J Wood Sci (2007) 53:314–319
DOI 10.1007/s10086-006-0867-3

ORIGINAL ARTICLE

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The monomer composition controls the Σ*β*-*O*-4/Σ*O*-4 end monomer ratio of the linear lignin fraction

Received: June 16, 2006 / Accepted: October 13, 2006 / Published online: March 31, 2007

Abstract Lignins are cell wall phenolic heteropolymers that result from the oxidative coupling of three monolignols bearing *p*-coumaryl (H), coniferyl (G), and sinapyl (S) units, in a reaction mediated by peroxidases. Here, we report the existence of a relationship between the Σ*β*-*O*-4/Σ*O*-4 end monomer ratio of the linear lignin fraction, released through the specific cleavage of the alkyl ether linkages by thioacidolysis, and the G/S ratio of lignins, when this was estimated in differentially evolved vascular land plants. Most importantly, in the case of angiosperms, Gnetales, and lycopods, the Σ*β*-*O*-4/Σ*O*-4 end monomer ratio was apparently predictable from the proportions at which the G and S units were mixed. In the case of G lignins (present in basal gymnosperms and ferns), the Σ*β*-*O*-4/Σ*O*-4 end monomer ratio decayed exponentially to increase the *O*-4-linked dihydroconiferyl alcohol (DHCA) content. The results obtained suggest that the Σ*β*-*O*-4/Σ*O*-4 end monomer ratio of the linear lignin fraction depends intimately on the lignin monomer composition, and, therefore, on the chemical nature of the radicals derived from three monolignols (coniferyl, dihydroconiferyl, and sinapyl alcohols), whose gain have been finely tuned during land plant evolution.

Key words *β*-*O*-4 Lineal fraction · DHCA · Lignin · Σ*β*-*O*-4/Σ*O*-4 End monomer ratio · Syringyl moieties

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Introduction

Lignins are phenolic heteropolymers that result from the oxidative coupling of the three *p*-hydroxycinnamyl alcohols (monolignols), *p*-coumaryl, coniferyl, and sinapyl alcohols, in a reaction mediated by peroxidases.[1] The cross-coupling reaction produces an optically inactive hydrophobic heteropolymer[2] composed of *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units, respectively. These phenylpropanoid units are interconnected by means of a series of ether and carbon–carbon linkages,[2] which lead to the main substructures of guaiacylglycerol-*β*-aryl ether, phenylcoumaran, diarylpropane, resinol, biphenyl, and diphenyl ether, as well as other substructures of lesser importance. The most frequent *β*-*O*-4 bonds are present in guaiacylglycerol-*β*-aryl ether substructures, which are the targets of lignin depolymerization processes, such as thioacidolysis.[2] In contrast, the other interunit bonds, such as *β*-5 (in phenylcoumaran), *β*-1 (in diarylpropane), *β*-*β* (in resinol), 5-5 (in biphenyl) and 5-*O*-4 (in diphenyl ether), are very resistant to chemical degradation.

Lignins represent the most abundant organic compound on the earth's surface after cellulose, accounting for about 25% of plant biomass.[3] Lignins are found specifically in vascular plants (tracheophyta) and occur selectively in greatest quantity in the secondary cell walls of particular cells that form parts of woody tissues, such as fibers, xylem vessels, tracheids, and sclereids. Lignins have been identified in pteridophytes (ferns, lycophytes, and horsetails), widely considered to be the first vascular plants, and are likely to have played a key role in the colonization of the terrestrial landscape by plants during the Ordovician to Silurian transition, 400 to 450 million years ago.[4] Thus, and from the botanical standpoint, the phenomenon of lignification is essentially associated with the acquisition of the vascular structure by plants.

The most distinctive variation in lignin monomer composition in vascular plants is that found between the two main groups of seed plants. In gymnosperms (softwoods), lignins are typically composed of G units, with a minor

proportion of H units, while in angiosperms (hardwoods) lignins are mainly composed of similar levels of G and S units.[5] In grasses,[2] lignins are more complex, because they also contain significant amounts of ester-bound *p*-coumaric acid. In the light of this scenario, it may be concluded that the chemical complexity of lignins has increased during the course of plant evolution from ancient pteridophytes and gymnosperms to the most evolved grasses.

The three-dimensional structure of lignin and its ultrastructural assembly within the polysaccharidic matrix of the plant cell wall remain poorly elucidated and are subjects of controversy in wood science.[6] This is mainly because any gain in our knowledge is severely hindered by the fact that the extraction and isolation of lignin requires chemical treatments that alter its native structure. In spite of this, recent evidence has pointed to the existence of a certain level of coherence in the ultrastructure of lignin in native woody tissues, where electrospray ionization mass spectrometry analyses have revealed the presence of certain repeatable blocks, such as S-(*β-O*-4′)-S and S-(*β-O*-4′)-S-(*β-β*′)-S,[7] as a probable consequence of an ordered and controlled process of assembly during lignin deposition.

The polymerization step, as it is known,[6,8,9] could in part explain some of these constraints. If substrates (monolignols and H_2O_2) are delivered to xylem cells by the neighboring xylem parenchyma cells, as appears probable,[10] one may expect the existence of a diffusion gradient of lignin building blocks from the plasma membrane of xylem parenchyma cells to the secondary cell walls of xylem vessels. This concentration gradient, which also occurs for peroxidase,[11] produces two clearly defined topographic zones in lignifying cell walls: one (characterized by high levels of peroxidase and a generous supply of diffusing substrates) located in the primary cell wall of xylem parenchyma cells and associated xylem vessels, and the other (characterized by low levels of peroxidase and a low rate of supply of diffusing substrates) located beyond the primary cell walls, that is, in the secondary cell wall thickenings of xylem vessels. In vitro (cell-free) assays suggest that this dual topography produces two types of polymerization: one fast (in the primary cell wall) and the other slow (in secondary cell walls).[11] Rapid "bulk" polymerization, as probably occurs in the middle lamella and primary cell walls, favors the C–C coupling of monolignols into highly branched three-dimensional polymers, rich in *β*-5, *β*-1, *β-β*, 5-5, and 5-*O*-4 interunit bonds; meanwhile, gradual "end-wise" polymerization, as may occur in secondary cell walls, favors *β-O*-4 coupling of monolignols into relatively linear polymers.[12]

That these two types of polymerization probably occur in lignifying cell walls is supported by both in situ studies and theoretical (modeling) calculations. Thus, lignins rich in H and G residues are mainly deposited in primary cell walls and are strongly cross-linked,[13,14] while lignins rich in G and S residues are mainly deposited in secondary cell walls, and the polymer formed is a linear polymer in which monolignol backbones are mainly linked by *β-O*-4 bonds.[14] Theoretical calculations[15] support these experimental results, because they predict that methoxyl substitution increases unpaired electron density on the phenolic oxygen of monolignol radicals and favors the formation of *β-O*-4 bonds. Spin density modeling of phenoxy radicals[15] also suggests the existence of these two clearly defined topographic zones for lignin building construction, and that these distinguishable topographic zones should lead to a transition of lignin structure from a preponderance of *β-β* and 5-5 bonding in the middle lamella, via an approximately equal proportion of *β*-5, *β-β* and *β-O*-4 linkages in the primary wall layer, to the largely *β-O*-4 lignins with fewer C–C linkages in the secondary wall.

The evidence that lignins may have domains of well-defined primary structure[16] comes mainly from the highly contrasted observation that the predominant interunit linkages in lignins from secondary cell walls are the *β-O*-4 bonds, the frequencies of which reportedly vary from 50% in gymnosperms to 80% in angiosperms.[5] Although these data have been frequently used to disprove a random coupling hypothesis for lignin assembly,[16] we show in this report that the Σ*β-O*-4/Σ*O*-4 end monomer ratio of the linear lignin fraction intimately depends on the monomer composition, and therefore on the chemical nature of the radicals derived from the three monolignols (coniferyl, dihydroconiferyl, and sinapyl alcohol), whose gain have been finely tuned during land plant evolution.

Materials and methods

Growth conditions

Seeds of *Capsicum annuum* L. (var. *annuum*) cv. Padrón were soaked overnight in tapwater before being sown in sterilized vermiculite. The seedlings were grown at 25°C under a 16-h photoperiod. *Verticillium dahliae* Kleb. (isolate VDL),[17] was cultivated in darkness on potato dextrose agar medium at 25°C. For inoculation, the roots of 24-day-old plants were cut approximately 0.5 cm from the apex and immersed in a *V. dahliae* conidia suspension of 10^6 conidia/ml. Roots of control (healthy) plants were immersed in sterilized water. After inoculation, plants were grown as described above in a vermiculite/humus mixture. Samples of the stems were taken 28 days post inoculation.

Young branches of *Ginkgo biloba*, *Cycas revoluta*, *Cupressus sempervirens*, *Taxus baccata*, *Pinus halepensis*, *Araucaria araucana*, *Araucaria heterophylla*, and *Populus alba* were harvested in April and May from trees at least 5 years old growing on the campus of the University of Murcia (in the southeast of Spain). Seedlings of *Zinnia elegans* L (cv. Envy) and *Ephedra viridis* (both from Chiltern Seeds, UK) were grown for 14 and 90 days, respectively, in a greenhouse under daylight conditions at 25°C on Humus King (type 3) (Impra S.L., El Ejido, Almería, Spain) containing 30% organic C, 0.5% organic N, and 52% total organic material, pH 5.5–6.0. Fertilizers present in the humus were 120–160 mg/l N, 100–130 mg/l P_2O_5, and 150–200 mg/l K_2O. *Selaginella martensii* cv jori was purchased from a local nursery. *Pteridium aquilinum* and *Dryopteris filis-max* were harvested in the field at positions 43°19.799′ N,

008°24.263′ W, and 43°15.871′ N, 008°09.765′ W, respectively, in the northwest of Spain.

Isolation of cell walls and thioacidolysis analyses

Cell walls were prepared by a Triton X-100 washing procedure.[17] Thioacidolysis of lignifying cell walls, which solubilizes the β-*O*-4 lignin core, was performed as described.[17] Gas chromatography-mass spectrometry (GC-MS) analyses were performed using a Hewlett Packard 5890 Series II gas chromatograph, an HP 5972 mass spectrometer, and an HP5 (30 m × 0.25 mm i.d.) column.[17] Mass spectra were recorded at 70 eV. Values were the mean of at least three independent determinations.

Results and discussion

Lignin monomer composition revealed by thioacidolysis

Thioacidolysis coupled to GC-MS analyses of lignifying cell walls of the plant species studied revealed a certain uniformity in the (β-*O*-4)-linked lignin monomer composition. For example, it demonstrated the presence of the thioethylated monomers (*erythro* and *threo* isomers) arising from the aryl-glycerol-β-aryl ether (β-*O*-4) structures derived not only from H and G units, typical of gymnosperm lignins, but also the presence of aryl-glycerol-β-aryl ether structures derived from S units, which are typical of the angiosperm lignins coexisting in almost all species with stilbene structures derived from coniferyl alcohol (Table 1).

A detailed fingerprint of the thioethylated monomers arising from thioacidolysis analysis of the plant species studied is shown in Table 1. It shows, as the main features, the almost all universal presence of aryl-glycerol-β-aryl ether structures derived from coniferyl alcohol, coniferyl aldehyde, and *p*-coumaryl alcohol (Table 1), present in almost all angiosperms (samples 1–4), gymnosperms (samples 5–12), lycopods (sample 13), and ferns (samples 14 and 15). It also shows the presence of aryl-glycerol-β-aryl ether structures derived from sinapyl alcohol, which was also observed in almost all the phyla with the exception of ferns (samples 14 and 15). Aryl-glycerol-β-aryl ether structures derived from sinapyl aldehyde were only seen in angiosperms (samples 1–4), gymnosperms belonging to Gnetales (sample 5), and lycopods (sample 13).

Within these lignin analyses, the species *Ephedra viridis* (Gnetales) and *Selaginella martensii* (Lycopodiophyta) deserve special attention. Lignins of these species contain significant amounts of S units, leading to lignins typical of angiosperms, because S units alone constitute 60%–70% of the lignin building blocks (Table 1). In the case of *E. viridis*, this observation is coherent with the fact that Gnetales show vessels like angiosperms and angiosperm-type lignins.[18] This is also the case for *S. martensii* (Table 1), one of the earliest divergent extant vascular plants, which also shows vessel elements.[19] The presence of S units in the lignins of *Selaginella* spp. has also been reported by means of ozonation, acidolysis, infrared spectroscopy, and ^{1}H nuclear magnetic resonance (NMR) spectroscopy,[20] so that the reported presence of S units in *S. martensii* lignins by thioacidolysis is far from being a false positive of the technique.

Thioacidolysis analyses in these species also revealed significant amounts of 4-*O*-linked end monomers (Table 1), mainly derived from coniferyl alcohol, coniferyl aldehyde, and vanillin (Table 1), and which were seen in angiosperms (samples 1–4), gymnosperms (samples 5–12), lycopods (sample 13), and ferns (samples 14 and 15). Furthermore, thioacidolysis analysis revealed the presence of the 4-*O*-linked dihydroconiferyl alcohol (DHCA) end unit (Fig. 1), a lignin monomer typical of gymnosperms,[21] lycopods, and ferns (Table 1, samples 6–15), which, however, may also be found in the thioacidolysis analyses of the angiosperm, *Cap-*

Table 1. Monomeric degradation products obtained by thioacidolysis of lignifying plant cell walls of the plant species studied

Sample	Species	β-*O*-4					Stilbene structures	*O*-4-end			
		CAlc	Cald	SAlc	SAld	CmAlc		CAlc	CAld	DHCA	V
1	*Zinnia elegans*	10.43	0.48	13.85	0.45	0.22	0.43	0.95	0.14		0.38
2	*Capsicum annuum* (healthy)	22.60	0.70	11.48	0.74	0.27	1.34	1.48	0.51	0.06	0.40
3	*Capsicum annuum* (unhealthy)[a]	2.30	0.10	0.81	0.05	0.04	0.10	0.16	0.04		0.05
4	*Populus alba*	1.40	0.05	2.05	0.09	0.05	0.06	0.09	0.05		0.07
5	*Ephedra viridis*	0.39	0.03	0.64	0.04	0.03	0.01	0.02	0.01		0.03
6	*Pinus halepensis*	2.26	0.40			0.12	0.11	0.12	0.08	0.12	0.12
7	*Araucaria araucana*	0.58	0.05	0.01		0.06	0.12	0.02	0.02	0.02	0.02
8	*Araucaria heterophylla*	3.06	0.29	0.14		0.34	0.30	0.30	0.13	0.16	0.05
9	*Cupressus sempervirens*	0.34				0.05	0.02	0.01		0.01	0.01
10	*Taxus baccata*	0.64	0.05	0.01		0.01	0.02	0.01	0.01	0.01	0.02
11	*Ginkgo biloba*	0.51	0.06	0.02		0.02	0.04	0.03	0.01	0.01	0.01
12	*Cycas revoluta*	0.66		0.01		0.03		0.06		0.01	0.01
13	*Sellaginella martensii*	0.64	0.07	1.90	0.21	0.10		0.04	0.02	0.02	0.02
14	*Pteridium aquilinum*	0.64	0.02			0.02	0.02	0.04	0.02		0.02
15	*Dryopteris filis-max*	0.57	0.03			0.02	0.03	0.03	0.01	0.01	0.01

Values are given in total ionic current (TIC) × 10^{-8}. *SD* values were within 5%

CAlc, Coniferyl alcohol; CAld, coniferyl aldehyde; SAlc, sinapyl alcohol; SAld, sinapyl aldehyde, CmAlc, *p*-coumaryl alcohol; DHCA, dihydroconiferyl alcohol; V, vanillin

[a] Inoculated with *Verticillium dahliae*

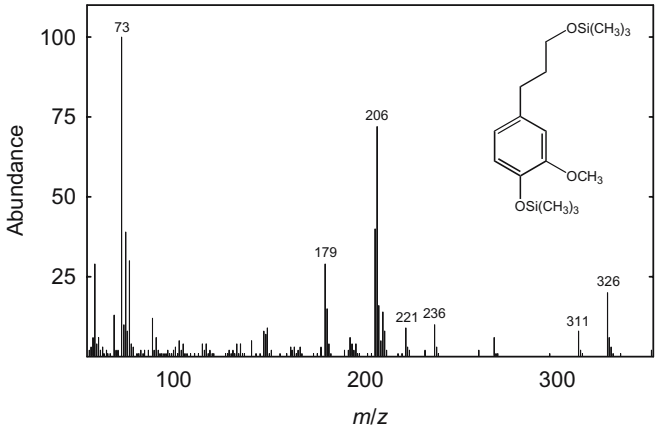

Fig. 1. Mass spectrum of the trimethylsilyl derivative of the thioethylated monomer arising from *O*-4-linked dihydroconiferyl alcohol (DHCA) in gymnosperm lignins

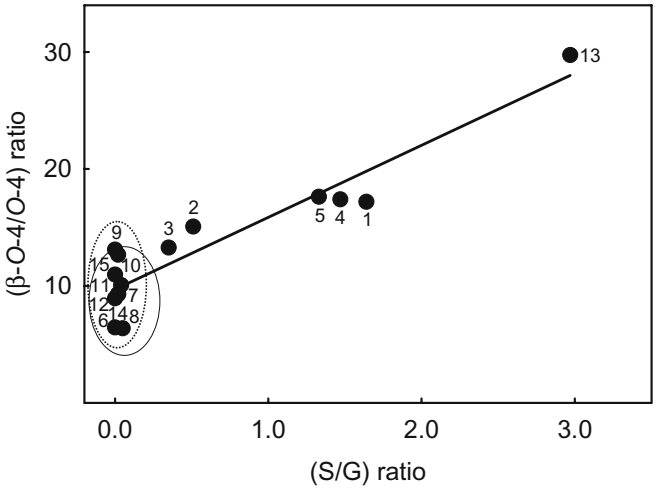

Fig. 2. Linear relationship between the Σ*β*-*O*-4/Σ*O*-4 end monomer ratio of the linear lignin fraction and the syringyl/guaiacyl (S/G) ratio of lignins in differentially evolved land plants. *Numbers* show species according to Table 1. Error bars showing standard deviations are obscured by symbols

sicum annuum (Table 1, sample 2). In this species, inoculation with the soil-borne pathogen *Verticillium dahliae* reduces the DHCA content to levels below the limit of detection (Table 1, sample 3).

With the exception of vanillin, which may arise from coniferyl aldehyde through an aldol reaction in cell walls,[22] the remaining *O*-4-linked phenolic end groups present in this lignin fraction deserve special attention. Most of these structures are thought to act as nucleation (initiation) points for lignin growth, because they may only arise from the coupling at the *β* position of a monolignol radical with the radical species of phenolics at the *O*-4 position.[9] In other words, these *O*-4 radicals of phenolics would act as nucleation points to which radicals of monolignols would be added in successive steps, which would allow the lignin network to grow by successive radical–radical additions.[9] In other words, these phenolic blocks fulfill all the requirements to be considered initiation sites (nucleation points) through which cell wall lignification would proceed.

The Σ*β*-O-4/Σ*O*-4 end monomer ratio of the linear lignin fraction and the monomer composition

When the Σ*β*-*O*-4/Σ*O*-4 end monomer ratio of the linear lignin fraction was studied as a function of the monomer composition in this group of differentially evolved land plants, a statistically significant relationship ($r^2 = 0.866$) was found between this ratio and the S/G ratio of lignins (Fig. 2). In other words, the Σ*β*-*O*-4/Σ*O*-4 end monomer ratio increases as the proportion of S units increases, regardless of the total amount of monomers that participate in *β*-*O*-4 bonds. This conclusion can easily be confirmed from data reported in Table 1, which shows that species with similar total amounts of monomers participating in *β*-*O*-4 bonds, as is the case for *Pinus halepensis* (sample 6, Σ*β*-*O*-4 = 2.89) and *S. martensii* (sample 12, Σ*β*-*O*-4 = 2.92), contain lignins with the lowest (Σ*β*-*O*-4/Σ*O*-4 end ratio = 6.45, S/G = 0) and the highest (Σ*β*-*O*-4/Σ*O*-4 end ratio = 29.74, S/G = 2.97) values, respectively.

This result agrees with our knowledge of the chemistry of sinapyl alcohol radicals:[15] the only coupling modes of sinapyl alcohol radicals are the *β*-*β* and *β*-*O*-4, because the only possible resonance structures of the sinapyl alcohol radical are the R_{O4} and the R_{β}. Because the *β*-*β* coupling mode is less favored than the *β*-*O*-4 coupling mode in sinapyl alcohol at low radical concentrations,[15] almost all the sinapyl alcohol resources utilized for lignin biosynthesis are mainly incorporated in polymers rich in *β*-*O*-4 bonds. This chemical reasoning would easily explain why the use of sinapyl alcohol for lignin biosynthesis allows the linear lignin fraction to reach maximum Σ*β*-*O*-4/Σ*O*-4 end monomer ratios independently of the total amount of monomers that participate in *β*-*O*-4 bonds. In support of a purely chemical control of the Σ*β*-*O*-4/Σ*O*-4 end monomer ratio of the linear lignin fraction, it should be mentioned that in vitro end-wise dehydropolymers (end-wise DHPs) prepared from copolymerization of *p*-coumaryl and coniferyl alcohols (in a 1:1 molar ratio), and from copolymerization of *p*-coumaryl, coniferyl, and sinapyl alcohols (in a 1:1:1 molar ratio) yielded DHPs with Σ*β*-*O*-4/Σ*O*-4 end ratios of 6 and 27,[23] which are in the range of those found in vivo (Fig. 2). These results firmly suggest the existence of an exclusive and purely chemical control of the Σ*β*-*O*-4/Σ*O*-4 end monomer ratio of the linear lignin fraction, in which the availability and further incorporation of S moieties lengthens the linear *β*-*O*-4 chain accordingly.

The above reasoning is valid for angiosperms (samples 1–4), Gnetales (sample 5), and lycopods (sample 13), all of which bear significant amounts of S lignins (see Table 1, Fig. 2). However, basal gymnosperms (samples 6–12) and ferns (samples 14 and 15) escape this reasoning due to their low, if any, S content. These species cluster together at the origin of the *x*-axis where S/G ratio values are represented (Fig. 2).

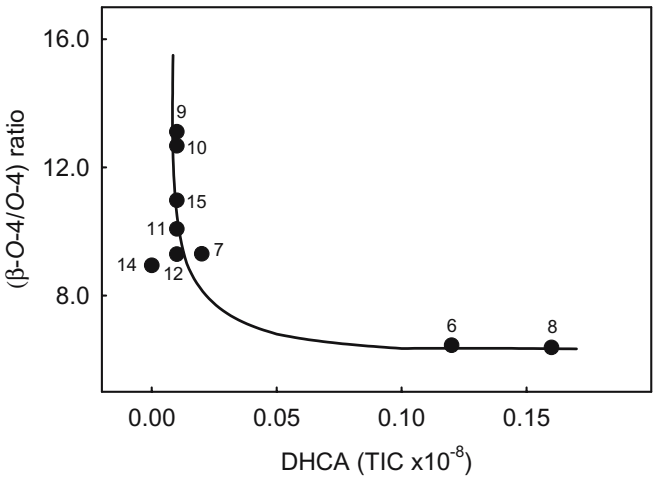

Fig. 3. Relationship between the Σ*β*-*O*-4/Σ*O*-4 end monomer ratio of the linear lignin fraction and the *O*-4-linked DHCA content of lignins in basal gymnosperms and ferns. *Numbers* show species according to Table 1. Error bars showing standard deviations are obscured by symbols

To find any relationship between the Σ*β*-*O*-4/Σ*O*-4 end monomer ratio of the *β*-*O*-4 lignin fraction and the levels of *O*-4-linked monomers in these species, the Σ*β*-*O*-4/Σ*O*-4 end monomer ratio was correlated with the DHCA content. This was because DHCA may compete with coniferyl alcohol during oxidation by peroxidases in the cell wall of the xylem but is unable to form *β*-*O*-4 bonds.[24] The results obtained (Fig. 3) suggest that in the case of basal gymnosperms and ferns, the Σ*β*-*O*-4/Σ*O*-4 end monomer ratio decays exponentially with the *O*-4-linked DHCA content, as can be predicted from the use and possible coupling modes of coniferyl alcohol and DHCA. It should be mentioned that DHCA is a minor component of gymnosperm lignins,[21] but its abundance is greatly enhanced in cinnamyl alcohol dehydrogenase down-regulated plants.[25]

Conclusions

A linear relationship was found to exist between the Σ*β*-*O*-4/Σ*O*-4 end monomer ratio of the linear lignin fraction, released through the specific cleavage of the alkyl ether linkages by thioacidolysis, and the G/S ratio of lignins, when this was estimated in differentially evolved vascular land plants differing in the nature of their lignins. Most importantly, in the case of angiosperms, Gnetales, and lycopods, the Σ*β*-*O*-4/Σ*O*-4 end monomer ratio was apparently predictable from the proportion in which G and S units are mixed. In the case of G lignins (present in basal gymnosperms and ferns), the Σ*β*-*O*-4/Σ*O*-4 end monomer ratio decays exponentially to increase the DHCA content. In such a scenario, the results obtained suggest that the Σ*β*-*O*-4/Σ*O*-4 end monomer ratio of the linear lignin fraction depends intimately on the lignin monomer composition, and therefore on the chemical nature of the radicals derived from three monolignols (coniferyl, dihydroconiferyl, and sinapyl alcohol), whose gain have been finely tuned during land plant evolution.

Acknowledgments This work was supported by grants from the Fundación Séneca (00545/PI/04), XUGA (PGIDIT-04RAG-503018PR), MCYT (BOS2002-03550), and MEC (BFU2006-11577)-FEDER (EU). LVGR holds a fellowship (FPI) from the MCYT.

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