Primary cell wall synthesis and expansion

Setting the boundaries

Mature plant cells typically have two-layered walls: a first-formed thin outer primary wall layer enclosing a later-formed thick inner secondary wall. The surface area of the primary wall limits the size of the cell and thus the maximum amount of biomass that can potentially be accumulated in the secondary wall. By controlling the shape and size of the cell, the primary wall therefore imposes the limits on the amount of inedible biofuel a plant cell can make.

Primary compared with secondary

Primary cell walls are usually much thinner than secondary walls, and constitute a smaller proportion of the biosphere. The cell synthesizes its primary wall before any secondary wall, so the primary wall ends up as the outermost layer of a plant cell. The distinction is that a primary wall is (or was, in its early life) susceptible to plastic extension, i.e. able to accommodate the cell’s irreversible growth in volume. A secondary wall never increases in area, although it can increase in thickness and mass as long as the cell remains alive.

Cellulose is the world’s most abundant organic substance, much of which is potentially processable into liquid biofuels. Most cellulose is found in plants’ thick secondary cell walls, such as those of xylem (wood). Indeed, pine logs are an excellent winter fuel requiring little biotechnology and used since well before the time of Good King Wenceslas.

Although not itself a bulk fuel, the primary wall is nevertheless pivotal because it dictates the shape and area of the plant cell surface, on to whose inner face a bulkier secondary wall may later be plastered. The more expansive the primary wall, the more massive the secondary wall can potentially become.

Two common misconceptions

It is often incorrectly asserted that primary cell walls are ‘rigid’ and ‘lack lignin’. Although it is true that they are often strong (resisting breakage) and may be inextensible (resisting stretching and thus limiting cell expansion), most primary walls are highly flexible (the opposite of rigid). If you freeze and thaw a young plant, you don’t damage its walls, but it does become extremely limp, illustrating the walls’ flexibility. The phrase ‘rigid cell wall’ should be expunged except in discussions of secondary walls. Likewise, primary walls may become heavily lignified in some tissues (especially wood); indeed, lignification of xylem cells generally begins in the primary wall and only later spreads into their secondary wall layers.

Not just cellulose

In land plants, the dry mass of a primary wall is mainly polysaccharides, comprising cellulose, pectins and hemicelluloses (~1:1:1 by weight, except for grasses and cereals)\(^1\). The cellulose occurs as microfibrils; the other two-thirds constitute the wall ‘matrix’. A variable minority is non-polysaccharides such as proteins, glycoproteins, phenolics and minerals: for example, xylem cell walls may become >30% lignin, an intractable phenolic polymer.

Cellulose is an insoluble linear homopolymer of (1→4)-linked β-d-glucose (Glc) residues. Hemicelluloses are diverse polysaccharides, generally capable of hydrogen-bonding to cellulose, built of xylose (Xyl), Glc, mannose (Man), arabinose (Ara) and numerous minor sugars. Pectins are GalA (galacturonic acid)-rich polysaccharides built of at least four distinct domains, the simplest of which is homogalacturonan, a linear homopolymer of (1→4)-linked a-d-GalA residues, partially methyl-esterified. For details, see Figure 1.

Uncharted pathways

Given the abundance of wall polysaccharides in the biosphere, it is astonishing that large gaps remain in our knowledge of the metabolic pathways supplying the sugar-nucleotides which act as the glycosyl donor substrates in their biosynthesis. For example, plants possess enzymes potentially enabling two distinct pathways to supply UDP-GlcA (glucuronic acid). Although GlcA itself is a minor polysaccharide component, UDP-GlcA is the precursor of four other intermediates [UDP-GalA, UDP-apiose (Api), UDP-Xyl and UDP-Ara] needed for polysaccharide biosynthesis; the oxidation necessary to get from the level of a neutral hexose (e.g. Glc) to a uronic acid (GlcA) involves UDP-Glc in one proposed pathway or inositol in the other. Either pathway can operate, but which pathway does operate? Answering this is feasible by a dual-radio-
Wall biosynthesis is often the plant's principal anabolic activity. Cellulose microfibrils are synthesized from UDP-Glc by cellulose synthases located in 'rosettes' within the plasma membrane, and are extruded on to the inner accreting face of the wall. Matrix polysaccharides, in contrast, are produced from diverse NDP-sugars by enzymes in the Golgi membranes. NDP-sugars, synthesized in the cytosol, may enter the Golgi lumen via specific transporters and then serve as sugar donors for the biosynthesis of wall-matrix-destined polysaccharides. As a generalization, cellulose synthase-like (CSL) processive enzymes produce the polysaccharides' backbones, and

**Synthesizing individual polysaccharides**

When applied to cultured Arabidopsis cells, this in-vivo dual-labelling approach showed that the UDP-GlcA oxidation pathway strongly predominates over the inositol pathway. The same experiment also showed that GDP-Man (the mannosyl donor for hemicellulose biosynthesis) arises via Man phosphates rather than by 2-epimerization of GDP-Glc. It will be of interest to apply the same methodology to other plant tissues to test the generality of these conclusions.
matrix polysaccharides are further built up into an exquisite composite material possessing all the necessary properties of strength, flexibility, permeability and extensibility. Indeed, although most matrix polysaccharides are water-soluble when pure, they are difficult to extract from the cell wall. This resistance to extraction is, of course, essential for the outermost part of a cell, especially one that lives in pond water or wet soil. Diverse cross-links, both covalent and non-covalent, integrate the matrix polymers firmly into the wall. In addition, making new cross-links some time after integration may cause wall 'tightening', negatively regulating wall extensibility and thus cell expansion.

Hydrogen-bonding

Hemicelluloses such as xyloglucans hydrogen-bond to the microfibrillar cellulose; and since xyloglucan chains are very long (~100–1000 nm) compared with the spac-
Among all land plants, appreciable MXE activity is confined to a single genus, *Equisetum* (the horsetails; Fig 4). *Equisetum* probably holds the record as the most evolutionarily isolated genus of all living land-plants, its closest extant relatives having diverged >370 000 000 years ago in the Upper Devonian. It has been evolving its own sweet way ever since, paying little attention to what other plants do. Perhaps not surprisingly, it has acquired (or retained) some biochemical peculiarities during this time, one of which is MXE.

*Equisetum* is not a major world crop. However, the other big group of plants possessing MLG are the Poales (including grasses and cereals). These are major world crops, currently lacking MXE; if they can be engineered to express this unique *Equisetum* enzyme, we could discover the biological role of MXE in cell wall assembly, and possibly enhance the mechanical properties of poalean crops.

**XET, a homotransglycanase activity**

The existence of a transglycanase activity (xyloglucan endotransglucosylase; XET), capable of ‘cutting and pasting’ mid-chain glucosyl linkages in the backbone of xyloglucan, was first demonstrated *in vivo* by studies of polysaccharide turnover and confirmed *in vitro* by studies of extracted enzymes. The activity is easiest demonstrated if a radiolabelled oligosaccharide is used as acceptor substrate (Figure 3).

In an *in-vivo* density-labelling isopycnic centrifugation experiment (modelled on the Meselson–Stahl experiment which 40 years earlier had demonstrated the semi-conservative replication of DNA), we showed that xyloglucan chains undergo this ‘cutting and pasting’ reaction during or immediately after secretion into the wall. Newly exocytosed xyloglucan molecules became glycosidically grafted on to pre-existing wall-bound ones, indicating a role in wall assembly.

The importance of XET action during diverse physiological processes such as cell expansion, organogenesis, seed germination and fruit ripening is underlined by the fact that about 20–60 genes predicted to encode the functional product have been found in all higher plants tested. For example, *Arabidopsis thaliana* has a family of 33 genes encoding XET-active proteins. Some members of the family also have xyloglucan endohydrolase (XEH) activity, and the proteins are therefore given the general term ‘xyloglucan endotransglucosylase/hydrolases’ (XTHs). They are carbohydrate-acting enzymes of CAZy class GH16.

An activity comparable with that of XET and found in some primary cell walls is mannan endotransglycosylase (MET), which is an endohydrolase possessing, in addition, significant transglycanase activity.

**MXE, a heterotransglycanase activity**

Whereas XET and MET activities catalyse homotransglycosylation, MXE (mixed-linkage glucan:xyloglucan endotransglucosylase) is a heterotransglycanase. Its preferred donor substrate (i.e. the one that gets cleaved) is a hemicellulose called mixed-linkage β-glucan (MLG; Figure 1), and its preferred acceptor substrate is xyloglucan or an oligosaccharide thereof (Figure 3). This activity may thus contribute to wall assembly by grafting two dissimilar hemicellulose chains together, creating a ‘hybrid’ polysaccharide which might be of unique significance in wall architecture. Purified normal XTHs exhibit slight MXE activity, amounting to about 1/500 of the XET activity.

Figure 3. Cutting and pasting cell-wall polysaccharides by a homo- and a hetero-transglycanase. XET activity cleaves xyloglucan (blue donor substrate; ↑ = bond cleaved) and grafts a portion on to the non-reducing end of an acceptor substrate, which can be xyloglucan or a radiolabelled oligosaccharide thereof (red). MXE, a unique heterotransglycanase found in *Equisetum*, cleaves MLG (yellow donor-substrate; ↓ = bond cleaved), and grafts a portion on to an acceptor substrate (as above)\(^{11}\).

Among all land plants, appreciable MXE activity is confined to a single genus, *Equisetum* (the horsetails; Figure 4). *Equisetum* probably holds the record as the most evolutionarily isolated genus of all living land-plants, its closest extant relatives having diverged >370 000 000 years ago in the Upper Devonian. It has been evolving its own sweet way ever since, paying little attention to what other plants do. Perhaps not surprisingly, it has acquired (or retained) some biochemical peculiarities during this time, one of which is MXE.

*Equisetum* is not a major world crop. However, the other big group of plants possessing MLG are the Poales (including grasses and cereals). These are major world crops, currently lacking MXE; if they can be engineered to express this unique *Equisetum* enzyme, we could discover the biological role of MXE in cell wall assembly, and possibly enhance the mechanical properties of poalean crops.

Figure 4. *Equisetum arvense*, a common representative of what is probably the most evolutionarily isolated living plant genus.
Figure 5. Di-N-galacturonoylputrescine: a proposed cross-link formed by PME acting in transacylase mode on two methyl-esterified homogalacturonan chains, with putrescine as acceptor substrate. The two shades of red represent GalA residues in the two pectin molecules (---continuation of polysaccharide chain); the putrescine group is in blue.

Our current work is exploring diverse donor/acceptor substrate permutations to look for other novel homo- and hetero-transglycosylases. There is no doubt that several new transglycosylase activities exist, and their biological and potential biotechnological significance will require unravelling.

PME in transacylation mode?

If Arabidopsis seems keen on XTHs, with 33 genes, it is quite fanatical about pectin methylesterases (PMEs), with at least 66. Little is known about any enzymological differences between the 66 putative PME isoforms. The classic reaction catalysed is the hydrolysis of homogalacturonan methyl ester groups to release methanol and leave the polysaccharide in a more negatively charged form that is readily cross-linked by apoplastic Ca$^{2+}$. In this sense, PME may be considered a ‘cross-linking’ enzyme, helping with wall assembly and/or tightening.

In principle, however, a ‘PME’ could act as a transacylase as well as a hydrolase, just as XTHs and METs can catalyse transglycosylation and/or hydrolysis. If the acyl donor substrate of a ‘PME’ is assumed to be homogalacturonan methyl ester, the acceptor substrate could theoretically be any of (i) H$_2$O, in a hydrolysis reaction; (ii) an alcohol (other than methanol) or a phenol, in a transterification reaction; or (iii) an amine, resulting in amide (isopeptide) bond formation (Figure 5). The alcohol in (ii) could potentially be a sugar residue in a polysaccharide chain, with the transacylation reaction creating an ester cross-link between the homogalacturonan and another polysaccharide. In (iii), the amine could be a lysine ε-amino group, forming an isopeptide cross-link, e.g. to the lysine-rich glycoprotein, extensin; alternatively, it could be a polycation such as putrescine, which also has the potential to serve as a cross-link because it is bifunctional (Figure 5). Model compounds representing such ester and amide cross-links have been synthesized and characterized, but not yet definitively demonstrated in plant cell walls.

The first boron enzyme?

An intriguing new type of covalent cross-link involves a borate diester. It has long been known that borate reversibly forms esters with most sugars, an observation exploited for paper electrophoresis of ‘neutral’ sugars. However, a cis-diol in a furanose residue (e.g. of ribose or Api) can form exceptionally stable borate esters which appear to act as covalent cross-links in the primary cell wall matrix. Ribose is not a known wall component, but Api residues occur in specific oligosaccharide side chains of the quantitatively minor evolutionarily conserved pectic domain, rhamnogalacturonan-II (RG-II) (Figure 1). Two such Api residues thus become cross-linked through a negatively charged tetrahedral boron atom, forming a ‘diol>B<diol’ bridge.

RG-II–borate cross-links, once formed, appear to be almost permanent under physiological conditions of pH and temperature. However, they form only very slowly in vitro (though much more rapidly in vivo). Boric acid will bond slowly to the Api residues of RG-II in vitro, the bonding being greatly accelerated by Pb$^{2+}$ or Sr$^{2+}$. It is not known what takes the place of these exotic metal ions to promote the reaction in vivo. One exciting possibility is a novel enzyme, which would be the first enzyme with a boron compound as substrate. Other possibilities to account for the bonding of boron to RG-II in vivo are that the boron is delivered not as simple inorganic H$_3$BO$_3$, but attached to an organic ligand, or that the RG-II is chaperoned in some way which facilitates the approach of either H$_3$BO$_3$ or ligand-bound borate. This important aspect of primary cell wall biosynthesis is currently very poorly understood.

Xyloglucan–pectin bonding

Some allegedly discrete polysaccharides may be covalently linked to each other through a glycosidic bond between the reducing terminus of one and a non-reducing terminus of another. For example, xyloglucan may be attached to an acidic RG-I, giving these otherwise neutral xyloglucan segments a net negative charge. In vivo radio-labelling studies show that the xyloglucan–RG-I linkage is a long-lived stable feature of wall architecture, and is formed co-synthetically (intraprototoplasmically) rather than after secretion into the wall.

They’re not all Arabidopsis and rice

Wall biosynthesis has been best studied in flowering plants, especially dicotyledons such as Arabidopsis thaliana. However, different plants have different primary wall compositions. Most importantly, because they are major world crops, the Poales (grasses, cereals etc., conveniently modelled by rice) have primary walls containing relatively little xyloglucan and pectic polysaccharides,
but possessing mixed-linkage glucan (MLG; Figure 1) which is absent from dicotyledons, although recently discovered in the non-flowering genus *Equisetum*.

Studies of algae, bryophytes, pteridophytes, gymnosperms and diverse flowering plants lead to the generalization that giant lifestyle leaps in plant evolution were often accompanied by drastic changes in cell-wall composition.

Not only polysaccharides, but also wall enzymes vary phylogenetically. Our laboratory has recently surveyed numerous land plants and found drastic differences in their repertoire of glycosidase and glycanase activities, as well as revealing several novel transglycosidase and transglycanase activities. Thus, despite their appealing tractability for molecular biological studies, *Arabidopsis* and rice should not be the sole focus of attention in explorations of the primary cell wall.

**Conclusions**

In conclusion, progress is beginning to be made towards understanding the biosynthesis of primary cell wall polysaccharides and their sugar-nucleotide precursors, but much remains to be discovered about the cross-linking and other types of re-structuring which enable the assembly of the primary wall and control its expansion. This matters because the primary cell wall dictates the final surface area and shape of a plant cell, and thus the extent of the outer framework on to which any secondary wall is subsequently deposited. Secondary wall layers, which offer most of the world’s inedible biofuel, can become much bulkier than the primary wall, but can never exceed it in area, and in this sense their biomass is limited by the primary wall.

We thank the UK BBSRC and the Leverhulme Foundation for grants in support of our work on primary cell walls.

**References**

1. Ulvskov, P. (ed.) (2010) Plant Polysaccharides. Annual Plant Reviews, vol. 41, Wiley-Blackwell, Oxford
2. Sharples, S.C. and Fry, S.C. (2007) Plant J. 52, 252–262
3. Reyes, F., Orellana, A. (2008). Curr. Opin. Plant Biol. 11, 244–251
4. Cocuron, J.C., Lerouxel, O., Drakakaki, G. et al. (2007) Proc. Natl. Acad. Sci. U.S.A. 104, 8550–8555
5. Cavalier, D.M., Lerouxel, O., Neumetzler, L., et al. (2008) Plant Cell 20, 1519–1537
6. Rose, J.K.C., Braam, J., Fry, S.C. and Nishitani K. (2002) Plant Cell Physiol. 43, 1421–1435
7. Thompson, J.E. and Fry, S.C. (2001) Plant J. 26, 23–34
8. Eklöf, J.M. and Brumer H. (2010) Plant Physiol. 153, 456–466
9. Hrmová, M., Farkaš, V., Lahnstein, J. and Fincher, G.B. (2007) J. Biol. Chem. 282, 12951–12962
10. Schröder, R., Wegzyn, T.F., Sharma, N.N. and Atkinson, R.G. (2006) Planta 224, 1091–1102
11. Fry, S.C., Mohler, K.E., Nesselrode, B.H.W.A. and Franková L. (2008) Plant J. 55, 240–252
12. Kosik, O., Auburn, R.P., Russell, S. et al. (2009) Glycoconjugate J. 27, 79–87
13. Lenucci, M., Piro, G., Miller, J.G. et al. (2005) Phytochemistry 66, 2581–2594
14. O’Neill, M.A., Ishii, T., Albersheim, P. and Darvill, A.G. (2004) Annu. Rev. Plant Biol. 55, 109–139
15. Popper, Z.A. and Fry, S.C. (2008) Planta 227, 781–794
16. Popper, Z.A. (2008) Curr. Opin. Plant Biol. 11, 286–292

**Stephen Fry** carried out his PhD in plant physiology at Leicester University, then moved into plant biochemistry as a postdoc at the Universities of Cambridge and Colorado. Since 1983, he has been at the University of Edinburgh, where he holds a personal chair in plant biochemistry. His research focuses on documenting enzyme action in vivo (as distinct from enzyme activity in vitro) by tracing the synthesis, cross-linking, restructuring and degradation of cell-wall polysaccharides in living plant cells. Email: s.fry@ed.ac.uk

**Lenka Franková** is a senior postdoctoral researcher in the Institute of Molecular Plant Sciences at the University of Edinburgh. She was awarded her MSc and PharmDr degree from the Faculty of Pharmacy ofComenius University in Bratislava, Slovakia. She completed her PhD in Plant Physiology and Biochemistry at the Institute of Botany of the Slovak Academy of Sciences. She is interested in cell wall glycobiology and biochemistry. Lenka’s previous project was focused on developing high-throughput screens for wall-modifying enzymes and their inhibitors and she is currently exploring novel wall enzyme activities in the closest living algal relatives of land plants. Email: lenka.frankova@ed.ac.uk

**Dimitra Chormova** is a postdoctoral researcher in the Institute of Molecular Plant Sciences at Edinburgh University. She received her BS degree in crop science and her PhD in agriculture and food science at the University of Newcastle. Her current research interests centre on discovering new inhibitors of cell-wall enzymes and characterizing the biochemical role of boron in plants. Email: dimitra.chormova@ed.ac.uk