

Litter decay rates are determined by lignin chemistry

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Abstract Litter decay rates are often correlated with the initial lignin:N or lignin:cellulose content of litter, suggesting that interactions between lignin and more labile compounds are important controls over litter decomposition. The chemical composition of lignin may influence these interactions, if lignin physically or chemically protects labile components from microbial attack. We tested the effect of lignin chemical composition on litter decay in the field during a year-long litterbag study using the model system *Arabidopsis thaliana*. Three *Arabidopsis* plant types were used, including one with high amounts of guaiacyl-type lignin, one with high aldehyde- and *p*-hydroxyphenyl-type lignin, and a wild type control with high syringyl-type lignin. The high aldehyde litter lost significantly more mass than the other plant types, due to greater losses of cellulose, hemicellulose, and N. Aldehyde-rich lignins and *p*-hydroxyphenyl-type lignins have low levels of cross-linking between lignins and polysaccharides, supporting the

hypothesis that chemical protection of labile polysaccharides and N is a mechanism by which lignin controls total litter decay rates. 2D NMR of litters showed that lignin losses were associated with the ratio of guaiacyl-to-*p*-hydroxyphenyl units in lignin, because these units polymerize to form different amounts of labile- and recalcitrant-linkages within the lignin polymer. Different controls over lignin decay and polysaccharide and N decay may explain why lignin:N and lignin:cellulose ratios can be better predictors of decay rates than lignin content alone.

Keywords Lignin · Decomposition · Cellulose · Nitrogen

Introduction

Lignin is likely to be an important control over litter decay, given that litter decomposition rates are often negatively correlated with the initial ratios of lignin:N or lignin:cellulose in litter (Aber et al. 1990; Aerts 1997; Cortez et al. 1996; Melillo et al. 1982; Moore et al. 1999; Trofymow et al. 2002; Zhang et al. 2008). However, the chemical mechanisms by which lignin—and its interactions with labile litter components like polysaccharides and N in the cell wall—controls the litter decay process in ecosystems are poorly understood. Identifying these mechanisms is

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key to understanding why lignin:N or lignin:cellulose ratios in litter consistently correlate with decomposition rates across a range of ecosystems.

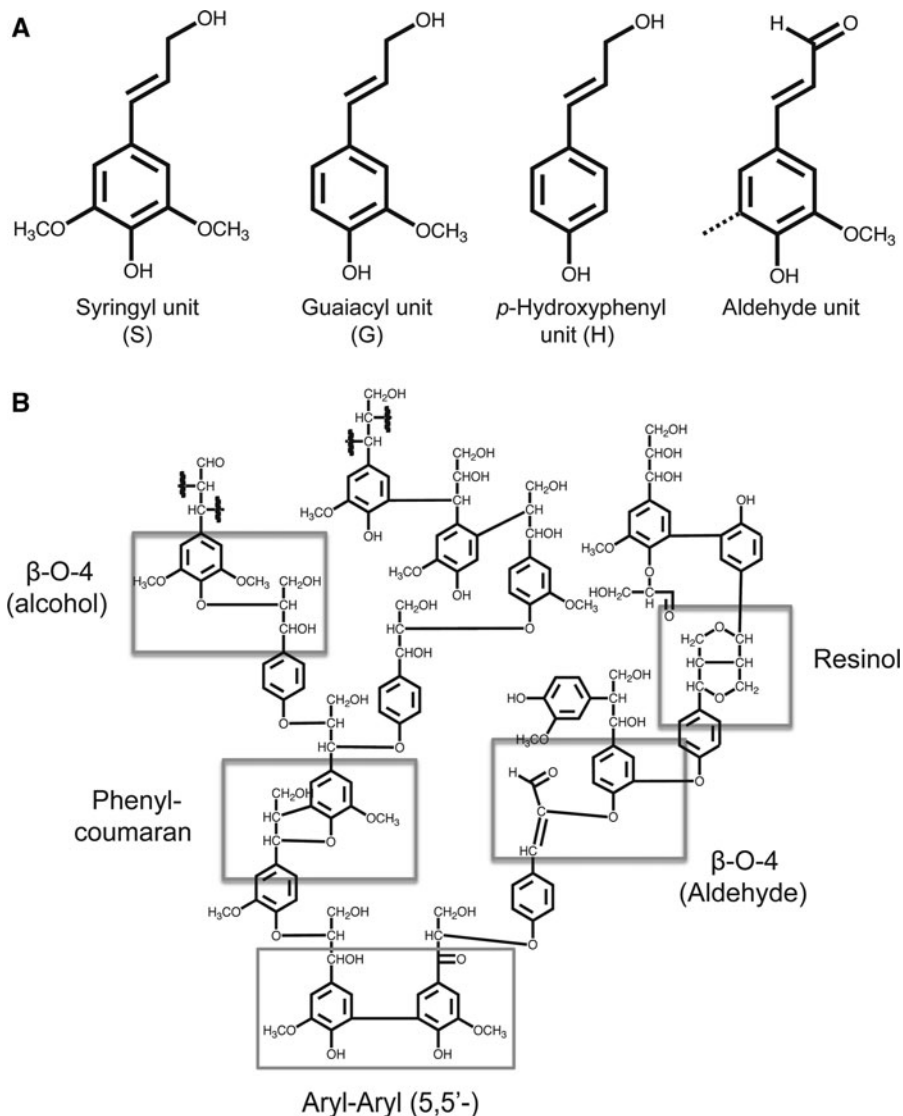
Historically, three mechanisms have been invoked to explain how lignin might influence decomposition. First, lignin could slow mass loss by its resistance to degradation by extracellular enzymes. Lignin is an aromatic polymer found in the cell walls of vascular plants, and it resists cleavage by most biological agents (Kirk and Farrell 1987). Since lignin comprises approximately 15–40% of terrestrial biomass (Berg and McClaugherty 2003), its retention in decaying tissues could substantially decrease rates of total mass loss (Berg et al. 1993; Meentemeyer 1978). Second, lignin is deposited in cell walls within the hemicellulose-protein matrices in which cellulose microfibrils are embedded (Boerjan et al. 2003), thus it could *physically* protect these more labile cell wall components from microbial attack during litter decomposition. Third, the lignin polymer can form covalent bonds (i.e. cross-link) to hemicelluloses and protein (Hammel 1997), potentially *chemically* protecting these labile compounds from hydrolysis during decay. Based on these mechanisms, rates of decomposition should theoretically correlate with the absolute amounts of lignin in leaf litter. However, within individual species at similar maturity stages, plant tissue decomposability is only weakly correlated with total lignin content (Jung and Vogel 1992). This observation suggests that other components of lignification may control lignin, polysaccharide, and protein loss in decomposing plant tissue.

The chemical structure of lignin could determine lignin-decay relationships by altering the magnitude of physical and chemical protection of labile litter components. Lignin is a structurally heterogeneous polymer; a variety of unit types can be incorporated into lignin via a number of inter-unit linkages, resulting in chemical structures that vary between plant taxa and throughout the developmental stages of a plant (Campbell and Sederoff 1996). For example, gymnosperm lignins are composed of guaiacyl-type (G) lignin, while angiosperm lignins contain guaiacyl- and syringyl-type (S) lignin, with smaller amounts of *p*-hydroxyphenyl-type (H) lignin (Boerjan et al. 2003). The monomer units in guaiacyl, syringyl, and *p*-hydroxyphenyl lignins differ in only a single methoxyl group (Fig. 1). However, this variation gives rise to lignin polymers with different

chemical structure that differ in their susceptibility to enzymatic oxidation and hydrolysis (Hedges et al. 1985). For example, guaiacyl units polymerize to form condensed (5,5') aryl-aryl linkages, while syringyl-type lignin is formed from more labile β -O-4 linkages (Fig. 1). Aryl-aryl linkages have been observed to persist during the process of soil development, while other linkages are degraded quickly in the initial stages of litter breakdown (Bahri et al. 2006; Dignac and Rumpel 2006; Goni and Hedges 1992; Opsahl and Benner 1995). If differences among plants in lignin structure also affect the physical or chemical protection of labile compounds from microbial attack, differences in lignin chemistry alone could explain a large portion of species-specific variation in litter decay rates.

Model plant systems provide the opportunity to explore how differences in lignin chemical structure affect the loss of different litter chemical components during decomposition. In these systems, lignin structures can be modified by altering the expression of genes coding for enzymes in the lignin biosynthesis pathway, or by chemically modifying the lignin as it polymerizes in the cell wall. Previous studies have used genetically or chemically modified plant systems in laboratory studies to examine lignin chemistry effects on cell wall degradation by rumen microbes (Grabber 2005; Grabber et al. 2009; Jung and Casler 1991; Jung et al. 1994; Jung and Buxton 1994; Jung et al. 1999; Vailhe et al. 1996), extracellular enzyme preparations (Grabber 2005; Grabber et al. 1998c; Grabber et al. 1997; Li et al. 2010), or microbes in soil microcosms (Hénault et al. 2006; Machinet et al. 2009; Webster et al. 2005). These studies have found mixed evidence for the hypothesis that lignin chemical composition affects plant cell wall degradation. Some documented strong effects of recalcitrant, condensed lignin structures on total decomposition rates (Bertrand et al. 2006; Grabber et al. 1998c), while others have found no such effects (Grabber 2005; Grabber et al. 1997; Li et al. 2010) or are unable to test for them because of covariation between lignin chemical composition and other aspects of litter chemistry (Machinet et al. 2009). Notably, several recent laboratory microcosm studies have found that polysaccharide breakdown and the rate of total C loss in grass litter are related to the abundance of phenolic units (*p*-coumaric acid and ferulic acid) that crosslink polysaccharides to lignins

Fig. 1 Structures of phenylpropanoid precursor units (a) and linkages between units (b) found in the lignin polymer. Precursors include alcohol subunits (monolignols) that make up syringyl-, guaiacyl-, *p*-hydroxyphenyl-lignins, as well as subunits that make up aldehyde-rich lignins. Linkages illustrated include a β -O-4 linkage between normal alcohol units, phenylcoumaran linkage between guaiacyl alcohol units, β -O-4 linkage between aldehyde units, and a resinol linkage that is often abundant in *p*-hydroxyphenyl lignin



in the cell wall (Bertrand et al. 2006; Grabber et al. 2009). These studies indicate that chemical protection of labile cell wall components could be an important mechanism of lignin control over rates of total litter decay, and that physical protection of these components may also play a role. However, it is still unclear how these mechanisms scale up to influence total decay rates and the chemical dynamics of litter during decomposition in natural ecosystems.

New methods in solution-state nuclear magnetic resonance (NMR) spectroscopy allow us to examine the chemical mechanisms responsible for variation in total litter decay rates more precisely than before. Dissolution techniques using perdeuterated dimethylsulfoxide and

N-methylimidazole can dissolve whole plant cell walls, with minimal chemical modifications to the lignocellulose structure, at concentrations suitable for solution-state 1D and 2D NMR experiments (Yelle et al. 2008a). In addition, advances in NMR instruments (e.g., with cryogenically cooled probes), pulse programs, and analysis software now allow more accurate quantification of individual monomer units and inter-unit linkages in the lignin polymer (Ralph et al. 2006b; Rencoret et al. 2009; Yelle et al. 2008b). These high-resolution NMR techniques have already been used to identify novel types of inter-unit linkages present in lignin polymers that are derived from substituted monomer units (Ralph et al. 1998; Ralph

et al. 1999). However, these techniques have never before been used to study lignin effects on decomposing litter in a natural field setting. By employing these techniques to study lignocellulose structure in plant tissues decomposing in the field, we can identify structural features in the lignin polymer that control the activity of in situ decomposer communities, determine how these structures are transformed throughout the decay process, and describe how individual aspects of lignin chemical structure contribute to early stages of soil development in a natural system.

To test how lignin chemistry influences total litter decay rates in the field, we used this new 2D NMR technique to study the in situ decay of genetically modified *Arabidopsis thaliana* plants that varied in lignin chemical composition. Our study has built upon previous laboratory studies by quantifying the effects of lignin chemistry on the decay rates of whole plant tissue in a natural ecosystem, as well as identifying the specific aspects of lignin structure that give rise to variation in rates of lignin accumulation in soils. Modified plants in our study had either high proportions of guaiacyl-type subunits or cinnamylaldehyde (“aldehyde”) subunits in lignin relative to the wild type (Fig. 1). Guaiacyl lignins are characterized by phenylcoumaran linkages and condensed linkages between aromatic rings (e.g. 5,5′ aryl–aryl structures) that are resistant to hydrolysis (Hedges et al. 1985). The more condensed, recalcitrant guaiacyl lignin could increase the physical protection of labile cell wall polysaccharides and protein from microbial attack. Therefore, we hypothesized that mutant plants with high amounts of guaiacyl lignin should have slow rates of litter decay compared to the wild type plant (which had high syringyl units) due to retention of lignin, polysaccharides, and N in decomposing tissues. The second plant type contained lignin rich in aldehyde units (Fig. 1), which typically have high levels of cross-coupling with other S and G units via β -0-4 bonds (Ralph et al. 2001). The high aldehyde plant also had higher levels of *p*-hydroxyphenyl-type lignin and resinol linkages in lignin (Fig. 1) compared to the other two plant types. Aldehyde-rich lignin is more hydrophobic and resistant to degradation compared to lignins polymerized from the typical monolignol units (Grabber et al. 1998c). Therefore, we expected lignin in this plant to be retained in decomposing tissue and offer more physical protection of labile cell wall compounds

compared to the wild type. However, plants with high aldehyde content and more resinol linkages have reduced lignin–polysaccharide cross-linking in the cell wall (Grabber et al. 1998c; Ralph et al. 2006a), which could decrease chemical protection of polysaccharides and protein and accelerate rates of total litter decay. As a result of these chemical differences between plant types, we predicted that total decomposition rates would decrease in the order wild type > high aldehyde content > high guaiacyl content.

Materials and methods

Study system: *Arabidopsis thaliana*

We tested the effects of lignin chemical composition on litter decay rates using lines of *Arabidopsis thaliana* that were manipulated to vary in lignin structural traits. The *Arabidopsis* plant types used included a wild type (Columbia ecotype) and two mutant plants, deficient in either a cytochrome P450-dependent monooxygenase or cinnamyl alcohol dehydrogenase (CAD). The cytochrome P450-dependent monooxygenase mutant was produced via chemical mutagenesis of the REF2 gene (Hemm et al. 2003). This mutant had high guaiacyl-type lignin and low syringyl-type lignin, resulting in a high ratio of guaiacyl-to-syringyl units compared to the wild type (Table 1). The CAD mutant was null-recessive for the *AtCAD-C* gene, produced via T-DNA insertion (Sibout et al. 2003). This modification resulted in elevated concentrations of cinnamylaldehydes in its lignin relative to the wild type (Table 1). Hereafter, we refer to the two modified lines as the high guaiacyl plant and the high aldehyde plant, respectively. The high guaiacyl plant was derived from the Columbia ecotype (Hemm et al. 2003) and the high aldehyde plant was isolated from the Wassilewskija ecotype (Sibout et al. 2003).

Arabidopsis plants were germinated from seeds provided by Dr. Clint Chapple (Purdue University) and Dr. Lise Jouanin (Institut national de la recherche agronomique). *Arabidopsis* seeds were stratified in deionized water for 24 h at room temperature (24°C) in the dark prior to planting in 3.5 inch square plastic nursery pots. Three seeds were sown per pot and then whole pots were stratified for an additional 4 days at 4°C in the dark before transfer to growth conditions.

Table 1 Lignin chemical structure in initial (undecomposed) stem tissue from *Arabidopsis thaliana* plants determined by quantitative 2D HSQC NMR

Plant type	Aromatic ring composition and alcohol: aldehyde ratios				Inter-unit linkages					
	% Syringyl (S)	% Guaiacyl (G)	% <i>p</i> -Hydroxyphenyl (H)	G:S	G:H	Alcohol: aldehyde	% β-O-4	% Phenylcoumaran	% Resinol	Phenylcoumaran: resinol
Wild type	21.6 (3.3)a	64.6 (0.5)b	10.6 (1.0)b	3.1 (0.5)b	6.1 (0.7)b	3.8 (0.2)a	85.5 (2.0)a	8.3 (1.5)b	6.5 (0.8)b	1.32 (0.07)b
High guaiacyl	9.2 (0.7)b	81.5 (1.5)a	9.0 (0.4)b	8.4 (0.6)a	9.1 (0.5)a	3.5 (0.1)ab	81.8 (0.7)a	12.2 (0.4)a	6.3 (0.4)b	1.94 (0.09)a
High aldehyde	21.0 (0.8)a	65.1 (0.7)b	14.0 (0.5)a	3.3 (0.2)b	4.6 (0.2)c	2.8 (0.2)b	81.6 (0.6)a	9.9 (0.8)ab	8.3 (0.06)a	1.19 (0.09)b

Standard errors are in parentheses

Significant differences ($P < 0.05$) among plant types are designated by different letters (Tukey groupings, $n = 3$)

Plants were cultivated in the UCI greenhouse for 7 weeks at 25–27°C under a 16 h light/8 h dark photoperiod in pots containing sphagnum peat-based growing medium (PRO-MIX PGX, Premier Horticulture, Québec, Canada). Every 7 days, pots were watered (by immersion of the base of the pots) in a solution containing potassium nitrate (3 mM), calcium chloride (0.5 mM), magnesium sulfate (0.25 mM), and sodium phosphate (0.25 mM). Pots were weeded 1 week after germination (2 weeks after stratification) to contain a single plant per pot. Approximately 3000 plants were cultivated to maturity (~1000 individuals/plant type). At the 7 week harvest date, stem tissue was isolated from each plant and dried at 60°C for 48 h. These plants did not reach senescence and therefore do not reflect litter produced by this plants. Nevertheless, we observed differences in lignin chemical composition with minor changes in other chemical components at the 7-week harvest date (Tables 1, 2), indicating that they could be used to isolate effects of lignin chemistry on decomposition rates. We measured surface area of a subsample (10 individuals/plant type) of oven-dried stems using a digital caliper prior to conducting the litterbag decomposition experiment. All stems of a plant type were pooled and subsamples were taken for litterbag construction and initial chemical analyses.

Study site

A litterbag decomposition experiment using the *Arabidopsis* plants was conducted in the mature black spruce (*Picea mariana* (P. Mill.) B.S.P) forest site described in Treseder et al. (2007). The forest is an upland boreal ecosystem in central Alaska, USA (63°55' N, 145°44' W). This site was chosen to test lignin chemistry controls over decay because of the critical need to understand controls over decomposition rates in northern ecosystems. Approximately 43% of the world's litter C is retained in the boreal zone (Schlesinger 1997), so small perturbations to litter decomposition rates in this region could strongly influence global carbon cycles (Goulden et al. 1998). Boreal systems are predicted to experience rapid and dramatic changes in climate over the next century (Soja et al. 2007), but whether or not decomposition rates will respond to these changes depends on the extent to which litter chemistry restricts microbial processing of litters in these areas.

Table 2 Chemical and physical characteristics of initial (undecomposed) stem tissue in *Arabidopsis thaliana* plants

Plant type	Total C ^a	Total N ^a	Lignin ^a	Cellulose ^a	Hemicellulose ^a	Soluble sugar ^a	C:N	Lignin:N	Stem surface area ^b
Wild type	414.7 (1.8)a	14.0 (0.6)b	93.5 (5.2)a	207.5 (17.0)a	96.7 (5.5)a	21.3 (3.2)a	29.7 (1.3)a	6.7 (0.2)a	0.55 (0.11)a
High guaiacyl	418.4 (0.8)a	15.1 (0.6)b	80.5 (2.9)a	224.6 (7.1)a	100.0 (5.2)a	24.9 (7.4)a	27.8 (1.1)ab	5.4 (0.3)b	0.52 (0.10)a
High aldehyde	415.8 (1.4)a	17.5 (0.2)a	81.7 (4.0)a	233.7 (9.2)a	104.9 (8.9)a	27.2 (3.6)a	23.8 (0.4)b	4.6 (0.2)b	0.59 (0.10)a

Standard errors are in parentheses

Significant differences ($P < 0.05$) among plant types are designated by different letters (Tukey groupings, $n = 3$)

^a Values are in units of $\mu\text{g mg stem}^{-1}$

^b Values are in units of $\text{cm}^2 \text{stem}^{-1}$

Although *Arabidopsis thaliana* does not occur naturally at this site, these plants are an ideal system to identify the potential for lignin chemistry to affect microbial decay processes. Using the *Arabidopsis* system in a litterbag decomposition experiment at this site allowed us to test the effects of lignin chemistry on the decomposition process in situ, by an intact microbial community, as would be expected to occur naturally at the site.

Soils at this site are gelisols and have an O-horizon thickness of 9.8 cm (King et al. 2002) with a soil pH of 4.9 (Allison and Treseder 2008). The vegetation understory is dominated by mosses, lichens, ericoid shrubs, and ectomycorrhizal shrubs (Mack et al. 2008). The growing season begins with bud break in mid-May and extends until leaf senescence in mid-September. The local climate is cold and dry, with a mean annual temperature of 2°C and a precipitation rate of 303 mm year⁻¹ (<http://weather.noaa.gov>). Concentrations of N in litter from this site range from 0.6 to 3% N (J. Randerson, unpubl. data). In a comparable site in interior Alaska, lignin content of foliar litter ranges from 6 to 13% (Yarie and Van Cleve 1996).

Litterbag decomposition experiment

Whole stems from each plant type were placed in 10 cm × 10 cm double-layered 1 mm mesh litterbags constructed from a single layer of nylon mesh inside a single layer of fiberglass mesh. One gram of stem tissue from a given *Arabidopsis* plant type was placed into a litterbag. To observe lignin chemistry

effects on natural decomposition processes by the local microbial community, litterbags were sterilized overnight via gamma-irradiation in the University of California-Irvine Medical Facility prior to deployment. Gamma irradiation is an effective, non-toxic sterilization technique (Howard and Frankland 1974) that has minimal effects on organic matter chemistry (e.g. phenolics, proteins, or total polysaccharides) compared to autoclaving, chemical fumigation, or cobalt-60 irradiation (Crocini et al. 1994; Trevors 1996; Wolf et al. 1989; Wu et al. 2004). Litterbags were affixed to the top of the forest floor on shaded, north-facing slopes at the field site to avoid photodegradation of the litter. Replicate bags of each plant type were placed in each of four plots, spaced 10 cm apart, resulting in a total of 12 litterbags deployed. All litterbags were deployed at the site in July 2008 and retrieved in July 2009. The retrieved litterbags were placed on ice, transported to the UCI campus, and freeze-dried within 48 h of retrieval time. Percent mass loss of litters was measured on fresh litterbag material and calculated as percent dry matter loss using the litter wet-weight to dry-weight ratios.

Chemical analyses of initial and decomposed litters

Initial and decomposed stem tissue samples were processed for total C and N, hemicellulose, cellulose, and lignin losses. Initial samples were also analyzed for soluble sugar content. For these analyses, freeze-dried samples were ground for 5–10 min on a Spex SamplePrep 8000D mixer/mill (Spex SamplePrep

LLC, New Jersey) using stainless steel vials and grinding media. Total C and N were determined on freeze-dried samples by combustion on an elemental analyzer (Flash EA 1112, Thermo Scientific, Waltham, MA). Samples were then fractionated following the International Association of Analytical Communities (AOAC International) official Uppsala method (Theander et al. 1995). Briefly, lipids/waxes/pigments were extracted (2×) with 100% petroleum ether, solubles were extracted (4×) with a 80:20 ethanol:water solution, and starch was removed by enzyme treatment (α -amylase followed by α -glucosidase) followed by washing with 95% ethanol and 100% acetone. Total soluble sugar contents were measured colorimetrically in the ethanol:water extract by the phenol–sulfuric acid method (Buysse and Merckx 1993). Together, the total lipid/wax/pigment and total starch components accounted for less than 3% total dry mass of the tissue, and so were not individually quantified for each *Arabidopsis* plant type.

Concentrations of lignin, cellulose, and hemicelluloses in the lipid-free, soluble-free, starch-free cell walls were analyzed by standard spectrophotometric methods. Total lignin content was determined by the acetyl bromide method (Hatfield et al. 1999; Iiyama and Wallis 1990), in which lignin is solubilized in a 1:4 acetyl bromide:acetic acid solution and quantified by measuring absorbance at 280 nm. Monterey pine reference material (NIST 8943) was used as a standard. Total cellulose content of cell walls was determined by the Updegraf method (Updegraf 1969), which involves removing hemicelluloses and lignin from the cell wall material by extraction with acetic acid/nitric acid, followed by solubilization of cellulose in 67% sulfuric acid. The released glucose is quantified by reaction with anthrone in sulfuric acid and the colored product is quantified by measuring absorbance at 620 nm. Avicel cellulose (FMC Biopolymer) was used as a standard. Total hemicellulose content was assayed in acetic/nitric extracts of the Updegraf procedure via an anthrone method similar to the Updegraf method (Hansen and Møller 1975). The hemicellulose calibration standard was prepared as a 10:7.5:7.5:7.5:7.5:5:5 mixture of glucose, xylose, arabinose, mannose, galactose, fucose, and rhamnose following the AOAC official hemicellulose analysis protocol (Theander et al. 1995). For initial samples, results are the means of triplicate measurements of a single pool of stems from each plant type. For final

samples, results are the means of 2–4 replicate litterbags for each plant type.

NMR experiments

To determine the chemical structure of the lignin polymer in each *Arabidopsis* plant type, we performed quantitative 2D heteronuclear single quantum coherence (HSQC) NMR spectroscopy on milled cell walls of the plants. In HSQC spectra, each single proton correlates with its directly attached carbon atom (Marita et al. 1999). This allows signals to be well dispersed (owing to the ^{13}C dimension) and chemical shift assignments to be made more accurately compared to traditional 1D spectra. The cell wall component used for the NMR experiments was isolated from the *Arabidopsis* tissue by successive extraction with 100% petroleum ether, 80:20 ethanol:water solution, and 100% acetone at room temperature to avoid heating-induced modifications to cell wall chemical structures (Ramiah 1970). Cell wall preparation for NMR experiments followed the procedure outlined in Yelle et al. (2008a). Approximately 100–190 mg of isolated cell wall sample was placed into a 10 ml yttrium-stabilized ZrO_2 vial along with yttrium-stabilized ZrO_2 grinding media (15 × 5-mm balls, 15 × 3-mm balls, and approximately 30 × 1-mm balls) and milled in a Spex SamplePrep 8000D mixer/mill programmed for 14 h (10 min pause every 10 min milling), giving an actual milling time of 7 h. Vial temperature remained below 60°C (Takacs and McHenry 2006). Milled cell wall (90 mg) was dissolved in 0.5 ml mixture of 4:1 (v/v) DMSO- d_6 :*N*-methyl imidazole- d_6 , stirred overnight, centrifuged for 1 h at 15,000 rpm, and the clear, dark brown-colored supernatant was transferred to a 5 mm NMR tube. Centrifugation removed <1 mg solid material from solution. The solid was bright white and similar in consistency to the ZrO_2 grinding media—no gel-like cell wall material was detected in the precipitate. Peak resolution was markedly improved in the 1H dimension after centrifugation (data not shown).

2D short-range ^{13}C - ^1H NMR spectra (gradient-selected HSQC) were acquired at 25°C on a Bruker DRX500 (^1H 500.13 MHz, ^{13}C 125.76 MHz) equipped with a TCI (three channel inverse) cryoprobe. The central solvent peak, DMSO (δ_{C} 39.5, δ_{H} 2.50 ppm), was used as an internal reference. An adiabatic pulse sequence (hsqcetgpsisp2.2) of a gradient-selected

sensitivity-improved inverse (^1H -detected) heteronuclear single quantum coherence (HSQC) experiment was used for acquiring the 2D spectra. The phase-sensitive HSQC spectra were determined with an acquisition time of 157 ms using an F_2 spectral width of 6510 Hz (13 ppm) in 2048 data points using 88 transients (16 dummy scans) for each of 512 t_1 increments of the F_1 spectral width of 27, 470 Hz (222 ppm). Processing used a Gaussian function for F_2 ($\text{LB} = -0.18$, $\text{GB} = 0.005$) and a sine squared function for F_1 prior to 2D Fourier transformation. The resulting data matrix was 1024×1024 points. ^{13}C -decoupling during acquisition was performed by GARP composite pulses from the high-power output decoupling channel. HSQC experiments were made quantitative by including a 1-s interscan delay and setting the d_{24} delay to 1.72 ms (Ralph et al. 2006b). Total acquisition time for each experiment was 5 h 24 min.

Initial spectra processing was performed using XWinNMR 3.5 software and peak integration calculations were conducted using MestReNova 6.1.1 software. Resonance assignments were confirmed by reference to the literature (Kim et al. 2008; Rencoret et al. 2009; Yelle et al. 2008a; Yelle et al. 2008b) and the “NMR data base of lignin and cell wall model compounds” (Ralph et al. 2005). Quantification of lignin inter-unit linkages, aromatic ring structures, and alcohol:aldehyde ratios were determined by automatically integrating selected cross-peaks in the HSQC spectra (Rencoret et al. 2009; Yelle et al. 2008a; Zhang and Gellerstedt 2007). The relative abundance of guaiacyl (G), syringyl (S), and *p*-hydroxyphenyl (H) units in lignin was determined by integrating well-resolved G-2, S-2/6, and H-2/6 resonances in the aromatic region, respectively (Fig. 4). The proportion of dominant inter-unit linkages in lignin, including β -O-4, phenylcoumaran, and resinol inter-unit linkages, were estimated from C_α – H_α correlations of each structure in the aliphatic region (not shown). Finally, the relative abundance of aldehyde and alcohol end units in lignin were estimated from C_γ to H_γ correlations of aldehyde and alcohol structures in the ketone/aldehyde region (9.2/187–9.8/197 ppm).

Data analysis

To test for differences in initial litter chemistry characteristics among plant types, we conducted a

one-way ANOVA with plant type as fixed effect (high guaiacyl plant, high aldehyde plant, and wild type) and the chemical components of stem tissue as dependent variables (concentrations of lignin, cellulose, hemicellulose, soluble sugar, total N, total C, lignin monomer units, and lignin inter-unit linkages). Total % mass loss and % loss of litter chemical components were analyzed by one-way ANOVA with plant type as main effect and % mass loss as the dependent variables. We calculated % change in lignin monomer units and inter-unit linkages during decay of each litter type as follows: [(Percent structure in decomposed tissues) – (Percent structure in un-decomposed tissues)]/(Percent structure in un-decomposed tissues)*100. An independent one-sample *t*-test was used to determine if these values were significantly different from zero. Where necessary, data were log-transformed to meet assumptions of normality and homogeneity of variance. Comparisons among means were analyzed by Tukey HSD Post-hoc contrasts. All data were analyzed using SAS statistical software (SAS v. 9.2, SAS Institute, Inc, 2008).

Results

The three *Arabidopsis* plant types used in this study varied in the chemical structure of lignin in stem tissue (Table 1), in terms of the types of monolignol subunits incorporated into lignin and the types of inter-unit linkages in the lignin polymer. 2D NMR experiments of un-decomposed stem tissue indicated that the wild type *Arabidopsis* had lignin typical of angiosperms (Boerjan et al. 2003) with high syringyl content and a high abundance of the dominant β -O-4 linkages in the lignin polymer (Table 1). By contrast, the high guaiacyl mutant had lignin with high guaiacyl content and low syringyl content, leading to significantly higher G:S ratios compared to the other two plant types ($P < 0.0001$). This plant also had a higher proportion of phenylcoumaran linkages in its lignin compared to the wild type and the high aldehyde plant ($P = 0.034$). Finally, the high aldehyde mutant had lignin with a significantly lower ratio of alcohol:aldehyde units compared to the other plant types ($P = 0.044$). In addition, the lignin of this plant type had high amounts of relatively rare *p*-hydroxyphenyl units ($P = 0.0060$) and resinol-type linkages ($P = 0.016$) compared to the other two plant

types. There was a significant difference among plant types in initial guaiacyl-to-*p*-hydroxyphenyl ratios ($P < 0.0001$) and initial phenylcoumaran-to-resinol ratios ($P < 0.0001$), such that these ratios increased in the order high aldehyde plant < wild type < high guaiacyl plant (Table 1).

Despite differences in lignin chemistry, the three plant types were similar to one another in other chemical and physical characteristics (Table 2). There were no significant differences between the plants in initial concentrations of lignin, cellulose, hemicelluloses, or soluble sugar in stem tissue, or in the surface area of stem tissue. The plant types varied in N content ($P = 0.001$), particularly between the wild type and high aldehyde mutant, which differed by $3.5 \mu\text{g N per mg stem tissue}$.

Plant type had a significant effect on total % mass loss of the litters. The high aldehyde plant had higher % mass loss than the other two plant types ($P = 0.0006$), while the high guaiacyl plant showed a % mass loss similar to the wild type plant (Fig. 2). The high aldehyde plant had greater % loss of cellulose, hemicelluloses, and N during decay compared to the other two plant types (Fig. 2). The high guaiacyl plant and the wild type did not differ significantly in % loss of these components.

Lignin degradation also varied significantly among plant types ($P = 0.020$). Percent lignin loss increased in the order high guaiacyl plant < wild type < high aldehyde plant (Fig. 3). This pattern was associated

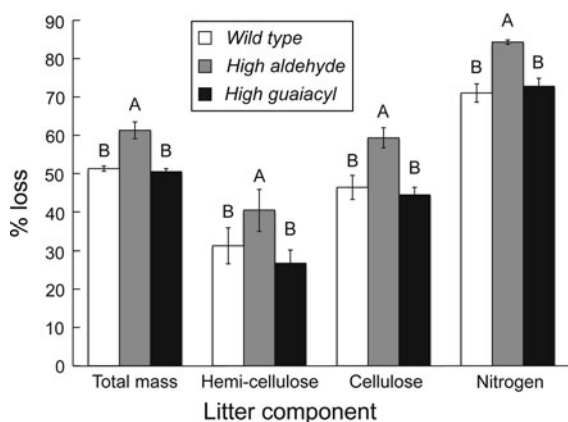


Fig. 2 Loss of total mass, lignin, hemicelluloses, cellulose, and N of stem tissue from different *Arabidopsis thaliana* plant types decomposed for one year in an Alaskan boreal forest. Bars are means ± 1 SE, $n = 2-4$. Letters represent Tukey groupings ($P < 0.05$)

with the initial ratio of guaiacyl-to-*p*-hydroxyphenyl units in lignin, such that plants with a high ratio had lower % lignin loss during decomposition (Fig. 3). Guaiacyl-based structures and *p*-hydroxyphenyl-based structures differed substantially in their resistance to degradation (Fig. 4). 2D NMR of the decomposed litters showed that across all plant types, *p*-hydroxyphenyl units declined by an average of 42% ($P = 0.0003$) and resinol linkages in lignin declined by an average of 32% ($P = 0.0022$) during decomposition (Table 3). By contrast, guaiacyl units increased by an average of 9% ($P = 0.0041$). This caused the guaiacyl-to-*p*-hydroxyphenyl ratio to increase significantly from an average of 6.6 in undecomposed litters to an average of 12.4 in decomposed litters ($P = 0.0068$). Guaiacyl-based phenylcoumaran linkages declined by 24% over the year of decay (Table 3). The ratio of guaiacyl-to-syringyl

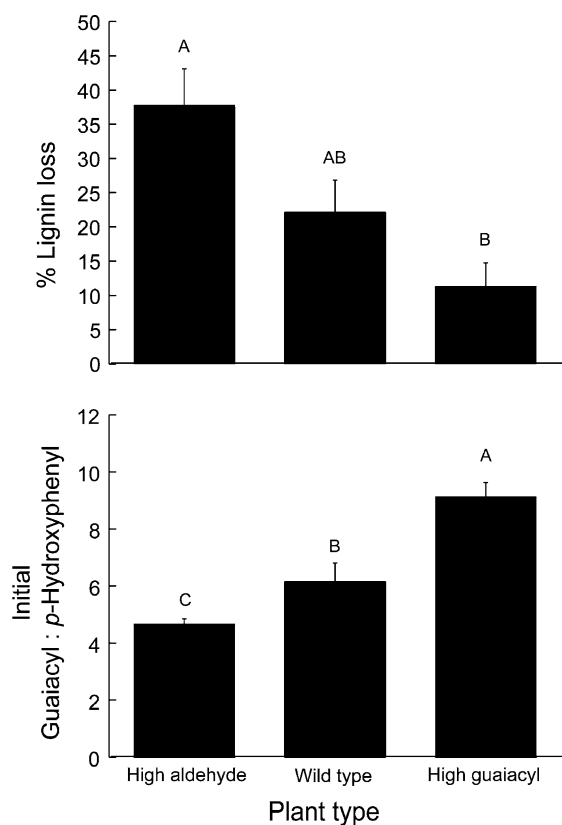


Fig. 3 Percent lignin loss after one year of decay (a) and initial ratio of guaiacyl-to-*p*-hydroxyphenyl units (b) of different *Arabidopsis thaliana* plant types. Bars are means ± 1 SE, $n = 2-4$. Letters represent Tukey groupings ($P < 0.05$)

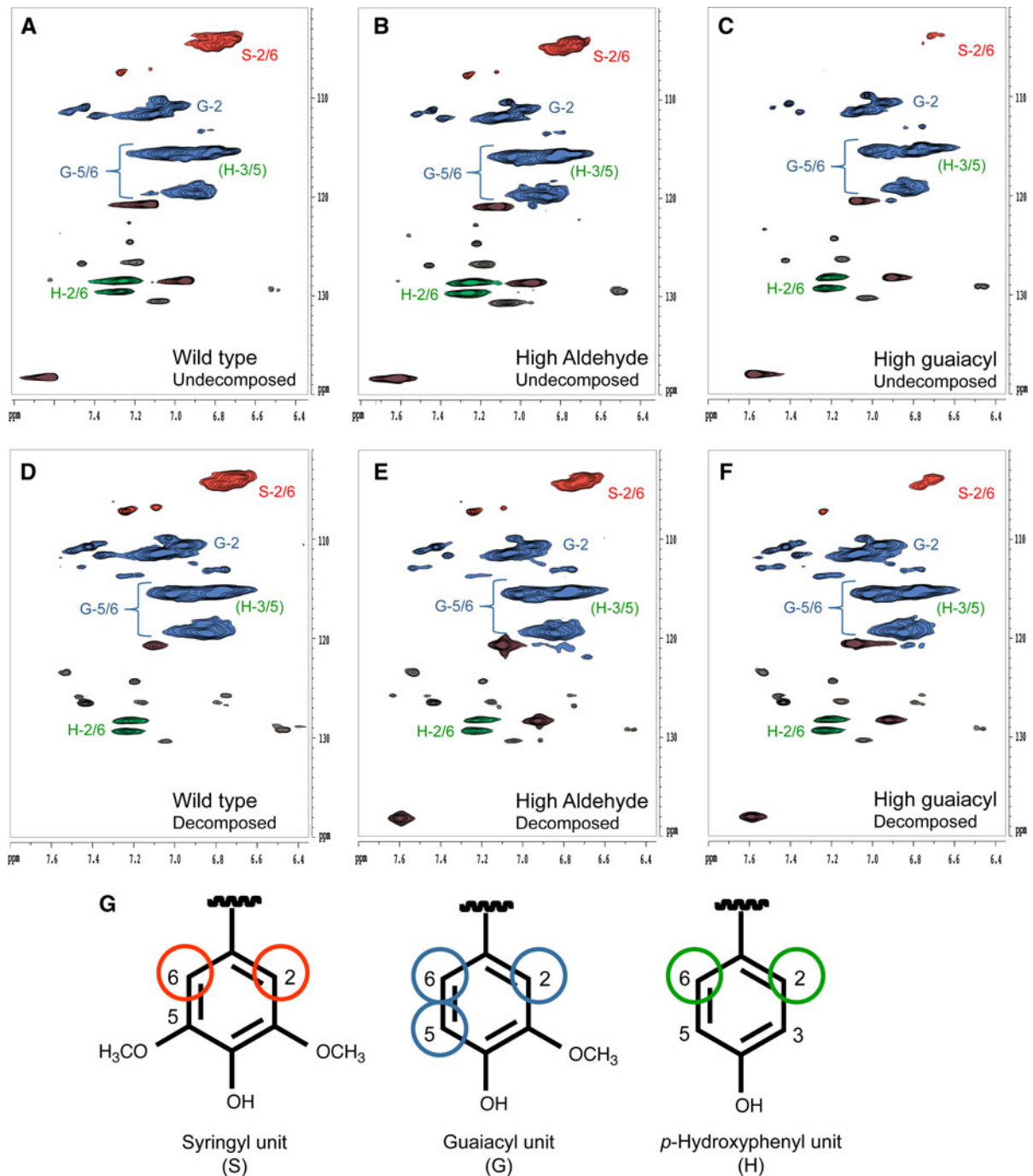


Fig. 4 Aromatic region of the whole plant cell wall HSQC spectra: **a** wild type un-decomposed; **b** high aldehyde plant un-decomposed; **c** high guaiacyl plant un-decomposed; **d** wild type decomposed; **e** high aldehyde plant decomposed; **f** high guaiacyl plant decomposed. The guaiacyl, syringyl, and *p*-hydroxyphenyl contours are a combination of free phenolic and etherified aromatic units. Contour colors and labels can be

matched to their respective structure (**g**). Brown contours are DMSO-*d*₆: *N*-methyl imidazole-*d*₆ solvent, gray contours are unassigned. Assignments are based on previous HSQC studies of whole plant cell walls (Kim et al. 2008; Rencoret et al. 2009; Yelle et al. 2008a; Yelle et al. 2008b) and model compound data in the NMR database of Lignin and Cell Wall Model Compounds (Ralph et al. 2005)

Table 3 Lignin chemical structure in decomposed stem tissue from *Arabidopsis thaliana* plants determined by quantitative 2D HSQC NMR

Plant type	Aromatic ring composition and alcohol:aldehyde ratios					Inter-unit linkages				Phenylcoumaran:resinol
	% Syringyl (S)	% Guaiacyl (G)	% <i>p</i> -Hydroxyphenyl (H)	G:S	G:H	Alcohol:aldehyde	% β -O-4	% Phenylcoumaran	% Resinol	
Wild type	22.35 (1.95)a	71.09 (2.0)b	6.6 (0.9)a	3.2 (0.4)b	11.4 (2.0)a	1.5 (0.1)a	87.7 (1.1)a	7.3 (0.6)ab	5.0 (0.5)a	1.46 (0.08)b
High guaiacyl	9.46 (1.42)b	84.4 (2.1)a	6.1 (0.6)a	9.2 (1.6)a	14.0 (1.8)a	1.2 (0.1)a	85.8 (1.3)a	9.7 (0.5)a	4.5 (0.8)a	2.19 (0.3)a
High aldehyde	19.91 (0.53)a	74.1 (0.7)ab	6.0 (0.1)a	3.7 (0.1)b	12.4 (0.4)a	1.1 (0.1)a	90.4 (1.4)a	5.4 (0.7)b	4.2 (0.7)a	1.29 (0.05)b
% loss of structure ^a	-0.9 (6.0)	-9.3 (2.4)*	42.0 (6.5)*	-10.7 (8.3)	-105.0 (30.7)*	50.9 (3.2)*	-5.6 (1.8)*	24.5 (7.8)*	32.2 (7.5)*	-10.5 (4.6)*

Standard errors are in parentheses

Significant differences ($P < 0.05$) among plant types are designated by different letters (Tukey groupings, $n = 2-4$)

^a Asterisks indicate significant ($P < 0.05$) loss or accumulation of chemical structure across all plant types after 1 year of decay ($n = 10$)

units in lignin did not change significantly during decomposition ($P = 0.1769$).

Discussion

The resistance of lignin to decomposition—and its ability to physically and chemically protect more labile litter components from degradation—are mechanisms that have long been invoked to explain correlations between lignin concentrations and decay rates (Alexander 1977). We found that lignin effects on rates of total litter decay in the field were determined by the chemical composition of lignin. Total litter decay rates were highest in the high aldehyde plant, which lost significantly more cell wall polysaccharides and N during decay compared to the other plant types (Fig. 2). In addition to aldehydes, this plant type had an increased abundance of relatively rare resinol linkages in its lignin. As neither aldehydes nor resinol structures form stable cross-links to the polysaccharide cell wall matrix (Grabber et al. 1998c; Ralph et al. 2006a), these results are consistent with the hypothesis that lignin controlled litter decay rates by chemically preventing hydrolysis of labile litter components by extracellular enzymes.

Physical protection of cell wall polysaccharides appeared to play less of a role in decay of the *Arabidopsis* plants than chemical protection. Guaiacyl-rich lignins are more resistant to oxidation and hydrolysis compared to syringyl-rich lignins (Boerjan et al. 2003), yet the high guaiacyl plant in our study lost polysaccharides and N at a rate similar to the wild type (Fig. 2). We also found that the retention of lignin in decaying tissues did not have a major effect on total mass loss. The high aldehyde plant had significantly higher % mass loss than the wild type, even though % lignin loss was not significantly different between these plants (Fig. 3). These results imply that the retention of lignin in decaying tissues and the physical protection lignin offers to polysaccharides and protein may be less influential in controlling total litter decay rates in this ecosystem compared to chemical protection of these labile litter components.

Our observation that chemical protection of polysaccharides is an important control over total decomposition rates is consistent with the results of other

studies that have used model plant systems (Grabber et al. 1998a; Grabber et al. 1998b; Grabber et al. 2009). In a recent experiment with chemically modified maize cell walls, Grabber et al. (2009) found that a reduction in lignin–polysaccharide cross-linking of 85% resulted in a 35% increase in polysaccharide fermentation rate by rumen microbes. The authors estimated that lignin–polysaccharide cross-linking accounts for over 50% of the inhibitory effect of lignin on cell wall degradation. Other laboratory studies have found that plants deficient in the enzyme cinnamyl alcohol dehydrogenase, similar to high aldehyde plant of the current study, have up to 50% greater cell wall degradability compared to unmodified plants (Baucher et al. 1999; Bernard Vailhé et al. 1998; Provan et al. 1997; Thorstenson et al. 1992; Webster et al. 2005). Hénault et al. (2006) found that when decomposed in soil microcosms, these cinnamyl alcohol dehydrogenase-deficient plants support higher biomass of decomposer fungi compared to unmodified plants or plants modified in other aspects of lignin chemistry. If these plants are similar in chemical composition to the high aldehyde plant in our study, low levels of lignin–polysaccharide cross-linking may contribute to elevated rates of total litter decay observed for these plants. By contrast, litter decay rates are typically insensitive to altering the ratios of syringyl- and guaiacyl-type subunits in lignin of model plants when used in laboratory decomposition studies (Bertrand et al. 2006; Grabber 2005; Grabber et al. 2009; Jung et al. 1999; Li et al. 2010; Machinet et al. 2009).

Lignin degradation and the loss of polysaccharides and N during decomposition appear to have been controlled by different properties of the lignin polymer. Polysaccharide and N losses were highest in the high aldehyde plant (Fig. 2), even though aldehyde subunits in lignin are more resistant to decay than alcohol subunits (Grabber et al. 1998c) and they accumulate in the lignin of decomposing plant tissues (Table 3). Lignin degradation, on the other hand, was not correlated with aldehyde content. Instead, lignin loss was negatively related to the initial ratio of guaiacyl structures-to-*p*-hydroxyphenyl structures in lignin (Fig. 3). Guaiacyl-type lignin and *p*-hydroxyphenyl-type lignin vary in their susceptibility to microbial attack; *p*-hydroxyphenyl units were preferentially lost during decay, while the relative abundance of guaiacyl units in lignin

increased (Fig. 4). This led to a large increase in the guaiacyl-to-*p*-hydroxyphenyl ratio of the *Arabidopsis* litter after decomposition (Table 3). Our results are consistent with a number of other studies that have reported an increase in guaiacyl units during decomposition of natural litter (Bertrand et al. 2006; Huang et al. 1998), during wood decay by white and brown-rot fungi (Haider et al. 1964; Hedges et al. 1988; Kirk et al. 1975; Nilsson and Daniel 1989; Tai et al. 1983), and during later stages of litter decay (Bahri et al. 2006; Chefetz et al. 2000; Dümig et al. 2009; Kögel 1986; Nierop and Filley 2007; Nierop et al. 2001; Opsahl and Benner 1995; Zech et al. 1997). However, several studies have shown the opposite trend during the early stage of litter decay—guaiacyl units are lost preferentially while the litter is still structurally intact (Christman and Oglesby 1971; Kögel 1986). The observation that guaiacyl-type lignin is lost quickly during initial stages of decay, but then is lost more slowly as decay progresses, has deterred the use of the guaiacyl-to-*p*-hydroxyphenyl ratio as an indicator of lignin degradation (Thevenot et al. 2010).

Variability in the loss of guaiacyl units during decay could be explained by differential stability of the various linkages formed by these units. In addition to β -O-4 linkages (Fig. 1), guaiacyl-type units also polymerize to form aryl–aryl linkages and phenylcoumaran linkages (Adler 1977), which differ dramatically in decomposability. Aryl–aryl linkages are resistant to decay—they tend to increase in abundance during wood decay (Goni et al. 1993) and during transformation of fresh litter into soil organic matter (Goni and Hedges 1992; Otto and Simpson 2006). By contrast, we found that phenylcoumaran linkages formed from guaiacyl units decreased in abundance during litter decay (Table 3). Depolymerization of phenylcoumaran linkages may drive the rapid loss of guaiacyl-type lignin during the early stage of litter decay, while accumulation of aryl–aryl linkages could contribute to the stability of guaiacyl-type lignin in litter over time.

Loss of *p*-hydroxyphenyl units was also driven by the lability of linkages that these units form in the lignin polymer. *P*-hydroxyphenyl units were correlated with high levels of resinol linkages in lignin (Table 1), consistent with observations in other plants (Ralph et al. 2006a; Ralph et al. 2006b). We found that across all plant types, over 30% of the resinol

linkages were lost during the year of decay, more than any other type of interunit linkage (Table 3). Increases in the ratios of aryl–aryl:resinol and aryl–aryl: β -O-4 linkages have also been reported to occur during decomposition of wood (Goni et al. 1993) and natural litter (Dignac and Rumpel 2006; Goni and Hedges 1992; Otto and Simpson 2006). These relationships indicate that the recalcitrance of interunit linkages in the lignin polymer formed by syringyl, guaiacyl, or *p*-hydroxyphenyl units is the primary mechanism underlying correlations between the abundance of these units and rates of lignin degradation. Other studies have reported that plants with modified expression of cinnamylalcohol dehydrogenase, like the high aldehyde mutant in this study, have lignin that is more easily extracted by alkali solution compared to unmodified plants (Halpin et al. 1994; Vaill   et al. 1998). If these mutant plants also have higher levels of *p*-hydroxyphenyl-type lignin compared to unmodified plants, higher alkali lability of their lignin could be due to the rapid degradation of resinol linkages formed by *p*-hydroxyphenyl units.

The susceptibility of various linkages in the lignin polymer to degradation supports recent advances in our understanding of lignin dynamics during later stages of soil development. Polymeric lignin typically decreases in abundance with soil depth and is often not present in fine particle size fractions of soil organic matter (Thevenot et al. 2010). In addition, numerous studies on the kinetics of lignin and soil organic matter degradation indicate that lignin fragments turn over more rapidly than bulk soil organic carbon in natural soils (reviewed in Thevenot et al. 2010). Our observation that the majority of lignin linkages are degraded during plant litter decomposition (Table 3) supports the new paradigm of soil development, in which lignin chemical recalcitrance is primarily important during the early-to-mid stages of pedogenesis while other C stabilization mechanisms like aggregation, mineral sorption, and substrate limitation become more important at later stages (Marschner et al. 2008).

In addition to differences in lignin chemistry among the *Arabidopsis thaliana* plants, the high aldehyde plant contained more N than the high guaiacyl and wild type plants (Table 2). Therefore, there may have been a positive influence of elevated N and lower C:N and lignin:N ratios on decomposition

rate of the high aldehyde plant. However, N differences between these plants were small (3.5 $\mu\text{g N/mg}$ stem tissue) and natural plants with this level of variation in N content often have similar rates of litter decay (e.g., Hobbie 2005; Melillo et al. 1982). Litter decay rate coefficients (*k*) correlate negatively with C:N ratio ($k = -0.0079 (\text{C:N}) + 1.2049$) and lignin:N ratio ($k = -0.0125 (\text{lignin:N}) + 1.0958$) across a wide range of natural plant species (Zhang et al. 2008). Based on these relationships, differences in initial C:N and lignin:N between plants in our study explain a small portion of variation in total % mass loss among the plants; together, differences in C:N and lignin:N account for a 4% increase in % mass loss of the high aldehyde plant compared to the wild type, out of the total 20% increase that we observed (Fig. 2). Furthermore, in an independent litterbag study, we found that wild type *Arabidopsis* plants that varied in N by this same amount, without detectable differences in other chemical characteristics, had equal amounts of mass loss during one year of decomposition in the field (J. Talbot, unpublished data). Based on these observations, it is likely that the decay of plants in the current study was driven primarily by differences in lignin chemistry, with minor influences of N content, C:N, or lignin:N ratios.

Differences in lignin chemical composition among the *Arabidopsis* plant types are within the range of variation found among natural plant species (Goni and Hedges 1992). Therefore, it is possible that variation in rates of litter decomposition among other plants might be explained in part by differences in their lignin chemical composition. Plant species vary in the relative amount of subunits and linkages in lignin (Hedges and Mann 1979) and this variation could be an important factor determining decay rates if differences in subunit composition result in more or fewer lignin–polysaccharide cross-links in the cell wall. For example, ferulic acid units often cross-link between lignin and hemicellulose units in grasses (Kato and Nevins 1985) and concentrations of ferulic acid in the cell wall can vary 25-fold among grass species (Cherney et al. 1989). This variation could lead to large differences in the chemical protection of structural polysaccharides in the cell wall and rates of total litter mass loss among grass species.

Similarly, differences among plants in the relative amount of labile and recalcitrant inter-unit linkages in lignin could account for species-specific differences

in lignin degradation during litter decay. The major inter-unit linkages in lignin vary among species, genera, and orders of plants, as well as among different types of plant vascular tissues (Goni and Hedges 1992; Yelle et al. 2008a). This variation correlates with rates of lignin degradation in the litter of different types of natural plants. Higher levels of lignin accumulate in soil organic horizons under gymnosperm species, which have primarily guaiacyl-type lignin that is rich in recalcitrant aryl–aryl linkages, compared to angiosperm trees that contain syringyl-type lignins dominated by more labile β -O-4 linkages (Goni and Hedges 1992; Kögel 1986). In fact, aryl–aryl linked lignin structures have been reported to accumulate during soil development in boreal forests (Otto and Simpson 2006), agricultural fields (Dignac and Rumpel 2006), and temperate lakes (Goni and Hedges 1992), as well as in wood samples degraded by white rot fungi (Goni et al. 1993). Contributions of aryl–aryl bound structures to more stable organic matter pools imply that the relative abundance of inter-unit linkages in fresh plant litter could be used to predict rates of C loss or accumulation during early stages of soil organic matter development under different plant communities.

Conclusion

Litter decomposition studies have primarily focused on using large, sometimes nebulously defined classes of compounds (i.e. lignin, cellulose, protein) as predictors of litter decay rates in a variety of ecosystems. Our study demonstrates that litter chemistry at a finer resolution, within a compound class (i.e. lignin), can also give rise to variation in total litter decay on an ecosystem-level. We found that lignin chemistry influenced total litter decay rates through its effects on chemical protection of more labile cell wall polymers (i.e. cellulose, hemicellulose, N), with lesser effects of physical protection or the retention of lignin in decaying tissues. Lignin chemistry also impacts lignin degradability through the resistance of interunit linkages to decay. Multiple mechanisms of lignin control over litter decomposition rates may explain why measures of litter lignin content are consistently correlated with litter decay rates around the globe. The use of new analytical

tools to identify additional structural variation in lignin or other litter compounds will provide more insight into how fine-scale litter chemistry influences the mechanisms of decomposition and how they vary by plant type and ecosystem.

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