Primary Cell Wall Structure in the Evolution of Land Plants

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Abstract

Investigation of the primary cell walls of lower plants improves our understanding of the cell biology of these organisms but also has the potential to improve our understanding of cell wall structure and function in angiosperms that evolved from lower plants. Cell walls were prepared from eight species, ranging from a moss to advanced gymnosperms, and subjected to sequential chemical extraction to separate the main polysaccharide fractions. The glycosyl compositions of these fractions were then determined by gas chromatography. The results were compared among the eight plants and among data from related studies reported in the existing published reports to identify structural features that have been either highly conserved or clearly modified during evolution. Among the highly conserved features are the presence of a cellulose framework, the presence of certain hemicelluloses such as xyloglucan, and the presence of rhamnogalacturonan II, a domain in pectic polysaccharides. Among the modified features are the abundance of mannosyl-containing hemicelluloses and the presence of methylated sugars.

Key words: cellulose; evolution; hemicellulose; methylated sugars; pectin; primary cell walls.


Knowledge regarding the structure of plant cell walls of angiosperms has increased considerably since about 1970 (Carpita and McCann 2000; O’Neill and York 2003). Current studies of cell walls often make use of mutants in the model plant Arabidopsis. When combined with molecular genetic approaches enabled by the complete sequencing of the Arabidopsis genome, studies of these mutants are yielding important new information, particularly in the area of cell wall biosynthesis (Reiter and Vanzin 2001; Doblin et al. 2003).

Although most of the current research on plant cell walls is focused on Arabidopsis and other angiosperms, some recent reports have focused on the cell walls of lower plants including algae (Popper and Fry 2003); seedless, nonvascular plants (liverworts, hornworts, mosses) (Ligrone et al. 2002; Popper and Fry 2003; Kremer et al. 2004; Matsunaga et al. 2004); and seedless, vascular plants (lycophytes and pteridophytes) (Popper and Fry 2003, 2004; Matsunaga et al. 2004). While these studies add to our knowledge about lower plants, it is also possible that our understanding of the cell walls of angiosperms will be increased by improving our knowledge of the cell walls of simpler organisms from which angiosperms evolved. Most evolutionary biologists consider pteridophytes to be the closest living relatives to seed plants (Pryer et al. 2001), with liverworts, hornworts, and mosses representing a more basal stage in evolution than the lycophytes and pteridophytes. Although some investigators consider liverworts to be the earliest land plants (Qui and Palmer 1999), the relationship among liverworts, hornworts, and mosses remains controversial. Likewise, the relationship between these early plants and the vascular plants remains uncertain (Qui and Palmer 1999; Pryer et al. 2001).

Interest in studying lower plants to facilitate our understanding of angiosperms has increased with the development of Physcomitrella patens as a model system. The discovery of efficient gene targeting through homologous recombination in this moss (Schaefer and Zrínyi 1997) and the application of this technique to understand plant function (Reski 1999; Schaefer 2002) were important advances that formed the basis for many current studies using Physcomitrella patens as a model system.

The present study was undertaken as a survey of the glycosyl composition of cell walls from a variety of lower land plants...
up through and including gymnosperms. Emphasis was placed on applying the same cell wall purification and sequential extraction procedures to all of the samples. It was hoped that this uniform approach would facilitate comparisons of cell wall structures across phyla to a greater extent than is possible from the existing published reports and would thereby provide a useful dataset for improving our understanding of the evolution of angiosperm cell walls. The samples were generally taken from green leaves or leafy tissues, with rachis or the central vein excised, except for the plants having only enations or very small leaves closely adherent to the stem, in which case young stem tissue was included in the sample. In the context of cell wall characterization, leaf tissue is considered to be parenchymatous with principally primary cell walls but with some amount of secondary cell wall and lignin present due to tracheids and fibers (Selvendran and O'Neill 1987). This predominance, but not purity, of the primary cell wall in the samples is noteworthy because much of the data on gymnosperm cell walls in the published reports are derived from studies of wood, which is principally secondary cell wall (Stephen 1983).

Results

The glycosyl compositions of the imidazole-soluble, NaOH-soluble, and NaOH-insoluble fractions are presented in Figures 1, 2 and 3, respectively, for the eight plants examined in this project.

For most of the eight plants examined, the imidazole-soluble fraction was rich in galacturonosyl, arabinosyl, galactosyl, and rhamnosyl residues. These sugars are the characteristic components of angiosperm pectic polysaccharides (O'Neill and York 2003), which are usually extractable with imidazole. Some noticeable differences, however, were evident among the eight plants. The fraction from Physcomitrella patens included more than 20 mol% glucose, which was likely due to starch because we have found that some amount of starch persists with the cell wall fraction of the moss even after repeated extraction with dimethylsulfoxide. Elimination of the 1% glucose from the culture medium might be a strategy to reduce this starch contamination, but the glucose addition is desirable because it considerably speeds moss growth (Basile and Basile 1988). It should also be noted in this context that while growth of moss under aseptic conditions in the laboratory ensures that the resulting cell wall fraction is free of contaminants from other organisms, it remains possible that moss grown in the wild might have somewhat different cell wall properties.

Other differences from typical angiosperm pectic polysaccharides were evident in the imidazole-soluble fractions from Psilotum nudum and Equisetum hyemale, both of which were particularly rich in galacturonosyl residues but with relatively little arabinosyl, galactosyl, and rhamnosyl residues. In contrast, the fractions from Psilotum nudum and Equisetum hyemale were relatively rich in mannnosyl and glucurono-syl residues, respectively. Considerable uniformity was evident in the imidazole-soluble fractions from Osmunda regalis, Selaginella lepidophylla, Encephalartos longifolius, Metasequoia glyptostroboides, and Gnetum gnemon. The glucosyl residues present in some of these fractions might again indicate residual starch contamination. Among the minor sugars, xylosyl residues were especially noticeable in Selaginella lepidophylla, Physcomitrella patens, Psilotum nudum, Osmunda regalis, and Gnetum gnemon. The unusual 3-O-methyl-rhamnosyl residue was detected in the imidazole soluble fractions from Physcomitrella patens, Encephalartos longifolius, Gnetum gnemon, Osmunda regalis, and Equisetum hyemale. The fraction from Metasequoia glyptostroboides contained a small amount of 4-O-methyl-glucuronosyl residues.

In the case of angiosperms, the NaOH-soluble fraction would be expected to contain predominantly hemicelluloses, which include both xyloglucans and gluconorarabinoyxylans. Xyloglucans are more abundant in eudicots and many monocots, while the gluconorarabinoyxylans are more abundant in the commelinoid monocots, which include grasses, cereals, palms, bromeliads, gingers, and cypresses (Carpita and McCann 2000). A third hemicellulose, the mixed (1→3),(1→4)-β-D-glucan, occurs in the cereals and grasses of the order Poales. Somewhat consistent with these expectations, xylosyl, glucosyl, arabinosyl, glucuronosyl, 4-O-methyl-glucuronosyl, galactosyl, and fucosyl (the latter two are present in some xyloglucans) residues were generally the predominant sugars in the NaOH-soluble fractions from the eight plants examined (Figure 2). Again, however, noticeable differences were evident among the group. Mannosyl residues were quite abundant in the fractions from Psilotum nudum and Physcomitrella patens, and to a lesser extent, in the fractions from the gymnosperms. Galacturonosyl residues were unexpectedly the most abundant in the fractions from Equisetum hyemale and Osmunda regalis, indicating that the pectic polysaccharides in these species were somewhat resistant to extraction with imidazole and thus appeared with the hemicelluloses in the NaOH-soluble fraction. Differences in pectin extractability occur even just among angiosperms, however, causing some investigators to favor a more complex procedure involving a sequence of steps with increasingly harsh reagents to extract the pectic polysaccharides prior to the hemicelluloses (Selvendran and O'Neill 1987; Fry 1988). The unusual 3-O-methyl-rhamnosyl residue was detected in the NaOH-soluble fractions from Physcomitrella patens, Encephalartos longifolius, Gnetum gnemon, Osmunda regalis, and Equisetum hyemale.

Cellulose is predominant in the NaOH-insoluble fraction of angiosperms, with some extensin glycoproteins also present (O'Neill and York 2003). Thus, the expected glycosyl composition is high glucosyl content with smaller amounts of arabinosyl
and galactosyl residues. The glycosyl contents of the NaOH-insoluble fractions from all eight lower plants were generally consistent with these expectations (Figure 3), although relatively high levels of mannosyl residues were present in the fractions from *Physcomitrella patens*, *Psilotum nudum*, *Equisetum hyemale*, and *Selaginella lepidophylla*, with lesser amounts of this sugar in the other plants.

**Discussion**

This project involved analysis of cell walls of lower plants through sequential chemical extraction to separate the walls into major polysaccharide fractions. Quantification of the glycosyl compositions of these fractions was then carried out through gas chromatography. The results thus obtained are complementary...
to those obtained in the recent extensive surveys by Popper and Fry (2003, 2004), where the approach largely involved enzymic digestion of whole walls and semi-quantitative analysis of the resulting monosaccharide and oligosaccharide products by paper chromatography and paper electrophoresis. Both approaches point to several interesting aspects in the evolution of primary plant cell walls. Among the features common to all of the terrestrial plants examined in these and other studies is an abundance of cellulose as the skeleton of the primary wall (Figure 3 in the present study but more generally Stephen 1983; Bacic et al. 1988; Carpita and McCann 2000). Popper and Fry (2003, 2004) presented strong evidence that xyloglucan is present in the primary cell walls of all land plants examined, but not in the cell walls of charophycean green algae, which are thought to be closely related to land plants (Qiu and Palmer 1999). Thus, xyloglucan appears to be important in the evolutionary step from aquatic to terrestrial plants. The glycosyl compositions of the NaOH-soluble fractions in the
Figure 3. Glycosyl compositions of NaOH-insoluble fractions from eight plants.

Ara, arabinosyl residues; Fuc, fucosyl residues; Gal, galactosyl residues; GaIA, galacturonosyl residues; GlcA, glucuronosyl residues; Glc, glucosyl residues; Man, mannosyl residues; MGlcA, 4-O-methyl-glucuronosyl residues; MRha, 3-O-methyl-rhamnosyl residues; Rha, rhamnosyl residues; Xyl, xylosyl residues.

The present study (Figure 2) would be consistent with the presence of xyloglucan in all of the plants examined, but the relative abundances of the xylosyl, glucosyl, arabinosyl, glucuronosyl, and 4-O-methyl-glucuronosyl residues in Selaginella lepidophylla, Encephalartos longifolius, Metasequoia glyptostroboides, and Gnetum gnemon suggest that glucuronoarabinoxylan might be the more important hemicellulose in these species.

Among the most interesting findings relative to evolutionary change in the cell walls of terrestrial plants is evidence of higher levels of mannose-containing polymers in the primary cell walls in lower plants than in angiosperms (Popper and Fry 2003, 2004; Figures 1–3 in the present study). Mannans and galactomannans are abundant in the cell walls of the endosperm of some seeds (Stephen 1983). In vegetative tissues, glucomannans, galactoglucomannans, and galactomannans are commonly found in angiosperm cell walls, but most often at only low levels (Carpita and McCann 2000). Galactoglucomannans and glucomannans have been most thoroughly studied as major
hemicelluloses in secondary cell walls forming the softwoods of gymnosperms such as Abies, Ginkgo, Larix, Picea, Thuja, and Tsuga where the galactoglucomannans tend to be extractable with NaOH but the glucomannans tend to remain with the cellulose (Stephen 1983; Bacic et al. 1988). Both of these polymers contain a linear backbone of mannosyl and glucosyl residues, both in \((1\rightarrow4)\) \(\beta\)-linkage, in various proportions but usually with the mannosyl residues in greater abundance. Galactosyl residues are also present, generally in \((1\rightarrow4)\) \(\alpha\)-linkage to either the backbone mannosyl or glucosyl residues (Stephen 1983). When the galactosyl side residues are significantly abundant, the name galactoglucomannan is used. Popper and Fry (2003, 2004) found that mannosyl residues were more abundant in the primary cell walls of charophytes, bryophytes, lycophytes, Psilotum, Equisetum, and an eusporangiate fern than in the more advanced leptosporangiate ferns, gymnosperms, and angiosperms. Abundant mannosyl, but less abundant galactosyl, residues were found with the glucosyl residues in the NaOH-insoluble fractions from Physcomitrella patens, Psilotum nudum, Equisetum hyemale, and Selaginella lepidophylla in the present study (Figure 3), probably indicating the presence of glucomannan. Osmunda regalis, a leptosporangiate fern, had relatively little mannosyl content in any of its cell wall fractions. Relatively high mannosyl content, together with glucosyl and galactosyl residues, in the NaOH-soluble fractions and even in the imidazole-soluble fractions from Physcomitrella patens and Psilotum nudum might indicate the presence of galactoglucomannan. Galactoglucomannans have been previously found in the cell walls of the aquatic moss Fontinalis antipyretica (Geddes and Wilkie 1971) and the bracken fern Pteridium aquilinum (Bremner and Wilkie 1971).

Pectic polysaccharides are abundant components of the primary cell walls of most angiosperms, although not in the cereals and grasses where the pectic content is only a few percent (Carpita 1996). Popper and Fry (2003, 2004) found galacturonosyl residues, the characteristic component of pectic polysaccharides, in all of the plants examined. The level of galacturonosyl residues was reported to be higher in the hydrolysates of bryophytes and charophytes than in any of the vascular plants examined (Popper and Fry 2003). The results of the present study (Figures 1–3) show galacturonosyl residues to be present in the cell walls of all eight species examined, but the results do not support a higher abundance in the bryophyte Physcomitrella patens than in the seven vascular plants. Within the group of pectic polysaccharides, at least four types of domains have been recognized: homogalacturonan, rhamnogalacturonan I, rhamnogalacturonan II, and xylogalacturonan. Three of these have a backbone of \((1\rightarrow4)\) \(\alpha\)-galacturonic acid, while rhamnogalacturonan I has a backbone of the repeating disaccharide \(\alpha\)-D-galactosyl-(1\(\rightarrow\)4)\(\alpha\)-D-galacturonic acid-(1\(\rightarrow\)) with about half of the rhamnosyl residues carrying long arabinitol, galactan, or arabinogalactan side chains (Carpita and McCann 2000). The results of the present study are consistent with the presence of these various sorts of pectic domains, but some particular differences among the plants are noteworthy. The glycosyl compositions of the fractions from Equisetum hyemale indicate substantial contents of homogalacturonan that is imidazole-soluble (Figure 1) and rhamnogalacturonan I that is NaOH-soluble (Figure 2) but not imidazole-soluble. In contrast, the composition of the imidazole-soluble fraction from Psilotum nudum seems to indicate the presence of considerable xylogalacturonan.

Among the most interesting findings about the structure of primary cell walls in recent years is the discovery that the rhamnogalacturonan II domain is a site of borate diester cross-linking between pectic polysaccharides (see review by O’Neill et al. 2004). This cross-link has been shown to be required for normal plant growth and development and resolves the longstanding puzzle regarding the role of boron as a required plant micronutrient. The rhamnogalacturonan II domain is structurally very complex, consisting of about 25 sugar residues of 12 different types. Remarkably, this structure is highly conserved across angiosperms, gymnosperms, lycophytes, and pteridophytes. Matsunaga et al. (2004) have recently conducted a detailed study and found rhamnogalacturonan II in a variety of lycophytes and pteridophytes, including both the sporoophyte and gametophyte generations of the fern Cyrtomium falcatum. Rhamnogalacturonan II was barely detectable in bryophytes, being present at less than 1% of the level in vascular plants, and was not detected in the cell wall of green algae. Rhamnogalacturonan II was isolated and partially structurally characterized from Psilotum nudum, Equisetum hyemale, two lycophyte club mosses, and four ferns. The structures were found to be very similar to that of rhamnogalacturonan II in angiosperms, although replacement of one or two terminal rhamnosyl residues by unusual 3-O-methyl-rhamnosyl residues was detected in several of these plants, including Psilotum nudum (Matsunaga et al. 2004). Popper and Fry (2003) found 3-O-methyl-rhamnosyl residues in the cell walls of several charophytes, bryophytes, and lycopodiophytes. Although the findings of Popper and Fry (2003) and Matsunaga et al. (2004) might indicate that the ability to synthesize polymers containing 3-O-methyl-rhamnosyl residues was lost with the evolution of seed plants, such is not the case. The present study (Figures 1 and 2) found 3-O-methyl-rhamnosyl residues in the cell walls of several charophytes, bryophytes, and lycopodiophytes. Although the findings of Popper and Fry (2003) and Matsunaga et al. (2004) might indicate that the ability to synthesize polymers containing 3-O-methyl-rhamnosyl residues was lost with the evolution of seed plants, such is not the case. The present study (Figures 1 and 2) found 3-O-methyl-rhamnosyl residues in the cell walls of Physcomitrella patens, Encephalartos longifolius, Gnetum gnemon, Osmunda regalis, and Equisetum hyemale, but not in Psilotum nudum. Earlier studies also reported 3-O-methyl-rhamnosyl residues in Osmunda japonica (Akiyama et al. 1988) and Encephalartos longifolius (Vogt and Stephen 1993). Thus, the ability to synthesize polymers containing 3-O-methyl-rhamnosyl residues was retained at least into the Gnetophyta, which are commonly regarded as the most advanced gymnosperms. In the case of Physcomitrella patens, we have found that 3-O-methyl-rhamnosyl residues account for approximately 15% of the glycosyl residues in arabinogalactan-proteins (in preparation).
The present and earlier studies have identified some aspects of primary cell wall structure that appear to have been conserved across all terrestrial plants and other aspects that have clearly evolved. Some of these features, such as rhamnogalacturonan II, are strongly supported by detailed structural and functional data. Other features, such as the higher levels of mannosyl-containing polymers in the primary cell walls of lower plants than in angiosperms, require further structural and functional studies. Also poorly understood is the functional significance of methylated sugars, such as the 3-O-methyl-rhamnosyl and 4-O-methyl-glucuronosyl residues discussed in the present report and the 3-O-methyl-galactosyl residues that seem to be unique to lycophyte primary cell walls (Popper et al. 2001). Methylation of DNA has a firmly established functional significance in the regulation of gene expression. Does methylation of polysaccharides also have functional significance?

Materials and Methods

Plant materials

Samples of *Psilotum nudum* (Phylum Pteridophyta, whisk fern), *Equisetum hyemale* (Phylum Pteridophyta, horsetail or scurrying rush), *Osmunda regalis* (Phylum Pteridophyta, fern), *Encephalartos longifolius* (Phylum Cycadophyta, cycad), *Metasequoia glyptostroboides* (Phylum Coniferophyta, dawn redwood), *Gnetum gnemon* (Phylum Gnetophyta, gnetum tree), *Cephalartos longifolius* (Phylum Cycadophyta, cycad), *Selaginella lepidophylla* (Phylum Lycopodiophyta, resurrection plant) was obtained from the Botanic Gardens at the University of California, Riverside. *Gnetum gnemon* (Phylum Gnetophyta, gnetum tree) was collected from specimens in the 1940s in Szechuan, China, and *Selaginella lepidophylla* (Phylum Lycopodiophyta, whisk fern), which have either enations or young stem tissue from growing shoot tips was included in the sample. *Physcomitrella patens* (Phylum Bryophyta, moss) was grown under aseptic conditions in the laboratory from a starting culture kindly provided by Karen Schumaker (University of Arizona). The growing tips (5 mm) of green leafy gametophytes were collected.

Cell wall purification

Specimens (5 g fresh weight) were frozen in liquid nitrogen and then homogenized by grinding to a fine powder in liquid nitrogen with a mortar and pestle. Proteins and other cytoplasmic materials were removed from the powder by extraction for 2.5 h with 35 mL solution A (200 mL 80% (w/w) phenol, 80 mL glacial acetic acid) and then centrifugation (always 10 min at 1200g), followed by re-extraction for 2 h with 35 mL of solution B (175 mL 80% (w/w) phenol, 70 mL glacial acetic acid, 35 mL distilled water) and centrifugation (Fry 1988). Residual phenol-acetic acid was removed by washing the pellet three times with 35 mL of 70% (v/v) ethanol. Extraction of the pellet with 35 mL 90% (v/v) dimethylsulfoxide for 12 h, followed by centrifugation, was carried out to remove starch (Fry 1988). The extraction with 90% dimethylsulfoxide was carried out two more times, and then residual dimethylsulfoxide was removed by washing the pellet twice with 35 mL 70% (v/v) ethanol. Extraction of the pellet with 35 mL 2:1 (v/v) chloroform:methanol for 20 min, followed by centrifugation, was carried out (twice) to remove residual lipids. After two washes (35 mL each) with acetone by centrifugation, the pellet was air-dried and then further dried by vacuum desiccation over P₂O₅ for 2 d to yield the cell wall fraction.

Cell wall fractionation by sequential extraction

An aliquot (0.2 g) of the dried cell wall fraction was twice treated for 10 h with 40 mL 0.5 mol/L imidazole-HCl, pH 7.0, to extract pectic polysaccharides (Mort et al. 1991). The supernatants from the two extractions were collected by centrifugation, pooled, filtered (Whatman GF/A filter disc, Maidstone, UK), dialyzed extensively against distilled water, and lyophilized to yield the imidazole-soluble fraction. The pellet remaining after imidazole extraction was twice treated for 11 h with 30 mL 6 mol/L NaOH with 1% (w/v) NaBH₄ to extract hemicelluloses (Fry 1988). The supernatants from the two extractions were collected by centrifugation, pooled, filtered (Whatman GF/A), titrated to pH 5.5–6.0 with glacial acetic acid, dialyzed extensively against distilled water, and lyophilized to yield the NaOH-soluble fraction. The pellet remaining after NaOH extraction, expected to be mostly cellulose, was washed by centrifugation with 40 mL distilled water seven times to achieve neutralization at pH 6.4–7.0 and was then lyophilized to yield the NaOH-insoluble fraction.

Analysis of glycosyl compositions of cell wall fractions

Glycosyl compositions of the imidazole-soluble and NaOH-soluble cell wall fractions were determined by gas chromatography of trimethylsilyl derivatives of methyl glycosides formed by methanalysis (Komalavilas et al. 1991). The NaOH-insoluble fraction was expected to be rich in cellulose, which does not cleave well by methanalysis, and was thus subjected to hydrolysis with H₂SO₄ (Fry 1988) prior to methanalysis. Identification of the trimethylsilyl methyl glycosides was based on comparison
to retention times of authentic standards and was confirmed by gas chromatography-mass spectrometry as needed.

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References


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