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Multidimensional NMR analysis reveals truncated lignin structures in wood decayed by the brown rot basidiomycete *Postia placenta*

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Summary

Lignocellulose biodegradation, an essential step in terrestrial carbon cycling, generally involves removal of the recalcitrant lignin barrier that otherwise prevents infiltration by microbial polysaccharide hydrolases. However, fungi that cause brown rot of wood, a major route for biomass recycling in coniferous forests, utilize wood polysaccharides efficiently while removing little of the lignin. The mechanism by which these basidiomycetes breach the lignin remains unclear. We used recently developed methods for solubilization and multidimensional ^{1}H–^{13}C solution-state NMR spectroscopy of ball-milled lignocellulose to analyse aspen wood degraded by *Postia placenta*. The results showed that decay decreased the content of the principal arylglycerol-β-aryl ether interunit linkage in the lignin by more than half, while increasing the frequency of several truncated lignin structures roughly fourfold over the level found in sound aspen. These new end-groups, consisting of benzaldehydes, benzoic acids and phenylglycerols, accounted for 6–7% of all original lignin subunits. Our results provide evidence that brown rot by *P. placenta* results in significant ligninolysis, which might enable infiltration of the wood by polysaccharide hydrolases even though the partially degraded lignin remains *in situ*. Recent work has revealed that the *P. placenta* genome encodes no ligninolytic peroxidases, but has also shown that this fungus produces an extracellular Fenton system. It is accordingly likely that *P. placenta* employs electrophilic reactive oxygen species such as hydroxyl radicals to disrupt lignin in wood.

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Introduction

Most terrestrial biomass is sequestered in lignocellulose, the principal structural material of vascular plants. Accordingly, the continuous biodegradation of this complex natural composite is a key step in the global carbon cycle (Zeikus, 1981). However, few organisms can metabolize lignocellulose because its cellulose and hemicelluloses, the components that can support microbial growth, are embedded in lignin. The lignin prevents saccharolytic enzymes from accessing the underlying polysaccharides, and is recalcitrant to hydrolytic depolymerization because it is a combinatorial, racemic polymer of phenylpropanoid subunits connected via ether and carbon–carbon bonds (Ralph *et al*., 2004a). Hence, any organism that depends on lignocellulose as a source of carbon and energy must have some mechanism to penetrate or disrupt this lignin barrier.

Wood decay basidiomycetes are the most efficient biodegraders of lignocellulose. Most of these, termed white rot fungi, cleave the encrusting lignin oxidatively to access the polysaccharides they require for growth, and are generally thought to employ several specialized peroxidases to promote ligninolysis (Martínez, 2002). However, brown rot basidiomycetes, a phylogenetically related group principally responsible for lignocellulose recycling in coniferous forests (McFee and Stone, 1966), utilize wood polysaccharides efficiently while leaving most of the lignin *in situ* (Cowling, 1961). They also generally lack detectable ligninolytic peroxidase activity (Eriksson *et al*., 1990).

The question of how brown rot fungi access wood polysaccharides without removing the lignin remains perplexing. Part of the explanation is probably that they produce extracellular Fe^{2+} and H_2O_2, which are small enough to infiltrate lignocellulose and react with each other within the wood cell wall via the Fenton reaction

($H_2O_2 + Fe^{2+} + H^+ \rightarrow H_2O + Fe^{3+} + {}^{\bullet}OH$) to produce hydroxyl radicals ($^{\bullet}OH$) that oxidatively cleave cellulose and hemicelluloses. Many brown rot basidiomycetes produce extracellular hydroquinones (Goodell *et al*., 1997; Hammel *et al*., 2002), which reduce Fe^{3+} and O_2 to drive Fenton chemistry (Guillén *et al*., 1997; Kerem *et al*., 1999; Gómez-Toribio *et al*., 2009). Brown rot fungi have also been reported to oxidize a variety of polymers, including cellulose, to products that suggest the involvement of $^{\bullet}OH$ (Kirk *et al*., 1991; Kerem *et al*., 1998; Cohen *et al*., 2002).

However, brown rot fungi also produce cellulases and hemicellulases (Eriksson *et al*., 1990), which suggests a requirement for ligninolysis to enable cell wall infiltration by these enzymes. In agreement, there are some indications that lignin is cleaved during brown rot: (i) the fungi cause low to moderate decreases in the acid-insoluble material (Klason lignin) that is generally interpreted as residual lignin after decay (Eriksson *et al*., 1990), (ii) ultrastructural changes characteristic of ligninolysis have been observed in the secondary cell wall of decayed wood specimens (Eriksson *et al*., 1990), (iii) a solid-state ^{13}C NMR spectrum of brown-rotted wood was reported to exhibit a signal attributable to lignin benzoic acid residues (Davis *et al*., 1994) and (iv) it has been observed that brown-rotted lignin is enriched in carbonyl and carboxyl groups (Kirk, 1975) while being depleted in major intermonomer linkages (Yelle *et al*., 2008a; Martínez *et al*., 2011). However, structures that are strongly diagnostic for ligninolysis have not yet been identified or quantified in brown-rotted wood.

The observation of cleaved lignin structures in biodegraded wood is technically challenging. Solvent extraction of lignins from lignocellulose is never quantitative, and thus results in sample fractionation that may obscure or magnify differences between degraded specimens and controls upon subsequent analysis. Solid-state NMR spectroscopy addresses the fractionation problem, but yields broad, overlapping signals that make it difficult to pinpoint structures diagnostic for ligninolysis. Recently, new methods for the solubilization of entire lignocellulose samples have addressed some of these problems by making it possible to exploit the enhanced signal dispersion provided by multidimensional solution-state NMR spectroscopy (Lu and Ralph, 2003; Ralph *et al*., 2006; Yelle *et al*., 2008a,b; Hedenström *et al*., 2009; Kim and Ralph, 2010). The principal caveat is that the samples must first be ball-milled, but research to date indicates that this procedure causes only minor increases in detectable cleaved lignin structures (Ikeda *et al*., 2002; Kim and Ralph, 2010). Here we have taken this approach to characterize changes that occur in lignin after wood decay by the brown rot fungus *Postia placenta*.

Results

Initial processing and analysis

We grew *P. placenta* on aspen wood, and after 16 weeks of decay we pooled 32 specimens exhibiting a dry weight loss of 69.7 ± 5.4%. The chemical composition of the biodegraded wood was typical of decay caused by this fungus (Table 1) (Eriksson *et al*., 1990). After grinding this material coarsely, we ball-milled 1 g of the resulting wood meal for 12 h at 300 r.p.m. For comparison, we pooled and ground 32 wafers of sound aspen sapwood, and then ball-milled 1 g of this material for 12 h at 600 r.p.m.

We then determined the methoxyl contents of the degraded and control samples, using an iodometric method that is quantitative with solid lignocellulosic specimens (Chen, 1992), so we could employ the lignin methoxyl NMR signal in each sample as an internal standard for quantification of other lignin structures. A loss of lignin methoxyls is typical after brown rot (Kirk and Adler, 1970; Kirk, 1975), and in this case their content declined from 1.82 to 1.00 mmol per g original wood. We next acetylated a portion of each sample to make it soluble in perdeuterated dimethylsulphoxide (DMSO-d_6) for solution-state NMR experiments (Lu and Ralph, 2003; Yelle *et al*., 2008a). In addition, we treated some of each ball-milled preparation with a cellulase mixture to remove most of the polysaccharides, which made the samples soluble in DMSO-d_6 without acetylation (Ralph *et al*., 2006).

Table 1. Chemical composition of sound and decayed aspen wood samples.

Sample	Klason lignin (mg)	ASL (mg)	Glucose (mg)	Xylose (mg)	Mannose (mg)	Galactose (mg)	Arabinose (mg)	Rhamnose (mg)
Sound wood								
Per g sound wood	231	34	409	168	17	5	4	3
Decayed wood								
Per g sound wood	158	14	63	14	1	0.4	0.2	nd
Per g decayed wood	522	47	207	47	3	1	1	nd

ASL, acid-soluble lignin; nd, not detected.

Depletion of arylglycerol-β-aryl ether structures in the lignin

Initially, we obtained two-dimensional one-bond ^{1}H–^{13}C heteronuclear single quantum coherence (HSQC) NMR spectra of the acetylated wood samples. This technique generates cross-peaks that correlate hydrogens in a molecule with carbons that are directly attached to them (Claridge, 1999). The results showed that decay depleted the arylglycerol-β-aryl ether units that account for approximately 80% of the intermonomer linkages discernible by NMR analysis of aspen sapwood lignin (Fig. 1A, structure **A**; Table 2). Integration of the HSQC contours showed that brown rot reduced the abundance of structure **A** to 82% of its original level relative to the methoxyls.

In good agreement with this result, quantitative ^{13}C NMR spectra of the cellulase-treated, non-acetylated samples showed that decay reduced the content of structure **A** to 83% of its original level relative to the methoxyls (Fig. 1B). After correction for the decrease in methoxyls that occurred during biodegradation, the quantitative ^{13}C NMR data show that the absolute content of structure **A** declined from 0.674 to 0.306 mmol per g original wood. In addition, the quantitative ^{13}C NMR results indicated that decay reduced the structure **A** content to 70% of its original level relative to C2 of the lignin's guaiacyl aromatic rings, and also decreased the syringyl/guaiacyl ratio of the lignin from 2.39 to 1.98 (data not shown). These results suggested that *P. placenta* degraded some of the lignin.

Products of C_α–C_β cleavage in the lignin

One diagnostic sign of oxidative ligninolysis is C_α–C_β cleavage of the propyl side-chain in structure **A**, as well as in other lignin subunits, to produce benzaldehyde and benzoic acid residues in the polymer (Eriksson *et al.*, 1990). To look for these structures in the decayed wood, we initially examined the quantitative ^{13}C NMR spectra of cellulase-treated, non-acetylated samples. We took this approach instead of using acetylated whole wood because the ^{13}C signal of the acetate carbonyl overlaps the corresponding signal from C_α in benzoic acids (Ralph *et al.*, 2004b).

The results showed that the decayed wood contained benzoic acid residues, whereas none of these structures was detectable in the undecayed wood. In addition, relative to the methoxyl signal, the brown-rotted sample contained more benzaldehydes than the undecayed sample (Fig. 1B). After correction for the decrease in methoxyl content that occurred during decay, the quantitative ^{13}C NMR data show that the quantity of degraded wood (0.303 g) derived from 1 g of sound wood contained 413% of the aryl carbonyl (benzaldehyde + benzoic acid) content that was present in the original sample. The absolute quantity of these truncated structures increased from 0.015 to 0.062 mmol per g original wood.

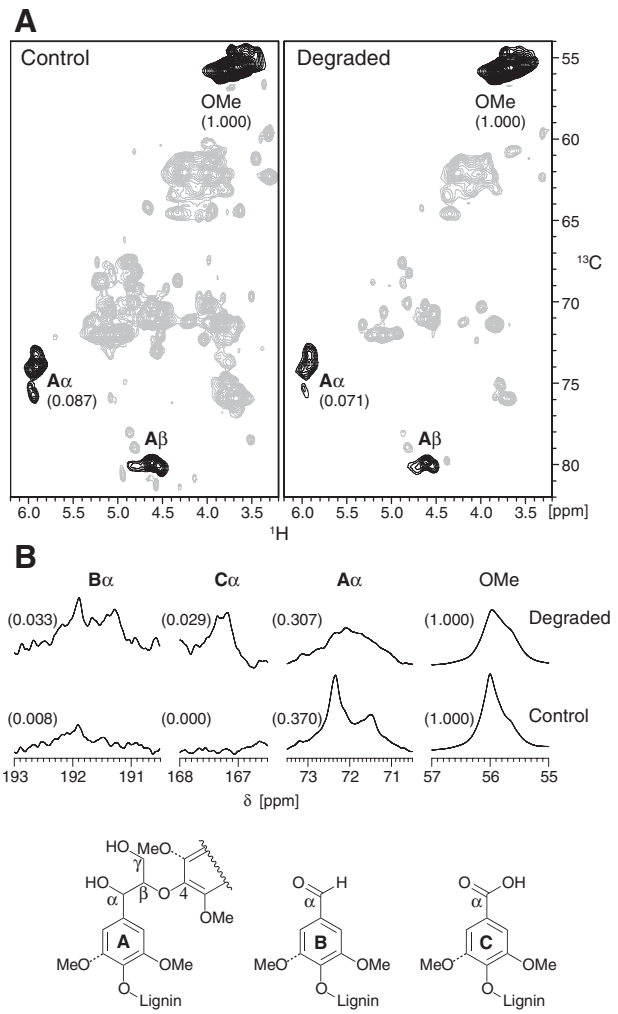

Fig. 1. A. Diagnostic portions of ^{1}H–^{13}C HSQC NMR spectra obtained on acetylated specimens of control and degraded aspen wood. Cross-peaks attributable to methoxyl (OMe) ^{1}H–^{13}C correlations, the structure **A** $^{1}H_\alpha$–$^{13}C_\alpha$ correlation and the structure **A** $^{1}H_\beta$–$^{13}C_\beta$ correlation are shown in black. Numbers in parentheses are integrations relative to the OMe signal. Unassigned contours, shown in gray, are principally due to polysaccharides.
B. Diagnostic portions of quantitative ^{13}C NMR spectra of non-acetylated aspen enzyme lignins showing signals for the degraded and control samples. To provide a semiquantitative visual impression of relative substructure abundances, the guaiacyl aromatic C2 peaks (not shown here), which changed relatively little during decay, have been set to equal heights for the degraded and control spectra. In addition, relative to the OMe signal in each spectrum, the height of the **A**α signal has been multiplied by 7, the height of the **B**α signal by 140 and the height of the **C**α signal by 56. Numbers in parentheses are integrations relative to the OMe signal, and should be used in conjunction with the iodometrically determined OMe contents for quantitative comparisons. Peak labels in the spectra (OMe, **A**α, **A**β, **B**α, **C**α) refer to the correspondingly labelled portions of chemical structures **A**, **B** and **C** under the figure.

We next confirmed the increased abundance of benzoic acids and benzaldehydes in the decayed wood by

Table 2. Approximate relative abundance of lignin interunit linkages in sound aspen wood.[a]

Structure	Average integral	Percent of the sum of integrals
β-Aryl ether (structure **A**)	1.00	84
Resinol[b]	0.11	9
Phenylcoumaran	0.046	4
Spirodienone	0.036	3

a. Determined by HSQC peak integration of three independent enzyme lignin samples.
b. This integral has been divided by two because two intermonomer units are required to produce one resinol linkage.

obtaining two-dimensional long-range ^{1}H–^{13}C heteronuclear multiple bond correlation (HMBC) spectra of the acetylated, whole wood cell wall samples. This method produces cross-peaks that correlate hydrogens with carbons located two to three bonds away, and is thus capable of detecting carbons such as those in carboxyl groups, which do not carry hydrogens (Claridge, 1999).

The HMBC spectra revealed correlations between the aryl carbonyls in the lignin and hydrogens at positions 2 or 6 on the associated aromatic rings (Fig. 2). The sound wood contained low levels of syringyl benzaldehydes (δ_H, $\delta_{^{13}C} = 7.3$, 192 p.p.m.), in agreement with earlier work (Kim *et al.*, 2000), but did not contain detectable levels of guaiacyl benzaldehydes (7.5, 191 p.p.m.), guaiacyl benzoic acids (7.5, 167 p.p.m.) or syringyl benzoic acids (7.2, 167 p.p.m.). By contrast, spectra of the decayed wood displayed cross-peaks at all the above coordinates, indicating that benzaldehydes and benzoic acids of both the guaiacyl and syringyl type were present. Additional correlations between aromatic H2/6 and various ring carbons are also apparent in the HMBC spectra and validate the structural assignments (Ralph *et al.*, 2004b).

Products of C_β-ether cleavage in the lignin

Another oxidative route for ligninolysis is cleavage of the ether linkage in structure **A** to produce phenylglycerol residues in the polymer (Eriksson *et al.*, 1990). It is difficult to identify phenylglycerols by solution-state NMR spectroscopy of acetylated whole wood because their side-chain signals overlap some of the polysaccharide signals (Ralph *et al.*, 2004b). This problem can be circumvented by using cellulase-treated wood, but a second difficulty is that one-dimensional ^{13}C NMR spectroscopy is not useful in this case because phenylglycerols and structure **A** exhibit similar ^{13}C chemical shifts for their alkyl side-chains (Ralph *et al.*, 2004b). Therefore, we employed two-dimensional ^{1}H–^{13}C HSQC spectroscopy of the cellulase-treated wood to provide the signal dispersion needed to look for phenylglycerols. This approach revealed a previously obscured cross-peak that was relatively more intense in the biodegraded sample and exhibited chemical shifts consistent with the H_α–C_α correlation for a guaiacyl or syringyl phenylglycerol (Ralph *et al.*, 2004b; 2006) (Fig. 3A, structure **P**).

We confirmed the phenylglycerol assignment by performing a three-dimensional total correlation spectroscopy–HSQC (TOCSY–HSQC) experiment. This method simultaneously correlates a hydrogen with the carbon to which it is bonded and with other hydrogens in the same proton–proton coupling network (i.e. in the same side-chain) in the molecule (Cavanaugh *et al.*, 1996). The results showed correlations for H_α–C_α, H_β–C_β and H_γ–C_γ that were well-dispersed in the H_α plane at $\delta_H = 4.37$ p.p.m. (Fig. 3B). In the H_β and H_γ planes, the H_γ–C_γ correlation was partially obscured, but the H_α–C_α and H_β–C_β cross-peaks were well-dispersed and had the same chemical shifts as in the H_α plane (data not shown).

By integrating the structure **P** H_α–C_α HSQC contours relative to the structure **A** H_α–C_α contours, and taking into account the previously calculated contents of structure **A** in the samples, we determined that the quantity of decayed wood (0.303 g) derived from 1 g of sound wood contained 280% of the wood's original phenylglycerol content. The absolute contents for phenylglycerols in the samples cannot be stated as precisely as the aryl carbonyl contents because comparisons between integrals from ^{1}H–^{13}C HSQC spectra may be less quantitative than those obtained by quantitative ^{13}C NMR spectroscopy. However, recent studies indicate that approximate quantifications can be obtained by this technique (Zhang and Gellerstedt, 2007; Stewart *et al.*, 2009; Wagner *et al.*,

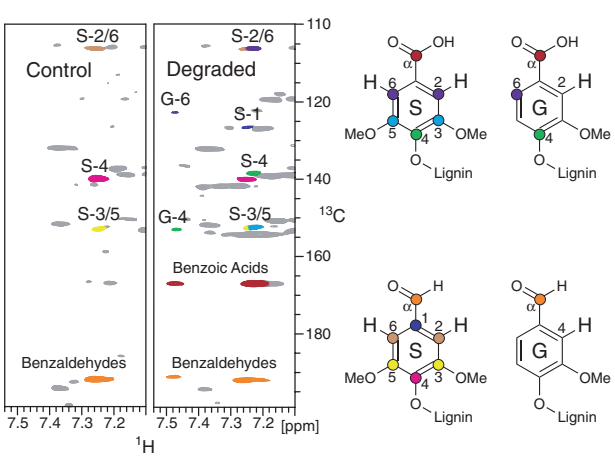

Fig. 2. Diagnostic portions of ^{1}H–^{13}C HMBC NMR spectra obtained on acetylated specimens of control and degraded aspen wood, showing correlations between aromatic H2/H6 and carbons 2 to 3 bonds away. Selected cross-peaks are colour-coded to their corresponding carbons in the chemical structures shown and unassigned contours are shown in gray. The H2/H6 protons are shown using a larger font in the chemical structures. Labels G and S in the spectra and chemical structures refer to guaiacyl and syringyl units respectively.

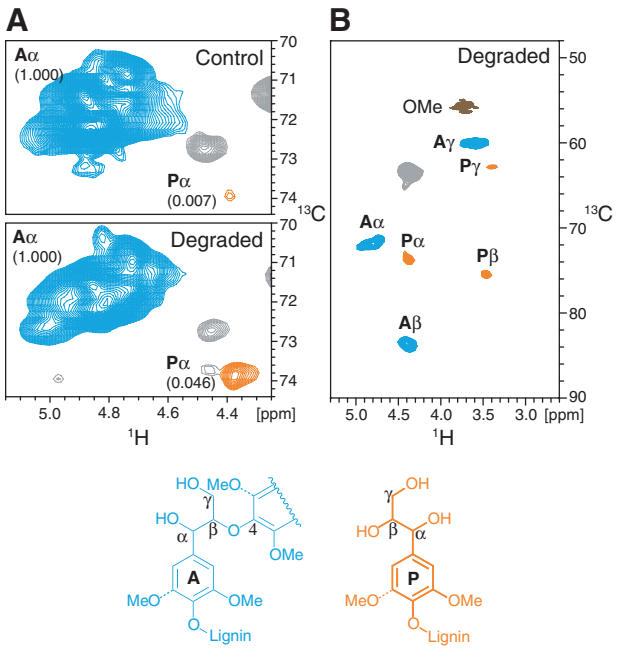

Fig. 3. A. Diagnostic portions of ^{1}H–^{13}C HSQC NMR spectra obtained on non-acetylated enzyme lignins from control and degraded aspen wood. Numbers in parentheses are integrals relative to the structure **A** signal.
B. Selection from the H_α plane (δ_H = 4.37 p.p.m.) of a three-dimensional TOCSY-HSQC spectrum obtained on non-acetylated, degraded aspen enzyme lignin. Peak labels in the spectra (OMe, **A**α, **A**β, **A**γ, **P**α, **P**β, **P**γ) refer to the correspondingly labelled portions of chemical structures **A** and **P** under the figure. Assigned cross-peaks are colour-coded to the chemical structures and unassigned contours are shown in gray.

2009; Koskela *et al.*, 2010). Accordingly, we estimate the phenylglycerol contents in the sound and degraded samples to be about 0.005 and 0.014 mmol, respectively, per g original wood.

Discussion

Selection of fungus and wood for the experiments

We selected *P. placenta* for this work because it is the first brown rot fungus to have its genome sequenced (Martinez *et al.*, 2009), which makes it possible to relate observed changes in lignin to putative biodegradative mechanisms. We selected aspen as the substrate for three reasons: (i) prior enzymatic removal of polysaccharides from ball-milled wood is a useful procedure in conjunction with solution-state NMR analysis because it makes acetylation unnecessary and can reveal previously obscured lignin structures. The extent of polysaccharide removal is generally greater with aspen than with other woods (Marita *et al.*, 2001). (ii) We have grown *P. placenta* on aspen in previous work, and already have some information about which enzymes and metabolites may contribute to decay in this culture system (Wei *et al.*, 2010). (iii) Although *P. placenta* attacks conifer wood most frequently, it also occurs naturally on aspen (Farr *et al.*, 1989).

Significance of truncated lignin structures

Our results show that aspen wood decayed by *P. placenta* was about 55% depleted in structure **A**, the major inter-monomeric unit in lignin. This decrease is considerably less than the result we obtained earlier with another brown rot fungus, *Gloeophyllum trabeum*, on spruce wood (Yelle *et al.*, 2008a). On the other hand, it is similar to the depletion reported recently for a field sample of ulmo wood that had been degraded by an uncharacterized brown rot fungus (Martínez *et al.*, 2011). In addition, our data show that the aspen decayed by *P. placenta* was enriched in three truncated lignin structures: benzaldehydes, benzoic acids and phenylglycerols. After subtraction of the quantities already present in sound aspen, these end-groups account for 8% of the **A** structures originally present, and thus for roughly 6–7% of all original lignin subunits (Table 2). We were unable to identify analogous cleaved lignin structures in our earlier study of spruce wood decayed by *G. trabeum*, but this negative result is probably attributable in part to extensive oxidation of the samples by this aggressive fungus, which left few recognizable structures in the residual lignin (Yelle *et al.*, 2008a).

One question that must be considered is whether the increased incidence of end-groups we found after decay in the present study was a chance event attributable to statistical variation in the levels of phenylglycerols and benzaldehydes that are already present in sound, ball-milled aspen. Some of these pre-existing structures are probably artefacts of ball-milling, but most are thought to occur naturally in lignins (Kim and Ralph, 2010). To assess the variability in pre-existing end-groups, we performed HSQC analyses of three independently prepared, cellulase-treated samples of sound, ball-milled aspen, and found that their phenylglycerol/methoxyl and benzaldehyde/methoxyl integral ratios exhibited standard deviations of 6.4% and 1.8% respectively (Table 3). These

Table 3. Variability in selected HSQC integrals for three independently prepared enzyme lignins from sound aspen wood.

Sample	OMe	**A**α	**P**α	**B**α
1	1.00	0.11	0.00059	0.0025
2	1.00	0.10	0.00066	0.0024
3	1.00	0.11	0.00067	0.0025
Average		0.11	0.00064	0.0025
Std. dev.		0.0051	0.000041	0.000044

OMe, methoxyls; **A**α, H_α–C_α signal for arylglycerol-β-aryl ethers; **P**α, H_α–C_α signal for phenylglycerols; **B**α, H_α–C_α signal for benzaldehydes.

values are much smaller than the three to fourfold increases in truncated structures we found after decay, and therefore we can exclude sampling error as the explanation for the result.

Another question is whether the increase in truncated lignin structures after decay can be attributed to ball-milling of the specimens. It is pertinent in this connection that we followed the usual protocol of milling each sample only to the minimum extent required for it to dissolve after acetylation (Lu and Ralph, 2003). Our sound wood required milling at twice the rate applied to the degraded wood, and previous work has shown that the energy imparted to a sample in a rotary ball mill increases approximately as the milling speed raised to the power of 1.3 (Iwasaki *et al*., 2006). Accordingly, the work done on our control sample was about 2.5 times the work done on our degraded sample.

Despite the milder treatment that the degraded sample received, it contained about four times as many truncated structures as were present in the sound sample. Thus, for the increase in truncated lignin structures we found after decay to be entirely an artefact of ball-milling, the lignin in brown-rotted wood would have to be roughly 10-fold more susceptible to mechanical depolymerization than the lignin in sound wood. Although further research on this question is undoubtedly needed, we consider such a large difference in physical properties unlikely.

Routes for ligninolysis

Instead, we attribute the new benzoic acids, benzaldehydes and phenylglycerols in the lignin of the brown-rotted wood to fungal ligninolysis. Research to date has identified three possible oxidative systems fungi could use to generate these cleaved structures: ligninolytic peroxidases, laccase/mediator couples and Fenton chemistry. We discuss each of these hypotheses below.

Specialized peroxidases are thought to have an important ligninolytic role in white rot fungi. Some of these enzymes, known as lignin peroxidases, oxidize aromatic structures directly. Others, known as manganese peroxidases, oxidize Mn^{2+} to produce a variety of oxidants, including Mn^{3+} and reactive oxygen species. An additional group, known as versatile peroxidases, has both capabilities (Martínez, 2002). All of these ligninolytic enzymes contain unique amino acid sequence motifs essential for catalysis – either an invariant tryptophan residue on the enzyme surface that is involved in electron transfer from an aromatic substrate to the oxidized haem, and/or three acidic residues near the haem propionates that form a site for binding and oxidation of Mn^{2+} (Martínez, 2002). The genome of *P. placenta* has been sequenced and encodes no peroxidases with these features (Martinez *et al*., 2009). Moreover, we found in a recent study that aspen wood colonized by *P. placenta* contained no detectable peroxidase activity (Wei *et al*., 2010). Therefore, it is unlikely that peroxidases were responsible for the truncated lignin structures we report here.

Laccases acting in conjunction with small electron transfer mediators have also been proposed to have a role in fungal ligninolysis (Li *et al*., 2001). *Postia placenta* produces at least one laccase (Wei *et al*., 2010), and it is well established that these copper radical oxidases oxidize a variety of synthetic nitrogenous compounds to produce ligninolytic reactive nitrogen species. Perhaps the best-known of these mediators is 1-hydroxybenzotriazole, which laccase oxidizes to yield nitroxide radicals that can cleave lignin (Rochefort *et al*., 2004). However, no natural analogue of these synthetic nitrogenous mediators has been found in any fungus so far (Li *et al*., 2001). Naturally occurring phenols might fill this gap by providing a source of laccase-generated phenoxyl radicals that could oxidize lignin, but recent work conducted with laccase and the naturally occurring phenol syringaldehyde showed that this system failed to cleave a lignin model compound based on structure **A** (Nousiainen *et al*., 2009). Therefore, although the hypothesis cannot yet be ruled out, it appears unlikely that a laccase-mediator couple accounts for ligninolysis by *P. placenta*.

Finally, Fenton chemistry in conjunction with the low pH that *P. placenta* produces extracellularly could account for ligninolysis (Cohen *et al*., 2002; Wei *et al*., 2010). Under these conditions, one of the expected reactions is electrophilic addition of $^{\bullet}OH$ to unsubstituted positions on aromatic rings in lignin, followed by protonation and loss of H_2O to give the same scissile cation radical intermediates that lignin peroxidases produce with greater selectivity (Fig. 4) (Snook and Hamilton, 1974; Kirk *et al*., 1986). Some of the benzaldehydes produced by this route then probably undergo facile oxidation by $^{\bullet}OH$ or oxyradicals derived from it to yield benzoic acids (Howard, 1973). Electrophilic addition of $^{\bullet}OH$, a very non-regioselective oxidant, is also expected to yield phenylglycerols when attack occurs at the aromatic 4-position to cleave the arylglycerol-β-aryl ether linkage in structure **A** (Fig. 4) (Tatsumi and Terashima, 1985). *Postia placenta* secretes a metabolite, 2,5-dimethoxyhydroquinone, which drives Fenton chemistry via redox cycling in the presence of Fe^{3+} (Cohen *et al*., 2002). Moreover, recent work shows that a laccase produced by *P. placenta* in wood accelerates this redox chemistry by catalysing the oxidation of 2,5-dimethoxyhydroquinone to produce semiquinone radicals that are precursors of H_2O_2 (Wei *et al*., 2010). Since components that support Fenton chemistry have been found in wood colonized by *P. placenta*, we consider this third route for ligninolysis the most likely one.

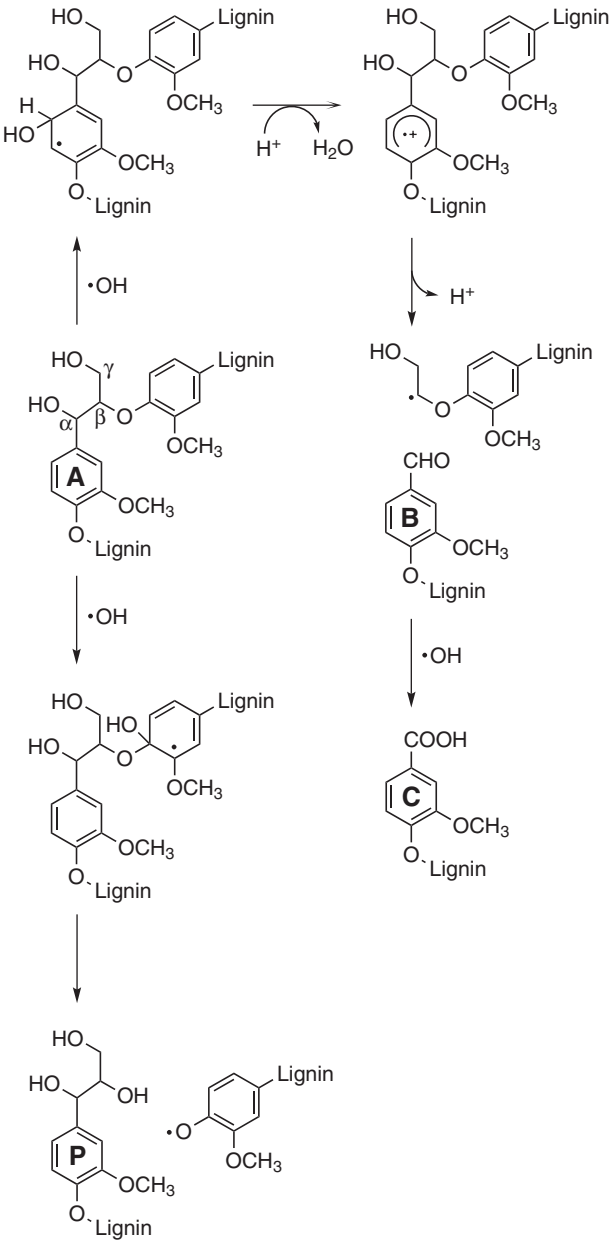

Fig. 4. Oxidation of structure **A** by hydroxyl radical (•OH) to give benzaldehydes (**B**), benzoic acids (**C**) and phenylglycerols (**P**).

Conclusion

Our results provide evidence that *P. placenta* is ligninolytic. Although most of the resulting lignin oligomers remain *in situ* and likely repolymerize to some extent under the oxidizing conditions of brown rot (Yelle *et al*., 2008a), we hypothesize that a transient, partial depolymerization suffices to allow infiltration by the polysaccharide hydrolases that this fungus has been shown to express on wood (Martinez *et al*., 2009; Vanden Wymelenberg *et al*., 2010). Previously, the specialized peroxidases of white rot fungi provided the only well-attested mechanism for fungal ligninolysis (Martínez, 2002), but it now appears that an additional mechanism contributes to the biodegradation of this recalcitrant polymer.

Experimental procedures

Reagents and fungal cultures

Chemicals were all of reagent grade or better. *Postia placenta* strain MAD-698-R (American Type Culture Collection No. 44394) was obtained from the USDA Forest Mycology Center (Madison, WI) and grown on two layers of aspen (*Populus* sp.) sapwood wafers with pre-determined dry weights (117 ± 17 mg each) over nutrient agar as previously described (Wei *et al*., 2010). After 16 weeks of growth, wafers were harvested from the top layer and vacuum-dried to determine losses in dry weight.

Sample preparation and wet chemical analyses

Thirty-two wafers with an average weight loss of 69.7 ± 5.4% were pooled, as were 32 sound control wafers. Each sample was ground to 80 mesh in a Wiley mill, and 1 g of each was then milled in a Retsch PM100 planetary ball-mill for 12 h, the degraded sample at 300 r.p.m. and the control sample at 600 r.p.m. as described previously (Yelle *et al*., 2008a).

The methoxyl (OMe) content of each sample was determined in duplicate by the standard iodometric titration procedure (Chen, 1992; Zakis, 1994). The replicate values for the control sample were 1.80 and 1.84 mmol OMe per g wood, whereas the degraded sample gave 0.99 and 1.00 mmol OMe per g original wood. Analyses of the sound and degraded samples for sugars, Klason lignin and acid-soluble lignin were conducted as described previously (Davis, 1998; Yelle *et al*., 2008a).

Approximately 100 mg each of decayed and control ball-milled wood was then dissolved in *N*-methylimidazole/DMSO, acetylated, precipitated in H_2O and redissolved in DMSO-d_6 for NMR analysis as described previously (Lu and Ralph, 2003; Yelle *et al*., 2008a). The remainder of each ball-milled wood sample was treated with a cellulase mixture (Cellulysin, Calbiochem) as described previously to remove most of the polysaccharides (Ralph *et al*., 2006), and the resulting enzyme lignin was dissolved in DMSO-d_6 for NMR analysis.

Solution-state NMR spectroscopy

NMR spectra were acquired on a 500 MHz Bruker DMX-500 instrument equipped with a cryogenically cooled 5 mm gradient probe (US Dairy Forage Research Center, Madison, WI). Samples of acetylated aspen (60 mg) and non-acetylated enzyme lignin (60 mg) were analysed in 500 µl of DMSO-d_6. Chemical shifts for lignin substructures were checked against authentic low molecular weight model compounds and against published values (Ralph *et al*., 2004b).

One-bond ^{1}H–^{13}C correlation (HSQC) spectra were acquired using adiabatic Bruker pulse program hsqcetgpsisp2.2 and processed as described previously (Kupče and Freeman, 2007; Yelle *et al*., 2008a). Three-

dimensional total correlation (TOCSY–HSQC) spectra were acquired using Bruker pulse program mleviietf3gs3d and processed using previously described parameters (Ralph *et al*., 1999; Ralph and Lu, 2004). Long-range ^{1}H–^{13}C correlation (HMBC) spectra were acquired using Bruker pulse program hmbclplrndqf and processed as described previously (Ralph *et al*., 2006), using an 80 ms long-range coupling delay. Quantitative inverse-gated, decoupled, NOE-suppressed ^{13}C spectra were acquired using Bruker pulse program zgig30 with a D1 delay of 4 s, a sweep width of 220 p.p.m. and 64 K data points. A line-broadening of 5 Hz was used for processing.

Quantification of lignin structures

We used the quantitative ^{13}C NMR spectra of enzyme lignins to quantify arylglycerol-β-aryl ether units (structure **A**), benzaldehydes (structure **B**) and benzoic acids (structure **C**) in the samples. The integral for C_α of each structure was divided by the OMe integral, and the resulting ratio was multiplied by the iodometrically determined molar quantity of OMe per g original wood (i.e. per 1.000 g for control wood and per 0.303 g for decayed wood). We then summed the values for structures **B** and **C**, and report them here as aryl carbonyls. We took this approach because sound lignin already contains some benzaldehydes, and the oxidation of a benzaldehyde to a benzoic acid is a facile reaction that involves no scission (Howard, 1973). Accordingly, the occurrence of structures **B** and **C** is evidence for ligninolysis only if the sum of their quantities is greater in the degraded sample than in the control.

As phenylglycerols (structure **P**) are not dispersed from other signals in ^{13}C NMR spectra, we used the HSQC spectra of enzyme lignins to quantify them. The integral for the H_α–C_α cross-peak of the phenylglycerols was divided by the integral for the H_α–C_α cross-peak of the arylglycerol-β-aryl ether units, and the resulting ratio was multiplied by the previously calculated molar quantity of arylglycerol-β-aryl ether units per g original wood (i.e. per 1.000 g for control wood and per 0.303 g for decayed wood). Although HSQC spectra are not strictly quantitative, the error in phenylglycerol determination is likely small because adiabatic pulse sequences were used to improve uniformity over the whole spectrum and to minimize effects due to coupling constant differences (Kupče and Freeman, 2007). Moreover, the chemical environment of the H_α–C_α bond in phenylglycerols is very similar to that of the H_α–C_α bond in arylglycerol-β-aryl ether units. Even if we are wrong in this assumption, it is only the absolute quantification of phenylglycerols that is in error – the factor by which phenylglycerols increased per g original wood remains correct.

Following the above reasoning, we also used integrals of HSQC H_α–C_α cross-peaks to estimate the relative frequencies of arylglycerol-β-aryl ethers (structure **A**), resinols, phenylcoumarans and spirodienones in enzyme lignins from sound aspen.

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References

Cavanaugh, J., Fairbrother, W.J., Palmer, A.G., and Skelton, N.J. (1996) *Protein NMR Spectroscopy: Principles and Practice*. San Diego, CA, USA: Academic Press.

Chen, C.-L. (1992) Determination of methoxyl groups. In *Methods in Lignin Chemistry*. Lin, S.Y., and Dence, C.W. (eds). Heidelberg, Germany: Springer, pp. 465–472.

Claridge, T.D.W. (1999) *High-Resolution NMR Techniques in Organic Chemistry*. Amsterdam, The Netherlands: Elsevier.

Cohen, R., Jensen, K.A., Houtman, C.J., and Hammel, K.E. (2002) Significant levels of extracellular reactive oxygen species produced by brown rot basidiomycetes on cellulose. *FEBS Lett* **531:** 483–488.

Cowling, E.B. (1961) Comparative biochemistry of the decay of sweetgum sapwood by white-rot and brown-rot fungi. *USDA Tech Bull* **1258:** 1–79.

Davis, M.F., Schroeder, H.A., and Maciel, G.E. (1994) Solid-state ^{13}C nuclear magnetic-resonance studies of wood decay. 3. Decay of Colorado blue spruce and paper birch by *Postia placenta*. *Holzforschung* **48:** 301–307.

Davis, M.W. (1998) A rapid modified method for compositional carbohydrate analysis of lignocellulosics by high pH anion-exchange chromatography with pulsed amperometric detection (HPAEC/PAD). *J Wood Chem Technol* **18:** 235–252.

Eriksson, K.E., Blanchette, R.A., and Ander, P. (1990) *Microbial and Enzymatic Degradation of Wood and Wood Components*. Berlin, Germany: Springer.

Farr, D.F., Bills, G.F., Chamuris, G.P., and Rossman, A.Y. (1989) *Fungi on Plants and Plant Products in the United States*. St Paul, MN, USA: American Phytopathological Society.

Gómez-Toribio, V., García-Martin, A.B., Martínez, M.J., Martínez, A.T., and Guillén, F. (2009) Induction of extracellular hydroxyl radical production by white-rot fungi through quinone redox cycling. *Appl Environ Microbiol* **75:** 3944–3953.

Goodell, B., Jellison, J., Liu, J., Daniel, G., Paszczynski, A., Fekete, F., *et al*. (1997) Low molecular weight chelators and phenolic compounds isolated from wood decay fungi and their role in the fungal biodegradation of wood. *J Biotechnol* **53:** 133–162.

Guillén, F., Martínez, M.J., Muñoz, C., and Martínez, A.T. (1997) Quinone redox cycling in the ligninolytic fungus *Pleurotus eryngii* leading to extracellular production of superoxide anion radical. *Arch Biochem Biophys* **339:** 190–199.

Hammel, K.E., Kapich, A.N., Jensen, K.A., and Ryan, Z.C. (2002) Reactive oxygen species as agents of wood decay by fungi. *Enzyme Microb Technol* **30:** 445–453.

Hedenström, M., Wiklund-Lindstrom, S., Oman, T., Lu, F.C., Gerber, L., Schatz, P., *et al*. (2009) Identification of lignin and polysaccharide modifications in *Populus* wood by chemometric analysis of 2D NMR spectra from dissolved cell walls. *Mol Plant* **2:** 933–942.

Howard, J.A. (1973) Homogeneous liquid-phase autoxidations. In *Free Radicals*. Kochi, J.K. (ed.). New York, NY, USA: Wiley, pp. 3–62.

Ikeda, T., Holtman, K., Kadla, J.F., Chang, H.M., and Jameel, H. (2002) Studies on the effect of ball milling on lignin structure using a modified DFRC method. *J Agric Food Chem* **50:** 129–135.

Iwasaki, T., Kim, J.H., and Satoh, M. (2006) Characterization of media mills based on mechanical energy applied to particles. *Chem Eng Sci* **61:** 1065–1073.

Kerem, Z., Bao, W., and Hammel, K.E. (1998) Rapid polyether cleavage via extracellular one-electron oxidation by a brown-rot basidiomycete. *Proc Natl Acad Sci USA* **95:** 10373–10377.

Kerem, Z., Jensen, K.A., and Hammel, K.E. (1999) Biodegradative mechanism of the brown rot basidiomycete *Gloeophyllum trabeum*: evidence for an extracellular hydroquinone-driven fenton reaction. *FEBS Lett* **446:** 49–54.

Kim, H., and Ralph, J. (2010) Solution-state 2D NMR of ball-milled plant cell wall gels in DMSO-d_6/pyridine-d_5. *Org Biomol Chem* **8:** 576–591.

Kim, H., Ralph, J., Yahiaoui, N., Pean, M., and Boudet, A.M. (2000) Cross-coupling of hydroxycinnamyl aldehydes into lignins. *Org Lett* **2:** 2197–2200.

Kirk, T.K. (1975) Effects of a brown-rot fungus, *Lenzites trabea*, on lignin in spruce wood. *Holzforschung* **29:** 99–107.

Kirk, T.K., and Adler, E. (1970) Methoxyl-deficient structural elements in lignin of sweetgum decayed by a brown-rot fungus. *Acta Chem Scand* **24:** 3379–3390.

Kirk, T.K., Tien, M., Kersten, P.J., Mozuch, M.D., and Kalyanaraman, B. (1986) Ligninase of *Phanerochaete chrysosporium*: mechanism of its degradation of the nonphenolic arylglycerol β-aryl ether substructure of lignin. *Biochem J* **236:** 279–287.

Kirk, T.K., Ibach, R., Mozuch, M.D., Conner, A.H., and Highley, T.L. (1991) Characteristics of cotton cellulose depolymerized by a brown-rot fungus, by acid, or by chemical oxidants. *Holzforschung* **45:** 239–244.

Koskela, H., Heikkilä, O., Kilpeläinen, I., and Heikkinen, S. (2010) Quantitative two-dimensional HSQC experiment for high magnetic field NMR spectrometers. *J Magn Reson* **202:** 24–33.

Kupče, E., and Freeman, R. (2007) Compensated adiabatic inversion pulses: broadband INEPT and HSQC. *J Magn Reson* **187:** 258–265.

Li, K.C., Horanyi, P.S., Collins, R., Phillips, R.S., and Eriksson, K.E.L. (2001) Investigation of the role of 3-hydroxyanthranilic acid in the degradation of lignin by white-rot fungus *Pycnoporus cinnabarinus*. *Enzyme Microb Technol* **28:** 301–307.

Lu, F.C., and Ralph, J. (2003) Non-degradative dissolution and acetylation of ball-milled plant cell walls: high-resolution solution-state NMR. *Plant J* **35:** 535–544.

McFee, W.W., and Stone, E.L. (1966) The persistence of decaying wood in the humus layers of northern forests. *Soil Sci Soc Am J* **29:** 432–436.

Marita, J.M., Ralph, J., Lapierre, C., Jouanin, L., and Boerjan, W. (2001) NMR characterization of lignins from transgenic poplars with suppressed caffeic acid *O*-methyltransferase activity. *J Chem Soc Perkin Trans 1* **2001:** 2939–2945.

Martínez, A.T. (2002) Molecular biology and structure-function of lignin-degrading heme peroxidases. *Enzyme Microb Technol* **30:** 425–444.

Martínez, A.T., Rencoret, J., Nieto, L., Jiménez-Barbero, J., Gutiérrez, A., and Del Río, J.C. (2011) Selective lignin and polysaccharide removal in natural fungal decay of wood as evidenced by *in situ* structural analyses. *Environ Microbiol* **13:** 96–107.

Martinez, D., Challacombe, J., Morgenstern, I., Hibbett, D., Schmoll, M., Kubicek, C.P., *et al*. (2009) Genome, transcriptome, and secretome analysis of wood decay fungus *Postia placenta* supports unique mechanisms of lignocellulose conversion. *Proc Natl Acad Sci USA* **106:** 1954–1959.

Nousiainen, P., Maijala, P., Hatakka, A., Martínez, A.T., and Sipilä, J. (2009) Syringyl-type simple plant phenolics as mediating oxidants in laccase catalyzed degradation of lignocellulosic materials: model compound studies. *Holzforschung* **63:** 699–704.

Ralph, J., and Lu, F.C. (2004) Cryoprobe 3D NMR of acetylated ball-milled pine cell walls. *Org Biomol Chem* **2:** 2714–2715.

Ralph, J., Marita, J.M., Ralph, S.A., Hatfield, R.D., Lu, F.C., Ede, R.M., *et al*. (1999) Solution-state NMR of lignins. In *Advances in Lignocellulosics Characterization*. Argyropoulos, D.S. (ed.). Atlanta, GA, USA: TAPPI (Technical Association of the Pulp and Paper Industry) Press, pp. 55–108.

Ralph, J., Lundquist, K., Brunow, G., Lu, F., Kim, H., Schatz, P.F., *et al*. (2004a) Lignins: natural polymers from oxidative coupling of 4-hydroxyphenylpropanoids. *Phytochem Rev* **3:** 29–60.

Ralph, S.A., Ralph, J., and Landucci, L.L. (2004b) *NMR Database of Lignin and Cell Wall Model Compounds* [WWW document]. URL http://ars.usda.gov/Services/docs.htm?docid=10491.

Ralph, J., Akiyama, T., Kim, H., Lu, F.C., Schatz, P.F., Marita, J.M., *et al*. (2006) Effects of coumarate 3-hydroxylase down-regulation on lignin structure. *J Biol Chem* **281:** 8843–8853.

Rochefort, D., Leech, D., and Bourbonnais, R. (2004) Electron transfer mediator systems for bleaching of paper pulp. *Green Chem* **6:** 14–24.

Snook, M.E., and Hamilton, G.A. (1974) Oxidation and fragmentation of some phenyl-substituted alcohols and ethers by peroxydisulfate and Fenton's reagent. *J Am Chem Soc* **96:** 860–869.

Stewart, J.J., Akiyama, T., Chapple, C., Ralph, J., and Mansfield, S.D. (2009) The effects on lignin structure of overexpression of ferulate 5-hydroxylase in hybrid poplar. *Plant Physiol* **150:** 621–635.

Tatsumi, K., and Terashima, N. (1985) Oxidative degradation of lignin. 7. Cleavage of the β-*O*-4 linkage of guaiacylglycerol-β-guaiacyl ether by hydroxyl radicals. *Mokuzai Gakkaishi* **31:** 316–317.

Vanden Wymelenberg, A., Gaskell, J., Mozuch, M., Sabat, G., Ralph, J., Skyba, O., *et al*. (2010) Comparative transcriptome and secretome analysis of wood decay fungi *Postia placenta* and *Phanerochaete chrysosporium*. *Appl Environ Microbiol* **76:** 3599–3610.

Wagner, A., Donaldson, L., Kim, H., Phillips, L., Flint, H., Steward, D., *et al*. (2009) Suppression of 4-coumarate CoA ligase in the coniferous gymnosperm *Pinus radiata*. *Plant Physiol* **149:** 370–383.

Wei, D.S., Houtman, C.J., Kapich, A.N., Hunt, C.G., Cullen, D., and Hammel, K.E. (2010) Laccase and its role in production of extracellular reactive oxygen species during wood decay by the brown rot basidiomycete *Postia placenta*. *Appl Environ Microbiol* **76:** 2091–2097.

Yelle, D.J., Ralph, J., Lu, F.C., and Hammel, K.E. (2008a) Evidence for cleavage of lignin by a brown rot basidiomycete. *Environ Microbiol* **10:** 1844–1849.

Yelle, D.J., Ralph, J., and Frihart, C.R. (2008b) Characterization of nonderivatized plant cell walls using high-resolution solution-state NMR spectroscopy. *Magn Reson Chem* **46:** 508–517.

Zakis, G.F. (1994) *Functional Analysis of Lignins and Their Derivatives*. Atlanta, GA, USA: TAPPI (Technical Association of the Pulp and Paper Industry) Press.

Zeikus, J.G. (1981) Lignin metabolism and the carbon cycle: polymer biosynthesis, biodegradation, and environmental recalcitrance. *Adv Microb Ecol* **5:** 211–243.

Zhang, L.M., and Gellerstedt, G. (2007) Quantitative 2D HSQC NMR determination of polymer structures by selecting suitable internal standard references. *Magn Reson Chem* **45:** 37–45.