

## Variations in the Cell Wall Composition of Maize *brown midrib* Mutants

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Most studies published thus far on the four *brown midrib* (*bm*) mutants (*bm1*, *bm2*, *bm3*, and *bm4*) in maize (*Zea mays* L.) have focused on one or two individual mutants, and comparisons between studies have been difficult because of variation in genetic backgrounds, maturity, and source of tissue. Detailed analyses of the stalks of the four *bm* single mutants and a *bm1–bm2* double mutant in a common genetic background (inbred A619) revealed structural and compositional changes in their isolated cell walls and lignins compared to the wild-type inbred. 2D-NMR revealed a significant presence of benzodioxane units in the *bm3* isolated lignin. 1D <sup>13</sup>C NMR revealed increased aldehyde levels in the *bm1* and *bm1–bm2* mutants compared to the wild-type inbred. The *bm3* and *bm1–bm2* mutants contained less Klason lignin in the isolated cell walls. The *bm1*, *bm3*, and *bm1–bm2* mutants contained ~50% less esterified *p*-coumaric acid with noticeably elevated levels of ferulate in the *bm3* mutant. A difference among *bm* mutants in the solubility of *p*-coumaric acid–lignin complexes during cellulase enzyme treatment was also discovered, suggesting that the *bm* mutations might also differ in the structural organization of lignin.

**KEYWORDS:** Maize; *Zea mays* L.; *brown-midrib* mutants (*bm1*, *bm2*, *bm3*, *bm4*); lignin; cell wall; NMR; benzodioxane

### INTRODUCTION

There are four *brown midrib* (*bm*) mutants known in maize (*Zea mays* L.). These mutants, *bm1*, *bm2*, *bm3*, and *bm4*, are believed to be Mendelian recessives and recognized by reddish-brown vascular tissue in the leaves and stems resulting from changes in lignin content and/or composition (1–3). The *bm* mutants are of interest because of their potentially higher nutritional value as a forage, presumably because of the lower lignin content and more digestible cell wall structure (4, 5).

Lignin itself is a complex polymer of phenylpropanoid units that hardens the cell walls of xylem tissue to provide mechanical strength to the stems and provides a physical barrier against pests and pathogens. Because lignin is hydrophobic, it decreases the permeability of cell walls and facilitates the transport of water through the xylem tissue (6).

The precursors of lignin are synthesized via the phenylpropanoid pathway. The first step in the pathway is the deamination of phenylalanine to produce cinnamic acid. Through a number

of hydroxylation and methylation reactions and a reduction of the carboxyl moiety, cinnamic acid is converted to *p*-hydroxycinnamyl alcohols, also referred to as monolignols, which can then undergo polymerization within the cell wall.

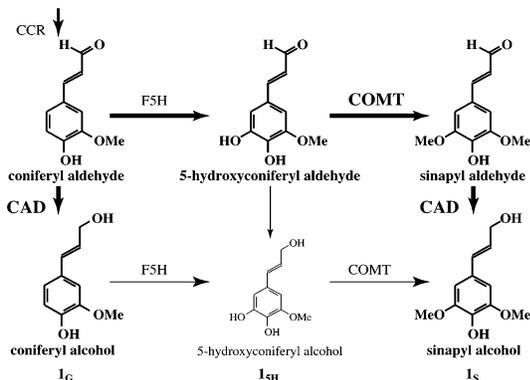
Detailed analysis of the cell wall composition of the *bm* mutants can provide information on the role of specific genes in lignin biosynthesis and can improve our understanding of lignification. The *bm1* mutation was shown to affect the activity of the enzyme cinnamyl alcohol dehydrogenase (CAD) (7), the last enzyme in the monolignol pathway (Figure 1). Using sequence data of the tobacco CAD gene, (Halpin et al.) cloned a maize cDNA encoding CAD. Although this *cad* cDNA mapped closely to the *Bm1* locus of maize, it is not yet clear whether the *Bm1* gene encodes CAD or controls its activity in some other way. Vermerris et al. believe that the *Bm1* locus acts in a dose-dependent manner on both *midrib* cell wall composition and the later stages of a plant's development (8). The exact function of the *Bm2* gene is still unknown, although there has been some speculation that it is associated with the overexpression of *O*-methyltransferase (OMT) (9). Pyrolysis–mass spectrometry studies indicated that the lignin in a *bm2* mutant showed a lower degree of cross-linking and that the vascular tissue in the mutant was lacking the tissue-specific cell wall composition observed in the wild-type plants (10). Vignols

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**Figure 1.** Partial diagram of the monolignol pathway in angiosperms. Bolder structures and large-diameter arrows represent preferred pathways as recently clarified (53–55). The *bm1* mutation affects the cinnamyl alcohol dehydrogenase (CAD) enzyme activity, and the *bm3* mutation affects the caffeic *O*-methyltransferase (COMT) enzyme activity.

et al. (11) identified two mutant alleles (*bm3*-1 and *bm3*-2) of the *Bm3* gene corresponding to an insertion and a deletion, respectively, in the caffeic acid *O*-methyltransferase (COMT) gene, one of the last enzymes involved in the production of syringyl units (Figure 1). These data substantiated the reason for lower OMT activity described earlier in *bm3* mutant lines (12, 13). Previously studied isolated lignins of *bm3* mutants contained 5-hydroxyguaiacyl (5-OH guaiacyl) subunits resulting from reduced methylation and exhibited a reduction in the proportion of syringyl lignin (12, 14, 15). Little is known about the changes in cell wall composition in the *bm4* mutant. Kuc et al. (2) reported a significant reduction in esterified *p*-coumaric acid (*p*CA) in *bm* mutants over normal plants and an increase in *p*-hydroxybenzaldehyde and vanillin as a result of nitrobenzene oxidation reactions. However, the nitrobenzene analysis, which is assumed to reflect the nature of lignin aromatic units, may be biased due to the oxidation of *p*CA and ferulic acid (FA) to *p*-hydroxybenzaldehyde and vanillin.

A number of investigations have studied *bm* mutants using a variety of techniques (7, 13, 14, 16–19). Typically, these studies would compare the *bm* mutants to normal (wild-type) plants; however, individual studies represented *bm* mutants in different genetic backgrounds, making direct comparisons between studies difficult. Different inbred lines can differ substantially in their lignin content and forage quality (20), so making direct comparisons between studies using different genetic backgrounds may not be valid. Furthermore, studies with the *bm1* (7) and *bm3* (14) mutations in different genetic backgrounds showed that the overall effect of the mutations was generally the same but that the extent of the changes varied.

Here we report the changes in cell wall composition in all four *bm* mutants (*bm1*, *bm2*, *bm3*, and *bm4*) and a *bm1*–*bm2* double mutant in near-isogenic backgrounds (A619), the exception being *bm3* for reasons described below.

## MATERIALS AND METHODS

**Seed Stocks.** Generation of the *bm1*, *bm2*, and *bm1*–*bm2* near-isogenic lines (NILs) was described in Vermerris and McIntyre (21). Essentially, *bm1* and *bm2* seed were in an A619 (wild-type) background following seven backcrosses and one generation of selfing and selection. The *bm1*–*bm2* double mutant was created by crossing plants from the original *bm1* seed (*bm1*, *anthocyaninless2*, *red aleurone1*) to derived-*bm2* plants (all in A619 background) followed by selfing of plants from the F<sub>1</sub> seed and selection in the F<sub>2</sub>. Double-mutants were confirmed by test crosses to the two single mutants (*bm1* and *bm2*). The *bm4* NIL was similarly generated in an A619 background. The *bm4* seed

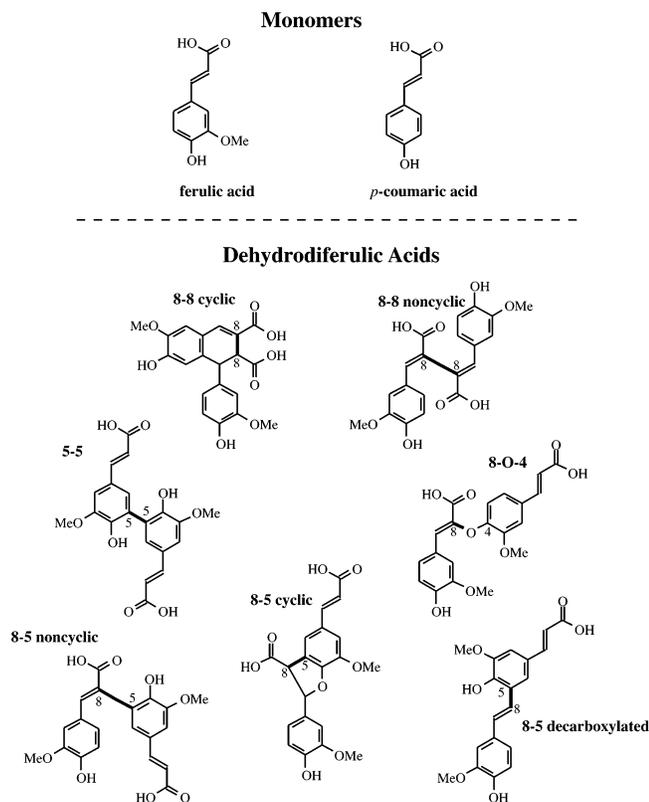
stock contained the linked mutation *Pl* (purple anthers). After the final backcross to A619, plants were selfed and progeny displaying the *bm4* but not the *Pl* phenotype were selected among the selfed backcross progeny (F<sub>2</sub>). The *bm3* seed was in a W22 background that also carried the sugary (*su*) mutation. Backcrosses to A619 were made for two generations, selecting *bm3* individuals that had normal seeds. These were the only *bm* plants not strictly NILs.

**Lignin Isolation and Preparation.** Wild-type (inbred A619), *bm1*, *bm2*, *bm3*, *bm4*, and *bm1*–*bm2* plants were grown in the field, and stalks were harvested when the tassels were just emerging (13-leaf stage). Entire stalks of four plants were cut in sections, frozen in liquid nitrogen and freeze-dried. Before isolation of maize cell wall material (CW), the stalk sections were cut into 2–3 cm pieces and ground to pass a 2.0 mm screen of a Wiley mill (Arthur H. Thomas Co., Philadelphia, PA) prior to grinding with a cyclone mill (Udy Corp., Fort Collins, CO). Cell wall and lignin isolations from the maize were essentially as previously described (22). Ground maize stems were extensively extracted with water, methanol, acetone, and chloroform. The isolated CWs were ball-milled, digested with crude cellulases (Cellulysin, Calbiochem-Novabiochem Corp., LaJolla, CA), and extracted into 96:4 dioxane/H<sub>2</sub>O. The dioxane/water fractions were lyophilized and saved as maize lignin extract (LE) and maize lignin residue (LR). A small amount (150 mg) of the freeze-dried LE fraction was acetylated overnight. Acetylated lignin samples were resuspended in 10 mL of methylene chloride (CH<sub>2</sub>Cl<sub>2</sub>) and run through a gel permeation column (GPC) to remove carbohydrates. The high molecular weight fractions from individual samples were collected and vacuum-dried before they were dissolved in ~400  $\mu$ L of acetone-*d*<sub>6</sub> for NMR analysis.

**Cell Wall Composition.** For all CW, LR, and LE samples, Klason lignin, total uronic acids, total neutral sugars, and phenolic compositions were determined. Klason lignin determinations were the ash-corrected residue remaining after total hydrolysis of cell wall polysaccharides by a modified Theander and Westerlund method (23, 24). Total uronic acids were estimated colorimetrically with galacturonic acid as the calibration standard (25, 26). Neutral sugars from total cell wall hydrolysis were determined by high-pressure liquid chromatography [high-performance anion exchange chromatography–pulsed amperometric detection (HPAE-PAD)]. Determination of phenolic acid esters followed a modified Ralph et al. (27) procedure. CW (~50 mg), LR (~55 mg), and LE (~30 mg) samples were analyzed using internal standards, 2-hydroxycinnamic acid (0.1 mg) for monomers, and 5-5-diferulic acid monomethyl ether (0.05 mg) for dimers (28). Derivatives of phenolic acids were separated by gas–liquid chromatography (GLC) as previously described (28). Phenolic monomers included *p*CA (*cis* and *trans*) and FA (*cis* and *trans*) (Figure 2). Dehydromonomers of ferulates (FA dimers) 8-8, 8-5, 8-*O*-4, and 5-5 are formed by oxidative radical coupling reactions. The total 8-8 diferulic acid (diFA) is the sum of 8-8 diFA (cyclic) and 8-8 diFA (noncyclic), and the total 8-5 diFA equals the sum of 8-5 diFA (cyclic), 8-5 diFA (noncyclic), and 8-5 diFA (decarboxylated) (Figure 2).

**Derivatization Followed by Reductive Cleavage (DFRC).** Release and determination of benzodioxane dimers by the DFRC method has been recently described (29). Benzodioxane lignin degradation products were quantified by GLC as previously described (30). Authentic guaiacyl and syringyl DFRC benzodioxane dimers were synthesized by Dr. F. Lu (University of Wisconsin, Madison, WI), as will be described elsewhere.

**Nuclear Magnetic Resonance (NMR).** 1D (<sup>1</sup>H and <sup>13</sup>C) and 2D NMR experiments were performed at 360 MHz on a Bruker DRX-360 instrument fitted with a 5 mm <sup>1</sup>H/broadband gradient probe with inverse geometry (proton coils closest to the sample). The conditions used for all samples were ~100 mg of acetylated isolated lignin in 0.4 mL of acetone-*d*<sub>6</sub>, with the central solvent peak as internal reference ( $\delta_H$  2.04 and  $\delta_C$  29.80). 2D NMR experiments used were standard Bruker implementations of gradient-selected versions of inverse (<sup>1</sup>H-detected) heteronuclear single quantum coherence (HSQC), HSQC–total correlation spectroscopy (HSQC–TOCSY), and heteronuclear multiple-bond correlation (HMBC) experiments. The TOCSY spin lock period was 100 ms; HMBC experiments used a 100 ms long-range coupling delay. Carbon/proton designations are based on conventional lignin



**Figure 2.** Chemical structures of phenolic acid monomers, *p*-coumaric acid (*p*CA) and ferulic acid (FA), and seven dehydrodiferulic acids (and derivatives) released from alkaline hydrolysis of maize cell walls (CW), dioxane/H<sub>2</sub>O residues (LR), and dioxane/H<sub>2</sub>O extracts (LE).

numbering, and lignin substructure numbering and colors are as previously described (31).

## RESULTS AND DISCUSSION

All four *bm* mutants (*bm1*, *bm2*, *bm3*, and *bm4*) and wild-type A619 maize were investigated for structural and compositional changes in their cell walls. In addition to the four individual mutants, a *bm1*–*bm2* double mutant was analyzed to examine the possible additive effects of two mutations on cell wall composition.

The isolated lignin amounts (percent of the CW and percent of the Klason lignin, respectively) were wild-type, 5 and 40%; *bm1*, 3 and 18%; *bm2*, 4 and 32%; *bm3*, 4 and 37%; *bm4*, 4 and 32%; and *bm1*–*bm2*, 3 and 28%. Despite differences in percentages isolated, if an extract from a mutant differs

substantially from that of the control, it is logical that such structural differences will be present in the whole polymer at some level.

**Maize Cell Walls.** Lignin was measured as an acid insoluble residue using a modified Klason lignin. The CWs from the *bm3* and *bm1*–*bm2* mutants had 20 and 10% lower lignin contents compared to wild-type, whereas the *bm1*, *bm2*, and *bm4* mutant CWs contained the same amount of lignin as wild type (**Table 1**). The *bm* mutants are generally recognized by a reduction in lignin content (32); however, as indicated above, the results from numerous studies vary to the same extent as the number of genetic stocks investigated. Consequently, differences in the lignin content of any given *bm* mutation compared to wild-type were quite variable. To what extent genetic or environmental effects change the structure and/or composition of lignin in *bm* mutants has not been addressed, although many studies come to the conclusion that such effects do exist (14, 19).

The sum of Klason lignins (acid insoluble residues), total neutral sugars, and uronic acids (**Table 1**) accounted for 92–95% of the *bm* mutant cell wall. The rest of the cell wall can be divided further into acid soluble components including structural and metabolic proteins and smaller phenolic components (e.g., acid soluble lignins, polymerized lignans, and wall-bound FA and *p*CA). Compositional data of all *bm* mutants and wild-type CWs (mg g<sup>-1</sup> of CW) are presented in **Table 1**. There were no dramatic differences in total neutral sugars or uronic acids levels between CW samples. The proportion of individual neutral sugars (g<sup>-1</sup> of CW) was generally the same for all *bm* mutants and wild-type CW, between 47 and 50% cellulose (as determined by glucose) and 22–27% xylan (as determined by xylose) with substitution with arabinose varying between 1:8 in the *bm3* mutant and 1:6 in wild-type maize. Minor shifts in rhamnose and mannose were observed for *bm3* and *bm4* CWs. The uronic acids remained constant at ~4% for all CWs.

CW hydroxycinnamates (**Figure 2**), esterified and etherified FA and *p*CA, were measurable following low- and high-temperature alkaline hydrolysis (**Table 2**). Low-temperature hydrolysis (room temperature, 20 h) releases esterified acids, whereas high temperature hydrolysis (170 °C, 2 h) releases also those acids phenol-etherified to lignins (33) but not the 8-*O*-4 diferulates (which are also ethers) (34). FA monomers and dimers (ester-linked) were consistent across all CW samples, with the exception of the *bm3* CW that had elevated levels of both. This would indicate a greater amount of FA esterified to arabinoxylans in the *bm3* cell walls, although the level of cross-linking between arabinoxylans, indicated by total FA dimers, was not higher. Equivalent amounts of FA monomers but a

**Table 1.** Cell Wall (CW) Composition of Wild-Type and *bm* Mutants of Maize in A619 Background Averaged over Two Replicates

	A619	<i>bm1</i>	<i>bm2</i>	<i>bm3</i>	<i>bm4</i>	<i>bm1</i> – <i>bm2</i>
Klason lignin (mg g <sup>-1</sup> of CW)	130 ± 3	130 <sup>a</sup>	131 ± 4	104 ± 2	138 ± 1	118 ± 3
uronosyls (mg g <sup>-1</sup> of CW)	41 ± 2	49 ± 2	43 ± 2	37 ± 0	41 ± 2	42 ± 0
neutral sugars (mg g <sup>-1</sup> of CW)						
fucose	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.3 ± 0.0
arabinose	36.7 ± 0.6	34.4 ± 0.1	32.6 ± 0.3	32.7 ± 1.0	34.4 ± 0.6	33.0 ± 0.5
rhamnose	1.1 ± 0.2	0.8 ± 0.2	0.9 ± 0.1	0.3 ± 0.4	1.1 ± 0.2	1.0 ± 0.0
galactose	7.0 ± 0.1	6.8 ± 0.2	6.5 ± 0.1	6.9 ± 0.4	6.7 ± 0.2	6.5 ± 0.2
glucose	479.1	471.8	469.1	491.2	485.7	472.5
xylose	226.6 ± 5.4	252.9 ± 20.3	252.0 ± 4.2	262.1 ± 3.5	242.0 ± 7.1	244.9 ± 9.7
mannose	4.7 ± 0.1	3.2 ± 0.0	3.3 ± 0.2	3.0 ± 0.1	2.0 ± 2.8	3.1 ± 0.2
total	755.4	770.1	764.6	796.5	772.2	761.2
cell wall total <sup>b</sup>	927	949	939	937	951	920

<sup>a</sup> Based on one replicate. <sup>b</sup> Cell wall total = Σ(Klason lignin + uronosyls + total neutral sugars).

**Table 2.** Cell Wall (CW) Phenolic Acids Released from Wild-Type and *bm* Mutants of Maize in A619 Background Averaged over Two Replicates

	A619	<i>bm1</i>	<i>bm2</i>	<i>bm3</i>	<i>bm4</i>	<i>bm1–bm2</i>
monomers (mg g <sup>-1</sup> of CW)						
esterified <i>pCA</i>	14.07 ± 0.20	8.46 ± 0.02	15.03 ± 0.18	7.90 ± 0.10	16.14 ± 0.03	7.89 ± 0.24
esterified FA	4.46 ± 0.07	5.09 ± 0.05	5.24 ± 0.24	7.29 ± 0.07	4.67 ± 0.09	4.89 ± 0.01
etherified FA	2.21 ± 0.05	1.85 ± 0.19	2.69 ± 0.24	2.51 ± 0.23	2.36 ± 0.11	1.78 ± 0.19
% wild-type	100	84	122	114	107	81
FA dimers (mg g <sup>-1</sup> of CW)						
8-8	0.25 ± 0.01	0.35 ± 0.00	0.27 ± 0.02	0.34 ± 0.01	0.26 ± 0.10	0.38 ± 0.03
8-5	1.16 ± 0.07	1.07 ± 0.06	1.23 ± 0.58	1.10 ± 0.12	1.32 ± 0.31	0.72 ± 0.11
8- <i>O</i> -4	0.27 ± 0.01	0.35 ± 0.02	0.20 ± 0.04	0.29 ± 0.03	0.20 ± 0.03	0.27 ± 0.05
5-5	0.17 ± 0.01	0.18 ± 0.00	0.10 ± 0.01	0.13 ± 0.01	0.11 ± 0.01	0.13 ± 0.01
dimer total	1.85	1.96	1.79	1.87	1.90	1.50
FA total <sup>a</sup>	8.52	8.90	9.72	11.67	8.93	8.17

<sup>a</sup> FA total = Σ(esterified FA + etherified FA + total FA dimer).

**Table 3.** Klason Lignin in Dioxane/H<sub>2</sub>O Residue (LR) and Dioxane/H<sub>2</sub>O Extract (LE) as a Percent Cell Wall (CW) Klason Lignin (Milligrams per Gram of CW)

	% CW					
	A619	<i>bm1</i>	<i>bm2</i>	<i>bm3</i>	<i>bm4</i>	<i>bm1–bm2</i>
Klason lignin (LR + LE)	92	47	75	63	79	67

decrease in FA dimers indicated a less cross-linked (between arabinoxylans) cell wall in the *bm1–bm2* compared to wild-type. For the *bm1* mutant (83% of etherified FA wild-type levels) and the derived *bm1–bm2* (81%) mutant, the FA totals (esterified plus etherified) were equivalent to those of wild-type. Higher levels of esterified FA had compensated for the lower levels of etherified FA (**Table 2**). Etherified levels in the double mutant more closely resemble levels found in the *bm1* parental line rather than *bm2*. All other *bm* mutants showed elevated amounts of etherified FA as a percentage of wild-type (*bm2*, 122%; *bm3*, 114%; and *bm4*, 107%).

*p*-Coumarate is thought to be mainly associated with lignins (35). The levels of esterified *pCA* differed depending on the mutant CW compared to wild-type (**Table 2**). Levels of esterified *pCA* in *bm2* and *bm4* CWs were comparable to those in wild-type; however, the *bm1*, *bm3*, and double *bm1–bm2* CW levels were ~50% of the wild-type, somewhat paralleling the decreases in Klason lignin levels. The decrease in esterified *pCA* in the *bm1–bm2* CW could suggest a dose effect inherited from the *bm1* parental line. Large differences in levels of esterified *pCA* among *bm* mutant cell walls have been reported previously (2, 9, 14, 36, 37).

**Maize Lignin Extract and Lignin Residue Fractions.** Striking differences between the *bm* mutants were revealed by compositional shifts in their LR and LE fractions. When enzyme-digested CWs are partitioned into the LE and LR fractions, the sum of their Klason lignin values should theoretically add up to the corresponding CW Klason lignin value. However, the sum for all samples, particularly the *bm* mutants, was lower (**Table 3**). Differences in the levels of acid soluble components lost during hydrolysis and/or differences in individual carbohydrate profiles may account for such discrepancies.

Parallel reductions in esterified *pCA* were observed in the same maize LR and LE fractions; every maize sample had lower total esterified *pCA* (LR + LE) than amounts released from their CW (**Table 4**). The *bm1* mutant displayed the greatest reduction at 46% of its CW level (68% of the wild-type level). This parallels the reduction observed in the *bm1* mutant Klason lignin (LR + LE) at 53% of its CW level (51% of the wild-

**Table 4.** Sum of Dioxane/H<sub>2</sub>O Residue (LR) and Dioxane/H<sub>2</sub>O Extract (LE) Alkaline Extractable Phenolic Acids (Monomers) of Wild-Type and *bm* Mutants of Maize in A619 Background

	A619	<i>bm1</i>	<i>bm2</i>	<i>bm3</i>	<i>bm4</i>	<i>bm1–bm2</i>
monomers (mg g <sup>-1</sup> of CW) (LR + LE)						
esterified <i>pCA</i>	11.20	4.55	10.02	4.59	10.66	4.42
esterified FA	1.13	0.86	1.35	0.88	1.34	1.03
% CW						
esterified <i>pCA</i>	80	54	67	58	66	56
esterified FA	25	17	26	12	29	21

type level). Similarly, the esterified *pCA* and Klason lignin totals (LR + LE) in the other *bm* mutants were comparatively lower than their CW or wild-type levels.

Previous reports suggested that little *pCA* is esterified to arabinoxylans in maize (35, 38); therefore, the *pCA* released during low-temperature hydrolysis must be predominantly esterified to lignin in the LR and LE fractions. These results suggest that *pCA*–lignin complexes were solubilized and lost in the supernatants during cellulase enzyme treatment. Low-temperature alkaline hydrolysis of the enzyme supernatants did reveal substantial amounts of esterified *pCA*. Furthermore, 0.1 N trifluoroacetic acid (TFA) hydrolysis of the maize CWs released *pCA*–arabinoxyl conjugates, detectable by GC-MS selective ion monitoring, but at insufficient levels for reliable quantification (data not shown). This mild hydrolysis method should be sufficient to release *pCA*–arabinoxyl conjugates given that released levels of FA–arabinoxyl conjugates were comparable to the levels of esterified FA released by low-temperature alkaline hydrolysis. Because only small amounts of *pCA* were found esterified to arabinoxylans in the maize samples, the esterified *pCA* measured after low-temperature hydrolysis from the supernatants clearly indicates that significant amounts of *pCA*–lignin complexes are being solubilized during cellulase enzyme treatment. These results help to explain why parallel reductions of *pCA* and lignin were observed in the *bm* mutant LR and LE fractions; specifically, the higher the solubility of *pCA*–lignin complexes in the *bm* mutants, the greater the loss after the cellulase enzyme treatment.

Compositional shifts in total neutral sugars existed between the LR and LE fractions. Cellulose (as measured by glucose) was greater in all of the maize LR fractions versus LE fractions. Total residual cellulose (LR + LE glucose) following cellulase enzyme treatment correlated to percent cell wall degradability [(1 – recovered weight/initial weight) mg g<sup>-1</sup> of CW] (**Table 5**). The *bm3* mutant with the greatest degradability (88%) had the lowest residual cellulose (LR + LE glucose; 40 mg g<sup>-1</sup> of

**Table 5.** Sum of Dioxane/H<sub>2</sub>O Residue (LR) and Dioxane/H<sub>2</sub>O Extract (LE) Total Neutral Sugars (TNS) of Wild-Type and *bm* Mutants of Maize in A619 Background and Percent Cell Wall Digested Following Cellulase Enzyme Treatment (Averaged over Two Replicates)

	A619	<i>bm1</i>	<i>bm2</i>	<i>bm3</i>	<i>bm4</i>	<i>bm1</i> – <i>bm2</i>
TNS – LR (mg g <sup>-1</sup> of CW)						
fucose	<0.1 ± 0.0	<0.1 ± 0.0	<0.1 ± 0.0	<0.1 ± 0.0	<0.1 ± 0.0	<0.1 ± 0.0
arabinose	6.6 ± 0.0	5.0 ± 0.1	6.4 ± 0.0	2.3 ± 0.1	7.4 ± 0.5	5.0 ± 0.0
rhamnose	0.4 ± 0.0	0.1 ± 0.0	0.3 ± 0.1	<0.1 ± 0.1	0.2 ± 0.3	0.3 ± 0.1
galactose	1.5 ± 0.0	0.9 ± 0.0	1.3 ± 0.0	0.7 ± 0.0	1.5 ± 0.1	1.1 ± 0.0
glucose	62.4 ± 11.4	67.9 ± 2.9	91.4 ± 1.3	38.0 ± 0.7	131.2 ± 5.4	66.7 ± 1.4
xylose	56.8 ± 1.0	60.4 ± 2.8	71.5 ± 0.7	26.3 ± 0.9	89.4 ± 4.4	50.2 ± 0.2
mannose	1.6 ± 0.0	0.7 ± 0.1	0.4 ± 0.6	0.7 ± 1.0	0.7 ± 1.0	1.4 ± 0.0
totals	129.4	135.2	171.3	68.0	230.7	124.7
TNS – LE (mg g <sup>-1</sup> of CW)						
fucose	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
arabinose	1.6 ± 0.1	0.9 ± 0.0	1.4 ± 0.0	1.5 ± 0.0	1.3 ± 0.1	1.1 ± 0.0
rhamnose	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
galactose	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
glucose	5.4 ± 0.3	1.9 ± 0.0	2.9 ± 0.2	1.9 ± 0.0	2.9 ± 0.2	4.4 ± 0.1
xylose	14.0 ± 0.0	8.3 ± 0.1	11.9 ± 1.0	10.8 ± 0.5	10.9 ± 0.6	8.8 ± 0.1
mannose	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
totals	21.2	11.3	16.3	14.2	15.3	14.4
% degradability <sup>a</sup>	76	82	77	88	71	82

<sup>a</sup> Degradability is [(1 – recovered wt/initial wt) mg g<sup>-1</sup> of CW].

CW), whereas the *bm4* mutant with the lowest degradability (71%) had the highest residual cellulose (LR + LE glucose; 134 mg g<sup>-1</sup> of CW). In general, most of the remaining neutral sugars, after treatment of the CWs with cellulase, were partitioned into the LR fraction. Individual neutral sugars were not partitioned equally among samples, implying that the hydrolysis of component polysaccharides was not consistent across *bm* mutants. For example, *bm4* had the lowest glucose (72%) and xylan (59%) releases, whereas *bm3* had the highest glucose (92%) and xylan (86%) releases. Equivalent neutral sugars levels (mg g<sup>-1</sup> of CW) were observed among the maize LE fractions, suggesting that the composition of the neutral sugars extracted in the soluble lignin fraction among all maize samples is the same regardless of the *bm* mutation. The presence and detection of neutral sugars in the LE fraction support an interaction between carbohydrates and lignin—a reasonable conclusion because arabinoxylans are known to be cross-linked to lignin by FA (39, 40). Uronic acids remained constant across all LR and LE samples.

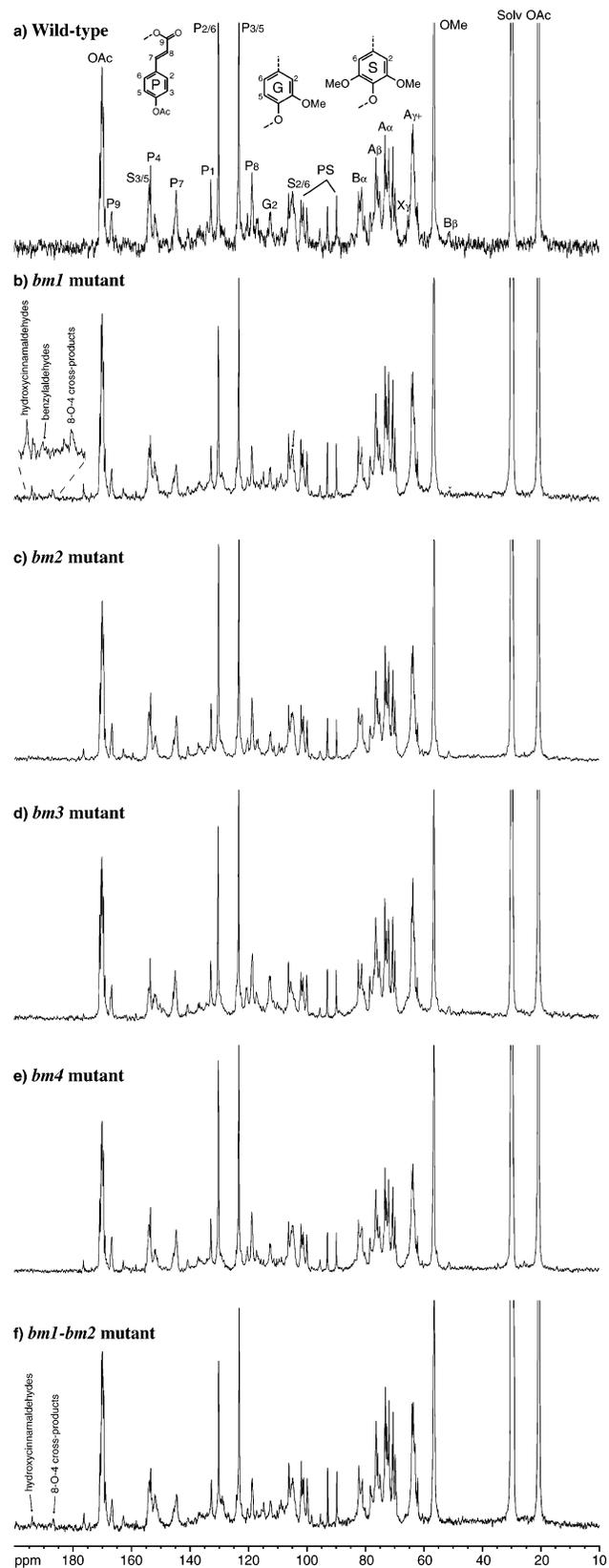
The reason neutral sugar components are partitioned predominantly in the LR fraction may be an organizational issue whereby their incorporation into the cell wall is dependent on the respective *bm* mutation being expressed. As Kuc and Nelson (1) alluded to in their studies of *bm* maize, perhaps plants carrying the mutant alleles lack some function controlling lignification. These data would suggest that the function lacking in some of the *bm* mutants does not control lignification per se but may control *elements* leading to lignification such as peroxidase activity or hydrogen peroxide production and availability. As a result, the lignin polymer is altered in such a way that incorporation of esterified *pCA* is reduced and consequently the structure of the lignin polymer altered at some organizational (“nonpolymeric”) level not detected by NMR (discussed below).

**<sup>13</sup>C NMR Spectra.** Qualitative 1D <sup>13</sup>C NMR spectra of isolated lignin LE from *bm* mutants are presented in **Figure 3**. These spectra substantiate the significant presence of *pCA* on maize lignins as evidenced from the large P<sub>3/5</sub> (~123 ppm) and P<sub>2/6</sub> (~130 ppm) peaks. The <sup>13</sup>C NMR spectra also confirm a decrease in *pCA* observed in the *bm1*, *bm3*, and *bm1*–*bm2* mutants. The S and G components are not strikingly different

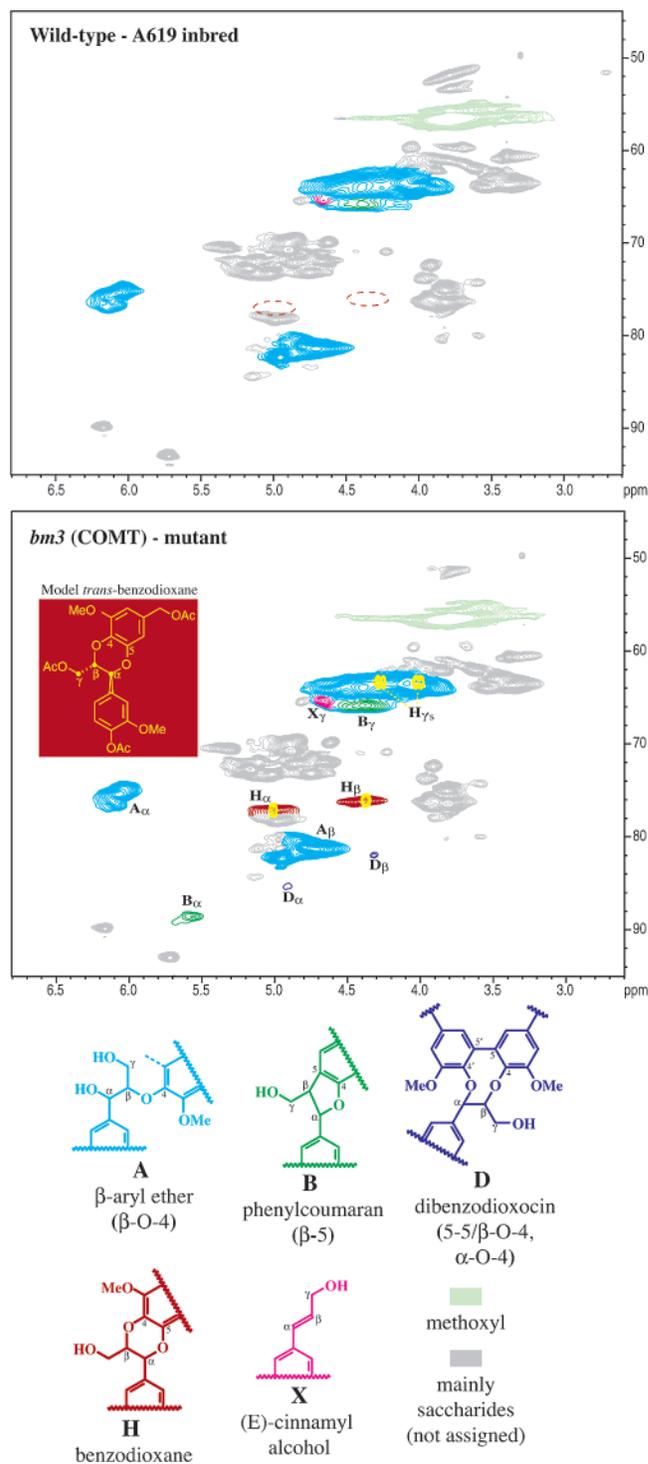
between *bm* mutants as evidenced by similar S (S<sub>2/6</sub> at ~105 ppm and S<sub>3/5</sub> at ~154 ppm) and G (G<sub>2</sub> at ~113 ppm) peak profiles in the <sup>13</sup>C NMR spectra. The exceptions being differences in the *bm1* and *bm3* peak profiles compared to wild-type with lower S peaks relative to G peaks. DFRC degradation products of the maize CWs confirmed the differences in their S:G ratios compared to wild-type. Whereas all other maize samples had DFRC monomer S:G ratios between 0.12 and 0.16, the S:G ratio of the *bm1* mutant was 0.09 and that of the *bm3* mutant was 0.06. The DFRC method releases S and G monomers by cleaving β-ether bonds flanking the unit in lignins.

On the basis of previous studies, the *bm1* mutation affects cinnamyl alcohol dehydrogenase (CAD) and typically has a reduced lignin content in mature plants (7). Studies of a variety of plants deficient in CAD (41–48) indicated a marked rise in aldehydes. The 1D <sup>13</sup>C NMR spectra of lignin in *bm1* stems confirmed modest increases in aldehydes relative to levels in wild-type or any other single *bm* mutant (**Figure 2**). Examination of this aldehyde region revealed the presence of hydroxycinnamaldehydes (~193 ppm), benzaldehydes (~191 ppm), and 8-*O*-4 cross-products (~187 ppm) between hydroxycinnamaldehydes and guaiacyl or syringyl units at much lower levels than seen in CAD-deficient plants previously studied. The only other *bm* mutant displaying an aldehyde presence was the double *bm1*–*bm2* mutant. The aldehyde levels in the *bm1*–*bm2* mutant were lower than in the contributing *bm1* parent but nevertheless present. The low levels of 8-*O*-4 cross-products do not allow easy identification of the S/G nature of units constituting these peaks as was possible in the <sup>13</sup>C-enriched tobacco samples (43).

**2D HMQC NMR Spectra.** Two-dimensional (2D) HMQC NMR experiments revealed similarity in the composition of the isolated lignins from the maize samples excluding the *bm3* mutant. Incorporation of 5-hydroxyconiferyl alcohol into the lignin of the *bm3* mutant produced novel benzodioxane units **H** (**Figure 4**). Correlations from a model *trans*-benzodioxane compound overlaid on the HMQC spectrum of the *bm3* mutant confirmed the benzodioxane unit **H** identity (**Figure 4**). Hwang and Sakakibara (49) first suggested that 5-hydroxyconiferyl alcohol could be incorporated into lignins after identifying a benzodioxane dimer in hydrogenolysis products from *Fraxinus*



**Figure 3.** 1D  $^{13}\text{C}$  NMR spectra of acetylated LE maize lignins from (a) wild-type, (b) *bm3* mutant, (c) *bm4* mutant, (d) *bm1* mutant, (e) *bm2* mutant, and (f) *bm1-bm2* mutant. Major peaks are shown with their assignments; the letters **A**, **B**, **X** correspond to structures **A**, **B**, and **X** in Figure 3; **G** and **S** are used to represent general guaiacyl and syringyl units; **P** represents abundant *p*-coumarate (*pCA*) esters acylating lignin side chains; **OAc** are acetate groups; **OMe** is the methoxyl peak; and **Solv** is the solvent peak.



**Figure 4.** Gradient-selected 2D HMQC spectra of acetylated LE maize lignins from wild-type and the *bm3* mutant. Standard lignin numbering is used along with the standardized color scheme from Ralph et al. 1999 (37). Lignin structures are **A**,  $\beta$ -aryl ether ( $\beta$ -O-4); **B**, phenylcoumaran ( $\beta$ -5); **C**, resinol ( $\beta$ - $\beta$ ); **D**, dibenzodioxocin (5-5/ $\beta$ -O-4,  $\alpha$ -O-4); **H**, benzodioxane; and **X**, cinnamyl alcohols. Novel benzodioxane units **H** resulting from incorporation of 5-hydroxyconiferyl alcohol into the lignin are emphasized in red with model *trans*-benzodioxane contours (in yellow) superimposed on the HMQC spectrum of the *bm3* mutant.

*mandshurica* Rupr. var. *japonica* Max. Later, 5-hydroxyguaiacyl monomers were discovered in thioacidolysis products in *bm3* maize (12). The incorporation of 5-hydroxyconiferyl alcohol into lignins as benzodioxane units was more recently reported in a COMT-deficient hardwood and legume (30, 41, 50, 51).

More predominant G units were also observed as evidenced by elevated phenylcoumaran units **B** compared to wild-type, as substantiated by the 1D <sup>13</sup>C NMR spectra and DFRC results. There were no marked compositional or structural differences revealed from the HMQC spectra of the remaining *bm* mutants (data not shown); β-ether units **A** predominated in all maize stem samples with small amounts of phenylcoumaran units **B** and cinnamyl alcohol endgroup units **X**.

The data presented here represent a comprehensive analysis of the effects of *bm* mutations on lignin in a single genetic background. There were variations in the *bm* mutational effects compared to results previously published in other genetic backgrounds. This may be attributed to the multiplicity of genes in maize whereby a mutational effect in one isoform is compensated for by other isoforms present and functioning or variable expression of a specific transgene having different impacts depending on the genetic background it is being expressed in. Gentinetta et al. (52) and Chabbert et al. (14) reported variations in the effect of the *bm3* mutation in different maize genetic backgrounds, supporting the hypothesis that phenotypic variation exists between inbreds with *bm* mutations.

The *bm1* and *bm3* mutational effects (elevated aldehyde levels and the presence of benzodioxane units **H**) were undeniably present. NMR revealed minor differences in the S and G components of the *bm1* and *bm3* lignins as corroborated by the DFRC results. An increase in aldehydes in the *bm1* mutant and to a lesser degree in the double *bm1*–*bm2* mutant was also observed. The most striking difference discovered by NMR was the significant presence of benzodioxane units in the *bm3* isolated lignin.

The most noticeable changes in cell wall composition were elevated levels of FA monomers in the *bm3* mutant with no change in the amount of cross-linking (as evidenced from etherified FA levels). The only maize samples to show a reduction in their level of cross-linking were the *bm1* and *bm1*–*bm2* mutants. Any attributable mutational effects in the *bm1*–*bm2* CWs appear to be *bm1* in origin, especially changes in cell wall composition; however, because exact *bm2* mutational effects are not known, parameters other than those examined may represent more closely the *bm2* mutational effect. Esterified *p*CA levels were variable among all maize samples, with the *bm1*, *bm3*, and double *bm1*–*bm2* mutants having ~50% less esterified *p*CA than all other CW maize samples.

Differences between the cell walls (and lignin fractions) among the *bm* mutants were evidenced by the solubility of *p*CA–lignin complexes during cellulase enzyme treatment and the possible partitioning of greater amounts of esterified FA (on arabinoxylans) versus etherified FA (on lignin) in the case of *bm1* and *bm1*–*bm2*. It appears that because of a difference in the structural organization of lignin there is less *p*CA incorporation and greater solubility of smaller-sized *p*CA–lignin complexes. Further study into greater solubility versus greater degradability could be of interest.

The data presented here represent the first detailed analysis of the *bm4* mutant. The NMR analyses did not reveal major changes in the lignin structure or composition of the *bm4* mutant, similar to what was observed for the *bm2* mutant. The data on the *bm4* mutant are not consistent with a specific block in the lignin biosynthetic pathway, and additional chemical and genetic analyses are necessary before the identity of the *Bm4* gene can be established.

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## LITERATURE CITED

- (1) Kuc, J.; Nelson, O. E. The abnormal lignins produced by the brown-midrib mutants of maize. *Arch. Biochem. Biophys.* **1964**, *105*, 103–113.
- (2) Kuc, J.; Nelson, O. E.; Flanagan, P. Degradation of abnormal lignins in the brown-midrib mutants and double mutants of maize. *Phytochemistry* **1968**, *7*, 1435–1436.
- (3) Gee, M. S.; Nelson, O. E.; Kuc, J. Abnormal lignins produced by the brown-midrib mutants of maize. II. Comparative studies on normal and brown-midrib-1 dimethylformamide-lignins. *Arch. Biochem. Biophys.* **1968**, *123*, 403–408.
- (4) Cherney, J. H.; Cherney, D. J. R.; Akin, D. E.; Axtell, J. D. Potential of brown-midrib, low-lignin mutants for improving forage quality. *Adv. Agron.* **1991**, *46*, 157–198.
- (5) Jung, H. 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.; ASA-CSSA-SSSA: Madison, WI, 1993; pp 315–346.
- (6) Higuchi, T. Biosynthesis of lignin. In *Biosynthesis and Biodegradation of Wood Components*; Higuchi, T., Ed.; Academic Press: Orlando, FL, 1985; pp 141–160.
- (7) Halpin, C.; Holt, K.; Chojecki, J.; Oliver, D.; Chabbert, B.; Monties, B.; Edwards, K.; Barakate, A.; Foxon, G. A. Brown-midrib maize (*bm1*)—a mutation affecting the cinnamyl alcohol dehydrogenase gene. *Plant J.* **1998**, *14*, 545–553.
- (8) Vermerris, W.; Thompson, K. J.; McIntyre, L. M. The maize *Brown midrib1* locus affects cell wall composition and plant development in a dose-dependent manner. *Heredity* **2002**, *88*, 450–457.
- (9) Chabbert, B.; Tollier, M. T.; Monties, B.; Barriere, Y.; Argillier, O. Biological variability in lignification of maize: expression of the brown midrib *bm2* mutation. *J. Sci. Food Agric.* **1994**, *64*, 455–460.
- (10) Vermerris, W.; Boon, J. J. Tissue-specific patterns of lignification are disrupted in the *brown midrib2* mutant of maize (*Zea mays* L.). *J. Agric. Food Chem.* **2001**, *49*, 721–728.
- (11) Vignols, F.; Rigau, J.; Torres, M. A.; Capellades, M.; Puigdomenech, P. The *brown midrib 3* (*bm3*) mutation in maize occurs in the gene encoding caffeic acid *O*-methyltransferase. *Plant Cell* **1995**, *7*, 407–416.
- (12) Lapierre, C.; Tollier, M. T.; Monties, B. A new type of constitutive unit in lignins from the corn *bm3* mutant. *C. R. Acad. Sci., Ser. 3* **1988**, *307*, 723–728.
- (13) Grand, C.; Parmentier, P.; Boudet, A.; Boudet, A. M. Comparison of lignins and of enzymes involved in lignification in normal and brown midrib (*bm3*) mutant. *Physiol. Veg.* **1985**, *23*, 905–911.
- (14) Chabbert, B.; Tollier, M. T.; Monties, B.; Barriere, Y.; Argillier, O. Biological variability in lignification of maize: expression of the brown midrib *bm3* mutation in three maize cultivars. *J. Sci. Food Agric.* **1994**, *64*, 349–355.
- (15) Gaudillere, M.; Monties, B. Biochemical and biosynthetic studies on lignification of Gramineae. *ACS Symp. Ser.* **1989**, *No. 399* (*Plant Cell Wall Polymers*).
- (16) Zuber, M. S.; Colbert, T. R.; Bauman, L. F. Effect of brown-midrib-3 mutant in maize (*Zea mays* L.) on stalk strength. *Z. Pflanzenzucht.* **1977**, *79*, 310–314.
- (17) Goto, M.; Sato, T.; Morita, O.; Takabe, K.; Inoue, N. Variations in anatomy and ultraviolet microspectrometry between normal and brown midrib maize possessing different rumen degradabilities. *J. Sci. Food Agric.* **1993**, *63*, 427–434.

- (18) Goto, M.; Matsuoka, J.; Sato, T.; Ehara, H.; Morita, O. Brown midrib mutant maize with reduced levels of phenolic acids ether-linked to the cell walls. *Anim. Food Sci. Technol.* **1994**, *48*, 27–38.
- (19) Provan, G. J.; Scobbie, L.; Chesson, A. Characterisation of lignin from CAD and OMT deficient *bm* mutants of maize. *J. Sci. Food Agric.* **1997**, *73*, 133–142.
- (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) Vermerris, W.; McIntyre, L. M. Time to flowering in *brown midrib* mutants of maize: an alternative approach to the analysis of developmental traits. *Heredity* **1999**, *83*, 171–178.
- (22) Marita, J.; Ralph, J.; Hatfield, R. D.; Chapple, C. NMR characterization of lignins in *Arabidopsis* altered in the activity of ferulate-5-hydroxylase. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 12328–12332.
- (23) Theander, O.; Westerlund, E. A. Studies on dietary fiber. 3. Improved procedures for analysis of dietary fiber. *J. Agric. Food Chem.* **1986**, *34*, 330–336.
- (24) 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.
- (25) Blumenkrantz, N.; Asboe-Hansen, G. New method for quantitative determination of uronic acids. *Anal. Biochem.* **1973**, *54*, 484–489.
- (26) Ahmed, A. E. R.; Labavitch, J. M. A simplified method for accurate determination of cell-wall uronide content. *J. Food Biochem.* **1977**, *1*, 361–365.
- (27) Ralph, J.; Quideau, S.; Grabber, J. H.; Hatfield, R. D. Identification and synthesis of new ferulic acid dehydromers present in grass cell walls. *J. Chem. Soc., Perkin Trans. 1* **1994**, 3485–3498.
- (28) Bunzel, M.; Ralph, J.; Marita, J. M.; Hatfield, R. D.; Steinhart, H. Diferulates as structural components in soluble and insoluble dietary fibre. *J. Sci. Food Agric.* **2001**, *81*, 653–660.
- (29) Ralph, J.; Marita, J.; Lu, F.; Hatfield, R. D.; Lapierre, C.; Ralph, S. A.; Vermerris, W.; Boerjan, W.; Jouanin, L. *11th International Symposium on Wood and Pulp Chemistry*, Nice, France; Association Technique de l'Industrie Papetière (ATIP): Paris, France, Vol. II, 2001, pp 27–30.
- (30) Marita, J. M.; Ralph, J.; Hatfield, R. D.; Guo, D.; Chen, F.; Dixon, R. A. Structural and compositional modifications in lignin of transgenic alfalfa downregulated in caffeic acid *O*-methyltransferase (COMT) and caffeoyl-coenzyme A *O*-methyltransferase (CCOMT). *Phytochemistry* **2002**, *62*, 53–65.
- (31) Ralph, J.; Marita, J. M.; Ralph, S. A.; Hatfield, R. D.; Lu, F.; Ede, R. M.; Peng, J.; Quideau, S.; Helm, R. F.; Grabber, J. H.; Kim, H.; Jimenez-Monteon, G.; Zhang, Y.; Jung, H.-J. G.; Landucci, L. L.; MacKay, J. J.; Sederoff, R. R.; Chapple, C.; Boudet, A. M. Solution-state NMR of lignins. In *Advances in Lignocellulosics Characterization*; Argyropoulos, D. S., Rials, T., Eds.; TAPPI Press: Atlanta, GA, 1999; pp 55–108.
- (32) Barriere, Y.; Argillier, O. Brown-midrib genes of maize: a review. *Agronomie (Paris)* **1993**, *13*, 865–876.
- (33) Iiyama, K.; Lam, T. B. T.; Stone, B. A. Phenolic acid bridges between polysaccharides and lignin in wheat internodes. *Phytochemistry* **1990**, *29*, 733–737.
- (34) Grabber, J. H.; Hatfield, R. D.; Ralph, J.; Zon, J.; Amrhein, N. Ferulate cross-linking in cell walls isolated from maize cell suspensions. *Phytochemistry* **1995**, *40*, 1077–1082.
- (35) 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.
- (36) Hartley, R. D.; Jones, E. C. Phenolic components and degradability of the cell walls of the brown midrib mutant, *bm3*, of *Zea mays*. *J. Sci. Food Agric.* **1978**, *29*, 777–782.
- (37) Ostrander, B.; Maillot, M. P.; Toillon, S.; Barriere, Y.; Pollacsek, M.; Besle, J. M. Cell wall phenolics and digestibility of normal and brown midrib maizes in different stem sections and across maturity stages. *J. Sci. Food Agric.* **1999**, *79*, 414–415.
- (38) Mueller-Harvey, I.; Hartley, R. D.; Harris, P. J.; Curzon, E. H. Linkage of *p*-coumaroyl and feruloyl groups to cell wall polysaccharides of barley straw. *Carbohydr. Res.* **1986**, *148*, 71–85.
- (39) Lam, T. B. T.; Iiyama, K.; Stone, B. A. Cinnamic acid bridges between cell wall polymers in wheat and phalaris internodes. *Phytochemistry* **1992**, *31*, 1179–1183.
- (40) Ralph, J.; Hatfield, R. D.; Grabber, J. H.; Jung, H. G.; Quideau, S.; Helm, R. F. Cell wall cross-linking in grasses by ferulates and diferulates. In *Lignin and Lignan Biosynthesis*; Lewis, N. G., Sarkanen, S., Eds.; American Chemical Society: Washington, DC, 1998; pp 209–236.
- (41) Ralph, J.; Lapierre, C.; Marita, J.; Kim, H.; Lu, F.; Hatfield, R. D.; Ralph, S. A.; Chapple, C.; Franke, R.; Hemm, M. R.; Van Doorselaere, J.; Sederoff, R. R.; O'Malley, D. M.; Scott, J. T.; MacKay, J. J.; Yahiaoui, N.; Boudet, A.-M.; Pean, M.; Pilate, G.; Jouanin, L.; Boerjan, W. Elucidation of new structures in lignins of CAD- and COMT-deficient plants by NMR. *Phytochemistry* **2001**, *57*, 993–1003.
- (42) Ralph, J.; MacKay, J. J.; Hatfield, R. D.; O'Malley, D. M.; Whetten, R. W.; Sederoff, R. R. Abnormal lignin in a loblolly pine mutant. *Science* **1997**, *277*, 235–239.
- (43) Kim, H.; Ralph, J.; Yahiaoui, N.; Pean, M.; Boudet, A.-M. Cross-coupling of hydroxycinnamyl aldehydes into lignins. *Org. Lett.* **2000**, *2*, 2197–2200.
- (44) Chabannes, M.; Barakate, A.; Lapierre, C.; Marita, J.; Ralph, J.; Pean, M.; Danoun, S.; Halpin, C.; Grima-Pettenatia, J.; Boudet, A.-M. Strong decrease in lignin content without significant alteration of plant development is induced by simultaneous down-regulation of cinnamoyl CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) in tobacco plants. *Plant J.* **2001**, *28*, 257–270.
- (45) Yahiaoui, N.; Marque, C.; Myton, K. E.; Negrel, J.; Boudet, A.-M. Impact of different levels of cinnamyl alcohol dehydrogenase down-regulation on lignins of transgenic tobacco plants. *Planta* **1998**, *204*, 8–15.
- (46) Ralph, J.; Hatfield, R. D.; Piquemal, J.; Yahiaoui, N.; Pean, M.; Lapierre, C.; Boudet, A.-M. NMR characterization of altered lignins extracted from tobacco plants down-regulated for lignification enzymes cinnamyl-alcohol dehydrogenase and cinnamoyl-CoA reductase. *Proc. Natl. Acad. Sci.* **1998**, *95*, 12803–12808.
- (47) MacKay, J. J.; Dimmel, D. R.; Boon, J. J. Pyrolysis MS characterization of wood from CAD-deficient pine. *J. Wood Chem. Technol.* **2001**, *21*, 19–29.
- (48) Halpin, C.; Knight, M. E.; Foxon, G. A.; Campbell, M. M.; Boudet, A.-M.; Boon, J. J.; Chabbert, B.; Tollier, M.-T.; Schuch, W. Manipulation of lignin quality by downregulation of cinnamyl alcohol dehydrogenase. *Plant J.* **1994**, *6*, 339–350.
- (49) Hwang, B. H.; Sakakibara, A. Hydrogenolysis of protolignin. XVIII. Isolation of a new dimeric compound with a heterocycle involving  $\alpha$ - $\beta$ -diether. *Holzforchung* **1981**, *35*, 297–300.
- (50) Marita, J. M.; Ralph, J.; Lapierre, C.; Jouanin, L.; Boerjan, W. NMR characterization of lignins from transgenic poplars with suppressed caffeic acid *O*-methyltransferase activity. *J. Chem. Soc., Perkin Trans. 1* **2001**, 2939–2945.
- (51) Ralph, J.; Lapierre, C.; Lu, F.; Marita, J. M.; Pilate, G.; Van Doorselaere, J.; Boerjan, W.; Jouanin, L. NMR evidence for benzodioxane structures resulting from incorporation of 5-hydroxyconiferyl alcohol into lignins of *O*-methyl-transferase-deficient poplars. *J. Agric. Food Chem.* **2001**, *49*, 86–91.

- (52) Gentinetta, E.; Bertolini, M.; Rossi, I.; Lorenzoni, C.; Motto, M. Effect of brown midrib-3 mutant on forage quality and yield in maize. *J. Genet. Breed.* **1990**, *44*, 21–26.
- (53) Humphreys, J. M.; Hemm, M. R.; Chapple, C. Ferulate 5-hydroxylase from *Arabidopsis* is a multifunctional cytochrome P450-dependent monooxygenase catalyzing parallel hydroxylations in phenylpropanoid metabolism. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 10045–10050.
- (54) Osakabe, K.; Tsao, C. C.; Li, L.; Popko, J. L.; Umezawa, T.; Carraway, D. T.; Smeltzer, R. H.; Joshi, C. P.; Chiang, V. L. Coniferyl aldehyde 5-hydroxylation and methylation direct syringyl lignin biosynthesis in angiosperms. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 8955–8960.
- (55) Li, L.; Popko, J. L.; Umezawa, T.; Chiang, V. L. 5-Hydroxyconiferyl aldehyde modulates enzymatic methylation for syringyl monolignol formation, a new view of monolignol biosynthesis in angiosperms. *J. Biol. Chem.* **2000**, *275*, 6537–6545.

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