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Distribution of lignin monomers and the evolution of lignification among lower plants

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ABSTRACT

Through application of chemical, biochemical and histochemical analyses, we provide new data on the absence/presence of syringyl lignins in the algal species *Mastocarpus stellatus*, *Cystoseira baccata* and *Ulva rigida*, the bryophytes *Physcomitrella patens* and *Marchantia polymorpha*, the lycophytes *Selaginella martensii*, *Isoetes fluitans* and *Isoetes histrix*, the sphenophyte *Equisetum telmateia*, the ferns *Ceratopteris thalictroides*, *Ceratopteris cornuta*, *Pteridium aquilinum*, *Phyllitis scolopendrium* and *Dryopteris affinis*, and the angiosperm *Posidonia oceanica*. Lignins, and especially syringyl lignins, are distributed from non-vascular basal land plants, such as liverworts, to lycopods and ferns. This distribution, along with the already reported presence of syringyl lignins in ginkgoopsids, suggests that syringyl lignin is a primitive character in land plant evolution. Here, we discuss whether the pathway for sinapyl alcohol recruitment was iterative during the evolution of land plants or, alternatively, was incorporated into the earliest land plants and subsequently repressed in several basal liverworts, lycopods, equisetopsids and ferns. This last hypothesis, which is supported by recent studies of transcriptional regulation of the biosynthesis of lignins, implies that lignification originated as a developmental enabler in the peripheral tissues of protracheophytes and would only later have been co-opted for the strengthening of tracheids in eutracheophytes.

INTRODUCTION

The xylem constitutes the longest pathway for water transport in vascular plants. It is a simple pathway of low resistance, which enables water to be transported in large quantities and with great efficiency from the roots to the leaves. Cell walls of mature xylem elements are impregnated with lignin (Boudet *et al.* 1995), a polyphenolic polymer that imparts water impermeability, including resistance against tensile forces of the water columns, and confers structural support and flexural stiffness to the aerial organs. Lignin can also provide protection against microbial degradation of cell walls. Lignins are three-dimensional, amorphous heteropolymers that result from the oxidative coupling of three *p*-hydroxycinnamyl alcohols (*p*-coumaryl, coniferyl and sinapyl alcohols) in a reaction mediated by laccases and class III plant peroxidases (Boudet *et al.* 1995; Ros Barceló 1997). The cross-coupling reaction produces an optically inactive hydrophobic heteropolymer (Ralph *et al.* 2004) composed of H (*p*-hydroxyphenyl), G (guaiacyl) and S (syringyl) units, derived from *p*-coumaryl, coniferyl and sinapyl alcohols, respectively.

The emergence of the ability to synthesise lignin in the context of a conducting system is a crucial event in plant terrestrialisation (Lowry *et al.* 1980; Niklas 1997; Bateman *et al.* 1998; Friedman & Cook 2000). Specialised conducting cells may occur in the sporophytes of living bryophytes (Scheirer 1980; Ligrone *et al.* 2000) and, although the cell walls of their conducting elements may be impregnated with polyphenolic compounds (Miksche & Yasuda 1978; Scheirer 1980; Takeda *et al.* 1990), no evidence supporting the existence of lignin in extinct or extant mosses has been found (Ligrone *et al.* 2000). It is worth stressing that few extensive biochemical surveys of algae have been conducted, and so the distribution of the biosynthetic pathway underlying the formation of lignin is uncertain, although the pioneering work of Gunnison & Alexander (1975), who reported 'lignin' in the walls of the desmids *Staurastrum*, represents a challenge in this respect.

Over the last few years, it has become evident that the cell wall composition of embryophytes may be a sign of significant evolutionary similarities and divergences. Biochemical studies of primary cell walls (Popper & Fry 2004) have shown that xyloglucans also occur in seedless tracheophytes (pteridophytes) as well as in liverworts, hornworts and mosses. In contrast, xyloglucans have not been detected in charophytes, the closest living algal relatives of the embryophytes (Graham *et al.* 2000). Likewise, rhamnogalacturonan II, a structurally complex borate-linked pectic polysaccharide present in the primary walls of gymnosperms and angiosperms (O'Neill *et*

al. 2004), was found to occur in pteridophytes in amounts comparable with those present in seed plants, and was detected in minor amounts in bryophytes (Matsunaga *et al.* 2004). In addition to these biochemical investigations, recent immunocytochemical studies have revealed that the polysaccharide, glycoprotein and lignin epitopes present in angiosperm cell walls also occur in algae, hornworts, mosses, liverworts and lycopods, where, as in angiosperms, they exhibit specific cell and tissue distributions (Ligrone *et al.* 2002, 2008; Carafa *et al.* 2005; Martone *et al.* 2009).

Lignin monomer composition also varies distinctively between the different groups of living vascular plants. In most gymnosperms studied, lignins are typically composed of G units, with a minor proportion of H units. In contrast, lignins of angiosperms, gnetophytes and some lycophytes, like *Selaginella* (Jin *et al.* 2005, 2007a; Gómez Ros *et al.* 2007b), are characterised by similar levels of G and S units. In Poaceae (Ralph *et al.* 2004), lignins are more complex, since they also contain significant amounts of ester-bound *p*-coumaric acid. This increased heterogeneity of lignin monomer composition might be related to the separation of water transport and support functions, although it has been reported that the presence of syringyl lignin is not necessarily linked to the presence of xylem vessels (Jin *et al.* 2007b; Martone *et al.* 2009).

Despite knowledge of the unique anatomical, morphological and biochemical characteristics of algae and embryophytes, and despite the existence of modern methods of lignin analysis, surprisingly little is known about the presence of lignins in non-vascular plants. Early studies on the presence of lignins in non-vascular plants were performed using chemical degradative methods (Siegel 1969; Miksche & Yasuda 1978; Logan & Thomas 1985), which yielded unspecific *p*-hydroxy-benzyl units. Other techniques used, such as infrared (Delwiche *et al.* 1989) and ^{13}C-RMN (Nimz & Tutschek 1977; Wilson *et al.* 1989; Edelmann *et al.* 1998) spectroscopy of carbonyl and methylene groups, are not specific for lignins. In contrast, thioacidolysis, a chemical de-polymerising method that yields *p*-hydroxy-phenylpropane units from lignins by releasing aryl-glycerol-β-aryl ether structures (*erythro* and *threo* isomers) derived from the three monolignols (Lapierre *et al.* 1995), appears to be a conclusive and unequivocal method (see Robinson & Mansfield 2009 and references therein) for lignin analysis. For example, thioacidolysis has revealed the presence of lignins, and more concretely of syringyl lignins, in the lycopod *Selaginella* (Jin *et al.* 2005; Gómez Ros *et al.* 2007b). The aim of this research was to use thioacidolysis to search for the presence of syringyl lignins (a structural character) in algae, mosses, liverworts, lycophytes and ferns, and to follow the trace that syringyl peroxidases (a functional character) may have left behind during the early stages of land plant evolution.

MATERIALS AND METHODS

Plant species studied

The list of studied species (Table 1) includes three algae: *Mastocarpus stellatus* (Stackhouse) Guiry (Rhodophyta, Florideophyceae), *Cystoseira baccata* (S.G. Gmelin) P.C. Silva (Ochrophyta, Phaeophyceae) and *Ulva rigida* C. Agardh (Chlorophyta, Ulvophyceae). Red algae are considered the first pluricellular organisms and green algae are accepted as the ancestors of land plants (Bhattacharya & Medlin 1998). Following the phylogenetic nomenclature proposed by Cantino *et al.* (2007), the non-vascular plant species studied also include two basal land plant species belonging to Bryopsida (*Physcomitrella patens* [Hedw.] Bruch & Schimp.) and Marchantiopsida (*Marchantia polymorpha* L.). The rest of the studied species were basal vascular plants belonging to Lycophyta (*Selaginella martensii* Spring, *Isoetes fluitans* Romero, Amigo & Ramil-Rego and *Isoetes histrix* Bory), Monilophyta (*Equisetum telmateia* Ehrh), Polypodiophyta (*Ceratopteris thalictroides* [L.] Brongn, *Ceratopteris cornuta* [P. Beauv.] Lepr., *Pteridium aquilinum* [L.] Kunhn, *Phyllitis scolopendrium* [L.] Newman and *Dryopteris affinis* [Lowe Fraser-Jenkins]), and Monocotyledoneae (*Posidonia oceanica* [L.] Delile). *C. thalictroides*, *C. cornuta*, *P. aquilinum*, *P. scolopendrium* and *D. affinis* show both tracheid and vessel elements. Both *C. thalictroides* and *C. cornuta* show helical thickenings (Carlquist & Schneider 2000) and are typical of aquatic habitats. *P. oceanica* is a secondarily aquatic, marine

Table 1. Plant species investigated, ranked by division, class and family.

division	class	family	plant species	organ selected	type of stele
Rhodophyta	Florideophyceae	Petrocelidaceae	*Mastocarpus stellatus*	Whole plant	–
Ochrophyta	Phaeophyceae	Cystoseiraceae	*Cystoseira baccata*	Whole plant	–
Chlorophyta	Ulvophyceae	Ulvaceae	*Ulva rigida*	Whole plant	–
Hepatophyta	Marchantiopsida	Marchantiaceae	*Marchantia polymorpha*	Whole young plant	–
Bryophyta	Bryopsida	Funariaceae	*Physcomitrella patens*	Whole plant (after 60 days of culture)	–
Pteridophyta	Lycopsida	Selaginellaceae	*Selaginella martensii*	Fronds	Plectostele
Pteridophyta	Lycopsida	Isoetaceae	*Isoetes fluitans* *Isoetes histrix*	Fronds	Plectostele
Pteridophyta	Equisetopsida	Equisetaceae	*Equisetum telmateia*	Fronds: central cylinder. Young plants	Eustele
Pteridophyta	Filicopsida	Parkeriaceae	*Ceratopteris thalictroides* *Ceratopteris cornuta*	Fronds	Dictyostele
Pteridophyta	Filicopsida	Aspleniaceae	*Phyllitis scolopendrium*	Fronds: central cylinder. Young plants	Dictyostele
Pteridophyta	Filicopsida	Dryopteridaceae	*Dryopteris affinis*	Fronds: central cylinder. Young plants	Dictyostele
Pteridophyta	Filicopsida	Pteridaceae	*Pteridium aquilinum*	Fronds: central cylinder. Young plants.	Dictyostele
Magnoliophyta	Liliopsida	Posidoniaceae	*Posidonia oceanica*	Leaves	Atactostele

monocotyledon (Larkum & Den Hartog 1989), which was used as control for the presence of lignins/syringyl lignins in aquatic habitats.

Sampling and growth conditions

M. stellatus and *U. rigida* were collected in A Coruña, NW Spain (43°22.366′ N, 8° 24.995′ W, altitude: 0 m), in April. *C. baccata* was collected from Mera beach during April (NW Spain, 43°23.011′ N, 8°20.479′ W, altitude: 0 m) after a strong swell. *M. polymorpha* was collected in Oleiros (NW Spain, 43°19.383′ N, 8°20.690′ W, altitude: 30 m). *P. patens* was provided by the Biotechnology Department of the Universidad Politécnica of Madrid, and was cultured as described (Benito & Rodriguez-Navarro 2003). *I. fluitans* was collected during May in Forxa River (NW Spain, 43°11.274′ N, 7°53.392′ W, altitude: 453 m). *I. histrix* was collected in Melide (NW Spain, 42°53.978′ N, 7°58.605′ W, altitude: 449 m) during May. *S. martensii*, *C. thalictroides* and *C. cornuta* were purchased from a local nursery. *E. telmateia* was collected in Arteixo (NW Spain, 43°19.417′ N, 8°29.911′ W, altitude: 26 m). *P. scolopendrium* and *D. affinis* were collected during May and June from the banks of the Mandeo River (NW Spain, 43°15.871′ N, 8°09.765′ W, altitude: 26 m). *P. aquilinum* was collected in A Zapateira (NW Spain, 43°19.799′ N, 8°24.263′ W, altitude: 135 m) during May and June. *P. oceanica* was collected off the coast of the Dehesa de Campoamor (SE Spain, 37°51.861′ N, 0°45.341′ W). The taxonomic position, organs selected and stele type of the samples is reported in Table 1.

Histochemical stains for monitoring lignins and peroxidase

Lignins were detected using the Wiesner test by soaking 0.5-mm thick sections in 1.0% (w/v) phloroglucinol in 25:75 (v:v) HCl:ethanol for 10–15 min (Pomar *et al.* 2002). Peroxidase was monitored using the 3,5,3′,5′-tetramethylbenzidine (TMB) endogenous H_2O_2-dependent method (Ros Barceló 2005). For this, sections were directly incubated for 10 min at 25 °C in a staining solution composed of 0.1 $mg \cdot ml^{-1}$ TMB-HCl in 50 mM Tris-acetate buffer (pH 5.0). Control incubations were performed in the presence of 0.1 mM ferulic acid (Ros Barceló *et al.* 2000), a competitive inhibitor of peroxidase, whose oxidation is strictly dependent on H_2O_2.

Lignin analyses

Cell walls were prepared by a Triton X-100 washing procedure that included as the last steps washing with ethanol (three times) and diethyl ether (also three times) (Novo Uzal *et al.* 2009). Lignin quantification was performed using acetyl bromide, as described by Iiyama & Wallis (1988). Alkaline nitrobenzene oxidation of lignifying cell walls and HPLC analyses were performed essentially as described (Gómez Ros *et al.* 2007b). The quantification of *p*-hydroxybenzaldehyde, vanillin and syringaldehyde was performed at 290 nm using the corresponding standards. Thioacidolysis of lignifying cell walls, which solubilises the β-*O*-4 lignin core, and GC-MS analyses were performed (Gómez Ros *et al.* 2007b) using a Thermo Finnigan Trace GC gas chromatograph, a Thermo Finnigan Polaris Q mass spectrometer and a DB-XLB, J&W (60 × 0.25 mm I.D.) column. Mass spectra were recorded at 70 eV.

Total peroxidase extraction

Plant samples were homogenised in 50 mM Tris–HCl buffer (pH 7.5) containing 1 mM EDTA, 1 M KCl, and 0.05 g PVPP per g tissue. The homogenate was filtered through nylon layers and centrifuged at 27,000 *g* for 30 min at 4 °C. The supernatant was desalted by dialysis on cellulose membranes.

Assay of peroxidase activity

Peroxidase activity was determined spectrophotometrically at 25 °C in a reaction medium containing 50 mM Tris-acetate buffer, pH 5.0, and 0.5 mM H_2O_2 with 4-methoxy-α-naphthol, syringaldehyde, coniferyl alcohol, coniferyl aldehyde, sinapyl alcohol or sinapyl aldehyde, as described elsewhere (Ros Barceló & Pomar 2001).

Chemicals

3,5,3′,5′-Tetramethylbenzidine, ferulic acid, coniferyl alcohol, coniferyl aldehyde, sinapyl alcohol and sinapyl aldehyde were purchased from Sigma-Aldrich (Madrid, Spain). The rest of the chemicals were obtained from various suppliers and were of the highest purity available.

RESULTS

Lignin-like polyphenolic content in non-vascular plants

The acetyl bromide method (Iiyama & Wallis 1988), which indiscriminately solubilises cell wall polyphenolic compounds, was used to estimate the total lignin content in purified cell walls. Table 2 shows how the amount of lignin-like polyphenolic material is highly variable among the species studied. Surprisingly, a substantial amount of lignin-like polyphenolic

Table 2. Lignin-like polyphenolic content measured by the acetyl bromide method in cell walls of algae, mosses, liverworts, lycopods and ferns. Values are means ± SD (n = 6).

species	lignin-like polyphenolic content ($mg \cdot g^{-1}$ cell wall)
Mastocarpus stellatus	53.2 ± 7.6
Cystoseira baccata	30.6 ± 4.2
Ulva rigida	62.1 ± 8.9
Marchantia polymorpha	185.8 ± 5.8
Physcomitrella patens	184.0 ± 20.3
Selaginella martensii	185.5 ± 7.0
Isoetes fluitans	59.6 ± 4.8
Isoetes histrix	60.8 ± 8.4
Equisetum telmateia	94.6 ± 12.9
Ceratopteris thalictroides	36.7 ± 1.1
Ceratopteris cornuta	54.9 ± 3.1
Phyllitis scolopendrium	90.6 ± 9.5
Dryopteris affinis	173.4 ± 2.6
Pteridium aquilinum	193.9 ± 17.0
Posidonia oceanica	45.0 ± 10.0

material was found in the algae studied, and in the bryophytes *Physcomitrella patens* and *Marchantia polymorpha*. In fact, these last two species had lignin-like polyphenolic material in their cell walls, with values that occasionally exceeded those found in the studied tracheophytes. Equally interesting was the low lignin-like polyphenolic content of *Isoetes fluitans*, *Isoetes histrix*, *Ceratopteris thalictroides*, *Ceratopteris cornuta* and *Posidonia oceanica*, species that are linked to aquatic (freshwater and marine) habitats.

Lignin monomer composition

Thioacidolysis of purified cell walls, which specifically solubilises the β-*O*-4-linked lignin fraction (Lapierre *et al.* 1995), was performed to confirm the presence of lignins. Thioacidolysis coupled to GC-MS revealed the presence of aryl-glycerol-β-aryl ether structures (*erythro* and *threo* isomers) derived from the three monolignols in all the species, with the exception of the three algae and the moss, *P. patens* (Table 3), suggesting that these four species are unable to undergo lignification. Thus, aryl-glycerol-β-aryl ether structures derived from *p*-coumaryl alcohol were present in *M. polymorpha*, *Selaginella martensii*, *C. cornuta*, *Phyllitis scolopendrium*, *Dryopteris affinis*, *Pteridium aquilinum* and *Posidonia oceanica*, while they were absent from *Equisetum telmateia*, *Isoetes* species and *Ceratopteris thalictroides* (Table 3).

Aryl-glycerol-β-aryl ether structures derived from coniferyl alcohol were present in *M. polymorpha*, *S. martensii*, the two *Isoetes* species, *C. thalictroides*, *C. cornuta*, *P. scolopendrium*, *D. affinis*, *P. aquilinum*, *E. telmateia* and *P. oceanica* (Table 3). Thioacidolysis also revealed the presence of monomers arising from aryl-glycerol-β-aryl ether structures derived from coniferyl aldehyde in *S. martensii*, *C. cornuta*, *P. scolopendrium*, *D. affinis*, *P. aquilinum* and *P. oceanica*.

The same technique also revealed lignin monomers arising from 4-*O*-linked coniferyl alcohol and 4-*O*-linked coniferyl aldehyde end groups in *S. martensii*, *I. fluitans*, *P. scolopendrium*, *D. affinis*, *P. aquilinum*, *E. telmateia* and *P. oceanica*, although only in trace amounts in the two last cases. Lastly, thioacidolysis analysis revealed the presence of the 4-*O*-linked dihydroconiferyl alcohol end unit, a monomer unit typical of gymnosperm lignins (Lapierre *et al.* 1995; Gómez Ros *et al.* 2007b), in *M. polymorpha*, *S. martensii* and *D. affinis*.

Finally, aryl-glycerol-β-aryl ether structures derived from sinapyl alcohol, considered typical of angiosperm lignins, were present in significant amounts in *M. polymorpha*, in the lycophytes, *S. martensii* and *I. histrix* (although not in *I. fluitants*), in the fern *C. cornuta* and in *P. oceanica*. Aryl-glycerol-β-aryl ether structures derived from sinapyl aldehyde were only present in the lycophyte *S. martensii*, and the angiosperm *P. oceanica*.

Table 4 summarises the lignin monomer composition of all the species studied, as obtained by thioacidolysis (a chemical de-polymerising method that yields *p*-hydroxy-phenylpropane units) and alkaline nitrobenzene oxidation (a chemical degradative method that yields *p*-hydroxy-benzyl units). Although the results obtained by alkaline nitrobenzene oxidation are consistent with those obtained by thioacidolysis, one feature of the thioacidolysis analysis was the lower amount of thioethylated monomers arising from *p*-coumaryl alcohol. However, this result is not surprising given that *p*-coumaryl alcohol is mainly incorporated in the condensed lignin fraction deposited in primary cell walls (Terashima & Fukushima 1988), which is not susceptible to thioacidolysis cleavage (Lapierre *et al.* 1995).

Table 3. Monomeric degradation products obtained by thioacidolysis of cell walls of algae, mosses, liverworts, lycopods and ferns.

	β-*O*-4					*O*-4-end			
Species	CAlc	CAld	SAlc	SAld	CmAlc	CAlc	CAld	DHCA	V
Mastocarpus stellatus	–	–	–	–	–	–	–	–	–
Cystoseira baccata	–	–	–	–	–	–	–	–	–
Ulva rigida	–	–	–	–	–	–	–	–	–
Marchantia polymorpha	10.94	Tr	0.82	-	1.88	–	–	Tr	–
Physcomitrella patens	–	–	–	–	–	–	–	–	–
Selaginella martensii	128.5	14.23	380.4	42.17	20.40	8.68	4.25	4.55	4.69
Isoetes fluitans	49.76	–	–	–	–	–	0.49	–	–
Isoetes histrix	13.90	–	5.15	–	–	–	–	–	Tr
Equisetum telmateia	8.08	–	–	–	–	Tr	Tr	–	–
Ceratopteris thalictroides	12.90	–	–	–	–	–	–	–	–
Ceratopteris cornuta	25.61	Tr	23.96	–	Tr	–	–	–	–
Phyllitis scolopendrium	36.22	Tr	–	–	Tr	1.48	Tr	–	–
Dryopteris affinis	153.53	7.45	–	–	5.43	8.55	1.69	2.69	3.99
Pteridium aquilinum	192.50	5.53	–	–	6.01	12.88	4.46	–	4.80
Posidonia oceanica	14.79	4.70	30.51	Tr	3.84	Tr	–	–	Tr

Values are given in total ionic current (TIC) × 10^{-8} mg^{-1} CW.

β-*O*-4 = aryl-glycerol-β-aryl ether structures derived from the β-*O*-4 cross coupling of *p*-hydroxycinnamyl alcohols and aldehydes; *O*-4-end = terminal 4-*O*-linked groups; Calc = coniferyl alcohol; CAld = coniferyl aldehyde; SAlc = sinapyl alcohol; SAld = sinapyl aldehyde, CmAlc = *p*-coumaryl alcohol, DHCA = dihydroconiferyl alcohol; V = vanillin. Tr = trace.

SD values were within 5% (n = 3).

Table 4. Lignin monomer composition determined by nitrobenzene oxidation (NBO) and thioacidolysis (TA) in cell walls of algae, mosses, liverworts, lycopods and ferns.

	H (%)		G (%)		S (%)	
species	NBO	TA	NBO	TA	NBO	TA
Mastocarpus stellatus	100	0	0	0	0	0
Cystoseira baccata	100	0	0	0	0	0
Ulva rigida	100	0	0	0	0	0
Marchantia polymorpha	29	14	66	80	5	6
Physcomitrella patens	100	0	0	0	0	0
Selaginella martensii	22[a]	3	34[a]	27	44[a]	70
Isoetes fluitans	11	0	89	100	0	0
Isoetes histrix	8	0	92	73	0	27
Equisetum telmateia	28	0	72	100	0	0
Ceratopteris thalictroides	17	0	83	100	0	0
Ceratopteris cornuta	6	Tr	56	52	38	48
Phyllitis scolopendrium	15	Tr	85	100	0	0
Dryopteris affinis	2	3	98	97	0	0
Pteridium aquilinum	1	3	99	97	0	0
Posidonia oceanica	8	8	36	30	56	62

Tr = trace.
[a]Values are taken from Gross (1980).

Histochemical localisation of lignin and peroxidase in basal plants

To study the localisation of lignins, sections were stained by means of the Wiesner reaction, which is specific for 4-*O*-linked *p*-hydroxy-cinnamaldehyde and *p*-hydroxy-benzylaldehyde end groups (Pomar *et al.* 2002). The staining pattern in the eustela of *E. telmateia* at the level of the node (Fig. 1A) and internode (Fig. 1B) was similar to that most often found in stems of gymnosperms (Gómez Ros *et al.* 2007b) and angiosperms (Ros Barceló *et al.* 2006; Gómez Ros *et al.* 2007a) and, as in the above-mentioned cases, it was limited to vascular tissues. However, the staining pattern differed greatly depending on the tissue: in the node, there was intense lignification related to the high degree of vascularisation (branching) of this tissue (Fig. 1A), while in the internode, phloroglucinol only stained the central vascular bundles located near the carinal canal and the two rows of cells surrounding the phloem (Fig. 1B). Similar results were found in *P. scolopendrium*, *I. histrix* and *S. martensii*, in which lignification was also restricted to vascular tissues (data not shown). The cases of *P. aquilinum* and *I. fluitans* were somewhat different. In these species, lignification was found in the vascular system and also in the epidermal and sub-epidermal/cortical tissues (Fig. 1C).

When the sections were stained with tetramethylbenzidine (TMB) to monitor peroxidase/H_2O_2 localisation (Ros Barceló *et al.* 2006), peroxidase was always localised in the vascular cordon and the epidermal/sub-epidermal cells of all the species. Figure 1D–F show the cases of *E. telmateia* and *P. aquilinum.* Peroxidase was located in both the node and the internode of *E. telmateia* (Fig. 1D,E), particularly in the vascular cells that envelop the carinal canal, a longitudinal channel located inside the metaxylem and formed by disintegration of the protoxylem. To a slightly lesser extent, peroxidase was also located in the vascular bundles that surround the vallecular canal, an air-filled intercellular channel running the length of each internode and positioned approximately between the vascular bundles. This staining pattern was due

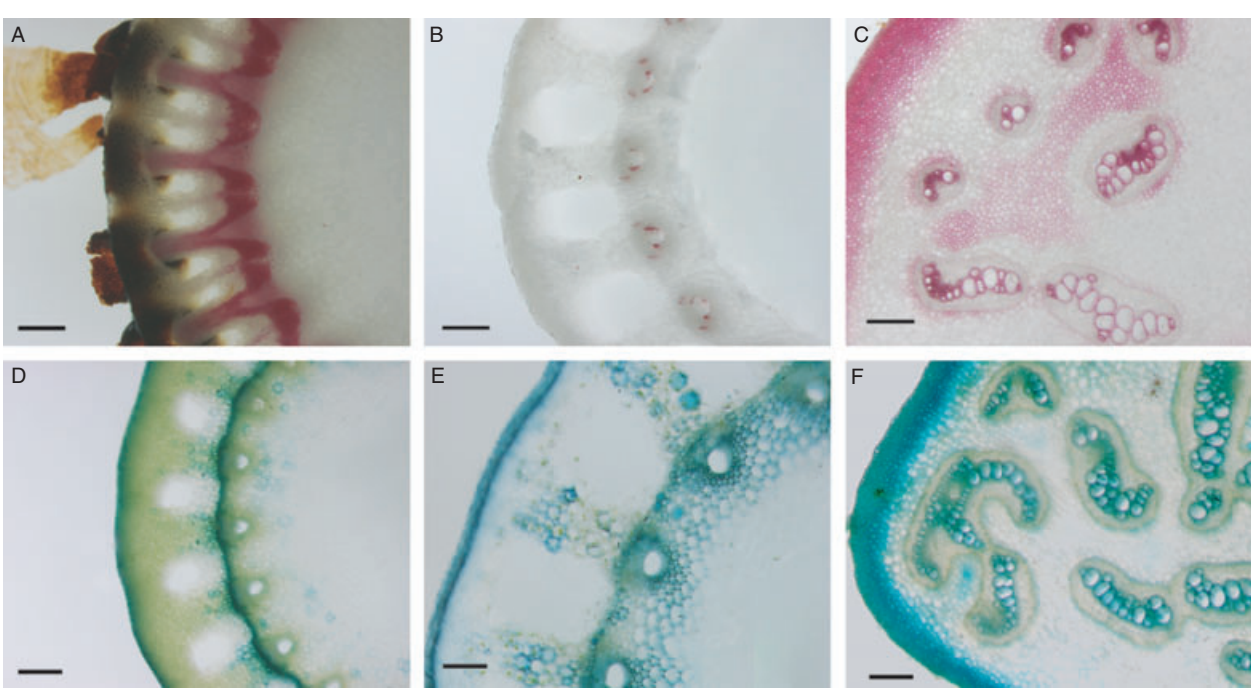

Fig. 1. Photomicrographs of transverse sections of the node (A), internode of *Equisetum telmateia* (B), and stalk of *Pteridium aquilinum* (C) after staining with phloroglucinol to reveal *p*-hydroxycinnamaldehyde-rich lignins. Bar = 300 μm. Photomicrographs of transverse sections of the node (D), internode of *Equisetum telmateia* (E), and stalk of *Pteridium aquilinum* (F) after staining with TMB, which reveals sites of peroxidase/H_2O_2 localisation. Bars = 300 μm.

to peroxidase, since all the reactions were sensitive to the peroxidase inhibitor ferulic acid.

Peroxidase activity in basal plants

From the results described above, it can be inferred that lignins are present not only in vascular plants, but also in some non-vascular plants and algae. It can be equally concluded that peroxidases are located in the same tissues as lignins. In other words, non-vascular plants, such as *M. polymorpha*, would not only contain lignins but also the enzymes responsible for their synthesis (Table 5). To confirm the ability of these peroxidases to oxidise lignin precursors (*p*-hydroxy-cinnamyl alcohols and aldehydes), peroxidase activity was measured in total tissue homogenates using many substrates, among them syringaldazine, the prototype substrate of syringyl peroxidases (Ros Barceló *et al.* 2002). The results showed that in nearly all species where these peroxidases were assayed (Table 5), the presence of peroxidases capable of oxidising coniferyl alcohol, coniferyl aldehyde, sinapyl alcohol and sinapyl aldehyde, as well as syringaldazine, was detected. Interestingly, peroxidase activity with lignin precursors, especially sinapyl alcohol and its analogue syringaldazine, was detected in species that do not lignify, such as the moss, *P. patens*, the green alga *U. rigida*, and the brown alga *C. baccata*. The only tested species that showed no peroxidase activity with any substrate related to lignification was the red alga *M. stellatus* (Table 5).

DISCUSSION

Peroxidases (class III plant peroxidases, EC 1.11.1.7) are the main enzymes involved in the process of monolignol assembly that leads to lignin biosynthesis. These enzymes are present in all land plants, but genes encoding this enzyme family occur most abundantly among angiosperms (Bakalovic *et al.* 2006). However, the presence of class III peroxidases in algae must be taken with caution. Although neither class III peroxidase-encoding sequences nor peroxidase activity have been found in the green alga *Chlamydomonas reinhardtii* (Passardi *et al.* 2004), several *Chara* species showed class III peroxidase activity (Greppin *et al.* 1986), and only recently it was shown that the genome of the red alga *Galdieria* encodes a small family of secreted class III peroxidases that might be involved in cell wall modification (Oesterhelt *et al.* 2008).

Certainly, the above-described results show (Table 5) that peroxidases capable of oxidising monolignols, as well as syringaldazine, are present in algae (*e.g. Ulva rigida*, *Cystoseira baccata*) but these peroxidases are not necessarily linked to cell wall lignification. In fact, although UV-absorbing poly-phenolic compounds are present in the cell walls of the three algae studied (Table 2), and are capable of yielding *p*-hydroxy-benzaldehyde upon alkaline nitrobenzene oxidation (Table 4), aryl-glycerol-β-aryl ether structures derived from *p*-coumaryl alcohol are lacking in these species (Table 3). Taking into account that thioacidolysis is the only method, used in our work, that unequivocally assures the presence of lignin, as it solubilises the β-*O*-4 lignin core, which is exclusive to lignins, the obtained results do not confirm the presence of lignins in the studied species. However, Martone *et al.* (2009), using DFRC confirmed the unequivocal presence of lignins in the red alga *Calliarthron cheilosporioides*, which is not only able to undergo lignification but also has lignins of the guaiacyl/syringyl type.

Phylogenetic studies have suggested that class III plant peroxidases appeared adaptively with the emergence of land plants (Duroux & Welinder 2003). The results in Table 5 also suggest that acquisition of the ability of plants to oxidise monolignols (not necessarily by a class III plant peroxidase itself) was gained prior to terrestrialisation. In this pre-adaptive scenario, it is not surprising that the enzymes responsible for monolignol oxidation (class III plant peroxidases) have been reported in mosses and liverworts (Table 5), and that these enzymes, as occurs for all highly expressed proteins that evolved slowly (Drummond *et al.* 2005), have been largely conserved during plant evolution (Ros Barceló *et al.* 2007).

Phylogenetic studies designed to use the structural determinants that control syringyl peroxidase activity (Gómez Ros

Table 5. Peroxidase activity measured with different substrates in algae, mosses, liverworts, lycopods and ferns. Values are means ± SD (n = 6).

	peroxidase activity (nkat·g^{-1} FW)					
species	4-Methoxy-α-naphthol	Coniferyl alcohol	Sinapyl alcohol	Coniferyl aldehyde	Sinapyl aldehyde	Syringaldazine
Mastocarpus stellatus	–	–	–	–	–	–
Cystoseira baccata	–	0.53 ± 0.07	3.17 ± 0.20	7.07 ± 0.35	0.95 ± 0.11	0.16 ± 0.05
Ulva rigida	–	–	0.18 ± 0.13	–	–	0.17 ± 0.09
Marchantia polymorpha	9.49 ± 1.89	35.56 ± 0.17	–	18.53 ± 1.03	0.81 ± 0.34	3.74 ± 0.52
Physcomitrella patens	1.41 ± 0.04	1.80 ± 1.24	1.75 ± 0.31	4.34 ± 0.50	1.09 ± 0.04	0.20 ± 0.13
Selaginella martensii	201.11 ± 29.96	956.02 ± 8.85	81.31 ± 1.99	798.98 ± 49.20	53.02 ± 6.10	–
Isoetes fluitans	35.92 ± 0.52	54.56 ± 6.40	0.51 ± 0.15	22.90 ± 0.61	0.34 ± 0.03	0.17 ± 0.05
Isoetes histrix	0.16 ± 0.01	1.23 ± 0.06	–	0.70 ± 0.17	–	–
Equisetum telmateia	36.45 ± 2.59	22.01 ± 0.88	0.20 ± 0.34	7.49 ± 0.59	1.61 ± 0.35	–
Ceratopteris thalictroides	1.86 ± 0.33	7.90 ± 0.82	–	4.00 ± 0.87	0.21 ± 0.01	0.09 ± 0.01
Ceratopteris cornuta	12.10 ± 0.17	20.78 ± 2.38	0.23 ± 0.06	14.53 ± 0.42	–	0.31 ± 0.11
Phyllitis scolopendrium	–	0.13 ± 0.03	–	–	–	0.09 ± 0.01
Dryopteris affinis	0.54 ± 0.13	1.29 ± 0.01	0.54 ± 0.05	0.59 ± 0.09	–	–
Pteridium aquilinum	4.82 ± 0.01	12.86 ± 0.82	1.58 ± 0.36	10.59 ± 0.24	–	0.54 ± 0.18
Posidonia oceanica	47.88 ± 1.55	87.02 ± 3.09	2.99 ± 0.41	48.84 ± 0.80	0.32 ± 0.08	3.93 ± 0.23

et al. 2007b; Ros Barceló *et al.* 2007) have predicted that class III plant peroxidases capable of oxidising syringyl moieties might occur not only in gymnosperms, lycophytes and polypodiophytes, but also in liverworts (Gómez Ros *et al.* 2007b; Ros Barceló *et al.* 2007). The results described in Table 5 lend experimental support to these molecular clocks, since the moss *Physcomitrella patens* and the liverwort *Marchantia polymorpha* show peroxidase activity capable of oxidising monolignols.

P. patens and *M. polymorpha* may be taken as representative of the most basal (non-vascular) land plants, and hence illustrate that the presence of a primitive vasculature is not necessarily required for cell wall lignification. Thus, while both *M. polymorpha* (Table 3) and *C. cheilosporioides* (Martone *et al.* 2009) are able to lignify, although lacking specialised water-conducting cells, *P. patens* does not lignify despite having an internal strand of imperforate water-conducting cells (hydroids) and a peripheral ring of thick-walled living cells (stereids) (Ligrone *et al.* 2000).

The results obtained for the lignin content using the acetyl bromide method in *P. patens* (Table 2) are consistent with previous studies that reported the presence of polyphenolic compounds (lignin-like material) in cell walls of mosses (Siegel 1969; Erickson & Miksche 1974; Nimz & Tutschek 1977; Miksche & Yasuda 1978; Logan & Thomas 1985; Delwiche *et al.* 1989; Wilson *et al.* 1989; Edelmann *et al.* 1998). Most of these compounds, such as *trans*-sphagnum acid (*p*-hydroxy-β-[carboxymethyl]-cinnamic acid) and hydroxybutenolide (2,5-dihydro-5-hydroxy-4-[4′hydroxyphenyl]furan-2-one) (Rasmussen *et al.* 1995), are derived from *p*-hydroxy-phenyl units, and usually yield *p*-hydroxybenzaldehyde by chemical degradative methods such as alkaline nitrobenzene oxidation (Table 4). These polyphenolic compounds can be solubilised by acetyl bromide and may be responsible for over-estimating lignin quantity, because they may also absorb in the UV range. In fact, the H units detected by alkaline nitrobenzene oxidation in the moss, *P. patens* (Table 4) might be attributed to cell wall polyphenolic compounds containing a *p*-hydroxyphenyl units unrelated to lignins, since thioacidolysis of this species (Table 3), unlike in *M. polymorpha*, did not reveal aryl-glycerol-β-aryl ether structures derived from either *p*-coumaryl alcohol or coniferyl alcohol, the latter considered the most helpful marker for the presence of lignins in plants.

The presence of lignins and, especially, of syringyl lignins, in Marchantiopsidae was reported by Logan & Thomas (1985) in *Conocephalum conicum* by means of cupric oxide alkaline oxidation. Here, the presence not only of lignins, but specifically of syringyl-type lignins, is demonstrated in *M. polymorpha* by means of alkaline nitrobenzene oxidation and, more conclusively, by thioacidolysis (Table 3). Syringyl units in *M. polymorpha* accounted for 6% of the lignin building blocks (Table 4).

The distribution of syringyl lignins among extant lycophytes is not uniform and not entirely understood. Syringyl lignin derivatives cannot usually be detected in extant Lycopodiaceae (*i.e. Lycopodium*) by chemical de-polymerising methods such as derivation followed by reductive cleavage (DFRC) (Weng *et al.* 2008), or by chemical degradative methods such as cupric oxide alkaline oxidation (Logan & Thomas 1987). It is perhaps evolutionarily significant that syringyl lignin derivatives have not been found in the extinct, arborescent lycophyte *Sigillaria ovata* (Logan & Thomas 1987). However, lignins, and more specifically, syringyl lignins were reported by chemical degradative methods in the herbaceous *Isoetes* and *Huperzia* (Towers & Gibbs 1953; Towers & Maass 1965) (see also Table 3) and, more conclusively, by chemical de-polymerising methods such as thioacidolysis in *Selaginella* and *Isoetes* (Table 3). In the case of *Selaginella*, the presence of syringyl units has already been confirmed by acidolysis, ozonation, ^{1}H-NMR spectroscopy (Jin *et al.* 2005, 2007a) and DFRC (Weng *et al.* 2008).

Both *Isoetes histrix* and *Selaginella martensii* showed syringyl units in their lignins, where they represented 27% and 70% of the total lignin building blocks (Table 4), a composition strongly recalling that of angiosperm lignins. The presence of syringyl units in their lignins is, however, consistent with the observation that lycophytes and aquatic ferns, together with angiosperms and gnetophytes, have xylem vessels (Carlquist & Schneider 2000; Schneider & Carlquist 2000). However, although *I. histrix* showed syringyl lignins, this was not the case with *Isoetes fluitans*, in which thioacidolysis revealed that lignins are exclusively constituted by G units (Table 4). Clearly, the case of *Isoetes* illustrates that the evolutionary gain of syringyl lignins is far from being understood, at least according to the current models for lignin evolution.

There were significant differences in the results obtained among monilophytes and polypodiophytes. The horsetail, *Equisetum telmateia*, only showed lignins of the guaiacyl type (Table 4), but did not show syringyl peroxidase activities (Table 5). Other *Equisetum* species, such as *Equisetum fluviatile*, have already been described as containing syringyl lignins (Logan & Thomas 1985). Extant ferns mainly show guaiacyl-type lignins (Table 4), while being capable of syringyl peroxidase activity (Table 5). However, among the ferns studied here, there is a significant exception. Although both *Ceratopteris thalictroides* and *Ceratopteris cornuta* are aquatic, the latter contains syringyl units in its lignins, reaching 48% of the total lignin building blocks (Table 4). However, *C. cornuta* is not the sole exception among ferns, since syringyl lignins have been reported in *Dennstaedtia bipinnata*, a tree fern, by means of cupric oxide alkaline oxidation (Logan & Thomas 1985). Neither the habit nor the ecology of the species seem to be, at first sight, connected with the distribution of this biochemical character.

This experimental study shows that syringyl lignins are distributed with an unknown pattern among non-vascular land plants, from liverworts to lycopods and ferns. Novo Uzal *et al.* (2009) have already described the presence of syringyl lignins in *Ginkgo biloba*. As far as it is known, angiosperms and gnetophytes have evolved two enzymes that catalyse the production of syringyl lignins and, given the presence of syringyl lignins in liverworts, lycopods, ferns and basal living gymnosperms, it is possible that the pathway for sinapyl alcohol has evolved (independently or not) at least several times during the evolution of land plants.

Alternatively, sinapyl alcohol may have been incorporated into the earliest land plants, having been subsequently lost or repressed (Novo Uzal *et al.* 2009) in some but not all of the extant diverging basal liverworts, lycopods, equisetopsids, ferns and even algae (Martone *et al.* 2009). Recruitment of sinapyl alcohol for lignin biosynthesis would thus be the 'primitive state.'

This working hypothesis is supported by recent advances in knowledge of transcriptional regulation of the biosynthesis of lignins. The AC elements are conserved cytosine- and adenosine-rich motifs that are present in the promoters of genes encoding enzymes of the phenylpropanoid pathway. AC elements contained in the promoter region of lignin biosynthetic genes are thought to enhance their expression in the xylem, and, at the same time, to prevent their expression in peripheral tissues (Peter & Neale 2004). Since the deletion of the AC element results in de-repression of these genes in tissues foreign to xylem tissues (Raes *et al.* 2003), it has been suggested that a tissue-specific repressor (transcription factor) is normally bound to the AC element, preventing expression in cells foreign to the xylem tissues (Leyva *et al.* 1992). The presence of such transcription factors in tissues other than the xylem might reflect the evolution of AC-rich element-containing promoters from a primitive non-specific to a specific vascular promoter through the introduction of a transcription factor that suppresses foreign tissue expression.

In such a scenario, the presence of lignins in the non-vascular tissues of *M. polymorpha*, *Pteridium aquilinum* and *I. fluitans*, an observation with no counterpart in gymnosperms or angiosperms (Gómez Ros *et al.* 2007a,b), supports the view (Friedman & Cook 2000; Boyce *et al.* 2003) that lignification might have originated in the peripheral tissues of protracheophytes and was only later co-opted for strengthening of tracheids in eutracheophytes. The presence of lignins in peripheral cells of the red alga *C. cheilosporioides* also seems to confirm this hypothesis (Martone *et al.* 2009). In this context, the observation that lignins in *P. aquilinum* and *I. fluitans* are putatively located in epidermal and sub-epidermal cells, which are not involved in water transport, seems to represent an inherited trait from their origin in the outermost tissues of an ancestral land plant lineage, a character that survives in the peripheral tissues of some extant lycophytes, like *I. fluitants*, and some filicopsids, like *P. aquilinum*. As a whole, this scenario is reminiscent of Gould's exaptation concept (Gould 2002), and probably reflects what Donoghue (2005) termed 'developmental enablers,' *i.e.* changes early in sequences that open up new design options.

However, the biochemical history of cell wall lignification may have been different. Modern lignin distributions may reflect early versatile biochemical pathways that were subsequently restricted by selection, developmental constraints, genetic or epigenetic mechanisms. Was cell wall lignification just another component of this early phase of diffusive evolution?

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