall Degradability

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**ABSTRACT:** In this study, eight maize recombinant inbred lines were selected to assess both the impact of lignin structure and the impact of cell wall reticulation by *p*-hydroxycinnamic acids on cell wall degradability independently of the main "lignin content" factor. These recombinant lines and their parents were analyzed for in vitro degradability, cell wall residue content, esterified and etherified *p*-hydroxycinnamic acid content, and lignin content and structure. Lignin structure and esterified *p*-coumaric acid content showed significantly high correlation with in vitro degradability ($r = -0.82$ and $r = -0.72$, respectively). A multiple regression analysis showed that more than 80% of cell wall degradability variations within these 10 lines (eight recombinant inbred lines and their two parents) were explained by a regression model including two main explanatory factors: lignin content and estimated proportion of syringyl lignin units esterified by *p*-coumaric acid. This study revealed new biochemical parameters of interest to improve cell wall degradability and promote lignocellulose valorization.

**KEYWORDS:** Cell wall, lignin content, lignin structure, esterified *p*-coumaric acid, ferulic acid, in vitro cell wall degradability

## INTRODUCTION

Strong worldwide demand for energy and concern over global climate change has led to a resurgence in the development of alternative energy to replace fossil transportation fuel. In response, many countries have initiated extensive research and development programs in biofuels. The U.S. Department of Energy Office of the Biomass Program has developed a scenario for supplying 30% of motor gasoline demand with biofuels by the year 2030. Similarly, the European Union has developed a strategy in which one-fourth of the EU transportation fuels will be derived from biofuels by 2030.

Presently, starch from corn and wheat grain and simple sugars from sugar cane and beets are used for the production of bioethanol by fermentation. These first generation biofuels may compete with food needs, and it is now imperative to make effective second generation biofuels based on lignocellulosic agricultural residues. Cellulose, the "green gold" of high industrial and nutritional interest, is mainly located in secondary lignified plant cell walls. Its biological conversion into fermentable sugars is hindered by its association with lignins, a phenolic polymer fairly resistant to chemical and microbiological attacks. To harness the structural sugars contained in lignocellulose, it is necessary to understand the reasons for lignocellulosic biomass recalcitrance related to the presence of lignins and other phenolic compounds in secondary cell walls.

Most studies related to genetics, genomics, and biochemistry of lignins have been performed on perennial or annual dicot or gymnosperm species, whereas most forage plants or bioenergetics resources are grasses. Today, the bioenergy boom justifies an investment in graminaceous cell walls. Cell walls are complex molecular assemblies involving polysaccharidic (cellulose and hemicelluloses) and phenolic (lignins and *p*-hydroxycinnamic acids) components. Grass lignins are polymers of mainly three types of constitutive units (*p*-hydroxyphenyl, guaiacyl, and syringyl units) interconnected by aryl ether bonds ($\beta$-*O*-4 linkages) and/or resistant carbon−carbon bonds. *p*-Hydroxycinnamic acids, namely, ferulic and *p*-coumaric acids are an important specificity of grass cell wall. These acids are able to establish covalent bonds with lignins and/or other cell wall polymers. Whereas *p*-coumaric acid is known to be ester-linked to the $\gamma$-position of the side chains of syringyl lignin units,[1] ferulic acid is linked both to polysaccharides through ester linkages and to lignins via radical coupling mechanisms, leading to various bonding patterns of the ether and carbon−carbon types.[2,3] Both lignins and *p*-hydroxycinnamic acids have been reported to limit forage cell wall degradability by increasing their resistance to enzymatic or microbial degradation.[4,5]

Undoubtedly lignin content is the first factor negatively correlated with cell wall degradability. However, it is not possible to construct plants with drastically reduced lignin contents since a minimum degree of lignification is needed to ensure plant posture, avoid lodging, allow sap flow in vessels, and protect the plant against parasitic attacks. It is therefore necessary to turn to other biochemical parameters to increase the potential for grass degradability improvement while preserving acceptable agronomical performances. The influence of such factors on degradability is difficult to assess since lignin content often screens their effects.

In the present study, we propose to eliminate the lignin content parameter to focus on other parameters potentially responsible for limited cell wall degradability. Among them, the lignin structure has already been addressed, but its effect is still

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much debated. Several studies carried out on tobacco,[6] alfalfa,[7] and *Arabidopsis*[8] suggest that at equal lignin content a lower syringyl/guaiacyl lignin units (S/G) ratio would be beneficial to degradability. We observed the opposite effect with maize,[9] whereas other studies performed on a F5H *Arabidopsis* mutant[10] and on artificially lignified maize cell wall[11] showed no effect of the S/G ratio on cell wall degradability. Given these conflicting results, we suspect that cell wall degradability is not exactly related to the monomeric composition of lignin but more precisely to specific structures linked to the lignin skeleton. Indeed, a crucial structural point needs to be examined when considering grasses and notably maize: *p*-coumaroylation of lignin syringyl units. Lu and Ralph[12] have established that sinapyl alcohol was acylated before its incorporation in lignin but until now, the impact of this *p*-coumaroylation on the incorporation of sinapyl alcohol in lignin polymers has been unknown. In addition to the *p*-coumaroylation of syringyl lignin units, cell wall cross-linking by ferulic acid accounts for grass originality. Ester-linked to hemicelluloses on the one hand and ether-linked to lignin on the other, ferulic acid acts as a bridge between the two cell wall constituents.

The present study focuses on grass cell wall structural specificities using maize as a grass model. We propose to take advantage of the genetic variability of a maize recombinant inbred line (RIL) population developed at INRA Lusignan, France, and already characterized for global parameters such as cell wall degradability or lignin content. To rule out the impact of lignin content, we selected individuals within this RIL population with a quite similar lignin level and variable cell wall degradability. This material is ideal to determine the impact of lignin structure, lignin organization, and cell wall reticulation by *p*-hydroxycinnamic acids on grass cell wall degradability.

## MATERIALS AND METHODS

**Plant Material and Cropping Conditions.** Eight RILs were selected within the F286 × F7012 RIL population developed at INRA Lusignan to present a similar lignin content and contrasted cell wall degradabilities as predicted by near-infrared reflectance spectrophotometer (NIRS). The eight RILs and their two parental lines were cropped at INRA Lusignan for two successive years (2008 and 2009). The trials were randomized block designs with three replicates. Each plot consisted of one 5.2 m long row, and the density was 90 000 plants per hectare. Irrigation was applied from mid-June to the end of August to prevent water stress. At the ensiling stage, plants without ears were sampled and chopped. The samples were oven-dried (70 °C) and ground with a hammer mill to pass through a 1 mm screen.

**Chemical Analysis.** The cell wall residue (CWR) was obtained after a three-stage extraction of the dry matter by toluene/ethanol (1:1, v/v), ethanol, and water.[13] The lignin content was estimated using two different methods: the Klason procedure[14] and the Goering and Van Soest procedure.[15] The lignin content was expressed as the weight percentage of Klason lignin (KL) or sulfuric acid detergent lignin (ADL) in CWR. The difference between KL content and ADL content was computed.

The *p*-hydroxycinnamic acid content was measured after treating CWR fractions with NaOH according to a previously described procedure,[9] which involves two alkaline treatments with different levels of severity. Esterified *p*-hydroxycinnamic acids were released by subjecting CWR samples (50 mg) to a mild alkaline hydrolysis (2 N NaOH, 5 mL, 20 h at room temperature). Other CWR samples (50 mg) were treated with 5 mL of 4 M NaOH for 2 h at 170 °C to release both esterified and etherified hydroxycinnamates. Samples recovered from mild and severe alkaline hydrolysis were subjected to the same precipitation procedure[16] prior to high-performance liquid chromatography−mass spectrometry (HPLC-MS) analysis. The concentration of esterified *p*-coumaric and ferulic acids corresponds to the amount of *p*-coumaric and ferulic acids released by mild alkaline hydrolysis, whereas etherified ferulic acid was calculated as the difference between ferulic acid amounts released by the severe and mild alkaline treatments.

The lignin monomeric composition was studied by thioacidolysis performed on 15 mg of CWR placed in a screw-cap glass tube together with 1 mL of internal standard (long-chain hydrocarbons C19, C21, and C22, 0.5 mg/mL each) and 10 mL of dioxane/ethanethiol mixture (9:1, v/v) containing 0.2 M boron trifluoride etherate, for 4 h in an oil bath at 100 °C.[17] After extraction of the lignin-derived monomers, the analysis of their trimethylsilyl derivatives was run by gas chromatography-mass spectrometry (GC-MS) (Varian Saturn 2100, polydimethylsiloxane capillary column [SPB1, Supelco, 30 m × 0.25 m, 0.25 μm], ion trap spectrometer detector [IE 70 eV, positive mode]). The molar yield in *p*-hydroxyphenyl, guaiacyl, and syringyl lignin-derived monomers was calculated on the basis of the KL content of the sample.

We also estimated the percentage of syringyl lignin units acylated by *p*-coumaric acid (S-PC) assuming that all of the *p*-coumaric acid was esterified to syringyl lignin units and extrapolating the percentage of syringyl units in the uncondensed lignin part to the whole lignin. Thus S-PC was calculated as mentioned above:

$$\text{S-PC} = (100 \times \text{esterified } p\text{-coumaric acid})/(\text{KL} \times \% \text{ syringyl lignin units})$$

In vitro dry matter degradability was determined by an adapted Libramont−Limagrain protocol. Briefly, 500 mg of initial sample (dry matter) in a small bag was incubated 24 h, at 40 °C, under rotation in a pepsin solution (7 g of pepsin in 1 L of 0.1 N HCl). After 30 min at 80 °C, bags were incubated 24 h, at 40 °C, and under rotation in a cellulase/amyloglucosidase solution (2.95 mL of acetic acid, 6.8 g of sodium acetate, 1 g of cellulase, and 1.5 mL of amyloglucosidase, supplemented to 1 L with $H_2O$). The sample was then profusely rinsed with water and dried at 70 °C for 48 h before being weighed.

In vitro CWR degradability was computed assuming that the non-CWR part was completely degradable:[18]

$$\text{in vitro CWR degradability} = 100 \times [\text{in vitro dry matter degradability} - (100 - \text{CWR})]/\text{CWR}$$

All of the analyses were performed in duplicate.

**Statistical Analysis.** To compare the relative influence of genetic, year, and plot effects, variance analysis was carried out using the GLM procedure in SAS according to the following model:

$$Y_{ijk} = \mu + A_i + G_j + AG_{ij} + B_k/A_i + R_{ijk}$$

where $Y_{ijk}$ is the value of genotype $j$ in year $i$ and for plot replicate $k$; $\mu$ is the overall mean; $A_i$ denotes the main effect of year $i$; $G_j$ denotes the effect of genotype $j$; $AG_{ij}$ denotes the interaction effect between genotype $j$ and year $i$; $B_k/A_j$ denotes the main effect of replicate $k$ nested in year $j$; and $R_{ijk}$ is the random residual term. Data for RILs were combined over year, field, and analysis replicates before determination of correlation coefficients and multiple regression analysis using *R*.

## RESULTS AND DISCUSSION

We report here on the variability in biochemical composition and in vitro degradability before addressing the question of lignin determination. Finally, we discuss two structural parameters accounting for degradability variations.

**Relative Influence of Genetic and Year Effects on Biochemical Composition and in Vitro Degradability Values.**

**Table 1. *F* Value of the ANOVA Test of Cell Wall Composition and Degradability for the Eight RILs and Two Parent Lines[a]**

| | *F* value year | Pr > *F* | *F* value genotype | Pr > *F* | *F* value genotype × year | Pr > *F* | *F* value block | Pr > *F* |
|---|---|---|---|---|---|---|---|---|
| DF | 1 | | 9 | | 9 | | 4 | |
| | | | in vitro degradability | | | | | |
| in vitro CWR degradability (%) | 67.5 | <0.0001 | 8.5 | <0.0001 | 1.67 | 0.1357 | 2.92 | 0.0354 |
| | | | cell wall composition | | | | | |
| CWR (% DM) | 160.73 | <0.0001 | 22.77 | <0.0001 | 6.65 | <0.0001 | 0.52 | 0.7185 |
| KL (% CWR) | 6.28 | 0.0172 | 16.59 | <0.0001 | 2.81 | 0.0138 | 3.07 | 0.0292 |
| ADL (% CWR) | 2.08 | 0.1582 | 2.93 | 0.011 | 2.85 | 0.013 | 3.14 | 0.0267 |
| | | | lignin structure | | | | | |
| *p*-hydroxyphenyl (μmol/g KL) | 1.19 | 0.2837 | 18.69 | <0.0001 | 3.32 | 0.0052 | 1.96 | 0.1236 |
| guaiacyl (μmol/g KL) | 13.41 | 0.0008 | 11.63 | <0.0001 | 2.84 | 0.0131 | 2.12 | 0.0997 |
| syringyl (μmol/g KL) | 30.96 | <0.0001 | 3.77 | 0.0022 | 3.07 | 0.0084 | 0.87 | 0.4923 |
| β-*O*-4 yield (μmol/g KL) | 25.88 | <0.0001 | 6.38 | <0.0001 | 3.04 | 0.0088 | 0.95 | 0.4457 |
| S/G | 9.72 | 0.0037 | 13.99 | <0.0001 | 4.22 | 0.001 | 4.37 | 0.0059 |
| | | | *p*-hydroxycinnamic acids | | | | | |
| esterified *p*-coumaric acid (mg/g CWR) | 16.7 | 0.0003 | 41.15 | <0.0001 | 1.93 | 0.0799 | 1.01 | 0.416 |
| etherified ferulic acid (mg/g CWR) | 3.87 | 0.00574 | 4.9 | 0.00003 | 2.66 | 0.0188 | 1.84 | 0.1439 |

[a] DF, degree of freedom.

**Table 2. Mean, Maximum, and Minimum Values and Variation Coefficients for Cell Wall Chemical Composition and in Vitro Degradability among the Eight RILs and Their Two Parent Lines[a]**

| | mean | max | min | P1 | P2 | VC |
|---|---|---|---|---|---|---|
| | | in vitro degradability | | | | |
| in vitro CWR degradability (%) | 45.4 | 50.5 | 39.9 | 45.7 | 48.1 | 7.6 |
| | | cell wall composition | | | | |
| CWR (% DM) | 69.2 | 74.8 | 62.7 | 64.8 | 70.9 | 5.6 |
| KL (% CWR) | 13.8 | 15.2 | 12.4 | 13.2 | 13.9 | 6.8 |
| ADL (% CWR) | 5.9 | 6.9 | 5.1 | 5.1 | 6.1 | 9.3 |
| KL-ADL (% CWR) | 7.9 | 9.0 | 6.6 | 8.1 | 7.8 | 10.5 |
| | | lignin structure | | | | |
| *p*-hydroxyphenyl (μmol/g KL) | 10.4 | 14.8 | 7.3 | 10.3 | 9.5 | 22.4 |
| guaiacyl (μmol/g KL) | 221.4 | 273.8 | 178.2 | 244.9 | 178.2 | 14.9 |
| syringyl (μmol/g KL) | 246.4 | 289.8 | 201.3 | 256.3 | 201.3 | 10.0 |
| β-*O*-4 yield (μmol/g KL) | 478.2 | 573.5 | 389.0 | 511.4 | 389.0 | 11.1 |
| S/G | 1.13 | 1.38 | 0.96 | 1.05 | 1.12 | 11.63 |
| | | *p*-hydroxycinnamic acids | | | | |
| S-PC | 12.4 | 15.8 | 8.2 | 15.8 | 9.3 | 19.0 |
| esterified *p*-coumaric acid (mg/g CWR) | 8.7 | 10.5 | 6.2 | 10.5 | 6.7 | 17.8 |
| etherified ferulic acid (mg/g CWR) | 2.3 | 2.7 | 1.8 | 1.8 | 2.3 | 14.0 |

[a] P1, P2, two parent lines.

The biochemical composition and degradability of maize plants showed very little changes due to field replicates (Table 1).

Clear-cut year effects were observed for in vitro cell wall degradability, CWR content, lignin structure, and *p*-hydroxycinnamic acid content (esterified *p*-coumaric acid and etherified ferulic acid). In contrast, no year effect or weak year effect was detected for lignin content (KL and ADL) (Table 1). In previous studies, the year factor has been reported to affect the lignin content (KL) but not the phenolic acid concentrations.[9,19] The plant material used in our study has been chosen to limit the variation in lignin content but to expand the range of variation for cell wall degradability and cell wall biochemical composition. This factor may explain the unusual absence of year effect on lignin content and in contrast the detected effect on *p*-hydroxycinnamic acid content.

All biochemical traits and degradability parameters reported in Table 1 were markedly affected by the genotype, in agreement with literature data. This genotype effect surpasses the year effect,

**Table 3. Correlation Coefficients between Cell Wall Composition Parameters[a]**

| | KL | ADL | KL-ADL | guaiacyl | syringyl | β-*O*-4 yield | S/G | esterified *p*-coumaric acid | S-PC | etherified ferulic acid |
|---|---|---|---|---|---|---|---|---|---|---|
| KL | 1.00 | 0.49 | 0.81[b] | 0.42 | 0.40 | 0.44 | −0.15 | 0.12 | −0.20 | 0.43 |
| ADL | | 1.00 | −0.11 | −0.26 | −0.22 | −0.28 | 0.20 | −0.64[a] | −0.80[b] | 0.34 |
| KL-ADL | | | 1.00 | 0.36 | −0.30 | 0.66[a] | −0.30 | 0.57 | 0.31 | 0.26 |
| guaiacyl | | | | 1.00 | 0.66[a] | 0.94[b] | −0.71[a] | 0.60 | 0.60 | 0.13 |
| syringyl | | | | | 1.00 | 0.87[b] | 0.05 | 0.73[a] | 0.51 | 0.07 |
| β-*O*-4 yield | | | | | | 1.00 | −0.43 | 0.71[a] | 0.62 | 0.14 |
| S/G | | | | | | | 1.00 | −0.14 | −0.36 | −0.11 |
| esterified *p*-coumaric acid | | | | | | | | 1.00 | 0.91[b] | −0.22 |
| S-PC | | | | | | | | | 1.00 | −0.34 |
| etherified ferulic acid | | | | | | | | | | 1.00 |

[a] $P < 0.05$. [b] $P < 0.01$.

except for syringyl monomer concentrations. Genotype × year interactions were found to be significant for some values, which is not unusual.[9,20] However, genotypic differences always accounted for more of the variation than did genotype × year interactions. This result highlights the widely predominant genotype effect on cell wall composition and degradability.

**KL and ADL Lignin Are Estimations of Two Different Parts of the Lignin.** There are many methods to solubilize cell wall carbohydrates and thus determine by gravimetric measurement the lignin content of the cell wall. The Klason procedure[14] developed in the early 1900s by Klason is the most commonly used. Goering and Van Soest[15] proposed using two sequential treatments to first solubilize hemicelluloses (ADF residue) and then solubilize cellulose to ultimately obtain a lignin residue. We retained these two common procedures to estimate the lignin content of the 10 selected lines.

The KL in the cell wall varied from 12.4 to 15.2% CWR and the ADL lignin content from 5.1 to 6.9% CWR (Table 2). As extensively presented in different studies,[18,21−24] KL values were higher than ADL values. Both methods may underestimate the lignin content due to the possible partial solubilization of lignin during the treatment. However, this underestimation is lower with the KL method,[18] which suggests that the loss of lignin is reduced with this method. Another hypothesis for the differential between KL and ADL contents could be due to the presence of contaminating proteins condensed onto the KL residue.[25] However, in grass, the difference between ADL and KL values was of too great a magnitude to be explained by protein condensed in the residues.[21] In fact, as presented in Hatfield and Fukushima,[24] when applied to grasses, the acid detergent solution used in the Van Soest procedure dissolves more than 50% of the lignin, which explains the drastically lower values encountered for the ADL lignin content. The correlation coefficient between KL (% CWR) and ADL (% CWR) was not significant ($r = 0.49$) (Table 3). This absence of correlation indicates that KL and ADL estimates correspond, in grasses, to two different lignin "realities". We calculated for each sample the difference between the KL and the ADL lignin content, a parameter that can be assigned to the fraction of lignin dissolved during the specific detergent treatments of the ADL procedure. This difference was found to vary between 6.6 and 9.0% CWR (Table 2) and to be positively and significantly correlated to the total yield in thioacidolysis monomers only involved in β-*O*-4 bonds (Table 3). This result suggests that the difference between KL and ADL residues would refer to the lignin fraction rich in β-*O*-4 bonds.

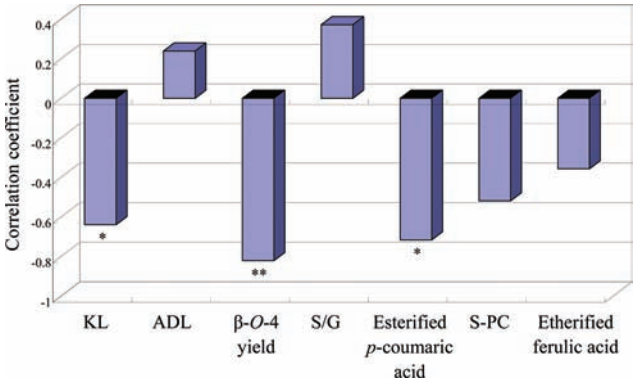


**Figure 1.** Correlation coefficient between in vitro CWR degradability and cell wall composition for the 10 selected lines (eight RIL and their two parents). KL, % CWR; ADL, % CWR; β-*O*-4 yield, total yield of thioacidolysis monomers (μmol/g KL); esterified *p*-coumaric acid (mg/g CWR); and etherified ferulic acid (mg/g CWR). *$P < 0.05$, and **$P < 0.01$.

Hatfield et al.[21] showed that syringyl-rich lignins were preferentially solubilized during the $H_2SO_4$ hydrolysis step of both KL and ADL procedures. It is to our knowledge the first time that a correlation has been established between the β-*O*-4 yield characterizing the lignin structure and its susceptibility to detergent solubilization.

Moreover, we illustrate that KL procedure is more suitable for the global determination of lignin, whereas the ADL procedure could be used to assess the proportion of the condensed lignin fraction. Our preliminary objective was to eliminate the lignin content parameter (based on KL NIRS prediction) in the selected lines. Although we have not completely satisfied this objective, we succeeded in considerably reducing the range of variation (three points of variation within the 10 lines) (Table 2), as compared to previous studies, namely, 8,[22] 5,[9] and 6[21] points of variation, respectively. Even in this narrow range, a negative and significant correlation ($r = −0.64$, $P < 0.05$) between in vitro cell wall degradability and KL lignin content was obtained. In contrast, and as previously reported,[23] no significant correlation between in vitro CWR degradability and ADL lignin content was detected. While KL lignin content partly accounts for in vitro cell wall degradability variation, it is not necessarily the best parameter to explain these variations. Indeed, unlike many studies presenting lignin content as the first impediment to cell wall degradability, several other parameters turned out here to be

significantly correlated to in vitro cell wall degradability (Figure 1). The KL-ADL parameter exhibited the highest significant correlation with in vitro CWR degradability ($r = -0.89$, $P < 0.01$).

**Uncondensed Lignin Is the First Obstacle to Cell Wall Degradability.** The proportion of *p*-hydroxyphenyl, guaiacyl, and syringyl lignin building units was evaluated by thioacidolysis and thus only reflects the units involved in $\beta$-*O*-4 bonds (the so-called "uncondensed" part of the lignin polymer). The total $\beta$-*O*-4 yield in thioacidolysis monomers, when calculated on the basis of the lignin content in the cell wall, provides an estimate of the proportion of lignin units only involved in $\beta$-*O*-4 bonds. This total yield ranged from 389 to 574 $\mu$mol/g KL (500 $\mu$mol/g KL corresponded to about 10% of the total KL content) and the S/G ratio range from 0.96 to 1.38 (Table 2).

In our study, the $\beta$-*O*-4 yield showed a high significant correlation coefficient with in vitro CWR degradability ($r = -0.82$, $P < 0.01$), whereas there was no strong relationship between the S/G ratio and the in vitro cell wall degradability ($r = 0.37$, ns). As observed for the total $\beta$-*O*-4 yield, the correlation between the two main lignin unit yields exhibited a negative and significant correlation with the in vitro cell wall degradability ($r = -0.77$, $P < 0.05$ for guaiacyl unit and $r = -0.72$, $P < 0.05$ for syringyl unit).

The influence of lignin composition on cell wall degradability is still controversial, and results can be very different when studying monocotyledons,[9] dicotyledons,[7,8,10] or artificially lignified cell walls.[11] Our results are in accordance with results obtained previously[26] on artificially lignified maize cell walls. The latter demonstrated that *p*-hydroxyphenyl, guaiacyl, and syringyl lignin units have similar inhibitory effects on degradability.[26] The effect of S/G ratio on cell wall degradability is still unclear. Similarly, the impact of lignin structure on cell wall degradability is still uncertain. In our study, $\beta$-*O*-4 content is an important impediment to cell wall degradability. It indicates that the more uncondensed (high $\beta$-*O*-4 yield) the lignin is the less degradable the cell wall. This result can be explained by considering the impact of interunit bonds on lignin conformation. In 2005, Besombes and Mazeau,[27] using molecular modelization, presented an illustrative picture of the lignin/cellulose association at the atomic scale. They showed that a lignin oligomer containing only $\beta$-*O*-4 interunit bonds adsorbs flat on the surface to maximize the interactions with cellulose. In contrast, an oligomer containing 5-5 and/or $\beta$-5 interunit bonds cannot straightforwardly orient parallel to the surface to optimize its interaction with cellulose. Thus, $\beta$-*O*-4-rich lignins would have a more extended shape likely to adsorb all along the cellulose fibers, whereas the more globular shape of condensed lignins leads to a lower coverage rate than $\beta$-*O*-4 rich lignins. For a given lignin content, an increase in the proportion of $\beta$-*O*-4 bonds in lignin may result in a lower accessibility of hydrolytic enzymes to cellulose fibers. By reducing the variation in lignin content within the selected group of lines, for the first time, we could show the clear-cut impact on cell wall degradability of the lignin interunit bonding pattern and the lack of impact of the S/G ratio in maize.

**Esterified *p*-Coumaric Acid Is Preferentially Encountered in the Uncondensed Lignin Part and Induces a Limitation of Cell Wall Degradability Whereas Ferulic Bridges Have No Impact on Cell Wall Degradability.** *p*-Hydroxycinnamic acids are known to be of importance in grass cell wall reticulation and organization. While *p*-coumaric acid is mainly ester-linked to S-lignin units,[1,28] ferulic acid is associated both with

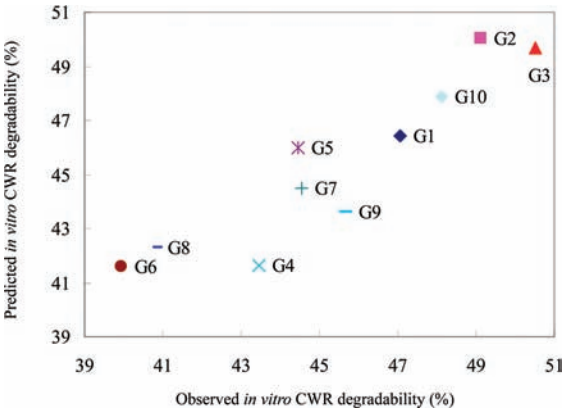


**Figure 2.** In vitro cell wall degradability observed vs in vitro CWR degradability predicted by applying the regression model using two variates, according to the following equation and for the eight RIL and their two parents (G1 to G10): in vitro CWR degradability = 96.6 − 2.84 KL − 0.98 S-PC.

hemicelluloses through ester linkages and with lignin via radical coupling mechanisms, which lead to a variety of bonding patterns (ether and C−C).[2,3] Thus, ferulate esters linked to hemicelluloses are nucleation sites for lignification in grass cell wall resulting in a bridge between hemicelluloses and lignin. At the beginning of this study, we hypothesized that cell wall reticulation by ferulic acid could be the most likely parameter to explain cell wall degradability variations in the context of a fairly constant lignin level. However, it is worth noting that etherified ferulic acid content was not correlated with in vitro CWR degradability ($r = -0.27$, ns) in this set of maize sister-lines unlike to results obtained in other studies.[29−31]

In contrast, esterified *p*-coumaric acid was found to be negatively and significantly correlated with in vitro CWR degradability ($r = -0.72$, $P < 0.05$). The negative impact of esterified *p*-coumaric acid has been reported in several studies.[32,33] The latter have suggested that the content in esterified *p*-coumaric acid indirectly and detrimentally affects cell wall degradability due to its positive correlation with lignin content.[19,33] However, as in previous studies,[9] we did not find any negative correlation between esterified *p*-coumaric acid content and KL lignin content ($r = 0.12$, ns) (Table 3). On the contrary, there was a significant correlation between esterified *p*-coumaric acid and total $\beta$-*O*-4 yield ($r = 0.71$, $P < 0.05$) or syringyl thioacidolysis units ($r = 0.73$, $P < 0.05$). On the whole, it appears that we have a correlation between the noncondensed lignin part and esterified *p*-coumaric acid content. Esterified *p*-coumaric acid could thus be preferentially linked to the syringyl lignin units only involved in $\beta$-*O*-4 bonds. Hence, it would be more accurate to say that S-PC units are more inclined to $\beta$-*O*-4 bonds and that esterified *p*-coumaric acid is thus preferentially encountered when associated with syringyl lignin units in the uncondensed lignin part. Indeed, Ralph et al.[28] showed that *p*-coumaric acid was not associated with syringyl lignin units after its incorporation in the polymer but that S-PC was imported in the grass cell wall to be incorporated in lignin. To conclude, we can assume that esterified *p*-coumaric acid is preferentially encountered in the uncondensed lignin and induces a decrease in cell wall degradability. This also explained the negative and significant correlation between ADL content and esterified *p*-coumaric acid since the lignin lost during the two step ADL procedure has been shown to be

richer in syringyl units[23] and more uncondensed. We thus demonstrated, in our set of lines, that the two main structural parameters, which explain cell wall degradability variations, were first the proportion of uncondensed lignin and second the content in esterified *p*-coumaric acid.

**Multiple Regression Analysis and in Vitro Cell Wall Degradability Estimation.** A multiple regression analysis was attempted to more comprehensively explain the in vitro CWR degradability observed variations. The best fitting regression equations were selected for in vitro CWR degradability, using KL, cellulose content, hemicellulose content, esterified *p*-coumaric acid, etherified ferulic acid, thioacidolysis total yield, thioacidolysis S/G ratio, and calculated parameter estimating the S-PC as explanatory variables. The best fitting regression model was obtained by a stepwise regression analysis. This model used two variables and significantly explained as much as 84% of the observed in vitro CWR degradability variations (Figure 2). The two variables were KL lignin content in the cell wall and the proportion of syringyl units acylated by *p*-coumaric acid, according to the following equation:

$$\text{in vitro CWR degradability} = 96.6 - 2.84\ \text{KL} - 0.98\ \text{S-PC}$$

where $R^2 = 84\%$ and $p = 5.5 \times 10^{-6}$.

Thus, when using more than one explanatory variable, the KL content came out together with S-PC, and none of the remaining variables could be significantly added in the regression. It was noteworthy that, even if limited, the variation in lignin content in the cell wall within the 10 selected lines was included in the best prediction equation together with a composite variable, which estimated the proportion of syringyl lignin units esterified by *p*-coumaric acid.

When only one variable to account for in vitro CWR degradability variations is used, the $\beta$-*O*-4 thioacidolysis yield appears to be the explanatory variable, whereas the S/G ratio had no impact. The second best explanatory variable was esterified *p*-coumaric acid content. When using a multivariable analysis, the best variables resulting from the experiments were KL content and a composite variable, which takes into account the proportion of syringyl lignin units acylated by *p*-coumaric acid. Thus, we highlighted the impact of both the proportion of uncondensed lignin and its acylation by *p*-coumaric acid in limiting cell wall degradability, whereas, in our plant material, ferulate bridges appeared to have no impact on the latter. Lignin condensation and cell wall *p*-coumaroylation were the two targets to take into consideration to increase cell wall degradability. This study offers applied perspectives for the development of second generation bioethanol by selecting plant material with improved susceptibility to biological conversion while maintaining a viable lignin content.

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## ABBREVIATIONS USED

ADL, acid detergent lignin; CWR, cell wall residue; KL, klason lignin; NIRS, near-infrared spectroscopy; RIL, recombinant inbred line; S/G, syringyl/guaiacyl lignin units ratio; S-PC, syringyl lignin units acylated by *p*-coumaric acid

## REFERENCES

(1) Grabber, J. H.; Quideau, S.; Ralph, J. *p*-Coumaroylated syringyl units in maize lignin: Implications for $\beta$-ether cleavage by thioacidolysis. *Phytochemistry* **1996**, *43*, 1189–1194.

(2) Lam, T. B. T.; Iiyama, K.; Stone, B. A. An approach to the estimation of ferulic acid bridges in unfractioned cell walls of wheat internodes. *Phytochemistry* **1994**, *37*, 327–333.

(3) Ralph, J.; Grabber, J. H.; Hatfield, R. D. Lignin−ferulate cross-links in grasses: Active incorporation of ferulate polysaccharide esters into ryegrass lignins. *Carbohydr. Res.* **1995**, *27*, 167–178.

(4) Besle, J. M.; Cornu, A.; Jouany, J. P. Roles of structural phenyl propanoids in forage cell wall digestion. *J. Sci. Food Agric.* **1994**, *64*, 171–190.

(5) Jung, H. J. G.; Deetz, D. A. Cell wall lignification and degradability. In *Forage Cell Wall Structure and Digestibility*; Jung, H. G., Buxton, D. R., Hatfield, R. D., Ralph, J., Eds.; American Society of Agronomy: Madison, WI, 1993; pp 315−346.

(6) Bernard-Vailhé, M. A.; Cornu, A.; Robert, D.; Maillot, M. P.; Besle, J. M. Cell wall degradability of transgenic tobacco stems in relation to their chemical extraction and lignin quality. *J. Agric. Food Chem.* **1996**, *44*, 1164–1169.

(7) Baucher, M.; Bernard, M. A.; Vailhe, B.; Chabbert, J. M.; Besle, C.; Opsomer, M.; Montagu, V.; Botterman, J. Down-regulation of cinnamyl alcohol dehydrogenase in transgenic alfalfa (*Medicago sativa* L.) and the effect on lignin composition and digestibility. *Plant Mol. Biol.* **1999**, *39*, 437–447.

(8) Goujon, T.; Ferret, V.; Mila, I.; Pollet, B.; Ruel, K.; Burlat, V.; Joseleau, J. P.; Barrière, Y.; Lapierre, C.; Jouanin, L. Down-regulation of the AtCCR1 gene in Arabidopsis thaliana: Effects on phenotype, lignins and cell wall degradability. *Planta* **2003**, *217*, 218–228.

(9) Mechin, V.; Argillier, O.; Menanteau, V.; Barrière, Y.; Mila, I.; Pollet, B.; Lapierre, C. Relationship of cell wall composition to *in vitro* cell wall digestibility of maize inbred line stems. *J. Sci. Food Agric.* **2000**, *80*, 574–580.

(10) Jung, H. J. G.; Ni, W.; Chapple, C. C. S.; Meyer, K. Impact of lignin composition on cell wall degradability in an Arabidopsis mutant. *J. Sci. Food Agric.* **1999**, *79*, 922–928.

(11) Grabber, J. H.; Hatfield, R. D.; Ralph, J. Apoplastic pH and monolignol addition rate effects on lignin formation and cell wall degradability in maize. *J. Agric. Food Chem.* **2003**, *51*, 4984–4989.

(12) Lu, F. C.; Ralph, J. Detection and determination of *p*-coumaroylated units in lignins. *J. Agric. Food Chem.* **1999**, *47*, 1988–1992.

(13) Effland, M. J. Modified procedure to determine acid-insoluble lignin in wood and pulp. *Tappi* **1977**, *60*, 143–144.

(14) Dence, C.; Lin, S. Y. The Determination of Lignin. In *Methods in Lignin Chemistry*; Lin, S. Y., Dence, C. W., Eds.; Springer: Berlin, 1992; pp 33−61.

(15) Goering, H. K.; Van Soest, P. J. Forage Fiber Analysis: Apparatus, Reagents, Procedures, and some applications. *USDA ARS Agricultural Handbook 379*; U.S. Government Printing Office: Washington, DC, 1970.

(16) Culhaoglu, T.; Zheng, D.; Baumberger, S.; Méchin, V. A green purification method for the analysis of ferulic and *p*-coumaric acids in maize cell-wall. *J. Chrom. B*, submitted for publication.

(17) Lapierre, C.; Monties, B.; Rolando, C. Preparative thioacidolysis of spruce lignin: Isolation and identification of main monomeric products. *Holzforschung* **1986**, *40*, 47–50.

(18) Méchin, V.; Argillier, O.; Barrière, Y.; Menanteau, V. Genetic variation in stems of normal and brown midrib 3 maize inbred lines. Towards similarity for *in vitro* digestibility and cell wall composition. *Maydica* **1998**, *43*, 205–210.

(19) Jung, H. J. G.; Ni, W. T.; Chapple, C. C. S.; Meyer, K. Forage quality variation among maize inbreds: Relationships of cell-wall composition and in vitro degradability for stem internodes. *J. Sci. Food Agric.* **1994**, *66*, 313–322.

(20) Lundvall, J. P.; Buxton, D. R.; Hallauer, A. R.; George, J. R. Forage quality variation among maize inbreds: In vitro digestibility and cell-wall components. *Crop Sci.* **1994**, *34*, 1672–1678.

(21) Hatfield, R. D.; Jung, H. G.; Ralph, J.; Buxton, D. R.; Weimer, P. J. A comparison of the insoluble residues produced by the Klason lignin and acid detergent lignin procedures. *J. Sci. Food Agric.* **1994**, *65*, 51–58.

(22) Jung, H. G.; Mertens, D. R.; Payne, A. J. Correlation of acid detergent lignin and klason lignin with digestibility of forage dry matter and neutral detergent fiber. *J. Dairy. Sci.* **1997**, *80*, 1622–1628.

(23) Fukushima, R. S.; Hatfield, R. D. Comparison of the acetyl bromide spectrophotometric method with other analytical lignin methods for determining lignin concentration in forage samples. *J. Agric. Food Chem.* **2004**, *52*, 3713–3720.

(24) Hatfield, R.; Fukushima, R. S. Can lignin be accurately measured. *Crop Sci.* **2005**, *45*, 832–839.

(25) Van Soest, P. J. *Nutritional Ecology of the Ruminant*, 2nd ed.; Cornell University Press: Ithaca, NY, 1994.

(26) Grabber, J. H.; Ralph, J.; Hatfield, R. D.; Quideau, S. *p*-Hydroxyphenyl, guaiacyl, and syringyl lignins have similar inhibitory effects on wall degradability. *J. Agric. Food Chem.* **1997**, *45*, 2530–2532.

(27) Besombe, S.; Mazeau, K. The cellulose /lignin assembly assessed by molecular modeling. Part 2: Seeking for evidence of organization of lignin molecules at the interface with cellulose. *Plant Physiol. Biochem.* **2005**, *43*, 277–286.

(28) Ralph, J.; Hatfield, R. D.; Quideau, S.; Helm, R. F.; Grabber, J. H.; Jung, H. J. G. Pathway of *p*-coumaric acid incorporation into maize lignin as revealed by NMR. *J. Am. Chem. Soc.* **1994**, *116*, 9448–9456.

(29) Grabber, J. H.; Ralph, J.; Hatfield, R. D. Ferulate crosslinks limit the enzymatic degradation of synthetically lignified primary walls of maize. *J. Agric. Food Chem.* **1998**, *46*, 2609–2614.

(30) Casler, M. D.; Jung, H. G. Relationships of fibre, lignin, and phenolics to in vitro fibre digestibility in three perennial grasses. *Anim. Feed Sci. Technol.* **2006**, *125*, 151–161.

(31) Jung, H. G.; Phillips, R. Putative Seedling Ferulate Ester (*sfe*) Maize mutant: Morphology, biomass yield, and stover cell wall composition and rumen degradability. *Crop Sci.* **2010**, *50*, 403–418.

(32) Taboada, A.; Novo-Uzal, E.; Flores, G.; Loureda, M.; Ros Barceló, A.; Masa, A.; Pomar, F. Digestibility of silages in relation to their hydroxycinnamic acid content and lignin composition. *J. Sci. Food Agric.* **2010**, *90*, 1155–1162.

(33) Gabrielsen, B. C.; Vogel, B. E.; Anderson, B. E.; Ward, J. K. Alkali-labile cell-wall phenolics and forage quality in switchgrasses selected for differing digestibility. *Crop Sci.* **1990**, *30*, 1313–1320.