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Identifying New Lignin Bioengineering Targets: Impact of Epicatechin, Quercetin Glycoside, and Gallate Derivatives on the Lignification and Fermentation of Maize Cell Walls

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Supporting Information

ABSTRACT: Apoplastic targeting of secondary metabolites compatible with monolignol polymerization may provide new avenues for designing lignins that are less inhibitory toward fiber fermentation. To identify suitable monolignol substitutes, primary maize cell walls were artificially lignified with normal monolignols plus various epicatechin, quercetin glycoside, and gallate derivatives added as 0 or 45% by weight of the precursor mixture. The flavonoids and gallates had variable effects on peroxidase activity, but all dropped lignification pH. Epigallocatechin gallate, epicatechin gallate, epicatechin vanillate, epigallocatechin, galloylhyperin, and pentagalloylglucose formed wall-bound lignin at moderate to high concentrations, and their incorporation increased 48 h in vitro ruminal fiber fermentability by 20−33% relative to lignified controls. By contrast, ethyl gallate and corilagin severely depressed lignification and increased 48 h fermentability by about 50%. The results suggest several flavonoid and gallate derivatives are promising lignin bioengineering targets for improving the inherent fermentability of nonpretreated cell walls.

KEYWORDS: *plant genetic engineering, cell wall, fiber, lignin, monolignol, flavan-3-ol, flavonol, gallate, fermentation, degradability, rumen, biofuel, biomass, forage*

INTRODUCTION

Although a vital structural component of plant cell walls, lignin is a major barrier hindering the ruminal or industrial fermentation of structural carbohydrates. To improve cell wall fermentability, plant breeding and biotechnology efforts have traditionally been aimed at reducing cell wall lignin content. Drastic reductions in lignin content greatly improve cell wall fermentability, but negative side effects often include decreased stem strength and stiffness, impaired vascular function, and reduced growth and survival of plants.[1,2] Increasingly, bioengineering of lignin to contain atypical building blocks from other metabolic pathways is being pursued to custom-design lignin that is easier to remove by biological or chemical pretreatments and less inhibitory toward polysaccharide fermentation. A wide variety of plant phenolics could participate in the oxidative coupling reactions involved in lignin polymerization, and recent studies have demonstrated that lignification in plants is extremely malleable. For example, perturbing single or multiple genes in the monolignol biosynthetic pathway can lead to dramatic shifts in the proportions of normal monolignols or monolignol pathway intermediates incorporated into lignin. Furthermore, various monolignol esters and ferulate xylan esters are substantial components of lignin in many plant species.[3−5]

Therefore, over a decade ago, we began using a well-characterized biomimetic cell wall model[6] to investigate the copolymerization of normal monolignols with coniferyl ferulate, which is a secondary metabolite containing two phenolic moieties connected by an alkali- and acid-labile ester linkage. As expected, cell walls lignified with coniferyl ferulate were more susceptible to pretreatment and more extensively saccharified by polysaccharidases.[7,8] This finding prompted the cloning of a monolignol ferulate acyltransferase gene[9] with the aim of expressing it in plants to enhance the production of biofuels, chemicals, or paper. Other model studies have recently demonstrated the utility of another biphenolic ester conjugate (rosmarinic acid) for enhancing cell wall delignification, saccharification, and fermentation.[10]

In addition to the aforementioned biphenolic ester conjugates, other types of natural phenolics such as flavan-3-ols, flavonol glycosides, and gallate esters hold promise as monolignol substitutes for modulating the adverse effects of lignin related to its hydrophobicity, cross-linking to structural polysaccharides, or recalcitrance to pretreatments. Although normally not associated with lignification, flavan-3-ols, flavonols, and gallate esters readily undergo oxidative coupling reactions,[11−15] and they are expected undergo radical cross-coupling reactions with monolignols to become integral components of lignin. As monolignol substitutes, *o*-diphenol

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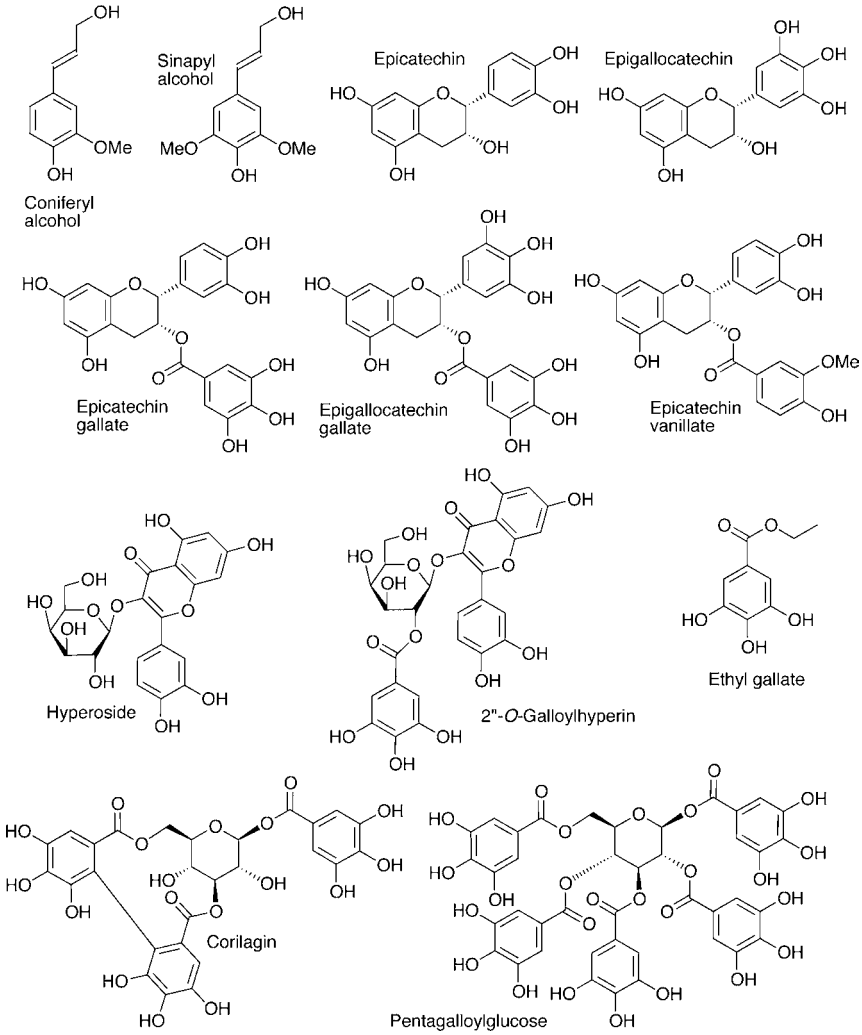

Figure 1. Coniferyl alcohol and sinapyl alcohol are the primary monolignols used by angiosperms to form lignin. In our studies, we examined how partial substitution of monolignols with epicatechin, quercetin glycoside, and gallate ester derivatives influenced lignin formation in maize cell walls and their fermentation by rumen microflora.

(catechol) and 1,2,3-triphenol (pyrogallol) groups on these molecules could provide an intramolecular pathway to trap lignin quinone methide intermediates that form cross-links between lignin and structural polysaccharides;[16] such cross-links appear to limit the enzymatic hydrolysis of cell walls.[17,18] Furthermore, extensive hydroxylation of flavan-3-ols, flavonol glycosides, and gallate esters might lower lignin hydrophobicity, aiding the penetration and hydrolysis of fiber by polysaccharidases. Such hydroxylation could also increase lignin solubilization in alkaline pretreatments, and cleavage of gallate ester substitutions by acid or base pretreatments could facilitate lignin depolymerization; both factors would be expected to improve saccharifiability of biomass. Because plants produce such a diverse array of flavan-3-ols, flavonol glycosides, and gallate esters, cell wall model studies should prove invaluable as a screening tool for identifying the most promising genetic engineering targets for modifying lignin. Plant genetic engineering studies will, of course, ultimately determine the feasibility and utility of modified lignins and their compatibility with plant growth and development.

Therefore, in this study we artificially lignified primary cell walls of maize (*Zea mays*) with monolignols plus various flavan-3-ols, flavonol glycosides, and gallate esters (Figure 1) to examine how their copolymerization with monolignols affects the formation of wall-bound lignin and the fermentability of cell walls prior to pretreatment. Cell wall fermentability was assessed by monitoring gas production during the in vitro incubation of cell walls with rumen microflora and by the analysis of residual nonfermented polysaccharides. Because gas production is directly linked to structural carbohydrate hydrolysis and fermentation by rumen microflora[19,20] and highly correlated with biomass fermentation to ethanol,[21] our findings should have broad application in plant bioengineering programs aimed at improving the utilization of fibrous feeds by livestock and the conversion cellulosic biomass into biofuels or other products.

MATERIALS AND METHODS

Lignin Precursors. All precursors used in lignification experiments exceeded 95% purity. The normal monolignols, coniferyl alcohol and sinapyl alcohol, were prepared as described previously.[22] The flavan-3-ols epicatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate were obtained from Biopurify (Chengdu, China). Epicatechin vanillate was synthesized by a high-yielding and simple method involving phenolic acetylation of epicatechin using acetic anhydride in methylene chloride, formation of the vanillate ester conjugate by coupling using 1-ethyl-3-(3-dimethylaminopropyl)-

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Table 1. Peroxidase Activity, pH, Lignin Content, and Fermentation Characteristics of Maize Cell Walls Artificially Lignified by Adding H_2O_2 with a Binary Mixture of Coniferyl Alcohol (CA) and Sinapyl Alcohol (SA) or Trinary Mixtures of CA and SA with Various Epicatechin Derivatives (Experiment 1)

				gas production[a]					
monolignol	peroxidase[b]	pH[c]	Klason lignin (mg/g)	8 h (mL/g)	24 h (mL/g)	48 h (mL/g)	NP[d] (mg/g)	GRL[e] (mL/mg)	NPAL[f] (mg/mg)
nonlignified control	very high	4.97 b[g]	26 c	275 a	346 a	361 a	20 e		
CA:SA low lignin control	high	5.45 a	149 b	112 c	240 c	257 c	174 b	0.85 b	1.26 ab
CA:SA normal lignin control	high	5.39 a	175 a	59 d	190 d	212 d	237 a	1.00 a	1.46 a
CA:SA:epicatechin	high	4.77 c	182 a	55 d	185 d	226 d	213 a	0.86 ab	1.24 b
CA:SA:epigallocatechin	very low	4.30 e	147 b	163 b	272 b	281 b	98 d	0.66 c	0.65 c
CA:SA:epicatechin gallate	moderate	4.41 d	179 a	91 c	241 c	258 c	146 bc	0.67 c	0.82 c
CA:SA:epigallocatechin gallate	low	4.29 e	173 a	109 c	249 c	264 c	114 d	0.66 c	0.64 c
CA:SA:epicatechin vanillate	moderate	4.93 b	153 b	98 c	256 bc	271 bc	117 cd	0.70 c	0.77 c

[a]Total volume of fermentation gasses produced during in vitro incubation with rumen microflora. [b]Visual rating of guaiacol and H_2O_2 reaction with cell wall peroxidase following lignification. [c]Final pH of cell walls suspended in water following lignification. [d]NP, nonfermented polysaccharides at 48 h. [e]GRL, gas reduction per unit lignin calculated as [48 h $gas_{nonlignified}$ − 48 h $gas_{lignified}$]/[Klason $lignin_{lignified}$ − Klason $lignin_{nonlignified}$]. [f]NPAL, nonfermented polysaccharide accumulation per unit lignin calculated as ($NP_{lignified}$ − $NP_{nonlignified}$)/[Klason $lignin_{lignified}$ − Klason $lignin_{nonlignified}$]. [g]Means within columns with unlike letters differ ($P = 0.05$).

carbodiimide hydrochloride, and subsequent deacetylation using pyrrolidine in methylene chloride. A report of this synthesis, including more detailed discussion of the chemistry and incorporation of epicatechin vanillate into in vitro dehydrogenation polymers, will be described in a future publication. The flavonol glycosides hyperoside and 2″-*O*-galloylhyperin were purchased from Biopurify, and gallate esters ethyl gallate, corilagin, and pentagalloylglucose were obtained from Biopurify or Ajinomoto (Louvain-la-Neuve, Belgium).

Cell Wall Lignification. Freshly prepared nonlignified primary cell walls (~2.2−3.4 g dry weight) were isolated from maize cell suspensions[23] and stirred in 300 mL of water containing 3 mM $CaCl_2$. Dilute H_2O_2 (0.44 mmol in 10 mL of water, ~2 equiv/mol of cell wall ferulate) was added by peristaltic pump over a 30 min period to dimerize ferulates by wall-bound peroxidases. After an additional 30 min of stirring, cell wall suspensions were artificially lignified via wall-bound peroxidase by dropwise addition of monolignols (prepared in 10 mL of dioxane and 90 mL of water) and dilute H_2O_2 (prepared in 110 mL of water) as described below. Monolignol and H_2O_2 additions were made by peristaltic pump, initially at 20 mL/h for 1 h, followed by 10 mL/h for 3 h, and then completed at 5 mL/h to mimic the proposed rapid to slow deposition of monolignols into plant cell walls.[24] Low and normal lignin controls were prepared by adding two-component equimolar mixtures of coniferyl alcohol (1 or 1.3 mmol) and sinapyl alcohol (1 or 1.3 mmol) to cell walls. In the first study, cell walls were also lignified by adding three-component mixtures of coniferyl alcohol (0.75 mmol) and sinapyl alcohol (0.75 mmol) with one of the following flavan-3-ols: epicatechin (0.75 mmol), epigallocatechin (0.75 mmol), epicatechin gallate (0.5 mmol), epigallocatechin gallate (0.5 mmol), or epicatechin vanillate (0.5 mmol). In the second study, the three-component mixtures consisted of coniferyl alcohol (0.75 mmol) and sinapyl alcohol (0.75 mmol) with one of the following flavonol glycosides or gallate esters: hyperoside (0.75 mmol), 2″-*O*-galloylhyperin (0.5 mmol), ethyl gallate (1.25 mmol), corilagin (0.425 mmol), and pentagalloylglucose (0.25 mmol). A treatment with epigallocatechin gallate (0.5 mmol) was again included in the second study to facilitate treatment comparisons between experiments. The quantity of flavan-3-ols, flavonol glycosides, or gallate esters added with normal monolignols was adjusted in both experiments so that the potential quantity of lignin formed (excluding glycoside units) would be comparable to the normal lignin control. In both experiments, 1.1 equiv of H_2O_2 was added per mole of monolignol, flavan-3-ol, flavonol, or gallate unit added to cell walls. Treatments in both studies were replicated twice in independent experiments, and nonlignified controls were stirred in a solvent mixture similar to the final makeup of the lignification reaction media.

At the end of monolignol additions, peroxidase activity in cell walls was visually assessed via guaiacol−H_2O_2 staining,[25] and the acidity of cell wall suspensions was measured with a pH meter. Following additions, cell walls were stirred for an additional 12 h, stored for several days at 4 °C, and then filtered and resuspended four times with 400 mL of 9:1 (v/v) acetone/water in fritted glass Buchner funnels (5 μm retention) to remove dehydrogenation products not bound to cell walls. Cell walls were then dehydrated by five 400 mL washes with acetone and briefly subjected to vacuum to remove excess acetone. Cell walls were then transferred to sample jars, set overnight in a hood to evaporate off acetone, and then dried at 55 °C. In the second study, dried lignified and nonlignified cell walls were weighed to estimate, by difference, the mass of monolignols polymerized into cell walls. Subsamples from acetone/water filtrates were dried in vacuo, redissolved in 1:1 (v/v) dioxane/water, and scanned from 200 to 700 nm with a spectrophotometer to estimate dehydrogenation products washed out of cell walls.

Lignin and Ferulate Analyses. Acid-insoluble Klason lignin in cell wall samples (75 mg) was determined in duplicate by a two-stage hydrolysis in 12 M H_2SO_4 at 25 °C for 2 h followed by 1.6 M H_2SO_4 at 100 °C for 3 h.[26] Whole cell walls (~50 mg) from selected lignification treatments were sonicated in 4:1 DMSO-d_6/pyridine-d_5 and subjected to gel-state 2-D NMR using a Bruker-Biospin (Billerica, MA, USA) 500 MHz AVANCE spectrometer equipped with an inverse gradient 5 mm TCI cryoprobe, as previously described.[27] 2-D NMR data were processed using Bruker Topspin v 3.1 for Mac OS X using DMSO-d_6 residual solvent as a reference for all samples (^{1}H NMR 2.49 ppm, ^{13}C NMR 39.5 ppm). Ester-linked ferulates released from duplicate cell wall samples (50 mg) by 2 M NaOH (20 h, 25 °C) were extracted with ethyl ether, silylated, and quantified by GLC-FID.[28]

Cell Wall Fermentations. Gas production from cell walls (100 mg) in 60 mL sealed bottles at 39 °C was monitored with pressure transducers for 48 h following the addition of 5.7 mL of phosphate−bicarbonate buffer, 0.3 mL of reducing agent, and 4 mL of diluted rumen inoculum.[29] Filtered inoculum was prepared with a 1:2 ratio (v/v) of rumen fluid and blended buffer-extracted rumen solids collected from four Holstein cows fed a total mixed ration of corn silage, corn grain, alfalfa hay, soybean meal, and supplemental vitamins and minerals.[29] Blank-corrected gas production data from three to four independent fermentation runs were fitted with a dual-pool exponential model to estimate the kinetics of microbial hydrolysis and fermentation of cell walls.[29] Kinetic parameters were then used to estimate the volume of gas produced at 8, 24, and 48 h. Freeze-dried residues remaining after 48 h of cell wall degradation by rumen

Table 2. Peroxidase Activity, pH, Lignin Content, and Fermentation Characteristics of Maize Cell Walls Artificially Lignified by Adding H_2O_2 with a Binary Mixture of Coniferyl Alcohol (CA) and Sinapyl Alcohol (SA) or Trinary Mixtures of CA and SA with Epigallocatechin Gallate, Quercetin Derivatives, or Gallate Esters (Experiment 2)

					gas production[a]					
monolignol	peroxidase[b]	pH[c]	Klason lignin (mg/g)	mass lignin (mg/g)	8 h (mL/g)	24 h (mL/g)	48 h (mL/g)	NP[d] (mg/g)	GRL[e] (mL/mg)	NPAL[f] ((mg/mg)
nonlignified control	very high	5.24 b[g]	16 e		262 a	332 a	350 a	23 e		
CA:SA low lignin control	high	5.73 a	162 bc	156.2 b	66 c	204 cd	236 d	212 b	0.78 a	1.30 b
CA:SA normal lignin control	high	5.74 a	194 a	176.2 ab	44 c	151 f	197 f	299 a	0.87 a	1.54 a
CA:SA:epigallocatechin gallate	low	4.31 d	186 a	172.6 ab	46 c	183 de	240 d	197 b	0.65 b	1.02 c
CA:SA:hyperoside	high	4.61 c	179 ab	193.6 a	50 c	164 ef	215 e	263 a	0.83 a	1.47 ab
CA:SA:galloylhyperin	moderate	4.55 c	159 c	173.3 ab	60 c	205 c	237 d	179 b	0.79 a	1.08 c
CA:SA:ethyl gallate	high	3.85 e	111 d	103.1 c	134 b	286 b	303 b	67 d	0.49 c	0.43 e
CA:SA:corilagin	moderate	3.66 e	111 d	118.9 c	135 b	277 b	290 b	64 d	0.63 b	0.41 e
CA:SA:pentagalloylglucose	low	4.22 d	160 c	178.9 ab	59 c	223 c	257 c	139 c	0.65 b	0.81 d

[a]Total volume of fermentation gasses produced during in vitro incubation with rumen microflora. [b]Visual rating of guaiacol and H_2O_2 reaction with cell wall peroxidase following lignification. [c]Final pH of cell walls suspended in water following lignification. [d]NP, nonfermented polysaccharides at 48 h. [e]GRL, gas reduction per unit lignin calculated as [48 h $gas_{nonlignified}$ − 48 h $gas_{lignified}$]/[Klason $lignin_{lignified}$ − Klason $lignin_{nonlignified}$]. [f]NPAL, nonfermented polysaccharide accumulation per unit lignin calculated as ($NP_{lignified}$ − $NP_{nonlignified}$)/[Klason $lignin_{lignified}$ − Klason $lignin_{nonlignified}$]. [g]Means within columns with unlike letters differ (P = 0.05).

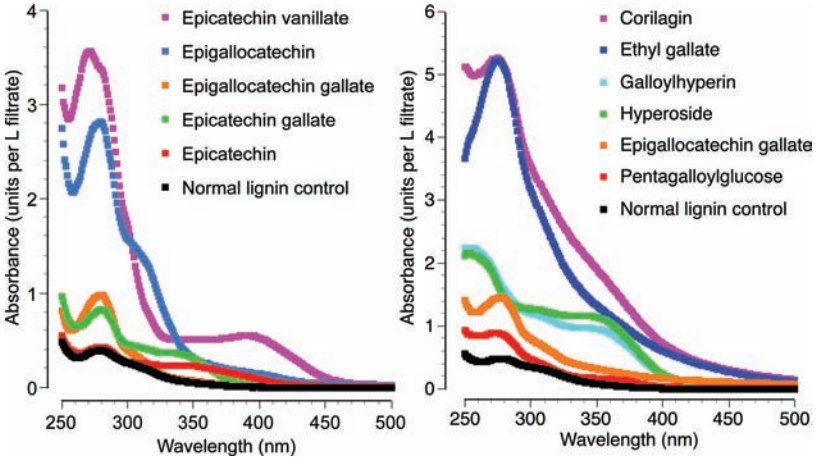

Figure 2. Spectrophotometric scans of filtrates collected after artificial lignification of maize cell walls.

microflora were dissolved in 12 M H_2SO_4 at 25 °C for 2 h and analyzed for nonfermented polysaccharides (NP) according to the phenol−sulfuric acid assay[30] with corrections for inoculum contamination and sugar recovery. The recovery of sugars from NP was estimated by running unfermented nonlignified cell walls through the 12 M H_2SO_4 dissolution/phenol−sulfuric acid assay procedure.

Statistical Methods. Data were subjected to an analysis of variance by running PROC MIXED[31] according to a randomized complete block design with two replications. If F tests were significant ($P \leq 0.05$), then least-squares means of fixed effects were compared by the PDIFF option at P = 0.05. Unless otherwise noted, all reported treatment differences were significant at P = 0.05.

RESULTS AND DISCUSSION

Cell Wall Lignification. In the current study, isolated maize cell walls containing bound peroxidases were stirred in water and artificially lignified with normal monolignols (coniferyl and sinapyl alcohols), added alone or in combination with various flavan-3-ols, flavonol glycosides, or gallate esters (Figure 1). When added, the monolignol substitutes comprised about 45% by weight of the precursor mixture, potentially yielding a shift in lignin composition comparable to that observed in some mutant or transgenic plants with altered lignin biosynthesis.[3−5] Previous studies suggest this biomimetic system successfully models many aspects of natural lignification in grasses;[23] thus, it is a valuable tool to test the compatibility of various monolignol substitutes with lignification and their potential effects on cell wall fermentation prior to attempting the bioengineering of plants.

Normal monolignols and most monolignol substitutes readily formed solutions in 9:1 (v/v) water/dioxane, but hyperoside (a 3-*O*-galactoside of quercetin) was only partly soluble and formed a finely dispersed suspension that fortunately could still be added by peristaltic pump to cell walls. Although capable of being oxidized by peroxidase,[12] we did not examine quercetin because its extremely poor aqueous solubility could hinder its incorporation into lignin and adversely increase lignin hydrophobicity.

Adding epicatechin, hyperoside, or ethyl gallate with normal monolignols had little effect on residual peroxidase activity in cell walls (Tables 1 and 2). By contrast, inclusion of epigallocatechin substantially reduced residual peroxidase activity to very low levels, but the effect was less pronounced with epigallocatechin gallate, suggesting that gallate ester substitution lessened peroxidase inactivation. Gallate ester

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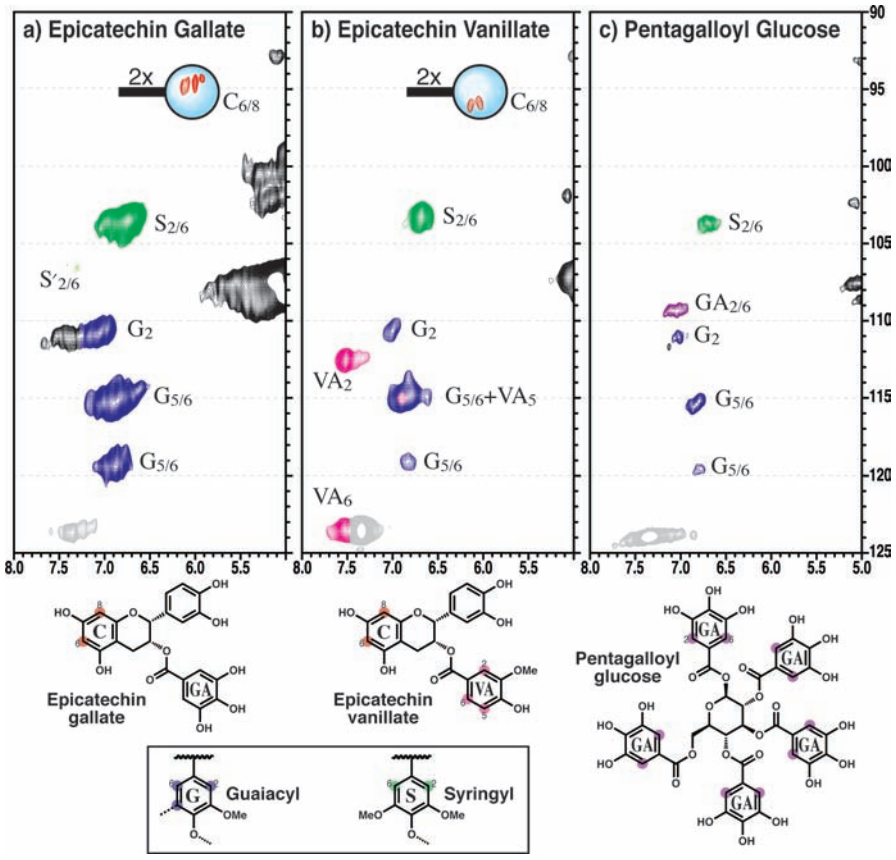

Figure 3. Aromatic regions from $^{13}C-^{1}H$ correlation gel-state 2D NMR spectra (HSQC) of whole cell walls in DMSO-d_6 and pyridine-d_5. Maize cell walls were artificially lignified with monolignols plus epicatechin gallate (a), epicatechin vanillate (b), and pentagalloylglucose (c).

substitution of hyperoside and epicatechin, however, reduced residual peroxidase activities to moderate levels. The other gallate esters, corilagin and pentagalloyl glucose, also diminished residual peroxidase activities to moderate or low levels. Thus, flavan-3-ols, flavonol glycosides, and gallates had variable effects on peroxidase activity, but the underlying mechanisms are not known.

Surprisingly, lignification with normal monolignols increased the pH of walls suspended in water from about 5.1 to 5.6 (Tables 1 and 2), even though lignin contains few functional groups affecting pH. The main matrix components influencing pH should be free carboxylic acid groups on uronosyls,[32] but nonlignified and lignified maize cell walls contain similar concentrations of both acidic and methyl esterified uronosyls.[17] Thus, this upward shift in pH during lignification might be attributed to the reaction of carboxylic acid groups on uronosyls with lignin quinone methide intermediates to form benzyl ester cross-links between structural polysaccharides and lignin.[17] By contrast, lignification with flavan-3-ols, flavonol glycosides, or gallate esters dropped the pH to between 3.6 and 4.9. Acidification was most pronounced with gallyl groups and gallate esters, particularly ethyl gallate and corilagin, which may be related in part to their more numerous phenolic groups undergoing single-electron oxidations to produce protons.[14]

In both experiments, we adjusted the quantity of flavan-3-ols, flavonol glycosides, or gallate esters added with normal monolignols so that the potential quantity of lignin formed (excluding glycoside units) would be comparable to the normal lignin control. The efficiency of lignin formation was then assessed by Klason lignin and gel-state NMR analyses of cell walls and by spectrophotometric scans of nonincorporated precursors recovered in filtrate. In the second study, we also estimated lignin concentrations in cell walls by a mass balance approach. On the basis of high Klason lignin and mass balance lignin concentrations (Tables 1 and 2) and very low filtrate absorbance at ~280 nm (Figure 2), epicatechin, epicatechin gallate, and epigallocatechin gallate all readily formed wall-bound lignins at levels comparable to the normal lignin control. Low filtrate absorbance and high mass balance lignin concentrations suggest that hyperoside, galloylhyperin, and pentagalloylglucose also readily formed wall-bound lignin, but their Klason lignin concentrations were lower than that of the normal lignin control. The relatively low Klason lignin concentrations for these treatments might in part be due to acid hydrolysis and loss of glycoside or gallate ester units from lignin during the Klason procedure. By contrast, Klason lignin, mass balance lignin, and filtrate scans indicated epigallocatechin and epicatechin vanillate modestly reduced lignin formation, whereas ethyl gallate and corilagin severely disrupted cell wall lignification. Compared to cream-colored lignified controls, cell walls lignified with epicatechin, quercetin glycoside, and gallate ester derivatives were highly colored in various shades of yellow, red, orange, or brown, all of which are characteristic of flavonoid and gallate coupling products.[14,33]

Although the analysis of whole cell walls by 2D $^{13}C-^{1}H$ correlation gel-state NMR is a powerful technique for characterizing the composition of lignin,[27] the method performed rather poorly for assessing the incorporation of flavonoid and gallate ester derivatives into lignin. For example, epicatechin gallate was added to cell walls at a 0.67:1:1 molar

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ratio with coniferyl and sinapyl alcohols, but the 6,8-position contours from epicatechin units were dwarfed by 2,6-position contours for syringyl units from sinapyl alcohol (Figure 3a). The gallate 2,6-position contours, seen previously in similar spectra,[34] were masked by the broader G2 correlation peak here. Similar additions of epicatechin vanillate with monolignols also yielded weak 6,8-position contours for the epicatechin moieties, but the 2-position contours for vanillate were readily detected and more prominent than 2-position contours for guaiacyl units from coniferyl alcohol (Figure 3b); the vanillate 5- and 6-position contours overlapped with the guaiacyl 5,6-contours and a contour from pyridine, but these are partially colored to designate their appearance in Figure 3b. Thus, the responses of epicatechin and gallate units were much lower than vanillate units in flavan-3-ols and lower than guaiacyl and syringyl units derived from normal monolignols. Other flavan-3-ols and flavonols were also difficult to detect in lignified cell walls by gel-state NMR (data not shown). In the case of pentagalloyl glucose, the 2,6-position contours for gallate units were comparable in size to those for syringyl units (Figure 3c). On a molar basis, however, the addition of gallate units exceeded that of sinapyl alcohol by 1.67-fold, suggesting that pentagalloyl glucose was also under-represented in the gel NMR spectrum. Inadequate detection of flavan-3-ols, flavonols, and gallates may in large part be related to their ability to undergo a wide variety of oxidative coupling reactions,[11,13−15] many of which involve the 6,8-positions of the epicatechin moiety and the 2,6-position of gallate units that were targeted in our $^{13}C-^{1}H$ correlation NMR experiments. Although not yet characterized, cross-coupling with monolignols or oligomers at these and other positions would further diminish or disperse signals of flavan-3-ols, flavonols, and gallates in gel-state NMR experiments. Thus, whereas gel-state NMR can detect flavonoid or gallate derivatives in lignin, alternative techniques will ultimately be required to more accurately assess their abundance in lignin of bioengineered plants.

Overall, whereas low pH adversely affects peroxidase activity and lignification in buffered reaction medium,[18] we observed that acidification associated with flavan-3-ols, flavonol glycosides, or gallate ester addition did not consistently affect peroxidase activity or lignin formation in cell walls. Normally, apoplastic acidification affects the growth of plant cells and their responses to hormonal and environmental cues,[32,35] but acidification during lignification may be of less consequence because it occurs at the final stages of cell development.[36]

Among the monomers examined, epicatechin, epicatechin gallate, epigallocatechin gallate, hyperoside, galloylhyperin, and pentagalloylglucose readily copolymerized with normal monolignols to form wall-bound lignin. The abundance of flavan-3-ol, flavonol glycoside, and gallate esters in lignin were, however, underestimated by 2D gel-state NMR techniques.

Cell Wall Hydrolysis and Fermentation by Rumen Microflora. Incubating artificially lignified cell walls in vitro with rumen microflora permits the effects of lignin alterations on cell wall fermentation to be assessed at the molecular level, free from confounding factors related to plant anatomy or deficiencies in key cell wall degrading enzymes.[34] The fermentability of cell walls was determined by continuously monitoring gas production by rumen bacteria and by the analysis of residual NP. Although the production of fermentation gases is substrate dependent, our use of a common source of cell walls for all lignification treatments ensures that shifts in gas production mirror the microbial degradation of cell walls. As noted in other studies, maize cell walls displayed a complex biphasic production of fermentation gases (see Tables S1 and S2 in the Supporting Information), which may reflect the digestion of two, as yet, poorly understood fractions in cell walls.[34,37] Therefore, to more simply illustrate the progression of fermentation, we used kinetic parameters to estimate gas production at 8, 24, and 48 h.

Nonlignified controls containing an average of 21 mg/g Klason lignin were rapidly and extensively degraded by rumen microflora, with 75% of the total gas produced within 8 h of incubation and only 22 mg/g of NP remaining after 48 h of incubation (Tables 1 and 2). By contrast, normal lignin controls averaging 185 mg/g of Klason lignin produced 81% less gas after 8 h, 50% less gas after 24 h, and 43% less gas after 48 h than nonlignified controls, and gas reduction per unit lignin (GRL) at 48 h averaged 0.94 mL/mg of lignin formed. Nonfermented polysaccharide concentrations at 48 h averaged 268 mg/g in normal lignin controls, and nonfermented polysaccharide accumulation per unit of lignin (NPAL) averaged 1.5 mg/mg, indicating each unit of lignin protected 1.5 units of cell wall polysaccharides from enzymatic hydrolysis and fermentation.

A modest reduction in lignin content from 185 mg/g in the normal lignin control to 155 mg/g in the low lignin control increased gas production by an average of 70% after 8 h, 32% after 24 h, and 20% after 48 h of incubation and reduced NP by 28% (Tables 1 and 2). This reduction in lignin content also reduced GRL and NPAL by about 14%, suggesting that simple reductions in lignification lessen the inhibitory effects of each unit of lignin formed in cell walls.

Among the monolignol substitutes that most readily formed wall-bound lignin, epicatechin gallate and epigallocatechin gallate increased 24 and 48 h gas production by about 25%, and they cut NP, GRL, and NPAL by 33−56% relative to the normal lignin control (Table 1). Epigallocatechin, epicatechin vanillate, galloylhyperin, and pentagalloylglucose, which formed less wall-bound lignin, increased gas production by an average of 40% after 24 h and 28% after 48 h of incubation. Klason lignin concentrations with epigallocatechin, epicatechin vanillate, galloylhyperin, or pentagalloylglucose were comparable to that of the low lignin control, yet they usually had lower NP, GRL, and NPAL values, suggesting that their incorporation also directly improved cell wall fermentability. Epicatechin and hyperoside also readily formed wall-bound lignins, but they had little or no impact on gas production, NP, GRL, or NPAL.

Improvements in fermentability with flavan-3-ols were associated with increased hydroxylation, but this response was not necessarily related to increased lignin hydrophilicity because flavonol glycosides and gallate esters with more extensive hydroxylation (e.g., hyperoside, galloyl hyperin, and pentagalloylglucose) had less pronounced effects on cell wall fermentability (Figure 4). Among flavan-3-ols, gains in cell wall fermentability were related to the presence of gallate and pyrogalloyl units. Recent work indicates pyrogalloyl and especially gallate units in epigallocatechin gallate readily form benzodioxane-linked structures with monolignols (Yuki Tobimatsu, personal communication). The formation of such structures should reduce benzyl ether and ester cross-linking in cell walls,[16] and this may in part account for the improved fermentability of cell walls lignified with epicatechin gallate and epigallocatechin gallate. However, other galloylated molecules that readily formed lignin (i.e., galloyl hyperin and pentagalloyl glucose) had less pronounced effects on cell wall fermentability.

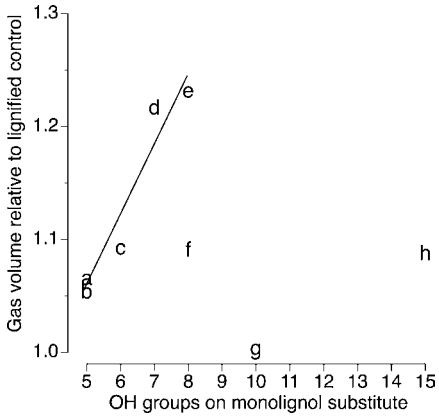

Figure 4. Relationship between total gas production after 48 h of fermentation with rumen microflora and the number of hydroxyl groups on epicatechin (a), epicatechin vanillate (b), epigallocatechin (c), epicatechin gallate (d), epigallocatechin gallate (e), hyperoside (f), galloylhyperin (g), and pentagalloylglucose (h). The regression line is for epicatechin derivatives (a–e).

Furthermore, *o*-diphenolic catechol units also form benzodioxane-linked structures with monolignols,[10] but the presence of such units in epicatechin and hyperoside appeared to have no effect on cell wall fermentability. Thus, the overall role of catechol, pyrogalloyl, and gallate units in modulating lignin's effect on cell wall fermentability is poorly understood. Quantifying benzyl ether and ester cross-links in cell walls would help to clarify the situation, but the analysis of such structures is problematic and limited to a small fraction of lignin that can be extracted from cell walls.[38,39]

By contrast, the extent of ferulate–lignin cross-linking in our cell wall model system can be simply calculated from the difference in alkali-labile ferulates recovered prior to and following biomimetic lignification.[28] An analysis of selected samples indicated that the copolymerization of monolignols with epicatechin gallate, epigallocatechin gallate, and, to a lesser degree, pentagalloyl glucose reduced the proportion of ferulates that underwent cross-linking with lignin (Table 3). On the basis of other studies,[40] these reductions in ferulate–lignin cross-linking should contribute to the improved cell wall fermentability.

Ethyl gallate and corilagin treatments yielded the most dramatic gains in gas production and reductions in NP, GRL, and NPAL, but their lignin concentrations were 40% lower than normal lignin controls and 30% lower than low lignin controls (Table 2). Thus, addition of these units probably acted indirectly to improve fermentability through severely reducing lignin content. Regardless of the mechanism, ethyl gallate and corilagin would likely be of limited value as bioengineering targets because they severely disrupted cell wall lignification. Such reductions in lignin content often reduce plant fitness[1,2] and can already be attained by down-regulating enzymes in the monolignol pathway.

In conclusion, several flavonoids are promising lignin bioengineering targets, but epicatechin gallate and epigallocatechin gallate are especially attractive because they substantially enhanced structural polysaccharide fermentation without disrupting lignin formation. Among the gallate ester derivatives examined (ethyl gallate, corilagin, and pentagalloylglucose), only pentagalloylglucose appeared to be suitable as a monolignol substitute because it improved fermentability while causing only modest reductions in lignification. Because they are ester conjugates with multiple phenolic groups, epicatechin gallate, epigallocatechin gallate, and pentagalloylglucose are also expected to greatly boost enzymatic saccharification of cell walls following ester-cleaving pretreatments. Therefore, in a forthcoming paper, we will examine the impact of these and other flavan-3-ols, flavonol glycosides, and gallate esters on the enzymatic saccharification of lignified cell walls, both before and after acid and alkaline pretreatments.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Tables S1 and S2 present data on the kinetics of cell wall hydrolysis and fermentation by rumen microflora as estimated by fitting gas production with a dual-pool exponential model. This material is available free of charge via the Internet at http://pubs.acs.org.

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Table 3. Alkali-Labile Ferulates in Maize Cell Walls Artificially Lignified to Similar Klason Lignin Concentration by Adding H_2O_2 with a Binary Mixture of Coniferyl Alcohol (CA) and Sinapyl Alcohol (SA) or Trinary Mixtures of CA and SA with Epicatechin Gallate, Epigallocatechin Gallate, Galloyl Hyperin, or Pentagalloyl Glucose

monolignol	Klason lignin (mg/g)	monomers (mg/g)	dimers (mg/g)	total (mg/g)	total cross-linked[a] (%)
		Experiment 1			
nonlignified control	26 b[b]	10.58 a	4.64 a	15.22 a	
CA:SA normal lignin control	175 a	0.86 c	0.53 c	1.39 c	89
CA:SA:epicatechin gallate	179 a	4.54 b	1.68 b	6.22 b	51
CA:SA:epigallocatechin gallate	173 a	4.21 b	1.68 b	5.89 b	53
		Experiment 2			
nonlignified control	16 b	16.85 a	6.39 a	23.24 a	
CA:SA low lignin control	162 a	1.46 b	1.46 d	2.92 d	85
CA:SA:galloylhyperin	159 a	2.81 c	2.37 c	5.19 c	73
CA:SA:pentagalloylglucose	160 a	4.92 b	3.13 b	8.05 b	59

[a]Values adjusted to account for the dilution of ferulates by the polymerization of lignin into cell walls. [b]Means within columns and experiments with unlike letters differ (P = 0.05).

constitute a guarantee or warranty of the product by the USDA and does not imply its approval to the exclusion of other products that may also be suitable.

Notes

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