

How Have Plant Cell Walls Evolved?¹

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Plant cell walls are highly sophisticated fiber composite structures that have evolved to fulfill a wide range of biological roles that are central to plant life. In so doing they have diversified not just between species, but also within plants, between cell types, and between cell wall microdomains. Some cell wall components have ancient prokaryotic origins and others are present in extant descendants of the algal ancestors of land plants. In some cases the emergence of certain polymers is correlated with specific land plant taxa or tissue types, but in general our current understanding of cell wall evolution is limited. This is primarily due to a lack of knowledge about cell wall diversity across the plant kingdom, a limited understanding of the structure/function relationships of many cell wall components, and limited information about cell wall-related genes in basal plant species. However, this important area of plant biology is poised to benefit from recent advances in techniques for high-throughput cell wall analysis and genome sequencing. This *Update* focuses on some of the information that is emerging from these new technologies and highlights some of the significant challenges that remain.

Terrestrial ecosystems are dominated by several hundred thousand plant species that display a great diversity of body plans, habitats, and adapted physiologies. Common to all land plants (embryophytes) though are carbohydrate-rich cell walls that provide support, act as defensive barriers, are conduits for information, and are a source of signaling molecules and developmental cues (Bacic et al., 1988; O'Neill et al., 1990; Carpita and Gibeau, 1993; Ridley et al., 2001). Although cell walls display considerable variability in their fine structures, most are essentially highly complex fiber composites based upon a load-bearing network that is infiltrated with matrix polymers. In the primary walls of growing plant cells, cellulose microfibrils are tethered together by cross-linking glycans (also known as hemicelluloses) and this assembly is embedded in matrix polysaccharides

and glycoproteins. In the secondary walls of woody tissues, the embedding material is the phenolic polymer lignin (Carpita and Gibeau, 1993; Fry, 2004; Cosgrove, 2005). Progress has been made in understanding some aspects of the structure/function relationships of cell wall components but many aspects of cell wall biology are poorly understood, including how these remarkable structures evolved.

It is generally recognized that the colonization of land by plants and their subsequent radiation and diversification are profoundly important episodes in the history of life, and it is reasonable to assume that cell walls have played crucial roles in this (Kenrick and Crane, 1997; Karol et al., 2001; McCourt et al., 2004). After all, cell walls are a defining feature of plants and make up much of the plant body. Much of the output of photosynthesis is channeled into cell wall production and in many species a large number of genes are dedicated to cell wall biosynthesis (Reiter, 2002; Scheible and Pauly, 2004; Pauly and Keegstra, 2008). One measure of the importance of cell walls is the degree to which mechanisms have evolved to maintain cell wall functionality in the face of extreme and diverse biotic and abiotic challenges (Pilling and Höfte, 2003). A manifestation of this is the extent to which plants can cope with the loss of seemingly important cell wall components, apparently by compensatory effects involving other cell wall polymers or by inherent redundancy. Advances in analytical techniques for plant cell wall research over the last two decades have been impressive, so much so that our ability to analyze cell walls often exceeds our ability to really make sense of what we find. What is clear though is that plants have remarkable glyco-engineering capacity that produces a unique diversity of complex polysaccharides and the global plant cell wall glycome is one of the richest bioresources on earth.

Until relatively recently the major focus of plant cell wall research has been directed at seed plants (spermatophytes), mostly angiosperms that are either model systems and/or crop plants. This has provided a wealth of data about cell wall composition and associated genes in one sector of the plant kingdom, but understanding cell wall evolution requires far broader genetic and biochemical sampling. A number of recent studies have provided information about the cell wall compositions of nonspermatophyte land plants and streptophyte green algae and these, together with emerging genetic data, enable us to start

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answering some of the important questions surrounding cell wall evolution in the streptophyta.

THE IMPORTANCE AND DIFFICULTIES OF SAMPLING CELL WALL DIVERSITY

A prerequisite to understanding cell wall evolution is to have a comprehensive knowledge of cell walls across the plant kingdom. However, our current understanding is limited to relatively few species analyzed by a variety of techniques that make direct comparisons difficult. This paucity of information is not surprising when one considers the enormity of undertaking a kingdom-wide analysis of cell wall structures, especially since a truly comprehensive survey would have to encompass not only a very large number of species but also include multiple developmental stages, organs, tissues, and cell types (Fig. 1). Moreover, the biochemical techniques that have served so well for the detailed analysis of a few species are not well suited to the comparative analysis of many. Partly in response to this, we have recently developed a microarray-based technique for the high-throughput screening of plant cell wall polysaccharides. The technique, known as Comprehensive Microarray Polymer

Profiling or CoMPP, involves the sequential extraction of cell wall components that are then spotted as microarrays and probed with monoclonal antibodies or carbohydrate-binding modules (Moller et al., 2007; Fig. 1, A and B). We have begun to use CoMPP to explore cell wall diversity across the plant kingdom with a particular focus on less-studied nonangiosperm species. Our findings so far have revealed phylogenetically correlated trends in the occurrence of some polysaccharides and the unexpected presence of others in certain species. For example, (1→4)- β -D-mannan that is generally not particularly abundant in the cell walls of angiosperms is a major cell wall component in several nonspermatophyte plants and appears to have evolved early in the land plant lineage (Moller et al., 2007; Popper, 2008). One surprising result was the discovery of mixed-linkage (1→3),(1→4)- β -D-glucan (MLG) in the horsetail *Equisetum arvense* (Fry et al., 2008a; Knox, 2008; Sørensen et al., 2008; Sørensen and Willats, 2008).

Within land plants, MLG had long been regarded as being unique to one family, the grasses and cereals (Poaceae), but was recently found in the cell walls of other families in the order Poales (Buckeridge et al., 2004; Trethewey et al., 2005). However, the Poales, including the Poaceae, are thought to have evolved

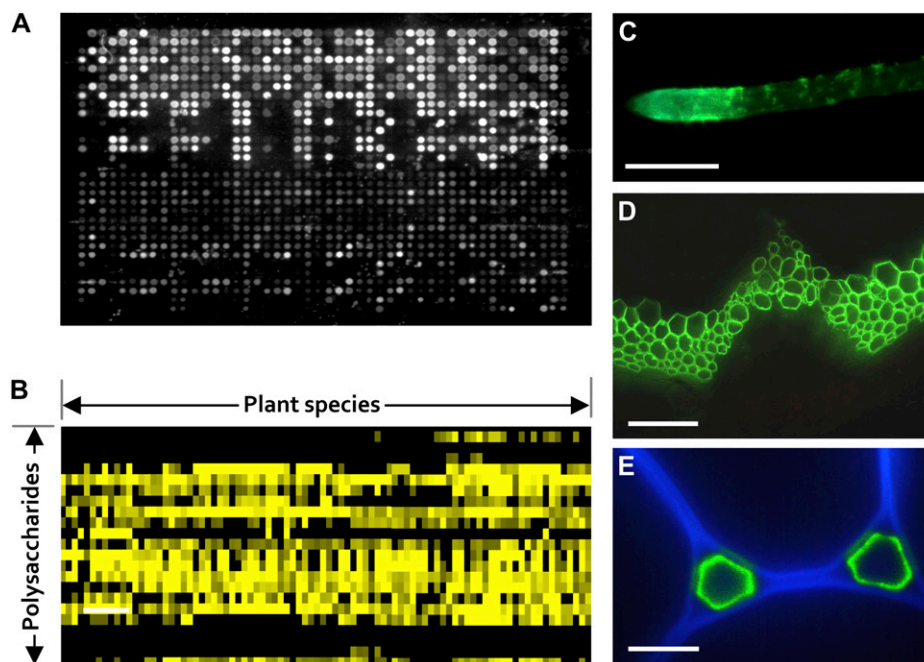


Figure 1. Cell walls display multiple levels of diversity. A and B, Across species—a microarray populated with polysaccharides extracted from >300 plant species and probed with an antibody with specificity for HG. The array (A) and the heatmap of the data derived from it (B) illustrate the differential occurrence of HG across the plant kingdom. The array was produced using the CoMPP technique described in the text. C, Within organs—labeling of an *Arabidopsis* (*Arabidopsis thaliana*) root with an antibody against β -(1→4)-galactan reveals the higher levels of this epitope in the distal portion of the root. Bar = 300 μ m. D, Between tissues—labeling of a section through an *Arabidopsis* stem with an antibody with specificity for β -(1→4)-xylan shows the presence of this epitope in the walls of xylem and interfascicular tissue but not phloem, pith, or cortical tissue. Bar = 20 μ m. E, Between cell wall microdomains—labeling of a section through a pea stem with an antibody with against HG reveals the restricted localization of this epitope in the lining of intercellular spaces. Bar = 10 μ m.

many million years after the horsetails. This finding highlights the importance of wide-scale sampling that encompasses less-well-studied species and raises the question of whether or not the biosynthetic mechanisms of MLG biosynthesis are deeply conserved, or rather have emerged by convergent evolution and involve nonrelated sets of glycosyl transferases (GTs).

The accurate mapping of cell wall diversity is important because it underpins much of our thinking about cell wall evolution and informs our interpretation of emerging genetic data. Although CoMPP is a useful new high-throughput method for cell wall analysis, it is limited by the availability of probes for cell wall components. Also, it provides information about the relative abundance of polysaccharide occurrence across a range of samples rather than information about their absolute levels (Møller et al., 2007). Several biochemical techniques such as methylation analysis and monosaccharide composition analysis do provide more quantitative data but are too low throughput for wide-scale sampling. Advanced techniques involving NMR and mass spectroscopy are undoubtedly powerful and can be used for carbohydrate sequencing but are expensive and highly time consuming. Another problem is that it can be hard to assign sugars and linkages to particular polysaccharides, particularly in cases where there is a paucity of prior knowledge about a particular cell wall type. In this regard we have found that a combinatorial approach is highly effective such that CoMPP is used for rapid polysaccharide screening to identify a subset of samples that are then analyzed in more detail using conventional biochemical approaches (Sørensen et al., 2008).

Immunolabeling of plant materials, although relatively low throughput, offers the unique advantage of providing information not just about the presence or absence of cell wall structural features (epitopes) but also about their cellular locations. Clearly this is immensely valuable in the context of understanding cell wall evolution because it can offer clues to how certain functional requirements may have driven the evolution of particular biosynthetic capacities. Although many antibodies are available for plant cell wall research, it is usually a considerable challenge to identify with precision the epitopes on polysaccharides to which they bind (Møller et al., 2008). Moreover, it should be borne in mind that although a particular antibody might be highly specific for a certain epitope, that epitope may occur on more than one class of polysaccharide. For example, in angiosperms (1→5)- α -L-arabinan is usually associated with the side chains of pectin but also occurs as a structural feature of certain arabinogalactan proteins in the moss *Physcomitrella patens* (Lee et al., 2005). Furthermore, recent work has illustrated that because of widespread epitope masking, great caution is needed when interpreting seemingly straightforward immunolabeling experiments (Marcus et al., 2008). In one example, a xyloglucan (XyG) epitope recognized by antibody LM15 was detected in just a few cell types in pea (*Pisum*

sativum) and tobacco (*Nicotiana tabacum*) stems, but following treatment with pectate lyase the epitope was revealed to be abundant across these organs (Marcus et al., 2008). Related effects have been reported in relation to xylan (Hervé et al., 2009) and mannan (P. Knox, personal communication), and these observations are alarming because they imply that the results and conclusions of numerous immunolabeling studies may need to be reevaluated. On the other hand, a combination of careful digestion of cell walls with well-characterized enzymes and antibody labeling may also be a powerful new approach for dissecting out the fine details of cell wall architectures.

CORRELATIONS BETWEEN CELL WALL STRUCTURES AND PLANT PHYLOGENY

Embryophyte evolution has been characterized by increasing complexity and diversity of body plans, organ, and tissue specialization and the emergence of highly specialized cellular systems (Niklas, 1997; Graham et al., 2000; Taylor et al., 2008). It is reasonable to suppose that to some extent these adaptive changes are underpinned by increasing diversity and complexity of cell compositions and architectures. It is true that in some cases, the appearance of specific cell wall components can be placed in a clear, if sometimes broad, phylogenetic contexts and these have been recently reviewed (Popper, 2008; Sarkar et al., 2009). For example, XyG is thought to be restricted to embryophytes (although this is discussed in more detail later) and rhamnogalacturonan II (RGII) is almost exclusively found in vascular plants (Matsunaga et al., 2004; Popper, 2008). RGII levels appear to have generally increased during the evolution of vascular plants, a trend that is probably correlated with upward growth and the ability to form lignified secondary walls (Matsunaga et al., 2004). RGII can be cross-linked by boron, and it is significant that grasses, which have a lower requirement for boron than dicots, also have reduced levels of RGII in their walls. Xylans are ubiquitous and often highly abundant in vascular plants, and their evolution may have been an important aspect of the preadaptation that enabled the emergence of vascular and mechanical tissues (Carafa et al., 2005; Popper, 2008). Further layers of complexity are revealed when one considers the variations in fine structure within polysaccharide classes. While cellulose and RGII are highly conserved (although some nonseed tracheophytes have RGII in which a L-rhamnosyl residue is replaced by a 3-O-methyl-rhamnosyl), many other cell wall components exhibit phylogenetically correlated variability. For example, the presence of highly substituted xylan epitope recognized by antibody LM11 in hornworts but not other bryophytes supports molecular studies that indicate a sister relationship between the hornworts and tracheophytes (Carafa et al., 2005). Also, detailed biochemical studies of XyGs have revealed intriguing variations in

the side chain structural motifs between taxa (Hoffman et al., 2005; Peña et al., 2008; Hsieh and Harris, 2009). For example, most vascular plants and hornworts produce a structurally homologous XXXG-type XyG with conserved branching patterns and fucosylated subunits. In contrast, the moss *P. patens* and the liverwort *Marchantia polymorpha* produce quite different XyGs that contain β -D-galactosyluronic acid and a branched xylosyl residue. The conservation of the XXXG XyG type in hornworts and tracheophytes is further evidence of a sister relationship between these groups (Peña et al., 2008). Even when cell wall analysis is narrowed down within a single plant group, there are often considerable variations in cell wall composition and/or cell wall architectures. A good example of this is provided by a study by Ligrone et al. (2002) that investigated the occurrence of cell wall polysaccharide and glycoprotein epitopes in water-conducting cells (WCCs) in bryophytes. This study showed that even within a single order, the Polytrichales, there was a remarkable degree of variability in the labeling of WCCs, leading to the conclusion that the cell wall characteristics of WCCs may have been modulated in response to very specific evolutionary pressures that applied differentially to species within the order (Ligrone et al., 2002).

Evolutionary relationships are most readily apparent when variations in cell wall components and phylogenetic distances are relatively small. In some cases though, a fainter line can be traced across taxa and the biochemical increments that underpinned the evolution of a particular polymer can be discerned. For example, lignin, which is an important component of the secondary walls of vascular tissues, is not present in the avascular bryophytes, but these are known to produce phenylpropanoid compounds related to lignin precursors (Popper, 2008). Moreover, the recent discovery of secondary walls and lignin in the red seaweed *Calliarthron cheilosporioides* also provides an example of the convergent or deeply conserved evolutionary history of a cell wall component (Martone et al., 2009).

The work described above and many other studies provide a wealth of data and intriguing correlations that in theory should provide a good basis for tracking the major processes that have underpinned cell wall evolution. In practice though, it is frustratingly difficult to reach further and make firm conclusions about the cell-, tissue-, organ-, and species-specific functional requirements and evolutionary pressures that have molded cell walls over time. One reason for this is that in many, indeed most, cases, we still have a poor understanding of the structure/function relationships of individual cell wall components. Another problem is that the analysis of cell walls across taxa is patchy. A series of careful investigations have provided intense detail in some sectors of the plant kingdom but analysis is altogether absent elsewhere. Also, the toolkit currently available to analyze plant cell walls, whether it be antibodies, genetic resources, or biochemical techniques, is heavily biased toward spermatophytes

and particularly angiosperms. This means that when a particular polysaccharide is deemed to be absent from a particular plant, it may in fact be present but in a context or variant form that renders it invisible to a particular analysis or that leads to equivocal results. For example, the recent discovery of MLG in horsetails was unequivocal because in this case MLG was also detectable by both an anti-MLG antibody and biochemical assays based on the lichenase enzyme that specifically cleaves MLG (Fry et al., 2008a; Sørensen et al., 2008). However, using CoMPP we have also detected relatively high levels of MLG in other species, including species from the of the Caryophyllales order (W.G.T. Willats, unpublished data), but in these cases we did not detect MLG using the lichenase-based MLG assay. This raises the possibility that these species have novel forms of MLG, possibly with unusual substitutions and/or cellotetraose/cellotriose block structures that are recognized by the antibody but not cleavable by the enzyme. Similarly, as discussed below, uncertainty surrounds the presence of XyG in certain streptophyte algae.

THE SPECIAL SIGNIFICANCE OF THE CHAROPHYCEAN GREEN ALGAE

The origins and early evolution of lands plants lie in the mid-paleozoic era between about 480 and 360 million years ago when certain freshwater green algae emerged from an aquatic habitat. If extant, these green algae would be classed among the charophycean green algae (CGA) that include several thousand mostly freshwater species with diverse morphologies ranging from unicells to complex erect branching forms (Kenrick and Crane, 1997; Karol et al., 2001; McCourt et al., 2004; Becker and Marin, 2009). Their phylogenetic position within the green plants means the CGA have a special significance for our understanding of the early origins of plant cell walls and they have already provided much valuable information. For example, analysis of the sites of cellulose synthesis and the sequences of cellulose synthase (*CESA*) genes reveal important evolutionary relationships between *CESA* sequences, terminal complex configuration, and microfibril structures (Tsekos, 1999; Roberts et al., 2002; Roberts and Roberts, 2007). The terminal complexes of the chlorophyte algae (sensu Lewis and McCourt, 2004) studied so far are linear while the rosette configuration that is conserved in land plants is thought to have arisen in the CGA. This is supported by the *CESAs* of the *Mesotaelium caldariorum* that are up to 59% identical at the amino acid level to those in spermatophytes (Roberts et al., 2002). These observations imply that although cellulose biosynthesis has very ancient cyanobacterial origins there were important specific developments in the *CESAs* that occurred in the ancestors of the CGA that are retained in the embryophytes.

A number of studies have provided evidence that in addition to cellulose, many CGA have cell walls that

contain components that are common to embryophyte walls (Popper and Fry, 2003; Domozych et al., 2007b; Eder and Lütz-Meindl, 2009). For example, homogalacturonan (HG) is abundant in the walls of the unicellular desmid *Penium margaritaceum* and in the charalean species, *Chara corallina* (Proseus and Boyer, 2006; Domozych et al., 2007b), where in both cases, as is frequently the case in embryophyte walls, it is complexed with calcium. Moreover, the spatially regulated distribution of HG epitopes with different degrees of methyl esterification (DEs) in *P. margaritaceum* and *Netrium digitus* suggest that as in embryophytes, DE is controlled in relation to specific events during cell development (Popper and Fry, 2003; Domozych et al., 2007b; Eder and Lütz-Meindl, 2009).

Recent work has also provided evidence for arabinogalactan proteins in the cell walls of several CGA where they appear to have functions related to cell development and adhesion (Domozych et al., 2007a; Eder et al., 2008). In addition, there is evidence for certain hemicelluloses, including XyG and MLG, in the walls of some CGA, for example *Micrasterias denticulata* (Eder et al., 2008). Although XyG oligomers have never been released from CGA cell walls following treatment with XyG-degrading enzymes, evidence is nevertheless mounting that certain CGA do, in fact, contain this polysaccharide. In addition to data from immunolabeling and linkage analysis, Fry et al. (2008b) have described the activity of an enzyme, mixed-linkage glucan/XyG endotransglucosylase, in certain CGA that grafts MLG to XyG oligosaccharides. Furthermore, XyG endotransglucosylase activity and a putative XyG endotransglucosylase/hydrolase enzyme have been reported in *Chara vulgaris* (Van Sandt et al., 2007).

We have recently undertaken a large-scale comparative analysis of the cell walls of 10 CGA species using a combination of monosaccharide linkage analysis, CoMPP, and immunolabeling (Domozych et al., 2009; W.G.T. Willats, unpublished data; Fig. 2). This study has shown that, collectively, the cell walls of members of the more evolutionarily advanced CGA orders, including the Charales, Coleochaetales, and Zygnematales, contain HG, XyG, (1→4)- β -D-xylan, MLG, and epitopes that are associated with arabinogalactan proteins and extensins in embryophytes. However, there appears to be a clear phylogenetically correlated transition of cell wall composition within the CGA since most of these polymers are either absent or present at far lower levels in more basal CGA species in the Klebsormidiales and Chlorokybales orders (W.G.T. Willats, unpublished data).

Taken together, analysis so far of the CGA cell walls suggests that the higher CGA have cell walls that to a large degree resemble those of embryophytes and this supports the notion that members of either the Charales or Coleochaetales are the closest living relatives to embryophytes. These findings are highly significant for understanding cell wall evolution since they imply that some features of land plant cell walls evolved prior to the transition to land, rather than having evolved as a

result of selection pressures inherent in that transition. Indeed, it is possible that the ability to synthesize such walls was an aspect of preadaptation that could explain in part why the ancestors of the CGA and not other algae gave rise to the land plant lineage.

EMERGING GENETIC EVIDENCE

A detailed understanding of how cell walls have evolved can only come with a comprehensive analysis of cell wall-related genes across key taxa in the plant kingdom.

The genomes of both the moss *P. patens* and the lycophyte *Selaginella moellendorffii* have now been sequenced and are especially important because they represent a nonvascular land plant and a basal vascular plant, respectively (Rensing et al., 2008; http://wiki.genomics.purdue.edu/index.php/Cell_wall_composition_and_glycosyltransferases_involved_in_cell_wall_formation, http://wiki.genomics.purdue.edu/index.php/Cellulose_synthase_superfamily). These genomes are now providing a wealth of information about the early evolution of cell wall-related GTs (Lee et al., 2005; Roberts and Roberts, 2007). For example, both *P. patens* and *S. moellendorffii* lack orthologs of the spermatophyte *CESA* genes that are specialized for primary and secondary cell wall biosynthesis, indicating that *CESA* diversification and specialization for primary and secondary cell wall biosynthesis was not necessary for the evolution of vascular tissues (http://wiki.genomics.purdue.edu/index.php/Cellulose_synthase_superfamily). In contrast to spermatophytes that contain seven or eight classes of *cellulose synthase-like (CSL)* genes, both *P. patens* and *S. moellendorffii* contain just three, *CSLD*, *CSLA*, and *CSLC*. The GTs encoded by *CSLAs* and *CSLCs* are known to synthesize the backbones of mannan and XyG, respectively, but the product of *CSLDs* is unknown and basal species with a limited set of *CSL* genes may prove useful for elucidating this. The diversification of *CSL* genes has been central to the evolution of hemicelluloses, and a recent analysis by Yin et al. (2009) has shed light on the origins of this gene group. They analyzed 17 land plant and algae genomes and found that six chlorophyte algae have a single-copy *CSL* gene that is most similar to the land plant *CSLAs* and *CSLCs*. They propose that while these two families arose as a result of duplication of an ancestral algal *CSL* gene, the other *CSL* classes have ultimately diversified from *CESA* and possibly *CSLs* of cyanobacterial origin (Yin et al., 2009). Yin et al. (2009) further suggest that the ancestral algae gene that gave rise to *CSLA* and *CSLC* classes may have been a mannan synthase and this accord with the high levels of mannan in the cell walls of many green algae. In the Poaceae, MLG synthesis is mediated by *CSLFs* and *CSLHs* and the absence of these *CSL* classes in *P. patens* and *S. moellendorffii* (and therefore presumably the CGA) raises interesting questions about the biosynthesis of MLG in the CGA. It is highly unlikely to be

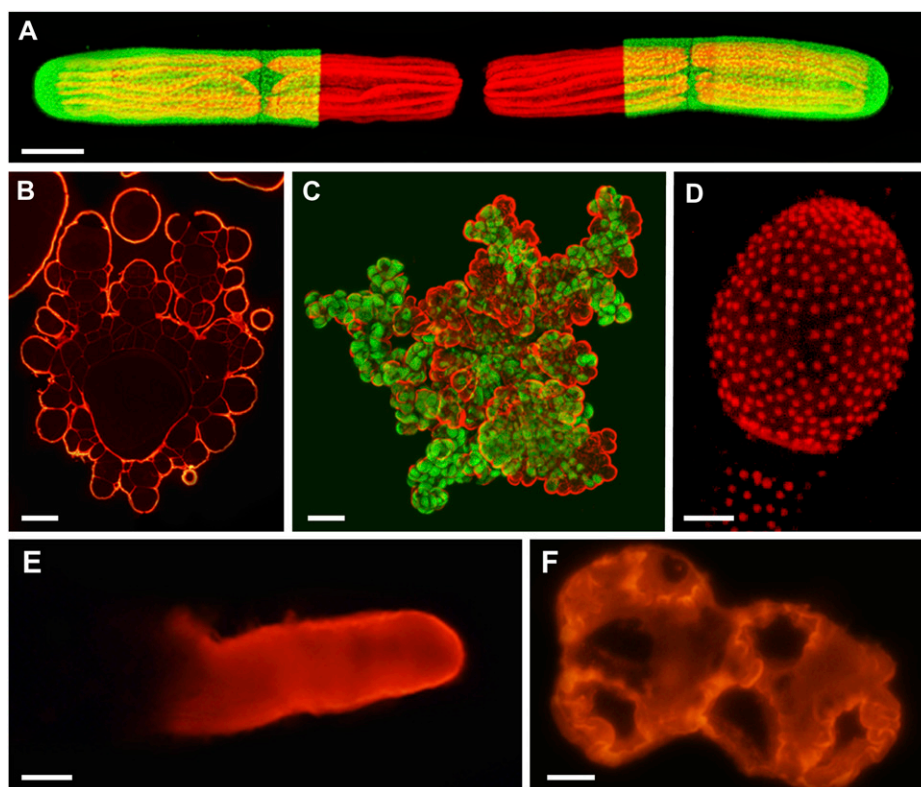


Figure 2. The cell walls of some charophycean green algae contain cell wall components common to land plants. A, A recently divided *P. margaritaceum* cell labeled (green) with the anti-HG antibody LM18. The red color results from the autofluorescence of chlorophyll. The live cell was imaged by confocal laser microscopy (CLSM) 12 h after labeling. Bar = 10 μm . B, A section through the apical region of *C. corallina* labeled (red) with the anti-HG antibody JIM5. Bar = 50 μm . C, The thallus of *Coleochaete orbicularis* labeled (red) with the anti-HG antibody JIM5. CLSM image of live cell 6 h after labeling. Autofluorescence of chlorophyll is also visible (green). Bar = 40 μm . D, The surface of *Pleurotaenium trabecula* labeled (red) with the anti-arabinogalactan protein antibody JIM13. The punctate labeling zones represent the aggregation of stiff hairs found on the outer region of wall pores found on the secondary cell wall. CLSM image of live cell 2 h after labeling. Bar = 25 μm . E, The rhizoid of *Spirogyra* sp. labeled (red) with the anti-arabinogalactan protein antibody JIM16. CLSM of live rhizoid. Bar = 25 μm . F, *Chlorokybus atmophyticus* labeled (red) with the anti-arabinogalactan protein antibody JIM13. CLSM of live cell. Bar = 15 μm .

the product of the ancestral CSLA/C proposed by Yin et al. (2009) and may be produced by a different, convergently evolved biosynthetic route that does not involve CSLs. However, the CSLD class also has ancient origins and may be represented in the CGA. The CSLDs have been proposed to include (1 \rightarrow 4)- β -glucan check for consistency synthases (Doblin et al., 2001) and could conceivably be involved in MLG biosynthesis in the CGA.

CONCLUSION

Although still fragmentary, a body of evidence is now emerging that enables a working model of land cell wall evolution to be tentatively proposed. It seems likely that the evolutionary origins of many, perhaps even most, cell wall components in embryophytes lie in an era prior to the colonization of land. This is certainly the case of cellulose, and the CGA suggest that it is also the case for many other polymers. Although some polysaccharides, notably RGII, do

appear to be embryophyte innovations, the story of cell wall evolution after the colonization of land appears to be characterized mostly by the elaboration of a preexisting set of polysaccharides rather than substantial innovation. The onset of new generation sequencing techniques, combined with new and established methods for wall analysis applied, in particular to basal species, is likely to herald a new age in our understanding of cell wall evolution.

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