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Report

Discovery of Lignin in Seaweed Reveals Convergent Evolution of Cell-Wall Architecture

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Summary

Lignified cell walls are widely considered to be key innovations in the evolution of terrestrial plants from aquatic ancestors some 475 million years ago [1–3]. Lignins, complex aromatic heteropolymers, stiffen and fortify secondary cell walls within xylem tissues, creating a dense matrix that binds cellulose microfibrils [4] and crosslinks other wall components [5], thereby preventing the collapse of conductive vessels, lending biomechanical support to stems, and allowing plants to adopt an erect-growth habit in air. Although "lignin-like" compounds have been identified in primitive green algae [6, 7], the presence of true lignins in nonvascular organisms, such as aquatic algae, has not been confirmed [2, 3, 8, 9]. Here, we report the discovery of secondary walls and lignin within cells of the intertidal red alga *Calliarthron cheilosporioides*. Until now, such developmentally specialized cell walls have been described only in vascular plants. The finding of secondary walls and lignin in red algae raises many questions about the convergent or deeply conserved evolutionary history of these traits, given that red algae and vascular plants probably diverged more than 1 billion years ago.

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Results and Discussion

The goal of this study was to explore the ultrastructure and chemical composition of cell walls in the coralline alga *Calliarthron cheilosporioides* (Corallinales, Rhodophyta), which thrives in wave-exposed rocky intertidal habitats along the California coast. Unlike fleshy seaweeds, *Calliarthron* fronds calcify, encasing cells in $CaCO_3$ [10], but have decalcified joints, called genicula, that allow calcified fronds to bend and avoid breakage when struck by incoming waves (Figure 1) [10, 11]. Early studies of genicula noted that as they decalcify and mature, genicular cells elongate up to 10-fold and their cell walls expand slightly [10, 12]. A recent histological analysis found that after elongation ceases, genicular cell walls undergo a second round of expansion and may double in thickness, up to 1.2 μm [13], strengthening genicular tissue [11]. This developmental pattern is analogous to secondary-cell-wall formation in xylem, because secondary walls are generated when cell walls expand after a discrete phase of cell elongation. Indeed, transmission electron microscopy (TEM) micrographs reveal that wall thickening in *Calliarthron* results from the deposition of distinct secondary walls (Figures 1D and 1E, S1 layer). Secondary cell walls do not appear to have been previously described in red algae, which are widely considered to have only primary walls [14]. In *Calliarthron*, distinct wall striations (Figure 1E) probably result from the annular deposition of secondary material inside primary walls. Secondary cell walls in genicula bear striking morphological resemblance to those in fiber elements in xylem tissues from terrestrial angiosperms (Figure 1F, S1–S3 layers). Moreover, cells nearest the genicular periphery have the thickest [13] and most developed secondary walls (Figures 1C and 1E), ideally positioned for resisting bending stresses [15], similar to the arrangement of thick-walled cells within stems of early fossilized vascular plants [16] and extant land plants [17].

Derivatization followed by reductive cleavage (DFRC) analyses showed that cell walls in *Calliarthron* contain components derived from the radical coupling reactions of hydroxycinnamyl alcohols (monolignols), the same components that characterize lignin in terrestrial plant tissues (Figure 2A). The DFRC method is considered diagnostic for lignins, because only cleavage of characteristic β-aryl ether linkages releases the acetylated monolignols (i.e., DFRC monomers; Figure 2B). The double bond in hydroxycinnamyl acetates is uniquely generated by reductive ether cleavage, while existing double bonds in lignins give rise to other structures [18]. Although there is some debate over what constitutes a "real" lignin polymer, we consider a polymer derived from radical coupling reactions involving monolignols to be lignin [19]. *Calliarthron* releases acetates of *p*-coumaryl and coniferyl alcohols, typically derived from *p*-hydroxyphenyl (H) and guaiacyl (G) units in gymnosperm tracheids and angiosperm protoxylem, and the acetate of sinapyl alcohol, typically derived from syringyl

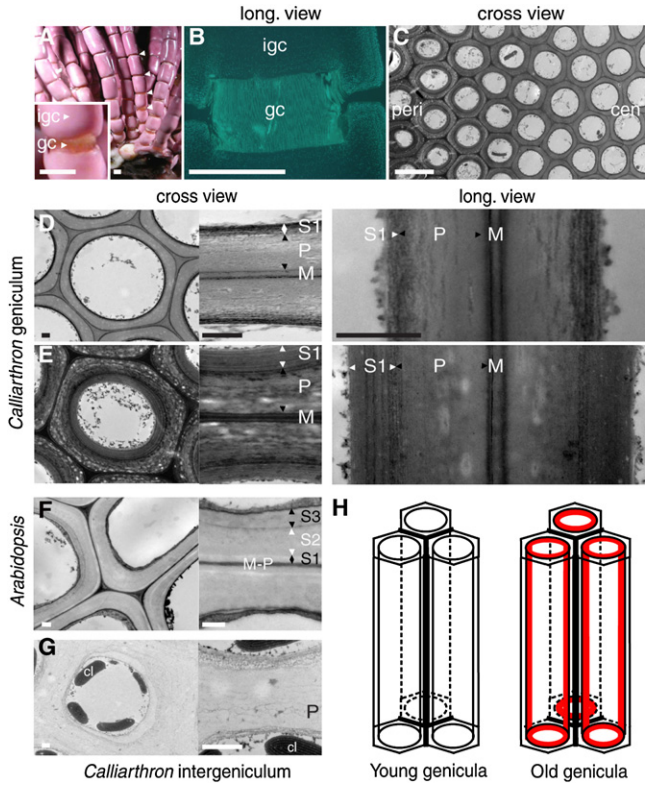


Figure 1. Morphology and Ultrastructure of *Calliarthron* Thalli in which Flexible Joints, Called Genicula, Separate Calcified Segments, Called Intergenicula

(A) Whole aspect, (B) long-section (viewed unstained under UV light), and (C) cross-section of geniculum.

(D) Immature genicular cells have primary cell walls and negligible secondary cell walls.

(E) Mature genicular cells develop thick secondary cell walls, resembling (F) fibers from *Arabidopsis* xylem with multiple secondary cell wall layers.

(G) Intergenicular cells develop only primary cell walls.

(H) Schematic of secondary-wall thickening (red) in genicular cells.

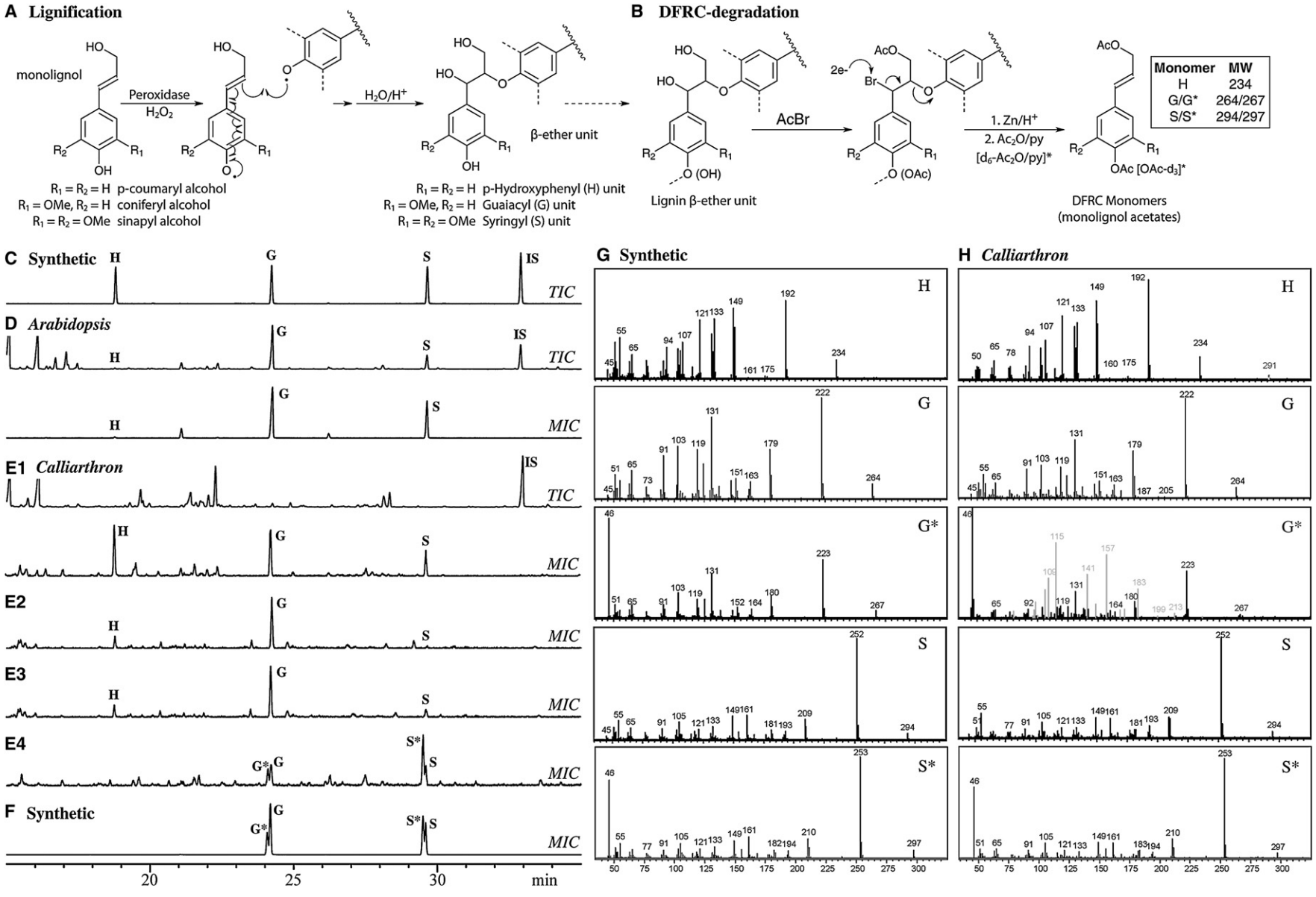
Images (C–G) were taken under TEM. Abbreviations are as follows: cen, central genicular cells; cl, chloroplast; gc, genicular cells; igc, intergenicular cells; M, middle lamella; p, primary cell wall; peri, peripheral genicular cells; S1–S3, secondary-cell-wall layers. Scale bar represents 1 mm (A and B), 10 μm (C), 0.5 μm (D–G).

(S) units in angiosperm metaxylem vessels and fibers (Figures 2C–2H). Per gram of tissue, *Calliarthron* genicula release approximately 0.25 mg DFRC monomers (molar ratio H:G:S = 1:1.5:trace), and uncalcified intergenicula release 0.19 mg DFRC monomers (molar ratio H:G:S = 1:2.4:0.1). Monomer levels and molar ratios varied among samples (e.g., one sample had 1:1 G:S levels and only trace amounts of H units), possibly reflecting differences in season, age, or life-history stage of samples or incomplete separation of genicular and intergenicular fractions, but all *Calliarthron* samples released characteristic DFRC monomers (Figure 2H). Molar ratios are unlike any proportions described for monolignols in terrestrial plants [20]. For example, DFRC monomers are released from gymnosperm (loblolly pine) walls at 50 mg/g and are essentially 100% G [21], *Arabidopsis* stems yielded 20 mg/g in a 4:1 G:S ratio with very few H units (Figure 2D), and the lycopod *Selaginella* releases 6 mg/g in a 1:1.2 G:S ratio [22]. Monolignols have previously been identified exclusively in terrestrial plants and have never been implicated in aquatic algal development [1–3, 9]; their presence in a marine red alga is, therefore, striking.

To further explore whether polymerized monolignols resemble lignin, the DFRC method was modified to provide information on the degree of etherification of units released from *Calliarthron* cell walls. Normally, DFRC monomers are released both from free-phenolic endgroup β-aryl ether structures and from polymerized units that are 4-O-β-etherified to adjacent units. By conducting the two-electron reductive cleavage step (via zinc) in deuteroacetic acid and utilizing perdeuteroacetic anhydride to achieve the final acetylation, we labeled monomers derived from 4-O-β-etherified units with trideuteroacetate groups, enabling them to be readily distinguished (by their 3-unit-higher masses) from units that were originally free-phenolic endgroup β-aryl ether structures (Figure 2). Gas-chromatography conditions even separate trideutero analogs (G* and S*) from unlabeled analogs (G and S monomers) in *Calliarthron* tissue, demonstrating that approximately 50% of G units and 75% of S units are etherified in *Calliarthron* (Figure 2E4). Similar trends are noted in typical lignins. For example, in *Arabidopsis*, approximately 88% of releasable G units and 99% of S units may be etherified [23]. That releasable DFRC monomers derive substantially from nonterminal β-ether units provides particularly compelling evidence for polymerized lignins within *Calliarthron* cell walls. Furthermore, this new "etherification" method successfully corroborates results from thioacidolysis following exhaustive methylation for several hardwoods [24], validating it as a useful tool for future lignin analyses.

Polyclonal antibodies designed to localize lignins within terrestrial plant tissues [25] label lignin epitopes within *Calliarthron* cell walls (Figure 3). Antibodies recognize lignin epitopes in both genicular and intergenicular walls, but lignin substructure, abundance, and distribution differ between the two tissues. Immunolabeling experiments detect G, H, and S units scattered throughout intergenicular walls and detect G and H units within genicular cell walls and middle lamellae. Labeling of condensed G linkages is most intense in secondary walls of peripheral genicular cells but is entirely absent within central cells. That condensed G-G dimer epitopes specifically bind to *Calliarthron* cell walls suggests that monolignols form at least dimeric structures, further supporting DFRC observations. Moreover, low molecular weight (MW) oligomers (degree of polymerization < 6) would have been removed by solvent-extraction steps, implicating the presence of higher MW oligomers or polymers.

The discovery of polymerized hydroxycinnamyl alcohols (lignin) within the cell walls of a red alga has major evolutionary implications (Figure 4). Because monolignol synthesis is exceptionally complex [19], it seems unlikely that *Calliarthron* and terrestrial plants evolved monolignol biosynthesis and polymerization completely independently. It seems more likely that relevant pathways, such as phenylpropanoid biosynthesis and polymerization by peroxidase-catalyzed oxidation [19], may be deeply conserved, having evolved prior to the divergence of red and green algae more than 1 billion years ago [26] (Figure 4). If this hypothesis is correct, we may expect to find conserved enzymatic pathways and, potentially, evidence of lignification among the multitude of evolutionary intermediates. For example, S peroxidases, capable of oxidizing S monolignols and assembling them into lignin polymers, have recently been found in basal land plants (Bryophytes) lacking both xylem tissue and lignin [27]. Moreover, given the sensitivity of the DFRC techniques described here,


A Lignification
monolignol
Peroxidase
H2O2
H2O/H+
β-ether unit
R1 = R2 = H p-coumaryl alcohol
R1 = OMe, R2 = H coniferyl alcohol
R1 = R2 = OMe sinapyl alcohol
R1 = R2 = H p-Hydroxyphenyl (H) unit
R1 = OMe, R2 = H Guaiacyl (G) unit
R1 = R2 = OMe Syringyl (S) unit
B DFRC-degradation
AcBr
2e-
1. Zn/H+
2. Ac2O/py
[d6-Ac2O/py]*
Lignin β-ether unit
DFRC Monomers
(monolignol acetates)
Monomer MW
H 234
G/G* 264/267
S/S* 294/297
C Synthetic
D Arabidopsis
E1 Calliarthron
E2
E3
E4
F Synthetic
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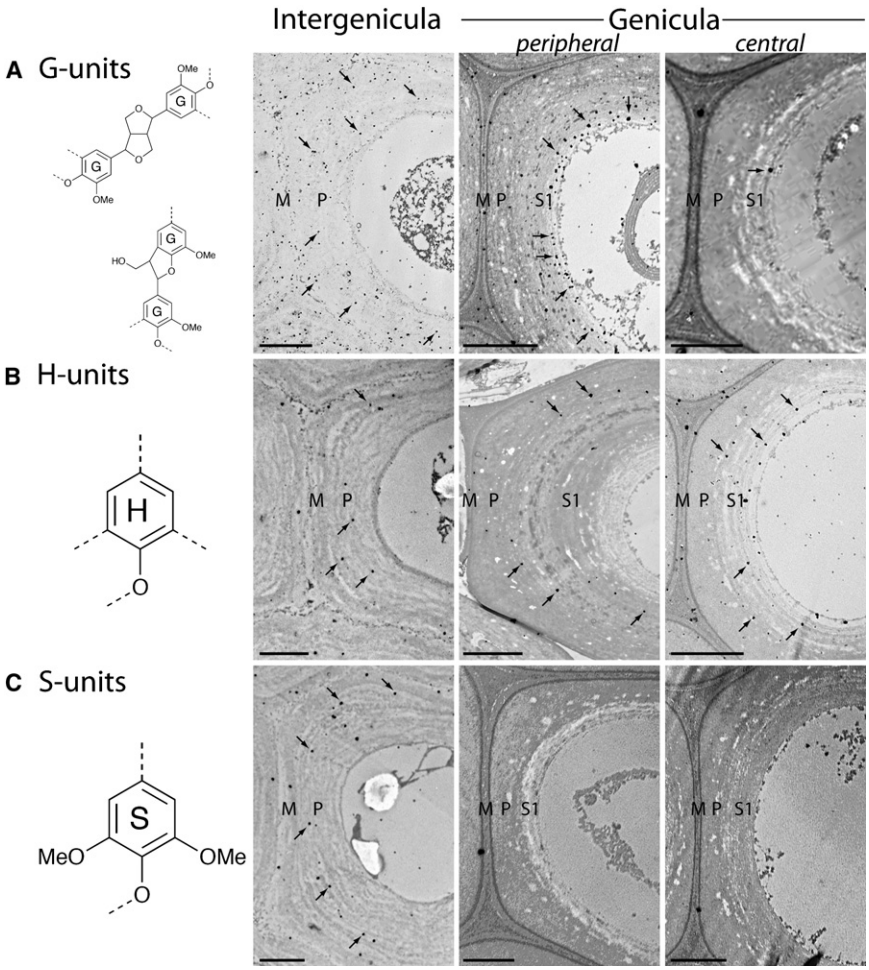

Figure 3. Immunocytochemical Localization of Lignin-Structure Epitopes within *Calliarthron* Tissues

Arrows point to a subset of the gold particles.

(A) Condensed G units are recognized throughout intergenicula and concentrate within secondary cell walls of peripheral genicular cells. Note that the gold particles are associated with the concentric lamellae in genicula. G units are not found in central genicular walls.

(B) H units are recognized throughout intergenicular and genicular walls.

(C) S units are recognized throughout intergenicular walls but are not found in genicular walls.

Scale bars represent 1 μm.

detecting lignins within other aquatic algae remains a distinct possibility.

Alternatively, lignin biosynthesis may have evolved convergently in *Calliarthron* and land plants to generate similar cell-wall structures. For example, angiosperms and the lycopod *Selaginella* synthesize S lignin via distinct and independently evolved cytochrome-P450-dependent monooxygenases [28], and production of S lignin in *Calliarthron* may reflect a third convergent pathway. Genes involved in the synthesis of monolignols have been identified in several vascular plants [2, 19, 29, 30], and experiments to search for homologous genes in *Calliarthron* are currently underway.

Lignins are thought to have evolved in the green algal lineage as adaptations to terrestrial habitats, facilitating hydraulic transport and contributing to the mechanical stability of upright stems. However, contrary to the current paradigm, our data indicate that H, G, and S lignins exist within a red alga's calcified cells that lack hydraulic vasculature and have little need for additional support. We speculate that lignin biosynthetic pathways may have functioned in the common unicellular ancestor of red and green algae, protecting cells from microbial infection or UV radiation [31–33], and in *Calliarthron*, lignins may orient the fibrillar scaffolding [34] that guides $CaCO_3$ deposition [35].

The presence of G lignin within the secondary walls of peripheral genicular cells may represent convergent evolution of cellular architecture in response to mechanical stress, given that G lignins also concentrate within secondary walls of terrestrial plant fibers. Selective pressures in the marine environment differ from those on land, but the wind-induced drag forces that presumably contributed to the evolution of wood in

Figure 2. Polymerization, DFRC Degradation, and Subsequent Detection of Monolignols with the Use of Total-Ion Chromatograms, Mixed-Selected-Ion Chromatograms, and Mass-Spectral Fragmentation Patterns

(A) Monolignols (*p*-coumaryl, coniferyl, and sinapyl alcohols) polymerize to form H, G, or S β-ether units in lignin via endwise radical coupling with the growing end of the polymer. β-ethers are considered diagnostic of lignins.

(B) DFRC degradation releases monolignols (as acetate derivatives) specifically from β-ether structures; the double bond in the DFRC monomer arises only via two-electron reductive cleavage of the bromo-ethers formed during AcBr derivatization. By conducting the reductive cleavage step in d_4-acetic acid and the final acetylation step with perdeutero-acetic anhydride, one can deduce the degree of etherification; phenolic deuteroacetate derivatives G* and S* arise from G and S units in the polymer that were originally etherified (see main text).

(C) Synthetic mixture of H, G, and S standards.

(D) DFRC monomers from *Arabidopsis* stems. *Arabidopsis* produces high-guaiacyl S-G lignin and very low H levels (<1%).

(E1) Monomers released from whole *Calliarthron* thalli. Monomers are not readily apparent in the *TIC* but can be diagnostically found via *MIC*. Whole thalli in this particular sample had relatively high H and S levels.

(E2) Monomers released from *Calliarthron* genicular walls. This sample had a relatively low S level.

(E3) Monomers released from *Calliarthron* intergenicular walls. In this sample, a slightly higher S level was observed.

(E4) Monomers and trideutero-analogs released from *Calliarthron* tissue (see the modified DFRC method described in the text), establishing that most DFRC monomers are released from fully etherified units within polymer chains.

(F) Synthetic G and S monomers and their trideutero-analogs G* and S*. Note that GC separated unlabeled monomers from labeled analogs. GC-retention times (not shown) and mass spectral fragmentation patterns of (G) authentic standard acetylated monolignols matched (H) DFRC monomers released from *Calliarthron*, confirming the identity of diagnostic DFRC products.

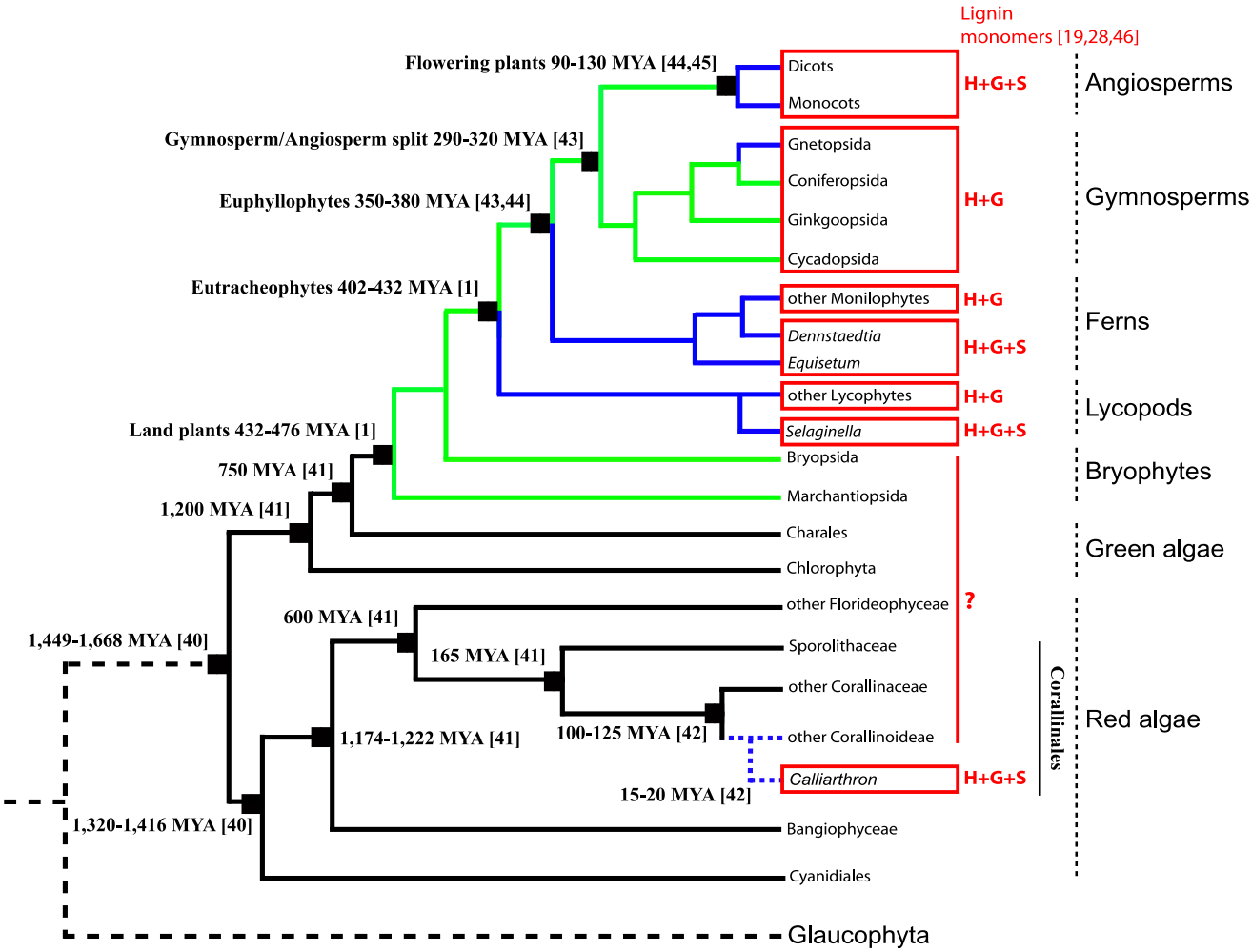

Figure 4. An Illustrated Summary of the Deep Evolutionary Relationship of *Calliarthron* to Lignin-Synthesizing Land Plants

Colored lines emphasize the ancestral development of water-conducting tracheids (green), the development of distinct fibers and vessels (blue), and the development of fiber-like cells in Corallinoid red algae (dotted blue). Like many derived land plants, *Calliarthron* synthesizes monolignols (red boxes), raising many questions about the precise evolutionary history of monolignol biosynthesis and H, G, and S lignins. While "lignin-like" compounds have been identified in some primitive green algae, the presence of true lignins within the wide diversity of evolutionary intermediates remains an open question at this time (denoted by question mark). See [1, 19, 28, 40–46].

terrestrial plants are mirrored by flow-induced drag forces on aquatic algae. On land, xylem lends mechanical support to erect stems [1–3], and in water, genicula provide mechanical support to *Calliarthron* fronds [11, 13]. As articulated fronds bend back and forth under breaking waves, bending stresses are amplified within peripheral genicular tissue [15], which develops thick secondary walls, apparently to resist breakage. Peripheral cells account for most of the lignin detected within genicula (Figure 3) and, given that peripheral cells make up approximately one-third to one-fifth the volume of genicula [13], the level of DFRC monomers released from this fraction may actually be 3–5 times higher (0.75–1.25 mg/g) than that released from intergenicula. We hypothesize that this putative 3- to 5-fold upregulation of lignin biosynthesis in peripheral genicular cells may be mechanically stimulated by bending stresses imposed by breaking waves. Similar mechanical on/off switches for lignin accumulation have been noted in terrestrial systems: plants grown in microgravity synthesize less lignin [36], whereas plants grown in hypergravity synthesize more lignin [37]. The mechanical consequences of such minute quantities of lignin on genicular material properties may be negligible. Nevertheless, that genicular tissue contains lignin and is also stronger, stiffer, and yet more extensible than other algal tissues [11, 15] is an intriguing coincidence, and lignin's potential role in these properties is an area of active research.

Convergent evolution of cell structure and development in *Calliarthron* genicula and terrestrial xylem may clarify lignin biosynthesis and lend insight into the early evolution of land plants. It is striking that *Calliarthron* contains lignified cell walls but evolved from calcified ancestors that lacked water-conducting tracheids or vessels. Vascular plants may have realized hydraulic transport by tapping into ancient biosynthetic pathways that initially evolved to fortify unicellular walls and were later adapted to provide biomechanical support.

Experimental Procedures

Sample Collection

Calliarthron cheilosporioides samples were collected from the wave-exposed, low intertidal zone at Hopkins Marine Station, Pacific Grove, CA, USA.

Sample Preparation

Calliarthron thalli were fixed at 4°C in 1% formaldehyde: 1% glutaraldehyde: 98% seawater and decalcified in 1 N HCl. Genicula were dissected from fronds, dehydrated through an ethanol series, and embedded with Spurr's low-viscosity resin. *Arabidopsis* stem sections were prepared and viewed as previously described [38]. Samples for DFRC analysis were simply ground in a cryogenic mill and extracted with EtOH, acetone, and chloroform for removal of low-MW soluble components, including oligolignols.

Transmission Electron Microscopy

Ultrathin sections were cut with a diamond knife (Diatome, Bienne, Switzerland) on a Leica Ultracut S (Leica Microsystems, Germany), mounted on formvar-coated grids, and stained for 20 s in 3% uranyl acetate in 50% acetone, followed by 3 min in 0.2% lead citrate. Images were taken with a JEOL 1230 TEM (Jeol, Akishima, Tokyo, Japan) at 80 kV with the use of a Gatan peltier-cooled Bioscan camera (Gatan, Pleasanton, California).

DFRC Procedures

DFRC was performed as described previously [39] with minor modifications. In brief, samples (50–70 mg) were suspended in acetyl bromide/acetic acid (20% v/v, 5 ml) in a 25 ml round-bottom flask and gently stirred at 50°C for 3.5 hr. The solid residues were filtered off, and filtrate was evaporated by a rotary evaporator at 50°C under reduced pressure. After evaporation, the residue was dissolved in dioxane/acetic acid/water (5/4/1 v/v, 2 ml), and zinc dust (50 mg) was added to the solution as it was stirred. Stirring was maintained for 30 min. Before gas chromatography-mass spectrometry (GC-MS) analysis, a solid-phase extraction was applied for collection of monomers while dimers and oligomers were removed. Thus, crude acetylated DFRC products in 50–100 μl dichloromethane were loaded onto a preconditioned SPE column (3 ml silica, normal phase, Supelco) and eluted with 12 ml cyclohexane/ethyl acetate (5/1, v/v). The eluant was concentrated to about 0.1 ml for GC-MS analysis.

A modified DFRC procedure was used for the detection of etherified aryl β-ether linkages, dominant interunit linkages present in lignin polymers. After replacement of the reductive cleavage solvent, dioxane/acetic acid/water, with dioxane/d_4-acetic acid/deuterium oxide and acetylation of the DFRC products with d_6-acetic anhydride, monomers released from phenol-etherified β-aryl ether moieties could be distinguished as having their phenolic acetates as trideutero-analogs and a consequently higher mass by 3 units.

GC-MS Analysis

GC-MS was run on a Shimadzu GCMS-QP2010 spectrometer. Specifications for the GC-MS analysis are as follows: column, 0.25 μm film; 0.25 mm × 30 m SHR5XLB (Shimadzu); helium carrier gas; linear-velocity flow-control mode at 35.0 cm/sec; 15:1 split ratio; injector temperature 250°C; initial column temperature 140°C; temperature ramped at 3°C/min to 250°C then ramped at 10°C/min to 280°C and held for 1 min, then finally ramped at 20°C/min to 300°C and held for 17 min.

Immunolabeling

Three specific, previously prepared and characterized [25] polyclonal antibodies were used as antisera. They were directed against (1) condensed homoguaiacyl (G) subunits with C-C interunit linkage, (2) H subunits, and (3) S subunits. TEM immunolabeling was performed on ultrathin transverse sections (500 Å) floating downward in plastic rings passed over 50 μl drops of reactives deposited on parafilm. The labeling process has been previously described [25]. In brief, sections were successively treated with 0.15 M glycine, rinsed with tris-buffered saline (TBS500), and blocked with nonfat dried milk in TBS500. Sections were then incubated on each antiserum, diluted 1: 50–1: 100 in the blocking buffer. They were carefully rinsed before being floated on the secondary marker (protein A-gold [pA G5], BioCell). The sections were then postfixed in 2.5% glutaraldehyde in H_2O. The diameter of the 5 nm gold particles was further enhanced with a silver-enhancing kit from Amersham. Finally, thin sections were transferred on carbon-coated copper grids and poststained in 2.5% aqueous uranyl acetate. Observations were performed at 80 kV with a Philips CM 200 Cryo-electron microscope. All comparative immunolabeling experiments were carried out in parallel in order to keep the same experimental conditions.

Acknowledgments

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