Annual Plant Reviews (2011) **41**, 1–42
doi: 10.1002/9781444391015.ch1

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Chapter 1

CELL WALL POLYSACCHARIDE COMPOSITION AND COVALENT CROSSLINKING

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Manuscript received February 2008

Abstract: Genetics now potentially lets us modify the production, crosslinking and degradation of cell wall polysaccharides. There remains, however, the need to test experimentally whether intended modifications of polysaccharide metabolism have successfully been effected *in vivo*. Simple methods for this are described, including *in-vivo* radiolabelling, enzymic dissection (e.g. with Driselase) and chromatographic/electrophoretic fractionation of dissection products.

After an overview of polysaccharide chemistry, I discuss the structures and taxonomic distribution of wall polysaccharides in charophytes and land plants. Primary and secondary walls are compared.

The major wall polysaccharides are cellulose [microfibrillar β-(1→4)-D-glucan], pectins (α-D-galacturonate-rich) and hemicelluloses (lacking galacturonate; hydrogen-bonding to cellulose; extractable by 6 M NaOH at 37 °C). Land-plant pectins are anionic polymers built of about four glycosidically interconnected domains (homogalacturonan, rhamnogalacturonans I and II, xylogalacturonan). Hemicelluloses occurring in most/all land plants are α-xylo-β-glucans, β-xylans (including α-arabino-β-xylans, α-glucurono-β-xylans, etc.) and β-mannans (including α-galacto-β-mannans, β-gluco-β-mannans, etc.). Another hemicellulose [mixed-linkage β-(1→3)(1→4)-D-glucan) is confined to *Equisetum* and some Poales.

Other taxonomically restricted features of angiosperm primary walls occur in Poales (xylose-poor xyloglucans; feruloylated arabinoxylans); Solanales and Lamiales (characteristic xyloglucans); Caryophyllales (feruloylated pectins); and Alismatales (apiogalacturonan). I also summarize characteristic wall features of charophytes, bryophytes, lycopodiophytes, fern-allies and gymnosperms.

Annual Plant Reviews Volume 41, Plant Polysaccharides, Biosynthesis and Bioengineering, First Edition, edited by Peter Ulvskov

The making or breaking of a 'crosslink' (defined as an individual chemical bond, not a whole 'tethering' chain) may cause wall tightening/loosening. Covalent crosslinks include phenolic coupling products, uronoyl esters and amides, and borate diesters.

Keywords: angiosperms; apiogalacturonan; bryophytes; cellulose; charophytes; chromatography; crosslinks (covalent); Driselase; electrophoresis (paper); gymnosperms; hemicellulose; homogalacturonan; hydrolysis; mannans; mixed-linkage glucans; pectins; polysaccharide chemistry; primary wall; pteridophytes; radiolabelling; rhamnogalacturonans; secondary wall; taxonomic variation; uronoyl esters/amides; xylans; xylogalacturonan; xyloglucan

1.1 Remit

This chapter discusses the main cell wall polysaccharides of streptophytes – i.e. land plants (embryophytes, from liverworts to angiosperms) plus charophytes (a group of algae sharing many subcellular features with land plants). Primary structures (sequences of sugar residues) and covalent crosslinks are discussed. A brief introduction to the vocabulary of polysaccharide ('glycan') chemistry, and the sugar abbreviations used, is included in the legend to Fig. 1.1; these abbreviations are used throughout the text without further definition. Further details of secondary and tertiary structures of polysaccharides can be found in Chapter 14. A major theme in the present chapter is the application of simple analytical methods by which polysaccharides can be identified, characterized and quantified, and their metabolism monitored *in vivo*. Another theme is the taxonomic distribution of various wall polysaccharides. Works complementing this chapter include Brett & Waldron (1996), Fry (2000), Schols & Voragen (2002), Mort (2002), O'Neill & York (2003) and Obel *et al.* (2006).

1.1.1 Some definitions

The best-known cell wall component is **cellulose** – a highly insoluble polysaccharide, of which the microfibrils ('scaffolding') of the wall are composed. Cellulose is, however, only one of many polysaccharides found in plant cell walls, usually accounting for less then half the wall's dry mass. The other, non-cellulosic, wall polysaccharides (matrix components) are categorized into **pectins** and **hemicelluloses**.

1.1.1.1 Pectins

These were traditionally defined by their extractability from the wall with chelating agents, often with the assistance of heating (though this inevitably causes partial degradation of pectins and should not be used if a determination of molecular weight is planned) and often followed by ice-cold aqueous Na_2CO_3. A more acceptable definition of pectins is wall polysaccharides rich in α-GalA residues.

1.1.1.2 Hemicelluloses

These are not extracted by chelating agents or ice-cold Na_2CO_3, but are by concentrated aqueous alkali. They generally share the property of hydrogen-bonding to cellulose, at least *in vitro*; and some hemicelluloses probably also do this *in muro*, tethering adjacent microfibrils (Fry 1989; Hayashi 1989). For this reason, the term 'crosslinking glycans' was suggested for hemicelluloses. However, this term is not used here because the proposed *in-muro* tethering role remains largely hypothetical in many cases, and also because some non-hemicellulosic polysaccharides (e.g. rhamnogalacturonan II, RG-II) do crosslink.

1.1.1.3 Crosslinks

A 'crosslink', as the term is used here, is an individual chemical bond, e.g. an ester linkage or a hydrogen bond, that joins together two otherwise separate polymers; it is not a whole molecular chain that joins together two structures (e.g. a xyloglucan chain tethering two microfibrils).

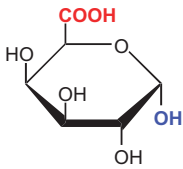
Definitions of polysaccharide classes by their extractability from the wall are far from perfect. One problem is that some polysaccharides chemically identical to hemicelluloses but not hydrogen-bonded to cellulose can sometimes be solubilized with hot neutral water – for example, the bulk xyloglucan present in some seeds as 'food reserves'. Another problem is that some hemicelluloses are covalently attached to pectins, resulting in hybrid polysaccharides that are difficult to classify. Nevertheless, the broad classification of wall polysaccharides into pectins, hemicelluloses and cellulose remains a useful convention.

1.1.1.4 Non-polysaccharide components

Also important in cell walls are non-polysaccharide components. First among these is water, typically accounting for around 60% of the wall's total fresh weight, and around 70% of the fresh weight of the wall matrix (Monro *et al.* 1976). Water confers important physical properties on (hydrated) wall polysaccharides, acts as a solvent for apoplastic solutes and enables the functioning of wall-located enzymes. Changes in the water content of the matrix may explain changes in wall extensibility (Ulvskov *et al.* 2005; Thompson 2008). Other wall constituents are highly variable between tissues, developmental stages and taxa: (glyco)proteins (e.g. extensins and arabinogalactan-proteins); the phenolic polymer lignin; the polyesters cutin and suberin; highly resistant cutan and sporopollenin; and silica (Fry 2001).

1.1.1.5 Primary wall

A primary wall layer is one whose cellulosic microfibrils were laid down while the cell was still (capable of) growing. Once deposited and the cell has stopped growing, a primary wall layer will not acquire more cellulose, although in certain cell types it later becomes impregnated with for example lignin or cutin. Such a wall layer is still 'primary', even if lignified.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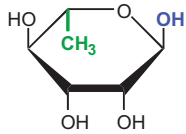
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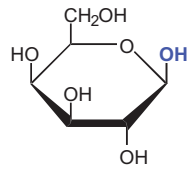
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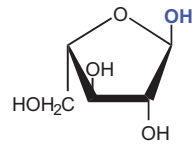
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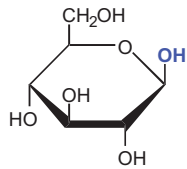
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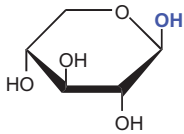
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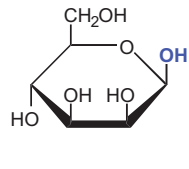
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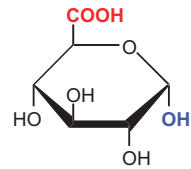
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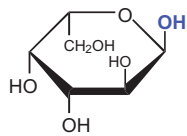
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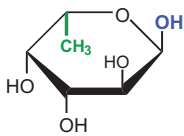
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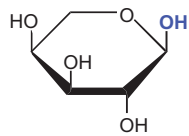
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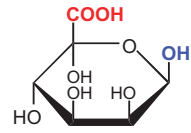
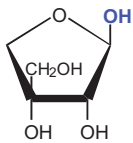

D-*lyxo*-5-Hexosulose uronic acid (Hua)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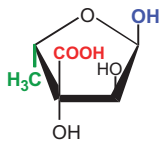
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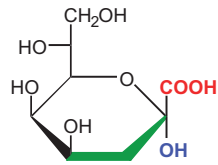
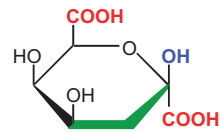

[2-Keto]-3-deoxy-α-D-*manno*-octulosonic acid (α-Kdo)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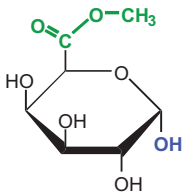
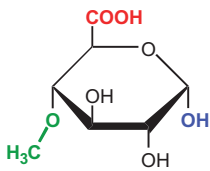
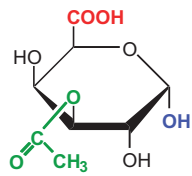

Methyl α-D-galacturonate [ester] (MeGalA)


4-*O*-Methyl-α-D-glucuronic acid [ether] (MeGlcA)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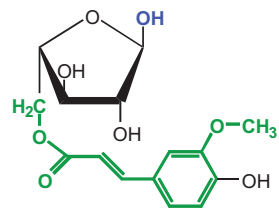
5-*O*-Feruloyl-α-L-arabinose [ester] (Fer-Ara)

Figure 1.1 Monosaccharide building blocks (shown as Haworth formulae) of plant cell wall polysaccharides. The figure shows all the known sugar residues of plant cell wall polysaccharides and a selection of their esters and ethers. Top row, major components of pectins; 2nd row, major components of hemicelluloses; 3rd row, minor sugars of various origins; 4th row, mainly or only known from RG-II; bottom row, a selection of sugars with non-carbohydrate substituents. Sugars with five and six C atoms are called pentoses and hexoses respectively; Rha and Fuc are deoxyhexoses.

The monosaccharides are shown as hemiacetal or hemiketal rings. However, within a polysaccharide, each sugar (except one, the reducing terminus) is present as an acetal or ketal residue, the term 'residue' implying that it is 'what remains' after losing the –OH group (shown in blue) from the anomeric carbon (the anomeric carbon is the one with single-bonds to *two* oxygens; it is here drawn as the right-hand extremity of the hexagon or pentagon). In a sugar residue of a polysaccharide, this particular –OH group has departed (in the form of H_2O), 'taking with it' one oxygen-linked H atom from the next sugar along the polysaccharide chain. The one sugar of the polysaccharide that is not strictly a residue is the reducing terminus, so called because it has not lost its anomeric –OH group and in aqueous solution can therefore equilibrate with the straight-chain form, which possesses an oxo group (C=O, which has reducing properties).

All but two of the sugars shown are aldoses (i.e. the anomeric carbon has only one additional C atom attached to it), but Kdo and Dha are ketoses (the anomeric C is attached to two other carbons). (Hua has two anomeric carbons (C-1 and C-5) and is both an aldose and a ketose.) In aqueous solution, each illustrated hemiacetal and hemiketal equilibrates with a small percentage of a straight-chain form possessing an oxo group (an aldehyde or ketone, in aldoses and ketoses respectively) – hence the slightly redundant term 'keto' in the names of Kdo and Dha.

Each named sugar could theoretically occur as two isomeric forms (enantiomers, designated D- and L-), distinguished by the orientation of the C–O bond of the penultimate C atom. Galactose is the only wall residue known to occur as both D- and L-enantiomer. Note that D- and L-Gal differ in orientation of the C–O bond at all four non-anomeric, chiral centres (= carbons 2, 3, 4 and 5; the difference at C-5 is indicated by the placement of the $–CH_2OH$ group).

The linkage between a sugar residue and the next building-block along a polysaccharide chain can be in either of two isomeric forms (anomers, designated α- and β-) defined by the orientation of the bond between the anomeric C atom and the oxygen atom (shown in blue) that bridges the two sugars: if this C–O bond has the same orientation as that of the penultimate C atom, then the residue is α-; if opposite, β-. This means that, in these Haworth formulae, the –OH of the anomeric carbon points down in α-D- and β-L-sugars, and up in β-D- and α-L-sugars.

The sugar ring can be 6-membered (pyranose; -*p*) or 5-membered (furanose; -*f*). Api and AceA *must* be -*f* because of the absence of an oxygen on a C-5, and MeGlcA can only be -*p*. Ara occurs in both forms. All the others could theoretically occur in either form, but in practice occur only in the -*p* form illustrated.

Each sugar residue is attached, via its anomeric carbon, to an –OH group on the following sugar unit in the polysaccharide chain. Usually, there are several such –OH groups to choose from (e.g., in the case of Glc*p*, on carbons 2, 3, 4 or 6: the linkage is designated (1→2), (1→3), (1→4) or (1→6), accordingly). However, a given sugar unit (either a residue or the reducing terminus) can and often does have more than one sugar residue attached to it.

Once it has become part of a polysaccharide chain, a given sugar residue is 'locked' in one of the four possible ring forms (α-*p*, β-*p*, α-*f*, or β-*f*). These ring forms have a huge impact on the polysaccharide, as is obvious from the enormous differences in physical, chemical and biological properties between amylose and cellulose (which are α-*p* and β-*p*, respectively, but otherwise identical).

Although illustrated here in unionized form, the free carboxy groups (–COOH, shown in red) would often be negatively charged ($–COO^-$) under physiological conditions of pH. Relatively hydrophobic (non-polar) groups are shown in green.

Abbreviations: The diagrams show (in parentheses) the shorthand used throughout this chapter. Thus, unless otherwise stated in the text, the ring-form (-*p* or -*f*) and enantiomer (D- or L-) are assumed to be as illustrated here; for example, 'β-Gal' implies β-D-Gal*p* unless specified as L-Gal. Other abbreviations used (not illustrated): MeXyl, 2-*O*-methyl-α-D-Xyl*p* (ether); MeFuc, 2-*O*-methyl-α-L-Fuc*p* (ether); MeRha, 3-*O*-methyl-α-L-Rha*p* (ether); MeGal, 3-*O*-methyl-D-Gal*p* (ether); 5AcAra, 5-*O*-acetyl-L-Ara*f* (ester); 6AcGal, 6-*O*-acetyl-D-Gal*p* (ester); 6AcGlc, 6-*O*-acetyl-D-Glc*p* (ester); ΔUA, a 4,5-unsaturated, 4-deoxy derivative of GalA or GlcA.

1.1.1.6 Secondary wall

A secondary wall layer is one whose microfibrils were laid down after the cell had lost the ability to expand. Since microfibrils are laid down adjacent to the plasma membrane, it follows that the primary wall is external to the secondary wall and that the most recently deposited layer of secondary wall is innermost. Xylem vessel elements, tracheids, sclerenchyma and cork are cell types depositing much secondary wall material. Like primary wall layers, secondary layers sometimes later become impregnated with non-polysaccharide substances, typically lignin and suberin.

1.2 The classic primary cell walls of dicots

For many years, rapidly growing and dividing suspension-cultured cells of sycamore (*Acer pseudoplatanus*) were the paradigm for primary cell wall studies. They do, however, have certain unusual features, e.g. an exaggerated extensin content (~20% of the dry weight) and – probably associated – resistance to digestion by 'protoplasting' enzymes.

Not all primary walls are chemically identical. There is taxonomic variation, surveyed later in this chapter. In addition, there are distinct differences between different primary walls within a given plant. Celery parenchyma, for example, has an unusually low xyloglucan content (Thimm *et al.* 2002). Changes in wall polysaccharide biosynthesis also occur during the cell cycle (Amino *et al.* 1984). Another example is provided by the duckweed *Spirodela*, which has a high proportion of apiogalacturonan in the walls of its vegetative fronds but almost none in its turions (organs of perennation). The frond:turion ratios (of rates of *de-novo* synthesis of polysaccharide residues) were 165:1 for β-Api, 11.5:1 for α-Rha and 1.7:1 for α-GalA. Thus, compared with fronds, developing turions continued to synthesize pectin, but much less rhamnogalacturonan and almost no apiogalacturonan (Longland *et al.* 1989).

1.2.1 Pectins

Much useful work on pectin characterization can be performed on whole isolated cell walls or alcohol-insoluble residue (AIR) (see Section 1.6). However, if necessary, pectins can be extracted from some tissues (especially ripe fruits) with chelating agents at neutral pH and 20 °C. This does not apply to most other plant tissues, especially actively growing ones. Extraction can be increased by heating, but with partial depolymerization. Heating at around pH 4 is least detrimental in this respect; at this pH, oxalate is a more effective chelator than EDTA or EGTA. Oxalate is also easier than EDTA, EGTA and hexametaphosphate to remove (e.g. by dialysis) after pectin extraction. Another agent increasing some pectins' extractability,

and maintaining their solubility, is 0.5 M imidazole (pH 7) (Zhang *et al.* 2007).

The 'pectins' described below (homogalacturonan, RG-I, RG-II, etc.) are probably conjoined domains, linked end-to-end by glycosidic bonds into a single polysaccharide; *in muro* each 'pectin' is not a separate polysaccharide in its own right. For example, high-M_r pectin extracted from sugar-beet roots with aqueous imidazole was degraded to RG-I, RG-II and free GalA by polygalacturonases (which hydrolyse homogalacturonan but not rhamnogalacturonans; Ishii & Matsunaga 2001). The simplest explanation is that the pectin was a long chain with RG-I and RG-II domains arranged like beads along a homogalacturonan 'string'. Further support for the domain concept came from the characterization of oligosaccharides released by mild acid hydrolysis, including GalA-GalA-GalA-Rha-GalA-Rha, which appears to be a fragment straddling part of a homogalacturonan domain and part of an RG-I domain (Coenen *et al.* 2007).

1.2.1.1 Homogalacturonan domains

These quantitatively major domains comprise an unbranched chain of anionic α-GalA and uncharged MeGalA (= methyl ester of α-GalA) residues joined by (1→4)-bonds. The MeGalA residues tend to occur contiguously, as neutral blocks, along the homogalacturonan chain; this arrangement probably arises by the progressive action of pectin methylesterase on a more fully methylesterified precursor chain. The backbone also includes some 2AcGalA and/or 3AcGalA residues, which may affect the polysaccharide's solubility but not its charge. After de-esterification, e.g. by ice-cold dilute alkali, homogalacturonan can be cleaved by endo-polygalacturonase (EPG) to small fragments with degree of polymerization (DP) 1–3, which serve as a simple quantitative assay for this domain.

Homogalacturonans are water soluble at neutral and alkaline pH, but insoluble at mildly acidic pH (especially after removal of any methyl ester groups) and in the presence of Ca^{2+}.

De-esterified homogalacturonans are digested by 'Driselase' (a mixture of numerous endo- and exo-hydrolases from the fungus *Irpex lacteus*; Fry 2000), giving a quantitative yield of free GalA. Driselase is preferred over acid hydrolysis for this purpose because (1) uronosyl linkages are only slowly acid-hydrolysed, and (2) the free GalA liberated is considerably less stable in hot acid than naturally occurring neutral monosaccharides, so recovery is low. Driselase also contains esterases that convert MeGalA to GalA unless a nearby *O*-acetyl group interferes. Driselase does not remove acetyl groups from homogalacturonan, and can thus yield structurally informative oligosaccharides containing AcGalA residue(s) (Perrone *et al.* 2002).

Commercial homogalacturonan ('polygalacturonic acid') is a useful model, e.g. for practising analyses, but the commercial material is of much lower M_r than natural homogalacturonan.

1.2.1.2 Rhamnogalacturonan-I domains

EPG digestion of walls or AIR solubilizes rhamnogalacturonan-I (RG-I). This high-M_r pectic domain (typically DP 1000–2000) has a backbone of the repeating disaccharide [–4)-α-GalA-(1→2)-α-Rha-(1→–]. To *O*-4 of roughly half the Rha residues, diverse neutral side chains are attached, which are rich in β-Gal (more abundant close to the Rha residue) and α-Ara (more abundant at the side-chains' non-reducing termini). Some very high-M_r side chains of RG-I (rich in Gal and/or Ara) may enable this pectic domain to hydrogen-bond to cellulose, a property not exhibited by most pectins (Zykwinska *et al.* 2005). Minor sugar residues also occur (Table 1.1). There are very many different side chains, their individual structures probably defined stochastically.

Table 1.1 Summary composition of plant cell wall polysaccharides

Polysaccharide	%[a]	Q[b]	Residue (s) of backbone	Linkage(s) within backbone	Main residue(s) of side chains	Main linkages between side chain and backbone
Microfibrillar						
Cellulose	30	0	βGlc	(1→4)	none	
Matrix						
Pectins						
Homogalacturonan domain	15	–	αGalA (±Me ester, ±Ac ester)	(1→4)	none	
Rhamnogalacturonan-I domain	10	–	αGalA (± Ac ester), αRha	[-GalA-(1→2)-Rha-(1→4)-]$_n$	βGal, αAra > αFuc, βXyl, βGlcA	Gal-(1→4)-Rha, Ara-(1→4)-Rha
Rhamnogalacturonan-II domain	1–4	–	αGalA (no Me-esters)	(1→4)	αAceA, βApi, αAra, αAra*p*, βAra, βDha, αFuc, βGal, L-Gal, αGalA, βGalA, βGlcA, α(?)Kdo, αMeFuc, αMeXyl, αRha, βRha	Api-(1→2)-GalA, Kdo-(1→3)-GalA, Dha-(2→3)-GalA, Ara-(1→3)-GalA

Two useful enzymes will cleave the backbone of RG-I into structurally informative fragments: rhamnogalacturonan lyase attacks near the reducing end, releasing oligosaccharides such as ΔUA-Rha-GalA-Rha; rhamnogalacturonan hydrolase attacks near the non-reducing end, releasing oligosaccharides such as GalA-Rha-GalA-Rha-GalA (Mutter *et al.* 1998).

RG-I lacks methyl esters, but it does have 2AcGalA, 2,3-Ac_2GalA (Ishii 1997) and 3AcGalA residues (Perrone *et al.* 2002), which can be isolated as components of Rha-containing oligosaccharides after Driselase digestion. De-esterified RG-I is thoroughly hydrolysed by Driselase, giving an almost 100% yield of the constituent monosaccharides. RG-I preparations from potato and soyabean are available commercially (Megazyme); gum karaya is also rich in RG-I.

Frequent linkages within side chains	**Major product(s) of Driselase digestion**	**Other enzymes that cleave backbone**	**Other notes**
	Glc		Water-insoluble. Hydrogen-bonded within microfibril
	GalA + MeOH, except where acetylated	Endo-polygalacturonase (EPG)	Very few (no?) Rha residues interrupting the backbone. Some GalA residues Me-esterified, some 2- and/or 3-*O*-acetylated, some both
Gal-(1→4)-Gal, Ara-(1→5)-Ara, Ara-(1→3)-Ara, Ara-(1→3)-Gal	GalA (except where acetylated), Rha, Gal, Ara, Fuc	RG lyase, RG hydrolase	Some GalA residues 2- or 3-*O*-acetylated, or 2,3-di-*O*-acetylated. EPG-resistant
complex; five main types of side-chain; see text	Trace of GalA and GlcA?; largely resisant to Driselase, with borate retained	none known	Some αRha present as 3-*O*-Me ether in some pteridophytes. MeFuc and AceA are *O*-acetylated. One Api residue can stably esterify with borate, crosslinking two RG-II molecules. EPG-resistant

Table 1.1 *Continued*

Polysaccharide	%[a]	Q[b]	Residue (s) of backbone	Linkage(s) within backbone	Main residue(s) of side chains	Main linkages between side chain and backbone
Xylogalacturonan domain	±	–	αGalA (± Me-ester)	(1→4)	βXyl > αFuc	Xyl-(1→3)-GalA
Apiogalacturonan (domain?)	0	–	αGalA	(1→4)	βApi, (Xyl)	Xyl-(1→2)-GalA, Xyl-(1→3)-GalA
Hemicelluloses						
Xyloglucans	20	0	βGlc (± Ac ester)	(1→4)	αXyl, βGal (± Ac ester), αFuc (±αAra, βAra, βXyl, α-L-Gal)	αXyl-(1→6)-Glc, αAra-(1→2)-Glc, βXyl-(1→2)-Glc
Xylans	8	–	βXyl (± Ac ester)	(1→4)	αAra, αGlcA, (±βXyl, β-D-Gal, ?-L-Gal, …)	Ara-(1→2)-Xyl, Ara-(1→3)-Xyl, GlcA-(1→2)-Xyl
Mannans	±	0	βMan	(1→4)	±αGal	Gal-(1→6)-Man
Glucomannans	±	0	βMan (± Ac ester), βGlc	(1→4)	±αGal	Gal-(1→6)-Man
Mixed-linkage glucans (MLGs)	0	0	βGlc	(1→4), (1→3)	none	
Callose	±	0	βGlc	(1→3)	none?	
Glucuronomannans	±	–	βGlcA, αMan	[-GlcA-(1→2)-Man-(1→4)-]$_n$	αAra, βGal, αFuc, Xyl	To *O*-3 of Man and GlcA

Ac, acetyl; Me, methyl.
See Fig. 1.1 for further abbreviations. Unless otherwise indicated, the sugar is the enantiomer (D- or L-) and ring-form (-*p* or -*f*) illustrated in Fig. 1.1.
±, not always present; ~, amount varies greatly.
[a]Rough guide to amount of polymer present, as % of dry weight of a typical dicot primary cell wall from a rapidly growing cell culture;
[b]Charge on polymer molecule (at physiological pH): –, negative; 0, uncharged.

Frequent linkages within side chains	Major product(s) of Driselase digestion	Other enzymes that cleave backbone	Other notes
Some Xyl-Xyl and/ or Fuc-Xyl.	?	Xylogalacturonan hydrolase	Major side-chains = single Xyl; some disaccharides
Api-(1→3)-Api	?	?	Only in certain monocots. Major side-chains = single Api; some disaccharide
Gal-(1→2)-Xyl, Fuc-(1→2)-Gal, α-Ara-(1→2)-Xyl	Isoprimeverose, Glc, Gal, Fuc, Ara	Endo-β-glucanase (cellulase), xyloglucan endo-glucanase (XEG)	See text for repeat units. Some 6AcGlc, 6AcGal and 5AcAra
Xyl-(1→2)-Ara, Ara-(1→2)-Ara, Xyl-(1→3)-Xyl	Xylobiose, Xyl, Ara, Fer-Ara-Xyl, Fer-Ara-Xyl-Xyl. No free GlcA?	Xylanases	Some 2AcXyl or 3AcXyl. In Poales, some Fer-Ara and 2AcAra
	Man, Gal, oligosaccharides.	Mannanase	Heavily galactosylated mannans in some seeds. Little studied in primary cell walls
	Man-Glc, Man-Man, Man, Glc	Mannanase	Well known in secondary walls of xylem. Some Man residues 2- or 3-*O*-acetylated
	Glc	Lichenase, cellulase	Only in Poales and *Equisetum*.
	Glc	Laminarinase	Mainly in wounded tissues, wall-regenerating protoplasts, and phloem sieve-tubes
	?	[Acid hydrolysis yields GlcA-Man disaccharide]	Some GlcA may be Me-esterified

1.2.1.3 Rhamnogalacturonan-II domains

RG-II is a small but exceedingly complex pectic domain. It has a backbone of at least eight α-GalA residues, to which are attached five different types of side chain: the first four are designated A–D, each with a precisely (not stochastically) defined primary structure (O'Neill *et al.* 2004). The side chains typically have the following composition:

A (octasaccharide), α-L-Gal, β-GlcA, α-MeXyl, α-Fuc, β-Rha, α-GalA, β-GalA, β-Api(⊗)
B (octa- or nonasaccharide), β-Ara, α-Rha (×1 or ×2), α-Ara*p*, β-Gal, α-MeFuc, α-AceA, β-Rha, β-Api(⊗)
C (disaccharide), α-Rha, α(?)-Kdo(⊗)
D (disaccharide), β-Ara, β-Dha(⊗).

where (⊗) indicates the residue attached to the oligo-GalA backbone.

All four of these side chains are themselves acidic (unlike those of RG-I) and contain very unusual residues, including some known only from RG-II. The fifth side chain is a single α-Ara residue (Melton *et al.* 1986). Side chain B has acetyl groups on the AceA and MeFuc residues (O'Neill *et al.* 2004). There are no reports of methyl esters in RG-II. If RG-II has one copy of each side chain, it is DP 30 (~5 kDa). The dimerization of RG-II by borate crosslinks is discussed later.

The linkages between the side-chains and RG-II's backbone are unusually acid-labile, especially the Api→GalA* and Kdo→GalA bonds; the side chains can therefore be pruned off the backbone by warm dilute acid. However, RG-II is largely resistant to Driselase, which provides a useful method for its purification.

A convenient source from which to purify RG-II is red wine, since the yeasts used in its manufacture cannot hydrolyse grape RG-II (O'Neill *et al.* 2004).

1.2.1.4 Xylogalacturonan domains

Another pectic domain, often quantitatively minor, is xylogalacturonan, briefly described in Table 1.1. A commercial preparation rich in xylogalacturonan is gum tragacanth (Zandleven *et al.* 2005).

1.2.2 Hemicelluloses

Hemicelluloses are conventionally extracted from the cell wall with aqueous alkali. NaOH acts as a chaotropic agent, minimizing hydrogen-bonding between hemicelluloses and cellulose. The high pH ionizes the –OH groups of carbohydrates,

*In this chapter, light arrows (e.g. →) indicate glycosidic bonds; heavy arrows (e.g. ➔) indicate the direction of a chemical reaction.

$$R\text{–}OH + OH^- \rightarrow R\text{–}O^- + H_2O,$$

so that the hemicellulose molecules become negatively charged and repel one another, helping to maintain their solubility. The alkali is usually supplemented with $NaBH_4$ as a precaution against polysaccharide oxidation or 'peeling'.

Hemicelluloses differ in the alkali concentration required for extraction. Dilute alkali may extract xylans with many side chains; higher concentrations are required for xylans with fewer side chains; and the highest concentrations are required for MLG and xyloglucans (Carpita 1984). Complete extraction of xyloglucans requires 6 M NaOH at 37 °C (Edelmann & Fry 1992). The more commonly used 4 M alkali is not fully effective, and is not recommended. Inclusion of 4% H_3BO_3 with the 6 M NaOH may facilitate extraction of mannans.

Once extracted from the wall with alkali, some hemicelluloses precipitate on neutralization (hemicellulose A), but usually the bulk do not (hemicellulose B).

Alkali treatment strips off all ester-bonded (e.g. acetyl and feruloyl) groups and should not be used if such groups are of interest.

1.2.2.1 Xyloglucans

The best-studied hemicelluloses of the primary cell wall are the xyloglucans. These possess a linear backbone of (1→4)-linked β-Glc residues (in this sense identical with cellulose). In many dicots, the backbone consists of a cellotetraose (G4G4G4G) repeat with the first three Glc residues (counting from the non-reducing end) carrying an α-Xyl residue on position 6. The repeating disaccharide, α-Xyl-(1→6)-Glc, is called isoprimeverose. The fourth Glc residue is unsubstituted. Some of the Xyl residues (especially the third from the non-reducing end) carry an additional β-Gal residue on position 2, and the β-Gal itself often carries α-Fuc on its 2-position.

Xyloglucans can be hydrolysed by endo-(1→4)-β-D-glucanase (cellulase) or by a xyloglucan-specific endo-glucanase (XEG). These two enzymes attack most xyloglucans at the same sites (the unsubstituted Glc residues); however, XEG is more specific and does not cleave cellulose and xylans. XEG hydrolyses the (1→4)-β-Glc linkage adjacent to and on the non-reducing side of an isoprimeverose unit. Endo-(1→4)-β-D-glucanase is available commercially (e.g. Megazyme) at acceptable purity; XEG may be available on request from Novozymes (Bagsværd) but requires purification by the method of Pauly *et al.* (1999). Either of these enzymes cleaves xyloglucan to yield oligosaccharides (XGOs), which can be purified and characterized, thus indicating the structure of the xyloglucan (though without information on the order in which the constituent XGOs occur).

The following code letters are used for describing concisely the sequence of side chains (and unbranched Glc residues) along the (1→4)-β-D-glucan backbone of xyloglucan:

G	a β-Glc residue of the backbone (or the reducing terminal Glc moiety) with no side-chain attached
Gol	glucitol (the former reducing terminus, if reduced with $NaBH_4$)
G	β-6AcGlc
X	α-Xyl-(1→6)-β-Glc (=β-isoprimeverose)
L	β-Gal-(1→2)-α-Xyl-(1→6)-β-Glc
L	β-6AcGal-(1→2)-α-Xyl-(1→6)-β-Glc
F	α-Fuc-(1→2)-β-Gal-(1→2)-α-Xyl-(1→6)-β-Glc
F	α-Fuc-(1→2)-β-6AcGal-(1→2)-α-Xyl-(1→6)-β-Glc
A	α-Ara-(1→2)-[α-Xyl-(1→6)]-β-Glc
B	β-Xyl-(1→2)-[α-Xyl-(1→6)]-β-Glc
C	α-Ara-(1→3)-β-Xyl-(1→2)-[α-Xyl-(1→6)]-β-Glc
J	α-L-Gal-(1→2)-β-Gal-(1→2)-α-Xyl-(1→6)-β-Glc
S	α-5AcAra-(1→2)-α-Xyl-(1→6)-β-Glc
S	α-Ara-(1→2)-α-Xyl-(1→6)-β-Glc
T	β-Ara-(1→3)-α-Ara-(1→2)-α-Xyl-(1→6)-β-Glc
U	β-Xyl-(1→2)-α-Xyl-(1→6)-β-Glc

The β-Glc in each structure is part of the (1→4)-β-glucan backbone xyloglucan. Square brackets indicate branching; for example S is Ara→Xyl→Glc, whereas A could be represented Ara→Glc←Xyl (both pentose residues being directly attached to the backbone glucose). The list of code letters will soon be extended by new XGOs discovered in bryophyte xyloglucans (Peña *et al.* 2007a).

The primary cell walls of *Acer*, and many other plants, yield two very predominant XGOs: XXFG and XXXG. Other widely observed sequences include XXLG, XLXG and XLFG.

Like most hemicelluloses, xyloglucans bind strongly to cellulose (e.g. filter paper *in vitro*) by hydrogen-bonding, especially after drying, but the XGOs mentioned above do not and can readily be washed off paper (even after drying) with water. In this sense, XGOs differ markedly from oligosaccharides of cellulose (e.g. GGGGGG), which have a very high affinity for cellulose.

XXFG often contains 6AcGal in place of the Gal in the F unit (the oligosaccharide is then designated XXFG), although the acetyl group can migrate non-enzymically to other positions of the same Gal residue. The acetate groups are stable during digestion with cellulase or XEG, though rapidly removed by NaOH.

Isoprimeverose [α-Xyl-(1→6)-Glc] is the most diagnostic repeat unit of xyloglucan, being unknown from any other polymer; it can be released quantitatively from xyloglucan by Driselase digestion, which thus serves as a simple assay for xyloglucan. The α-Xyl residues are completely stable to Driselase, which lacks detectable α-xylosidase activity. Essentially all the other residues are released as free monosaccharides; however, the 6AcGal residues appear to be released by Driselase as galactose + free acetate.

Commercially available xyloglucan (Megazyme) is from tamarind seed. This polysaccharide broadly resembles *Acer* xyloglucan except that fucose is absent; the major XGOs released by XEG are XLLG > XXLG > XXXG > XLXG. A small proportion of Ara residues is present.

1.2.2.2 Xylans

The second most abundant hemicelluloses in dicot primary cell walls are xylans, which have a backbone of (1→4)-linked β-Xyl residues. Dicot xylans carry side chains, especially α-Ara, α-GlcA and α-MeGlcA attached predominantly to position 2 of some Xyl residues – hence the use of fuller names such as glucuronoarabinoxylans (GAXs), but there is probably a continuum of compositions, and the term 'xylans' will generally be used here to cover all such hemicelluloses. *O*-Acetyl groups are also present, especially on the Xyl residues. Xylans can hydrogen-bond to cellulose, though generally more slowly and less strongly than do xyloglucans and MLGs.

When cell walls or AIR are digested with Driselase, the xylan backbone is cleaved to yield a mixture containing xylose and xylobiose, the yield of which is a valuable indication of xylan content. Xylobiose is a particularly useful diagnostic fragment which enables assay of xylan – as with isoprimeverose for xyloglucan. The yield is maximized by prior treatment with NaOH or mild acid. Xylobiose is completely stable in the presence of Driselase. The endo-xylanase activity of Driselase cleaves xylan's backbone to yield progressively smaller oligosaccharides, the last permitted reaction being hydrolysis of the trisaccharide:

$$\text{Xyl–Xyl–Xyl} + H_2O \rightarrow \text{Xyl–Xyl} + \text{Xyl}$$

forming a stable disaccharide, xylobiose. Other enzymes in Driselase remove the Ara residues as monosaccharide. However, Driselase lacks α-D-glucuronidase; the GlcA and MeGlcA residues of xylans probably end up in oligosaccharides.

1.2.2.3 Mannans

Dicot primary cell walls contain relatively small proportions of mannans, the broad term used here for polysaccharides with a backbone rich in (or composed entirely of) (1→4)-linked β-Man residues. Some mannans include β-Glc residues within the backbone and/or α-Gal residues as side chains (Table 1.1). Terms such as glucomannans, galactomannans and galactoglucomannans can specify this.

Endo-mannanases digest the backbone of mannans yielding useful diagnostic oligosaccharides (McCleary & Matheson 1983; Handford *et al.* 2003); some are available commercially (Megazyme).

1.2.2.4 Glucuronomannans

Perhaps better classed as mucilages than hemicelluloses, and completely distinct from the mannans described above, are glucuronomannans, which

contain α-Man instead of β-Man. They have a backbone of the repeating disaccharide [–4)-β-GlcA-(1→2)-α-Man-(1→–]. To position 3 of many GlcA and Man residues are attached diverse side chains rich in α-Ara, β-Gal, α-Fuc (Redgwell *et al.* 1986) and sometimes Xyl. Some GlcA residues are reported to be methyl-esterified (Honda *et al.* 1996); if confirmed, this is a unique feature.

The simplest tool for glucuronomannan identification is partial acid hydrolysis, which yields the relatively acid-stable disaccharide β-GlcA-(1→2)-Man, detectable by chromatography or electrophoresis and further characterized by digestion with β-glucuronidase (Sotiriou *et al.* 2007). Preparations rich in glucuronomannan are gum ghatti and leiocarpan A (Aspinall *et al.* 1969) – useful sources of this disaccharide.

1.2.3 Cellulose

Microfibrils are composed of cellulose, possibly with small amounts of entrapped hemicelluloses. In aerial organs (stems, hypocotyls, leaves), the orientation of microfibrils in the outer wall of the epidermis governs the direction of organ growth (length versus girth), this specific wall being much thicker than the others in a growing organ (Kutschera 2008).

Cellulose can be partially purified as the 'α-cellulose' fraction – wall material left insoluble after exhaustive extraction of the matrix polysaccharides e.g. with 6 M NaOH at 37 °C. Prepared in this way, cellulose is contaminated by glycoproteins (especially extensins) and some pectic material (possibly covalently linked to the extensins).

Cellulose is relatively resistant to hydrolysis in 2 M TFA at 120 °C for 1 h, although a small proportion can be released as glucose, especially if the crystallinity of the cellulose has been compromised by severe alkali pretreatments. Good conversion of cellulose to glucose can be achieved by a two-stage acid hydrolysis (Saeman method: stirring in 72% w/w H_2SO_4 at room temperature to dissolve the polysaccharide, then heating in 2 M H_2SO_4 at 120 °C for 1 h to hydrolyse it; subsequently the H_2SO_4 is neutralized with a small excess of $BaCO_3$). More convenient, however, is digestion of the α-cellulose fraction with Driselase, which usually gives an excellent yield of glucose. A short-cut, omitting the preparation of α-cellulose, is sequential treatment of cell walls with 2 M TFA (120 °C, 1 h) and then Driselase, giving a useful estimate of non-cellulosic and cellulosic Glc residues respectively (O'Looney & Fry 2005; Fig. 1.2); note, however, that contaminating starch would be recorded as non-cellulosic Glc in this procedure.

1.3 Secondary cell walls

Cells change their repertoire of polysaccharide synthesis during the differentiation of cambium into xylem (Thornber & Northcote 1962). Whereas

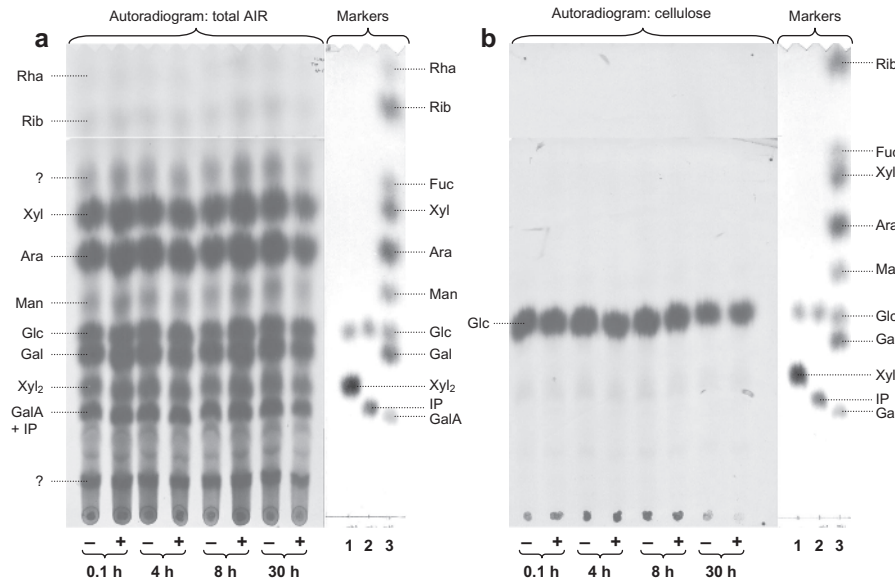

Figure 1.2 Exploring the effect of a new herbicide on cell wall polysaccharide biosynthesis. Maize cell-cultures were pretreated with (+) or without (–) 480-nM oxaziclomefone for 0.1–30 h and then pulse-labelled with [U-^{14}C]glucose for a further 2 h. The results, showing no effect of the herbicide, illustrate the reproducibility of the analytical method. (a) Total polysaccharide biosynthesis. The radiolabelled AIR was treated with Driselase, and the resultant sugars were separated by PC and autoradiographed. The ^{14}C-labelled products were Glc (cellulosic plus wall matrix-derived); Gal; Ara; Xyl and xylobiose (Xyl_2, diagnostic of xylans); GalA and Rha (diagnostic of pectins); isoprimeverose (IP, diagnostic of xyloglucan); Man, and Rib. Ribose is not a cell wall sugar, and the polymeric component of AIR from which it was released by hydrolysis was probably RNA. (b) Cellulose biosynthesis. The radiolabelled AIR was freed of pectins and hemicelluloses by TFA hydrolysis (2 M TFA, 120 °C, 1 h) followed by washing. The remaining insoluble material was then Driselase-digested, yielding [^{14}C]glucose, which indicates how much [^{14}C]cellulose had been synthesized.

From O'Looney & Fry (2005). 1, 2, 3: Marker lanes.

cambium has only primary walls, basically similar to those described in the above generalizations, the major polysaccharides of mature xylem cell walls are cellulose, xylans and mannans. Angiosperm xylem (hardwood) contains 33–50% w/w cellulose (dry weight basis; Willför *et al.* 2005). 4-*O*-Methylglucuronoxylan and glucomannan (constituting roughly 20–30% and 0.5–5.0% of the dry weight of the wood, respectively; Willför *et al.* 2005) are both partially *O*-acetylated (Teleman *et al.* 2003). The glucomannans are acetylated on position 2 of some Man residues and position 3 of others; some Man residues and the Glc residues are non-acetylated. Recent work (Peña *et al.* 2007b) has confirmed the older report (Andersson *et al.* 1983) that xylans of secondary walls have a backbone whose reducing terminus is β-Xyl-(1→3)-α-Rha-(1→2)-α-GalA-(1→4)-Xyl. In this sequence the Rha-GalA bond

is (1→2), whereas in RG-I it is (1→4). It seems likely that the Xyl-Rha-GalA-Xyl moiety is a primer on which xylan backbone biosynthesis is initiated.

In conifer xylem (softwood), the (galacto)-glucomannans are acetylated but the xylans are not (Teleman *et al.* 2003).

Xyloglucans may be present in small proportions in xylem secondary walls (Nishikubo *et al.* 2007). Arabinogalactans are abundant in larch wood (Sigma catalogue), but these are water-soluble rather than structural wall components. Wood also contains pectic residues (GalA + Rha ≈ 1.5–3.5% of dry wood; Willför *et al.* 2005), though probably mainly in the primary walls of tracheary elements and ray cells.

Cork (phellem) has thick secondary walls. Mature oak cork contains 26% w/w polysaccharide, which on Saeman hydrolysis yields 42 mol% Glc (mainly cellulosic), 30 mol% Xyl, 12 mol% uronic acids and 8 mol% Ara (suggesting xylans). There is only 3 mol% Man, 3 mol% Gal and 2 mol% Rha, indicating small amounts of mannans and pectins (Rocha *et al.* 2000). NaOH-extractable hemicellulose contains xylans with MeGlcA and β-Xyl residues attached to *O*-2 of some of the Xyl residues (Asensio 1987).

A very different secondary wall is that of cotton seed trichomes ('fibres'). At maturity, this thick wall is almost pure cellulose.

1.4 Taxonomic consideration of primary cell walls

The description of primary walls given above relates to dicots – the plants most intensively studied because of their agricultural importance. Similar primary walls, described as 'type I', are also found in many monocots. However, a substantially different primary wall, 'type II', occurs in the monocot order Poales. Other plant taxa also exhibit characteristic compositional features. A brief survey follows.

1.4.1 Poalean primary cell walls

Type II primary walls are characterized by a lower content of pectins and xyloglucans, a correspondingly higher proportion of (feruloylated) xylans, and the presence of a hemicellulose called mixed-linkage (1→3,1→4)-β-D-glucan (MLG), which is absent in dicots.

1.4.1.1 Poalean xyloglucans

Poalean xyloglucan also differs qualitatively from that of dicots. Although possessing a backbone rich in G and X units (see above), the X:G ratio is substantially lower than in dicots (e.g. only ~32 and ~50% of the Glc residues bear a Xyl side chain in the xyloglucan of rice shoots (Kato *et al.* 1982) and barley coleoptiles (Gibeaut *et al.* 2005) respectively), and the Gal, Ara and Fuc contents are very low. Poalean xyloglucans do possess a few Fuc residues – readily detected if the cells are prelabelled by feeding with [^{3}H]fucose (McDougall & Fry 1994). Digestion of alkali-extracted rice seedling xyloglucan with partially purified cellulase yielded XGOs from which Kato *et al.*

(1982) deduced major sequences to have been XXGGG and XGGG. Gibeaut *et al.* (2005) isolated numerous XGOs from XEG digests of barley coleoptile cell walls.

Because of the low X:G ratio, NaOH-extracted poalean xyloglucans have a limited water solubility; they may fall into the hemicellulose A class, precipitating upon neutralization. However, *in muro* many of the unbranched Glc residues in the backbone of poalean xyloglucans are 6AcGlc (designated G); this acetylation increases water solubility, like xylosylation. Major oligosaccharides obtained upon XEG digestion of alkali-untreated barley coleoptile walls include XXGGG, XXGG, XXGGGG, XXGGGG and XXGGG (Gibeaut *et al.* 2005). Indeed, (Xyl-free) cellulose that artificially carries 0.5–1.0 *O*-acetyl groups per Glc residue is water-soluble – unlike cellulose itself and unlike commercial cellulose acetate, which typically has approximately 2.5 *O*-acetyl groups per Glc residue. It is interesting that artificial water-soluble cellulose acetate is an effective donor substrate for xyloglucan endo-transglucosylase (XET) activity, in this sense evidently resembling xyloglucan (Fry *et al.*, unpublished).

1.4.1.2 Poalean (feruloylated) xylans

Poalean xylans differ from dicot xylans in having the α-Ara residues attached mainly to position 3, rather than 2, of some backbone β-Xyl residues. The Ara residues are frequently also further substituted with oligosaccharide side chains containing β-Xyl, D-Gal, L-Gal, and acetyl groups (Carpita 1996). The α-GlcA and/or MeGlcA are mainly on *O*-2 of the Xyl residues, as in dicots.

Poalean xylans have feruloyl and 4-coumaroyl groups esterified to *O*-5 of some Ara residues (forming Fer-Ara; Fig. 1.1). This applies to very diverse members of the Poales (*sensu lato*), from grasses to pineapples (Smith & Harris 2001) and also to some related monocot orders (Commelinales, Zingiberales, Arecales – the commelinoid group), but not to the Asparagales or Liliales. The position of feruloylation can be investigated by isolation and characterization of feruloyl-oligosaccharides. Mild acid hydrolyses furanosyl more rapidly than pyranosyl linkages, so the Ara*f*→xylan bond is selectively cleaved, yielding free arabinose and Fer-Ara; in addition, larger feruloylated side chain oligosaccharides are also obtained, always with Ara at the reducing terminus (Wende & Fry 1996). These structures show that some of the feruloyl groups were attached to Ara residues which themselves also bore additional sugar side chains. Xylans can also be hydrolysed with Driselase, releasing feruloylated oligosaccharides in which the reducing terminus is a Xyl that was formerly a backbone residue (Kato & Nevins 1985).

1.4.1.3 Poalean mixed-linkage glucans

Mixed-linkage (1→3,1→4)-β-D-glucan (MLG) is a hemicellulose of poalean primary walls, being especially abundant during periods of rapid cell expansion. It often decreases in quantity per cell (indicating breakdown,

rather than simply dilution by continued deposition of different polysaccharides) when cell expansion is complete. Once extracted in alkali, MLG is a flexible molecule, soluble in neutral water (Buliga *et al.* 1986) – unlike (1→4)-β-glucan (cellulose), which is insoluble. In growing cells, MLG is very firmly hydrogen-bonded to the cellulose (Carpita *et al.* 2001), and may tether microfibrils (Labavitch & Ray 1978; Wada & Ray 1978; Fry 1989). MLG also occurs abundantly in cereal grains, where it is largely not microfibril-bonded. Barley flour MLG is available commercially (Megazyme, Sigma).

Poalean MLG is an unbranched chain of β-Glc residues linked by (1→3) and (1→4) bonds. The polysaccharide typically takes the form:

…G3G4G4G3G4G4G4G3G4G4G3G4G4G3G…,

reading from non-reducing to reducing terminus, where 'G' is β-Glc, and '3' and '4' indicate (1→3) and (1→4) bonds respectively. The underlined domains are effectively cellotriose and (fewer) cellotetraose units, interlinked by single (1→3) bonds (Meikle *et al.* 1994).

MLG can be characterized by digestion with lichenase [(1→3),(1→4)-β-D-glucan 4-glucanohydrolase; EC 3.2.1.73], which cleaves the (1→4) bond in the sequence …G3G4G…, yielding analytically informative oligosaccharides, always with a (1→3) bond at the reducing end (Meikle *et al.* 1994; Grishutin *et al.* 2006; Li *et al.* 2006). The major oligosaccharide released by lichenase digestion of poalean MLG is G4G3G, but appreciable amounts of G4G4G3G are also obtained. The trisaccharide:tetrasaccharide molar ratio varies between samples, usually within the range 1.5:1 to 4:1. Progressively smaller proportions of G4G4G4G3G, G4G4G4G4G3G etc. also occur, certain larger oligosaccharides being favoured, especially a nonasaccharide and a dodecasaccharide (Woodward *et al.* 1983). These short 'cellulose-like' domains within MLG probably help the MLG to hydrogen-bond to microfibrils. Very little (sometimes 'no') disaccharide (G3G; laminaribiose) is obtained, which would have indicated the sequence …G4G3G4G3G…, and there is no strong evidence for consecutive (1→3)-bonds.

Cellulase digests MLG by cleaving mainly the (1→4) bond in …G4G3G…, yielding oligosaccharides with a (1→3) bond at the non-reducing terminus. However, cellulase also attacks some other hemicelluloses.

1.4.1.4 Other poalean polysaccharides

Members of the Poales also possess (gluco)mannans, concentrated in the epidermal walls (Carpita *et al.* 2001) – a location playing a particularly important role in restraining organ growth.

Most poalean cells contain relatively small proportions of pectin (thus a low GalA and Rha content). However, cultured maize and rice cells have RG-I that is remarkably similar to that of dicots, except for a lower Fuc content. Grass RG-IIs are typically present at less than 10% of the concentra-

tion seen in dicot primary walls, but are qualitatively indistinguishable from those of dicots (Thomas *et al.* 1989).

1.4.2 Taxonomically restricted features of non-poalean angiosperm walls

Although all dicots have xyloglucan in their primary cell walls, there is some distinct taxonomic and developmental specificity in the repeat units liberated by XEG digestion (Hoffman *et al.* 2005). The classic ('XXXG-type') of xyloglucan has a backbone in which three contiguous xylosylated β-Glc residues are followed by a single non-xylosylated one. The polysaccharide is thus built of Glc_4-based repeat-units, usually mainly XXXG and XXFG, with less XXLG, XLFG, XLLG and others.

In some dicots – the Solanales and Lamiales – 'XXXG-type' xyloglucan is scarce or absent. In these two orders, both in the asterid clade of eudicots, most xyloglucan has two or three contiguous non-xylosylated Glc residues. Examples are Glc_4-based (XXGG-type) repeat units, e.g. XXGG, XSGG, SXGG, SSGG, LXGG, LSGG, LLGG, LTGG; and Glc_5-based (XXGGG-type) repeat units, e.g. XLGGG, XSGGG, SXGGG and SSGGG. However, xyloglucan in olive fruit (Lamiales) is 'XXXG type' (predominant repeat units being XXSG and XLSG). Some S units (designated S) have 5AcAra in place of Ara.

Many of the XXGG- and XXGGG-type repeats are 6-*O*-acetylated, most commonly in the sequence XXGG and XXGGG, underlining indicating that an *O*-acetyl group that effectively replaces the 'missing' α-Xyl residue(s), as discussed for Poales.

In the order Caryophyllales (e.g. *Spinacia*, *Beta*, *Sagina*, etc.), pectins carry feruloyl and 4-coumaroyl ester groups linked to certain α-Ara and β-Gal residues, predominantly at the non-reducing termini of the Ara- and/or Gal-rich side chains of a pectic domain, probably RG-I (Fry 1983). Diagnostic oligosaccharides (Fer-Gal-Gal and Fer-Ara-Ara) can be released from spinach pectin by Driselase digestion (Fry 1982).

Another taxonomically restricted polysaccharide (domain?) is apiogalacturonan, found only in certain aquatic monocots of the order Alismatales, e.g. *Lemna*, *Spirodela* and *Zostera*.

1.4.3 Cell walls of non-angiosperms

To date, most cell wall research has understandably been focused on angiosperm crops and coniferous wood. Much less detail is available about the wall polysaccharides of non-angiosperms, although progress is beginning towards a description of the evolutionary history of the plant cell wall.

1.4.3.1 Charophytic algae

Charophytes are the closest living algal relatives of land plants. Indeed charophytes plus land plants are now often classified as a single taxon, the

Streptophyta, well resolved from other green algae. There is debate as to which charophytic order (Charales or Coleochaetales) is most closely related to the land plants. Unfortunately, very little is known about charophyte wall composition. They contain cellulose; they are also usually rich in GalA residues, probably as partially methylesterified homogalacturonans (Cherno *et al.* 1976), much of which is readily extracted from the wall with oxalate buffer, pH 4.3.

Acid hydrolysis yielded roughly equal amounts of Xyl and Glc from walls of *Klebsormidium* and *Chara* (Popper & Fry 2003). However, Driselase digestion of *Chara*, *Coleochaete* and *Klebsormidium* walls (or hemicelluloses) yielded no isoprimeverose, indicating the absence of conventional xyloglucan (Popper & Fry 2003). In fact, *Coleochaete* walls yielded very little Xyl on acid hydrolysis, precluding an appreciable xyloglucan content (Popper & Fry 2003). Lichenase digestion also failed to yield oligosaccharides from *Chara* or *Coleochaete*, indicating undetectable MLG.

Charophytic walls have a moderate content of GlcA, Man and MeRha residues (from unidentified polysaccharides), but no detectable MeGal (Popper & Fry 2003). Much remains to be discovered about charophytic cell walls – information that would promote our understanding of the primordial features of the land-plant wall.

1.4.3.2 Bryophytes

Bryophytes are non-vascular land plants, traditionally divided into liverworts, hornworts and mosses, although some 'mosses' such as *Sphagnum* and *Andreaea* seem sufficiently isolated that they should be placed in separate classes. Unlike all other land plants, the bryophytes have the gametophyte (haploid) phase of the life cycle dominant. The sporophyte (diploid) phase tends to be shorter-lived, smaller and dependent on a supply of nutrients from the gametophyte. Few chemical analyses of bryophyte sporophyte walls have been published; therefore, in comparisons between bryophytes and vascular plants, the effects of taxonomy and life-cycle stage are usually conflated.

Like all land plants, bryophytes possess xyloglucan, albeit often in smaller proportions than in vascular plants. The simplest demonstration of this is the production of isoprimeverose on Driselase digestion of bryophyte AIR. Work is in progress (Peña *et al.* 2007a) to characterize the bryophyte XGOs. Intriguingly, XGOs from the moss *Physcomitrella* have branched side chains containing acidic sugar residues. Despite these major differences between bryophyte and angiosperm xyloglucans, AIR from cell cultures of the hornwort *Anthoceros* yields the nonasaccharide XXFG (unpublished observations), a highly specific structure that has evidently been preserved unchanged throughout land-plant evolution.

All bryophyte walls investigated contain GalA and Rha, characteristic of pectins, and Driselase digestion yields xylobiose, indicative of xylans.

Bryophytes usually give a very high yield of Man on acid hydrolysis, suggesting a high mannan content. They contain MeRha (approximately equalling Rha), but little or no MeGal.

Driselase digestion of walls from bryophyte gametophytes yielded small quantities of a borate-containing polysaccharide (0.004–0.025% of the wall dry weight) that coelutes with RG-II dimer on gel-permeation chromatography (Matsunaga *et al.* 2004). The same bryophyte walls also contained traces of MeFuc and MeXyl, characteristic of RG-II; however, Api and AceA were below the limit of detection (Matsunaga *et al.* 2004). Thus, bryophytes may well contain RG-II or a similar polysaccharide, albeit about 100 times less than in flowering plants. Bryophyte sporophytes have not yet been tested in this respect.

On acid hydrolysis, gametophyte walls of the hornwort *Anthoceros caucasicus*, unlike mosses and liverworts, give a very high yield of an unusual disaccharide, α-GlcA-(1→3)-L-Gal, from an unidentified polysaccharide (Popper *et al.* 2003). The same disaccharide was obtained from cultured cells of *Anthoceros agrestis* (unpublished), suggesting that it is taxonomically rather than developmentally stipulated. The existence of a unique polysaccharide in this evolutionarily isolated genus supports the view that major steps in plant evolution were accompanied by significant changes in wall composition.

Extraction of bryophyte cell wall polysaccharides is often difficult by standard agents, e.g. chelating agents and alkali, possibly because of the presence of Hua residues (Fig. 1.1) acting as crosslinks – see later. A pretreatment with mild acid may be beneficial, as may acidified $NaClO_2$ (Painter 1983).

1.4.3.3 Lycopodiophytes

Lycopodiophytes, e.g. *Lycopodium*, *Selaginella* and *Isoetes*, are early-diverging vascular plants, traditionally treated as pteridophytes although clearly distinct from the euphyllophytic pteridophytes (see below). Modern lycopodiophytes are the few remaining progeny of a much more diverse and abundant flora that throve in the Devonian. They are split into homosporous (e.g. *Lycopodium*) and heterosporous plants (e.g. *Selaginella*).

MeGal is abundant in all lycopodiophytes tested but not in bryophytes or euphyllophytes. MeRha is abundant in homosporous (but not heterosporous) lycopodiophytes. It is a component of their RG-II, in place of Rha (Matsunaga *et al.* 2004); however, the total MeRha content (Popper & Fry 2004) seriously exceeds what would be required for the modest RG-II content, suggesting that the MeRha of homosporous lycopodiophyte walls is not confined to RG-II.

Many lycopodiophyte walls are rich in Man, although the polysaccharides involved have not been characterized.

1.4.3.4 Euphyllophytic pteridophytes

All vascular plants possessing megaphylls (leaves with true veins) instead of microphylls are called 'euphyllophytes'. Living euphyllophytic pteridophytes are *Equisetum*, *Psilotum* and *Tmesipteris* (which are all eusporangiate) and ferns (both eusporangiate and leptosporangiate, the latter being later-diverging). They contain RG-II remarkably similar to that of angiosperms, though in some groups (ferns and *Psilotum* but not *Equisetum*) Rha is replaced by MeRha (Matsunaga *et al.* 2004). No functional consequences of the Rha/MeRha swap were detectable.

It has recently been found that, apparently unique outside the Poales, horsetails (*Equisetum*) contain MLG (Fry *et al.* 2008b; Sørensen *et al.* 2008). *Equisetum* MLG broadly resembles that of the Poales, except that the major product of lichenase is the tetrasaccharide, not the trisaccharide, and that laminaribiose is also a noticeable lichenase product. *Equisetum* MLG resembles poalean MLG in having appreciable quantities of a nonasaccharide repeat unit, but appears to lack the dodecasaccharide. *Equisetum* possesses not only the polysaccharide MLG but also a novel endo-transglucosylase whose favoured action appears to be cleaving MLG and grafting a segment of it on to a xyloglucan (acceptor substrate) chain (Fry *et al.*, unpublished).

For such an enzyme to act *in vivo*, *Equisetum* would need also to possess xyloglucan. This it clearly does, on the basis of Driselase and XEG digestion products; however, these digests include several unusual oligosaccharides, indicating that *Equisetum* xyloglucan has unique structural features.

Man residues are more abundant in eusporangiate pteridophyte walls (extreme in the case of *Psilotum*) than in leptosporangiate fern walls (Popper & Fry 2004). Conversely, proanthocyanidins are more abundant in leptosporangiate fern AIR than in eusporangiate species.

1.4.3.5 Gymnosperms

Gymnosperms studied to date (mainly conifers) seem to have wall components resembling those of dicots, including xyloglucan with the repeat units XXXG, XXFG, XLFG, XXLG and XLLG. A high mannan content is a recurrent wall feature of conifers (Edashige & Ishii 1996) and cycads, though not gnetophytes (Popper & Fry 2004). Gnetophytes may be the gymnosperms closest to angiosperms. All four extant gymnosperm classes possess feruloyl esters in their primary walls (Carnachan & Harris 2000), but the feruloylated polysaccharide is unidentified.

1.5 Covalent bonds between wall polysaccharides

The primary cell wall has the strength to resist expansion (and rupture) in the face of, typically, 0.5-MPa turgor pressures, while permitting controlled creep and thus cell growth. The ability of the wall to restrain irreversible expansion (i.e. the wall's plastic extensibility) can be regulated *in vivo*, e.g.

hormonally, and this is assumed to involve the making or breaking of inter-polymeric crosslinks in the wall (see above regarding definition of crosslink).

Decreasing wall extensibility may involve the formation of additional crosslinks and is termed wall **tightening**. This term is preferred over 'stiffening' or 'rigidification', which would wrongly imply that the inextensible wall becomes difficult to bend; it usually does not. Conversely, an increase in plastic extensibility is described as wall **loosening**.

It is therefore important to document the polymer–polymer crosslinks existing in the primary cell wall. This chapter considers covalent crosslinks, non-covalent ones being discussed in Chapter 14.

1.5.1 Glycosidic bonds joining polysaccharides into molecular 'trees'

Glycosidic bonds are not regarded as true 'crosslinks'. A glycosidic bond has a well-defined directionality, indicated for example by the arrow in the systematic name of isoprimeverose: α-D-Xyl*p*-(1→6)-D-Glc. This is described as Xyl attached to Glc (not Glc to Xyl) because the linkage involves the anomeric centre of the Xyl, not the Glc. With one postulated exception (Hua; see Section 1.5.2), each sugar residue can form only a single glycosidic bond to another sugar unit, whereas a given sugar unit can in principle have anything from zero up to three (in a C_5 sugar), four (in a C_6 sugar) or even five (in Kdo) additional sugar residues attached to it – potentially leading to a branched tree-like structure (e.g. RG-I, RG-II or xyloglucan); the reducing terminus is the base of the 'tree trunk' (ignore the tree's roots!). It is chemically feasible for the reducing terminus of one polysaccharide to form a glycosidic bond to a residue within another (identical or non-identical) polysaccharide, thus covalently joining the two polysaccharides. However, this is not a true crosslink because the linkage does not differ in its fundamental nature from any glycosidic linkage within either 'individual' polysaccharide. The product is effectively a single larger polysaccharide, albeit maybe with qualitatively dissimilar domains.

Examples of this type of structure are found in pectins, where the reducing terminus of xylogalacturonan or RG-I can be glycosidically linked to the non-reducing end of homogalacturonan. It is likely that pectins *in muro* have structures such as

…RG-I→HGA→XGA→HGA→RG-II→HGA…

where → denotes a glycosidic bond, HGA is homogalacturonan and XGA is xylogalacturonan (Ishii & Matsunaga 2001; Coenen *et al.* 2007).

In suspension-cultured angiosperm cells, roughly half the xyloglucan is covalently bonded to acidic pectic domains, probably RG-I (Thompson & Fry 2000; Popper & Fry 2005). The precise chemistry of this xyloglucan–pectin bond is still uncertain; it is stable to prolonged treatment in concentrated

NaOH and urea and a tenable model is a glycosidic bond between the reducing end of the xyloglucan and the Ara/Gal-rich side chains of RG-I. The xyloglucan–RG-I association is most easily demonstrated by ion-exchange chromatography or high-voltage electrophoresis on glass-fibre 'paper': despite angiosperm xyloglucans containing no acidic residues, 44–75% of the 6 M NaOH-extractable xyloglucan (recognized by its conversion to isoprimeverose by Driselase) binds to an anion-exchange column (Popper & Fry 2005) or migrates towards the anode (Thompson & Fry 2000). The formation of these xyloglucan–RG-I bonds has been shown by *in-vivo* pulse-radiolabelling to occur cosynthetically (probably within the Golgi system), rather than by heterotransglycosylation in the wall (Popper & Fry 2008).

Hrmová *et al.* (2007) have shown that a barley XTH can catalyse the formation of an MLG→xyloglucan bond by using MLG as donor substrate and a xyloglucan oligosaccharide (e.g. XXXGol) as acceptor substrate in a heterotransglycosylation reaction:

…GGGGGGGGGGGGGGGGGGGG… + XXXGol

↓

…GGGGGGGGG→XXXGol + GGGGGGGGGGG…

where …GGGGGG… is MLG, → is a glycosidic bond, and ↓ indicates the direction of the enzymic reaction. However, the barley enzyme catalysed this reaction at approximately 0.2% of the rate of the classic XET reaction (where the donor is a xyloglucan). This is slower than with any of various artificial substrates, e.g. hydroxyethylcellulose or cellulose sulphate; the biological significance of the heterotransglycosylation reaction therefore remains unclear.

Recently, an enzyme activity was detected in *Equisetum* for which the formation of MLG→xyloglucan bonds is the preferred reaction, well exceeding the classic XET reaction rate (Fry *et al.* 2008a). Thus, at least in *Equisetum*, the formation of interpolysaccharide glycosidic bonds may be a major *in-vivo* reaction.

1.5.2 Glycosidic bonds forming true ('lateral') crosslinks between polysaccharides?

The inextractability of *Sphagnum* (moss) polysaccharides may be due to the presence of D-*lyxo*-5-hexosulopyranuronic acid (Hua) residues. This is an unusual monosaccharide with two anomeric carbons, potentially capable of simultaneously forming glycosidic bonds to two different sugars and thus acting as a true glycosidic crosslink between polysaccharide chains (joining two trees through their 'branches') (Painter 1983). This intriguing hypothesis requires future evaluation.

1.5.3 Oxidative coupling products as crosslinks or intrapolymeric loops

The feruloyl residues of poalean xylans and caryophyllalean pectins undergo oxidative phenolic coupling *in vivo*, forming dimers (dehydrodiferuloyl residues), probably catalysed by peroxidases (Encina & Fry 2005). The linkages between the two participating feruloyl residues are C–O–C (ether) or C–C (e.g. biphenyl) bonds. Some of this coupling occurs well after the feruloylated polysaccharides have been deposited in the wall, but pulse-radiolabelling experiments show that a substantial percentage of it occurs within 1–2 min of the feruloyl groups' becoming attached to the polysaccharide, thus presumably still within the Golgi system (Fry *et al.* 2000).

It is widely assumed that the two feruloyl residues that couple are from separate polysaccharides, so that the coupling reaction makes an interpolymeric crosslink. Recent data support this hypothesis (Lindsay & Fry 2008), which would provide a plausible mechanism for wall tightening, restraining further growth (Ortega *et al.* 2006). However, it is currently not possible to eliminate the additional possibility that sometimes both consenting feruloyl residues are on the same polysaccharide chain, coupling to form an intrapolymeric loop. Such loops are less likely in the case of ferulate trimers and larger coupling products, which in cultured maize cells considerably exceed the dimers (Fry *et al.* 2000). Some specific trimers and tetramers have been characterized (e.g. Funk *et al.* 2005), though this is challenging because of the steeply increasing number of structural permutations with increasing size.

Coumaroyl esters are much less susceptible than feruloyl esters to oxidative coupling *in vivo* (Fry *et al.* 2000).

1.5.4 Uronoyl esters and uronoyl amides

Theoretically, the –COOH group of an acidic sugar residue could become ester-bonded to an alcohol (e.g. another sugar residue) or amide-bonded to an amino compound (e.g. glucosamine, lysine or putrescine), thus potentially forming a crosslink. In the case of methylesterified GalA residues, a plausible crosslinking mechanism is transacylation whereby a GalA–methanol bond in homogalacturonan is cleaved and the 'uronoyl' residue transferred (still glycosidically linked within a homogalacturonan chain) on to an acceptor substrate (alcohol or amine). In principle, a pectinmethylesterase-like enzyme could catalyse such a reaction, if the enzyme could be coerced into favouring transacylation over hydrolysis; and it is intriguing that there are about 98 and 53 genes encoding putative pectinmethylesterases in *Arabidopsis* and rice respectively (few of which have been tested for enzymic activity).

Authentic model *O*-galacturonoyl–sugar esters (Brown & Fry 1993a), N^{ε}-galacturonoyl–lysine amide (Perrone *et al.* 1998) and *N,N′*-di-galacturonoyl–putrescine amide (Lenucci *et al.* 2005) have been synthesized chemically and their properties investigated. Notably, these ester and amide bonds are

resistant to Driselase, suggesting an obvious method of documenting their presence *in muro*. Preliminary evidence was compatible with GalA–Lys bonds in cultured cells (Perrone *et al.* 1998). Corresponding evidence was originally interpreted as suggesting GalA–sugar ester bonds *in muro* (Brown & Fry 1993b) and was, with hindsight, probably due to the inability of Driselase to cleave the glycosidic bonds of methylesterified GalA residues that are also *O*-acetylated (Perrone *et al.* 2002). Independent evidence was obtained for non-methyl GalA esters in maize coleoptile cell walls: namely, the yield of methanol obtained on alkaline hydrolysis was only 50–75% of the total quantity of apparently esterified (NaB^2H_4-reducible at pH7) GalA residues (Kim & Carpita 1992). However, it is difficult to imagine that, as these data suggest, cell walls could contain one to three ester crosslinks for every three MeGalA residues. Covalent crosslinks are expected to be strong but few (cf. weak but many in the case of non-covalent crosslinks). The nature of the putative non-methyl galacturonoyl esters remains an intriguing mystery.

1.5.5 Borate diesters

A proportion (usually high) of the RG-II present *in muro* is dimerized via a crosslink formed by a single borate group that is esterified to four –OH groups: those at positions 2 and 3 of the Api residue of side chain A of each of two RG-II domains (O'Neill *et al.* 2004). This arrangement can be represented as

$$\text{RG-II}>(B^-)<\text{RG-II}.$$

The formation of this structure is probably the main reason why plants require borate as a micronutrient.

Most common sugars extremely rapidly form borate esters in the presence of inorganic borate – the basis for separating neutral sugars by electrophoresis in borate buffer. However, such electrophoresis is routinely performed at pH9.4 (Fry 2000), and the esters are immediately hydrolysed at slightly lower pH values. Furanose residues possessing a diol group (e.g. the ribofuranose residue of RNA and the Api*f* residue of methyl apioside) form borate esters that are more acid-resistant than those of pyranose residues. However, the ester bonds in RG-II>(B^-)<RG-II are clearly in a different league. They form, slowly, *in vitro* at pH3.7, the bonding being promoted by large divalent cations, especially Pb^{2+} (although possibly Ca^{2+} suffices *in vivo*) (Matsunaga *et al.* 2004). Once formed, however, RG-II>(B^-)<RG-II bonds are sufficiently stable for RG-II dimers to be isolated by column chromatography; they are hydrolysed only by quite strong acid (e.g. pH1 at room temperature).

The formation of RG-II–borate–RG-II bonds is thus not due to the Api residues alone; other structural features of RG-II also contribute, including what might at first sight appear rather minor details. For example, the *mur1*

mutant of *Arabidopsis* produces an RG-II with L-Gal and 2-O-Me-L-Gal in place of the deoxyhexose residues Fuc and MeFuc respectively: the mutant RG-II makes borate crosslinks significantly more slowly and less stably than the wild type (O'Neill *et al.* 2001).

1.6 Methodology

1.6.1 Specific and non-specific radiolabelling *in vivo*

In-vivo radiolabelling offers access to much information that would be difficult or impossible to acquire without it (Fry 2000). By straightforward methods, radiolabelling renders detectable minute traces (e.g. 1–10 pg) of the polysaccharides of interest; it is non-destructive, so the sample can be further analysed later; and it enables studies of the dynamics of synthesis, modification, secretion, crosslinking and degradation of polysaccharides in living cells.

Non-specific radiolabelling results in all newly synthesized organic components of the cell becoming radioactive (Fig. 1.3). This can be useful for the detection, quantification and characterization of novel or little-known residues or substituents, which will be detectable by their radioactivity whether or not their presence was anticipated. Specific radiolabelling permits attention to be focused on a chosen group of metabolically related residues without interference from a large excess of other accompanying residues (Table 1.2).

Both specific and non-specific radiolabelling can give valuable information about the partitioning of metabolic flux between competing intermediary pathways (Sharples & Fry 2007), and between the diverse end-products that stem from a common pool of precursors. Thus, feeding of [^{14}C]glucose can indicate the relative rates of synthesis (during the time-frame for which the exogenous [^{14}C]glucose was available in the medium) of all organic components of the wall (Sotiriou *et al.* 2007). Similarly, feeding of [2-^{3}H] mannose can indicate the relative rates of synthesis of Man, Fuc and L-Gal residues (three quantitatively minor hexoses), without interference from the large excess of the major hexoses (Glc and D-Gal).

[2-^{3}H]Mannose feeds ^{3}H into the metabolic network *upstream* of the branch-point leading to polysaccharide-bound Man, Fuc and L-Gal residues (Fig. 1.3a), so these three residues can be expected to acquire the same specific radioactivity (e.g. measured in MBq μmol^{-1}). This contrasts with the situation when [^{3}H]arabinose is fed (Fig. 1.3b). [^{3}H]Arabinose selectively labels Ara and Xyl residues, such that pentosan metabolism can be specifically examined; however, because of the tritium enters metabolism downstream of the branch point leading to polysaccharide-bound Xyl and Ara residues (the radioisotope is infiltrating the UDP-Xyl pool against the bulk metabolic flux, which is indicated by large arrows in the figure), the Ara residues of polysaccharides will acquire a higher specific radioactivity than the Xyl residues, so it is not possible to quantify the partitioning of material

Table 1.2 Precursors found useful in the author's laboratory for radiolabelling cell wall polysaccharides in plant cell-suspension cultures

Radioactive precursor fed[a] (and references[e])	Wall polysaccharide residue(s) radiolabelled												
	Glc, Rha[b]	Gal	GlcA	GalA	Ara	Xyl	Api	Man	Fuc	L-Gal	Me	Ac	Fer
Specific radiolabelling													
[2-^{3}H]Man (1,2)	–	–	–	–	–	–	–	+	+	+	–	–	–
Fuc (3)	–	–	–	–	–	–	–	–	+	–	–	–	–
[6-^{14}C]GlcA (4)	–	–	+	+	–	–	–	–	–	–	–	–	–
[^{3}H]GlcA (5)	–	–	+	+	+	+	+	–	–	–	–	–	–
Ara (6)[c,d]	–	–	–	–	++	+	–	–	–	–	–	–	–
L-[Me-^{14}C]methionine (7)	–	–	–	–	–	–	–	–	–	–	+	–	–
trans-Cinnamate (7,8)	–	–	–	–	–	–	–	–	–	–	–	–	+
Semi-specific[c]													
[^{14}C]Man (2)	+	+	+	+	+	+	+	++	++	++	+	+	+
[1-^{3}H]Gal (9)	+	++	+	+	+	+	+	±	±	±	±	±	±
Acetate (7)	+	+	+	+	+	+	+	+	+	+	+	++	+
Non-specific[f]													
Glc (9–11)	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Fructose (9)	+	+	+	+	+	+	+	+	+	+	+	+	+

[a] Where no isotope or position of labelling is stated, these variables are thought to be inconsequential.
[b] No reliable method for specific radiolabelling of Rha or Glc residues has been found.
[c] The number of +s is a guide to the specific radioactivity (e.g. measured in kBq/nmol) achieved, not activity (kBq).
[d] In some plants (e.g. *Arabidopsis*), radiolabel from Ara can re-enter central metabolism via pentose phosphate pathways, leading to moderate labelling of hexoses.
[e] References: (1) Baydoun & Fry 1988; (2) Sotiriou *et al.*(2007); (3) McDougall & Fry (1994); (4) Brown & Fry (1993b); (5) Longland *et al.*(1989); (6) Kerr & Fry (2003); (7) Miller *et al.*(1995); (8) Fry *et al.*(2000); (9) Sharples & Fry (2007); (10) Fry (1982); (11) O'Looney & Fry (2005; Fig. 1.2).
[f] Note that inclusion of certain non-radioactive polysaccharide-precursors in culture media, particularly *myo*-inositol, can render radiolabelling with [^{14}C]glucose and [^{14}C]fructose semi-specific. Plant cells can utilize exogenous non-radioactive inositol to produce GlcA, GalA, Api, Ara and Xyl residues, thus decreasing their radiolabelling.

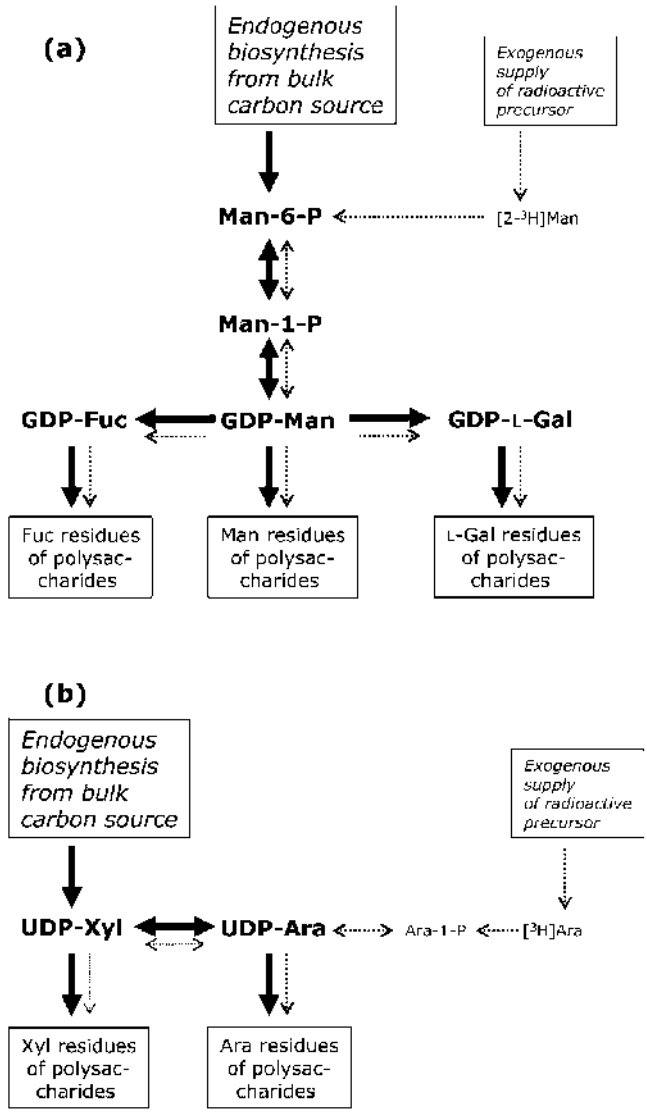

Figure 1.3 Incorporation of radioisotopes for specific radiolabelling of polysaccharide residues *in vivo*. In (a), the isotope (dotted arrows) enters the network upstream of the branch point so the three radiolabelled residues acquire approximately equal specific radioactivities. In (b), the isotope enters downstream of the branch point: ^{3}H enters the UDP-Xyl pool only by travelling against the bulk carbon flux, and in so doing decreases in specific radioactivity.

Solid arrows denote flux from bulk carbon source, dotted arrows flux from radiolabelled tracer.

between these two pentose residues. For example, when [1-^{3}H]arabinose was fed to cultured maize cells, the polysaccharide-bound [^{3}H]Ara and [^{3}H]Xyl residues acquired specific radioactivities of 1.68 and 0.78 MBq μmol^{-1} respectively (Wende & Fry 1996). Nevertheless, it is reasonable to assume that all polysaccharide-bound Xyl residues will have approximately the same specific activity, so the partitioning of material between the Xyl residues of xyloglucan and the Xyl residues of xylans can be validly monitored by analysis of the [^{3}H]isoprimeverose and [^{3}H]xylobiose released from these two polysaccharides, respectively, by Driselase (Kerr & Fry 2003).

1.6.2 Chemical and enzymic 'dissection' of wall polysaccharides

Much valuable work can be done without extraction of intact polysaccharides from the cell wall. For example, plant tissue can be homogenized in 75% ethanol (preferably acidified with 10% formic acid to inactivate endogenous wall-digesting enzymes) and washed to yield an AIR which is comprised of the total cellular polymers, of which polysaccharides usually predominate. Phenol/acetic acid/water (PAW, 2:1:1, w/v/v final composition; Fry 2000) is useful for denaturation and removal of proteins; it does not extract polysaccharides, and a PAW treatment often seems to render wall polysaccharides more vulnerable to subsequent digestion by deliberately added enzymes. Starch can be removed by prolonged stirring in 90% dimethylsulfoxide (though this may dissolve a small proportion of some wall polysaccharides, e.g. MLG and *O*-acetylated hemicelluloses) or in α-amylase solution.

For analysis of total sugar residues, it is recommended that the walls (or extracted polysaccharides) are first hydrolysed in 2 M TFA at 120 °C for 1 h. This yields monosaccharides as the major products from most matrix polysaccharides; however, because of the relative acid stability of uronosyl linkages, uronic acid-rich polysaccharides will additionally yield disaccharides (e.g. GalA→GalA from homogalacturonan, GalA→Rha from RG-I, GlcA→Xyl from xylans, GlcA→Man from glucuronomannans, and GlcA→L-Gal from *Anthoceros* polysaccharides). TFA does not efficiently hydrolyse cellulose; therefore the TFA-insoluble residue is subsequently treated with Driselase, which yields free cellulosic glucose.

For 'dissection' of wall polymers, retaining more structural information about sugar→sugar linkages, mild acid or enzymes are used. Mild acid, sufficient to hydrolyse furanose linkages but not the majority of pyranose residues (Kerr & Fry 2003), cleaves the Ara*f*→Xyl bonds of xylans and thereby releases much free Ara and also intact side chains that had been linked to the xylan backbone via an Ara*f* residue. Examples are Fer-Ara, Xyl-(Fer)-Ara, and more complex feruloylated side chains (Wende & Fry 1996).

Driselase digests all the common polysaccharides of the plant cell wall, and in several cases gives analytically useful 'diagnostic' fragments that define what polysaccharides were present – e.g. isoprimeverose from

xyloglucans, xylobiose from xylans (Kerr & Fry 2003), GalA-3AcGalA-3AcGalA(Me-ester)-GalA from acetylated homogalacturonan, GalA-Rha-3AcGalA-Rha-3AcGalA from RG-I (Perrone *et al.* 2002) and essentially intact RG-II from whole pectin (Matsunaga *et al.* 2004) (Table 1.1). Some substituents on polysaccharides may hinder Driselase digestibility, and this problem can be overcome by a preliminary treatment of the AIR with alkali and/or mild acid (Kerr & Fry 2003); TFA is the acid of choice because it can easily be removed, by drying, before Driselase addition.

Purified endo-acting enzymes (Table 1.1) attack polysaccharides at specific sites along the backbone to yield larger fragments than does Driselase. Such enzymes are endo-hydrolases (e.g. XEG, EPG, RG hydrolase, lichenase) and RG lyase. Several of these are available commercially (e.g. Megazyme). An extensive battery of wall-digesting enzymes, produced in the fungus *Pichia pastoris*, is also now available (Bauer *et al.* 2006).

It is important to distinguish the enantiomeric forms of monosaccharide residues. D-Gal is metabolically quite unrelated to L-Gal, and both occur in wall polysaccharides. Enantiomers can be distinguished if the monosaccharide is made to interact with another chiral molecule, which can be either an enzyme or a low-M_r chiral alcohol. For example, the Gal is treated with ATP + D-galactokinase (which phosphorylates D- but not L-Gal) or O_2 + D-galactose oxidase (Popper *et al.* 2003). The enzymic technique is particularly useful when then the Gal in question is radiolabelled and available only in minute quantities: for example, [^{3}H]Gal-1-P (formed by galactokinase) is readily separated from remaining unreacted [^{3}H]Gal by chromatography or electrophoresis. Alternatively, Gal (either enantiomer) will react with hot (+)-butan-2-ol (containing 1 M acetyl chloride, which forms HCl on contact with butanol) to produce a series of galactosides, the D-Gal and L-Gal versions of which are not enantiomers (mirror images) of each other and can therefore be resolved chromatographically (Gerwig *et al.* 1979).

1.6.3 Fractionation and characterization of mono- and oligosaccharides

Once the polysaccharides have been dissected into monosaccharides and oligosaccharides, these fragments can be fractionated and characterized. Depending on the aims of the research, various types of chromatography or electrophoresis are employed. Use the simplest technique that works.

1.6.3.1 Paper chromatography (PC)

PC gives excellent resolution of most of the common monosaccharides and disaccharides obtained from wall polysaccharides (Fry 2000). They can be detected by staining, either with $AgNO_3$ (which detects ~0.1 µg Ara) or with aniline hydrogen phthalate (which detects ~0.4 µg Ara). The latter gives different colours with different monosaccharide classes (aldohexoses, brown; aldopentoses, red; ketohexoses, yellow; uronic acids, orange); in

disaccharides, the colour is dictated by the reducing terminus, so isoprimeverose (brown) is easily distinguished from xylobiose (red). PC is also suitable for larger oligosaccharides (e.g. XEG digestion products, and feruloyl oligosaccharides), which it effectively resolves into size-classes. Radioactive analytes can be located by autoradiography (for ^{14}C) or fluorography (^{3}H), or, if these film-based methods are not sensitive enough, the paper is cut into strips, which are assayed by scintillation counting. To exploit maximally the resolving power of PC, the sample is mixed with an internal authentic marker (radioactive if the sample is non-radioactive, and vice versa); any mismatch between the sample spot and the internal marker spot, even by as little as 1–2 mm, proves that the sample is not the same substance as the marker.

A few important oligosaccharides, e.g. MLG-, cello- and manno-oligosaccharides, cannot satisfactorily be run on PC if their DP exceeds about 3. This is because of their high affinity for paper (cellulose) – they fail to migrate from the origin, or do so only as an diffuse streak.

Suitable papers for PC are Whatman 1 CHR for general-purpose rapid runs (typically 16–36 h, with about 60 samples per tank), the thicker Whatman 3MM Chr for preparative work, and the denser Schleicher and Schüll 2045B for better-resolution slow runs (2–9 days). The best general-purpose solvent for exploratory work with mono- and disaccharides is butan-1-ol/acetic acid/water (12:3:5 by volume, freshly prepared); for larger oligosaccharides it is ethyl acetate/acetic acid/water (10:5:6). These acidic solvents tolerate quite heavy contamination with salts and proteins. For better discrimination between different neutral mono- and disaccharides, ethyl acetate/pyridine/water (8:2:1) is excellent (Fry 2000).

1.6.3.2 Thin-layer chromatography (TLC)

TLC on silica gel gives similar resolution to PC but on a smaller area (typically 20 × 20 cm as opposed to 46 × 57 cm) (Fry 2000). TLC is more useful for large oligosaccharides, including those that bind paper (MLG-, cello- and manno-oligosaccharides). Suitable solvents include butan-1-ol/acetic acid/water (2:1:1) and propan-1-ol/nitromethane/water (5:2:3). A useful stain for carbohydrates on silica gel is freshly prepared 0.5% (w/v) thymol in 96% ethanol/H_2SO_4 (20:1 v/v) – the plate is quickly dipped in this solution, dried, and then heated in an oven at 105 °C for 3–6 min.

1.6.3.3 Paper electrophoresis (PE)

High-voltage PE (Fry 2000) is a surprisingly little-used technique that can, however, give excellent resolution of charged carbohydrates e.g. amino sugars, uronic acids, aldonic acids, aldaric acids, sugar phosphates and NDP sugars (e.g. Green & Fry 2005; Sharples & Fry 2007; Takeda *et al.* 2008; see also http://homepages.ed.ac.uk/sfry/service.html). Even neutral sugars can be analysed by PE if a borate buffer is used, with which the sugar reversibly forms an anionic borate ester (e.g. O'Looney & Fry 2005). Electrophoretic

mobility depends on both the charge (Q) of the compound (which at pH 6.5 will be about –1 in the case of GalA and GlcA→Man, and nearly –2 in the case of GalA→GalA) and its molecular weight (M_r). The migration rate towards the electrode (relative to a neutral marker) is proportional to $Q/M_r^{2/3}$ (Offord 1966). Therefore, if several compounds with known Q and M_r are run on the same electrophoretogram, the mobility of an unknown radioactive spot can provide valuable data – if its charge is known, then its M_r can be estimated, and if its approximate M_r is known (e.g. that it is a disaccharide), then its charge can be estimated.

1.6.3.4 High-pressure liquid chromatography (HPLC)

For details, see El Rassi (1995). Several forms of HPLC are useful for the resolution of mono- and oligosaccharides. In particular, a form of anion-exchange chromatography developed by Dionex Inc. enables excellent separation of all the common monosaccharides and numerous diverse oligosaccharides. The analytes are readily quantified with a pulsed amperometric detector if it has been calibrated for the compounds under investigation. Samples require more cleaning up than for PC. Radioactivity monitors designed for HPLC eluates are not sensitive enough (compared with autoradiography or scintillation counting in vials) to be useful unless unusually high levels of radiolabelling are employed.

1.6.3.5 Methylation analysis and gas chromatography (GC)

Methylation analysis is particularly useful when no suitable enzymes are available for 'dissection' of wall polysaccharides, or when detailed structural information is required about novel polysaccharides. Details are outside the scope of the present chapter (Waeghe *et al.* 1983; Harris *et al.* 1984; Carpita & Shea 1989). GC of sugars (or partially methylated sugars) requires them to be converted to volatile derivatives. Usually this involves reduction of the monosaccharide to an alditol with $NaBH_4$ (or NaB_2H_4 so that the original reducing end can be followed during MS) followed by acetylation with acetic anhydride. Resolution of partially methylated alditol acetates by GC is excellent, as is quantification; GC is, however, rarely attempted with radiolabelled samples because of the difficulties involved in measuring and disposing of radiolabelled volatiles.

1.6.3.6 Mass spectrometry (MS)

GC and HPLC are both frequently combined with MS, giving information about the masses of ions (strictly, the M_r/Q ratio, referred to in MS work as 'm/z') of the analytes and of the fragments formed from them inside the mass spectrometer. The m/z values can reveal the number and often the sequence of sugar residues of each class (e.g. pentose, hexose, deoxyhexose), but not the identity of the specific monosaccharide(s) (e.g. Glc, Gal and Man all have the same mass).

1.7 Conclusions

The recent development of convenient methods for genetic manipulation of plant cells, by which the genes for chosen wall-related enzymes can be targeted for up- or down-regulation in a spatially and temporally precise fashion, has given us the potential ability to alter artificially the production, modification, secretion, crosslinking and degradation of wall polysaccharides. There remains, however, the need to test experimentally whether the intended change in polysaccharide metabolism has actually been effected. Therefore, there is a need for simple methods by which wall polysaccharide composition and metabolism can be documented *in vivo*. A range of suitable methods for such endeavours has been compiled in this chapter. Of particular value are techniques of *in-vivo* radiolabelling (by which even quantitatively minor components can be detected, and the dynamics of their metabolism explored), enzymic dissection of wall polysaccharides, and separation of the digestion-products by chromatography and electrophoresis.

Acknowledgements

I thank the UK BBSRC for funding in support of this work.

References

Amino, S., Takeuchi, Y., Komamine, A. (1984) Changes in cell wall constituents during the cell cycle in a synchronous culture of *Catharanthus roseus*. *Physiologia Plantarum*, **60**, 326–332.

Andersson, S.I., Samuelson, O., Ishihara, M., Shimizu, K. (1983) Structure of the reducing end-groups in spruce xylan. *Carbohydrate Research*, **111**, 283–288.

Asensio, A. (1987) *Quercus suber* polysaccharides. 1. Structural studies of the hemicellulose-A from the cork of *Quercus suber*. *Carbohydrate Research*, **161**, 167–170.

Aspinall, G.O., Carlyle, J.J., McNab, J.M., Rudowski, A. (1969) *Anogeissus leiocarpus* gum. II. Fractionation of gum and partial hydrolysis of leiocarpan A. *Journal of the Chemical Society C* **1969**, 840–845.

Bauer, S., Vasu, P., Persson, S., Mort, A.J., Somerville, C.R. (2006) Development and application of a suite of polysaccharide-degrading enzymes for analyzing plant cell walls. *Proceedings of the National Academy of Sciences of the U S A*, **103**, 11417–11422.

Baydoun, E.A.-H., Fry, S.C. (1988) [2-^{3}H]Mannose incorporation in cultured plant cells: investigation of L-galactose residues in the primary cell wall. *Journal of Plant Physiology*, **132**, 484–490.

Brett, C.T., Waldron, K.W. (1996) *Physiology and Biochemistry of Plant Cell Walls*, 2nd edn. Chapman & Hall, London.

Brown, J.A., Fry, S.C. (1993a) The preparation and susceptibility to hydrolysis of novel *O*-galacturonoyl derivatives of carbohydrates. *Carbohydrate Research*, **240**, 95–106.

Brown, J.A., Fry, S.C. (1993b) Novel *O*-D-galacturonoyl esters in the pectic polysaccharides of suspension-cultured plant cells. *Plant Physiology*, **103**, 993–999.

Buliga, G.S., Brant, D.A., Fincher, G.B. (1986) The sequence statistics and solution conformation of a barley (1→3, 1→4)-β-D-glucan. *Carbohydrate Research*, **157**, 139–156.

Carnachan, S.M., Harris, P.J. (2000) Ferulic acid is bound to the primary cell walls of all gymnosperm families. *Biochemical Systematics and Ecology*, **28**, 865–879.

Carpita, N.C. (1984) Fractionation of hemicelluloses from maize cell-walls with increasing concentrations of alkali. *Phytochemistry*, **23**, 1089–1093.

Carpita, N.C. (1996) Structure and biogenesis of the cell walls of grasses. *Annual Review of Plant Physiology and Plant Molecular Biology*, **47**, 445–76.

Carpita, N.C., Shea, E.M. (1989) Linkage structure of carbohydrates by gas chromatography–mass spectrometry (GC–MS) of partially methylated alditol acetates. In: *Analysis of Carbohydrates by GLC and MS* (eds C.J Biermann, G.D. McGinnis). CRC Press, Boca Raton.

Carpita, N.C., Defernez, M., Findlay, K., *et al.* (2001) Cell wall architecture of the elongating maize coleoptile. *Plant Physiology*, **127**, 551–565.

Cherno, N.K., Dudkin, M.S., Areshidze, I.V. (1976) Pectin substances of *Chara aculeolata*. *Khimiya Prirodnykh Soedinenii*, **6**, 702–705.

Coenen, G.J., Bakx, E.J., Verhoef, R.P., Schols, H.A., Voragen, A.G.J. (2007) Identification of the connecting linkage between homo- or xylogalacturonan and rhamnogalacturonan type I. *Carbohydrate Polymers*, **70**, 224–235.

Edashige, Y., Ishii, T. (1996) Structures of cell-wall polysaccharides from suspension-cultured cells of *Cryptomeria japonica*. *Mokuzai Gakkaishi*, **42**, 895–900.

Edelmann, H.G., Fry, S.C. (1992) Factors that affect the extraction of xyloglucan from the primary cell walls of suspension-cultured rose cells. *Carbohydrate Research*, **228**, 423–431.

El Rassi, Z. (ed.) (1995) *Carbohydrate Analysis: High-Performance Liquid Chromatography and Capillary Electrophoresis*. *Journal of Chromatography Library*, Vol. 58. Elsevier, Amsterdam.

Encina, A., Fry, S.C. (2005) Oxidative coupling of a feruloyl-arabinoxylan trisaccharide (FAXX) in the walls of living maize cells requires endogenous hydrogen peroxide and is controlled by a low-M_r apoplastic inhibitor. *Planta*, **223**, 77–89.

Fry, S.C. (1982) Phenolic components of the primary cell wall: feruloylated disaccharides of D-galactose and L-arabinose from spinach polysaccharide. *Biochemical Journal*, **203**, 493–504.

Fry, S.C. (1983) Feruloylated pectins from the primary cell wall: their structure and possible functions. *Planta*, **157**, 111–123.

Fry, S.C. (1989) Cellulases, hemicelluloses and auxin-stimulated growth: a possible relationship. *Physiologia Plantarum*, **75**, 532–536.

Fry, S.C. (2000) *The Growing Plant Cell Wall: Chemical and Metabolic Analysis*, reprint edition, The Blackburn Press, Caldwell, NJ.

Fry, S.C. (2001) Plant cell walls. In: *Encyclopedia of Life Sciences*. Nature Publishing Group, London (also electronic edition 1999, http://www.els.net).

Fry, S.C., Mohler, K.E., Nesselrode, B.H.W.A., Franková, L. (2008a) Mixed-linkage β-glucan : xyloglucan endotransglucosylase, a novel wall-remodelling enzyme from *Equisetum* (horsetails) and charophytic algae. *Plant Journal*, **54**, 451–521.

Fry, S.C., Nesselrode, B.H.W.A., Miller, J.G., Mewburn, B. (2008b) Mixed-linkage (1→3,1→4)-β-D-glucan is a major hemicellulose of *Equisetum* (horsetail) cell walls. *New Phytologist*, **179**, 104–115.

Fry, S.C., Willis, S.C., Paterson, A.E.J. (2000) Intraprotoplasmic and wall-localised formation of arabinoxylan-bound diferulates and larger ferulate coupling-products in maize cell-suspension cultures. *Planta*, **211**, 679–692.

Funk, C., Ralph, J., Steinhart, H., Bunzel, M. (2005) Isolation and structural characterisation of 8–*O*–4/8–*O*–4-and 8–8/8–*O*–4-coupled dehydrotriferulic acids from maize bran. *Phytochemistry*, **66**, 363–371.

Gerwig, G.J., Kamerling, J.P., Vliegenthart, J.F.G. (1979) Determination of the absolute-configuration of monosaccharides in complex carbohydrates by capillary GLC. *Carbohydrate Research*, **77**, 1–7.

Gibeaut, D.M., Pauly, M., Bacic A., Fincher, G.B. (2005) Changes in cell wall polysaccharides in developing barley (*Hordeum vulgare*) coleoptiles. *Planta*, **221**, 729–738.

Green, M.A., Fry, S.C. (2005) Vitamin C degradation in plant cells via enzymatic hydrolysis of 4-*O*-oxalyl-L-threonate. *Nature*, **433**, 83–88.

Grishutin, S.G., Gusakov, A.V., Dzedzyulya, E.I., Sinitsyn, A.P. (2006) A lichenase-like family 12 endo-(l→4)-β-glucanase from *Aspergillus japonicus*: study of the substrate specificity and mode of action on β-glucans in comparison with other glycoside hydrolases. *Carbohydrate Research*, **341**, 218–229.

Handford, M.G., Baldwin, T.C., Goubet, F., et al. (2003) Localisation and characterisation of cell wall mannan polysaccharides in *Arabidopsis thaliana*. *Planta* **218**, 27–36.

Harris, P.J., Henry, R.J., Blakeney, A.B., Stone, B.A. (1984) An improved procedure for the methylation analysis of oligosaccharides and polysaccharides. *Carbohydrate Research*, **127**, 59–74.

Hayashi (1989) Xyloglucans in the primary cell wall. *Annual Review of Plant Physiology and Plant Molecular Biology*, **40**, 139–168.

Hoffman, M., Jia, Z., Peña, M.J., *et al.* (2005) Structural analysis of xyloglucans in the primary cell walls of plants in the subclass Asteridae. *Carbohydrate Research*, **340**, 1826–1840.

Honda, Y., Inaoka, H., Takei, A., Sugimura, Y., Otsuji, K. (1996) Extracellular polysaccharides produced by tuberose callus. *Phytochemistry*, **41**, 1517–1521.

Hrmová, M., Farkaš, V., Lahnstein J., Fincher, G.B. (2007) A barley xyloglucan xyloglucosyl transferase covalently links xyloglucan, cellulosic substrates, and (1,3;1,4)-β-D-glucans. *Journal of Biological Chemistry*, **282**, 12951–12962.

Ishii, T. (1997) *O*-Acetylated oligosaccharides from pectins of potato tuber cell walls. *Plant Physiology*, **113**, 1265–1272.

Ishii, T., Matsunaga, T. (2001) Pectic polysaccharide rhamnogalacturonan II is covalently linked to homogalacturonan. *Phytochemistry*, **57**, 969–974.

Kato, Y., Ito, S., Iki, K., Matsuda, K. (1982) Xyloglucan and β-D-glucan in cell walls in rice seedlings. *Plant and Cell Physiology*, **23**, 351–364.

Kato, Y., Nevins, D.J. (1985) Isolation and identification of *O*-(5-*O*-feruloyl-α-L-arabinofuranosyl)-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose as a component of *Zea* shoot cell walls. *Carbohydr Res*, **137**, 139–150.

Kerr, E.M., Fry, S.C. (2003) Pre-formed xyloglucans and xylans increase in molecular weight in three distinct compartments of a maize cell-suspension culture. *Planta*, **217**, 327–339.

Kim J-B., Carpita, N.C. (1992) Changes in esterification of the uronic acid groups of cell wall polysaccharides during elongation of maize coleoptiles. *Plant Physiology*, **98**, 646–653.

Kutschera, U. (2008) The growing outer epidermal wall: design and physiological role of a composite structure. *Annals of Botany* **101**, 615–621.

Labavitch, J.M., Ray, P.M. (1978) Structure of hemicellulosic polysaccharides of *Avena sativa* coleoptile cell walls. *Phytochemistry*, **17**, 933–937.

Lenucci, M., Piro, G., Miller, J.G., Dalessandro, G., Fry, S.C. (2005) Do polyamines contribute to plant cell wall assembly by forming amide bonds with pectins? *Phytochemistry*, **66**, 2581–2594.

Li, W., Cui, S.W., Kakuda, Y. (2006) Extraction, fractionation, structural and physical characterization of wheat β-D-glucans. *Carbohydrate Polymers*, **63**, 408–416.

Lindsay, S.E., Fry, S.C. (2008) Control of diferulate formation in dicotyledonous and gramineous cell-suspension cultures. *Planta*, **227**, 439–452.

Longland, J.M., Fry, S.C., Trewavas, A.J. (1989) Developmental control of apiogalacturonan biosynthesis and UDP-apiose production in a duckweed. *Plant Physiology*, **90**, 972–976.

Matsunaga, T., Ishii, T., Matsumoto, S., *et al.* (2004) Occurrence of the primary cell wall polysaccharide rhamnogalacturonan II in pteridophytes, lycophytes, and bryophytes. implications for the evolution of vascular plants. *Plant Physiology*, **134**, 339–351.

McCleary, B.V., Matheson, N.K. (1983) Action patterns and substrate-binding requirements of β-D-mannanase with mannosaccharides and mannan-type polysaccharides. *Carbohydrate Research*, **119**, 191–219.

McDougall, G.J., Fry, S.C. (1994) Fucosylated xyloglucan in suspension-cultured cells of the gramineous monocotyledon, *Festuca arundinacea*. *Journal of Plant Physiology*, **143**, 591–595.

Meikle, P.J., Hoogenraad, N.J., Bonig, I., Clarke, A.E., Stone, B.A. (1994) A (1→3,1→4)-β-glucan-specific monoclonal antibody and its use in the quantification and immunocytochemical location of (1→3,1→4)-β-glucans. *Plant Journal*, **5**, 1–9.

Melton, L.D., McNeil, M., Darvill, A.G., Albersheim, P., Dell, A. (1986) Structural characterization of oligosaccharides isolated from the pectic polysaccharide rhamnogalacturonan II. *Carbohydrate Research*, **146**, 279–305.

Miller, J.G., Buchanan, C.J., Eastwood, M.A., Fry, S.C. (1995) Specific ^{14}C-labelling of spinach cell walls in their methyl, acetyl or uronic acid residues. *Carbohydrate Letters*, **1**, 129–136.

Monro, J.A., Penny, D., Bailey, R.W. (1976) The organization and growth of primary cell walls of lupin hypocotyl. *Phytochemistry*, **15**, 1193–1198.

Mort, A.J. (2002) Interactions between pectins and other polymers. Pp 30–51 in *Pectins and their Manipulation*, ed. by G.B. Seymour and J.P. Knox, Blackwell Publishing Ltd., Oxford.

Mutter, M., Renard, C.M.G.C., Beldman, G., Schols, H.A., Voragen, A.G.J. (1998) Mode of action of RG-hydrolase and RG-lyase toward rhamnogalacturonan oligomers. Characterization of degradation products using RG-rhamnohydrolase and RG-galacturonohydrolase. *Carbohydrate Research*, **311**, 155–164.

Nishikubo, N., Awano, T., Banasiak, A., *et al.* (2007) Xyloglucan endo-transglycosylase (XET) functions in gelatinous layers of tension wood fibers in poplar – a glimpse into the mechanism of the balancing act of trees. *Plant and Cell Physiology*, **48**, 843–855.

O'Looney, N., Fry, S.C. (2005) Oxaziclomefone, a new herbicide, inhibits wall expansion in maize cell-cultures without affecting polysaccharide biosynthesis,

xyloglucan transglycosylation, peroxidase action or apoplastic ascorbate oxidation. *Annals of Botany*, **96**, 1097–1107.
O'Neill, M.A., York, W.S. (2003) The composition and structure of plant primary cell walls. In: *The Plant Cell Wall* (ed. J.K.C. Rose), pp. 1–54. Blackwell Publishing Ltd., Oxford.
O'Neill, M.A., Eberhard, S., Albersheim, P., Darvill, A.G. (2001) Requirement of borate cross-linking of cell wall rhamnogalacturonan II for *Arabidopsis* growth. *Science*, **294**, 846–849.
O'Neill, M.A., Ishii, T., Albersheim, P., Darvill, A.G. (2004) Rhamnogalacturonan II: structure and function of a borate cross-linked cell wall pectic polysaccharide. *Annual Review of Plant Biology*, **55**, 109–139.
Obel, N., Neumetzler, L., Pauly, M. (2006) Hemicelluloses and cell expansion. In: *The Expanding Cell* (eds J.-P. Verbelen, K. Vissenberg), Plant Cell Monograph 5, pp. 57–88. Springer, Berlin.
Offord, R.E. (1966) Electrophoretic mobilities of peptides on paper and their use in the determination of amide groups. *Nature*, **211**, 591–593.
Ortega, L.I., Fry, S.C., Taleisnik, E. (2006) Why are *Chloris gayana* leaves shorter in salt-affected plants? Analyses in the elongation zone. *Journal of Experimental Botany*, **57**, 3945–3952.
Painter, T.J. (1983) Residues of D-*lyxo*-5-hexosulopyranuronic acid in *Sphagnum* holocellulose and their role in cross-linking. *Carbohydrate Research*, **124**, C18–C21.
Pauly, M., Andersen, L.N., Kauppinen, S., *et al.* (1999) A xyloglucan-specific endo-β-1,4-glucanase from *Aspergillus aculeatus*: expression cloning in yeast, purification and characterization of the recombinant enzyme. *Glycobiology*, **9**, 93–100.
Peña, M.J., Darvill,A.G., York,W.S., O'Neill M.A. (2007a) Structural diversity of xyloglucans in the cell walls of land plants. *Physiologia Plantarum*, 130, Abstract 18, XIth Cell Wall Meeting.
Peña, M.J., Zhong, R.Q., Zhou, G.K., *et al.* (2007b) *Arabidopsis irregular xylem8* and *irregular xylem9*: implications for the complexity of glucuronoxylan biosynthesis. *Plant Cell*, **19**, 549–563.
Perrone, P., Hewage, C., Sadler, I.H., Fry, S.C. (1998) *N*α- and *N*ε-D-galacturonoyl-L-lysine amides: properties and possible occurrence in plant cell walls. *Phytochemistry*, **49**, 1879–90.
Perrone, P., Hewage, C.M., Thomson, A.R., Bailey, K., Sadler, I.H., Fry, S.C. (2002) Patterns of methyl and *O*-acetyl esterification in spinach pectins: new complexity. *Phytochemistry*, **60**, 67–77.
Popper, Z.A., Fry, S.C. (2003) Primary cell wall composition of bryophytes and charophytes. *Annals of Botany*, **91**, 1–12.
Popper, Z.A., Fry, S.C. (2004) Primary cell wall composition of pteridophytes and spermatophytes. *New Phytologist*, **164**, 165–174.
Popper, Z.A., Fry, S.C. (2005) Widespread occurrence of a covalent linkage between xyloglucan and acidic polysaccharides in suspension-cultured angiosperm cells. *Annals of Botany*, **96**, 91–99.
Popper, Z.A., Fry, S.C. (2008) Xyloglucan–pectin linkages are formed intraprotoplasmically, contribute to wall-assembly, and remain stable in the cell wall. *Planta*, **227**, 781–794.
Popper, Z.A., Sadler, I.H., Fry, S.C. (2003) α-D-Glucuronosyl-(1→3)-L-galactose, an unusual disaccharide from polysaccharides of the hornwort *Anthoceros caucasicus*. *Phytochemistry*, **64**, 325–335.

Redgwell, R.J., O'Neill, M.A., Selvendran, R.R., Parsley, K.J. (1986) Structural features of the mucilage from the stem pith of kiwifruit (*Actinidia deliciosa*). 1. structure of the inner core. *Carbohydrate Research*, **153**, 97–106.

Rocha, S.M., Coimbra, M.A., Delgadillo, I. (2000) Demonstration of pectic polysaccharides in cork cell wall from *Quercus suber* L. *Journal of Agricultural and Food Chemistry*, **48**, 2003–2007.

Schols, H.A., Voragen, A.G.J. (2002) The chemical nature of pectins. In: *Pectins and their Manipulation* (eds G.B. Seymour, J.P. Knox), pp. 1–29. Blackwell Publishing Ltd., Oxford.

Sharples, S.C., Fry, S.C. (2007) Radioisotope ratios discriminate between competing pathways of cell wall polysaccharide and RNA biosynthesis in living plant cells. *Plant Journal*, **52**, 252–262.

Smith, B.G., Harris, P.J. (2001) Ferulic acid is esterified to glucuronoarabinoxylans in pineapple cell walls. *Phytochemistry*, **56**, 513–519.

Sørensen, I., Pettolino, F.A., Wilson, S.M., *et al.* (2008) Mixed linkage (1→3),(1→4)-β-D-glucan is not unique to the Poales and is an abundant component of *Equisetum arvense* cell walls. *Plant Journal*, **54**, 451–521.

Sotiriou, P., Fry, S.C., Spyropoulos, C.G. (2007) Protoplast isolation and culture from carob (*Ceratonia siliqua* L.) hypocotyls: ability of regenerated protoplasts to produce mannose-containing polysaccharides. *Physiologia Plantarum*, **130**, 11–22.

Takeda, T., Miller, J.G., Fry, S.C. (2008) Anionic derivatives of xyloglucan function as acceptor but not donor substrates for xyloglucan endotransglucosylase activity. *Planta*, **227**, 893–905.

Teleman, A., Nordström, M., Tenkanen, M., Jacobs, A., Dahlmana, O. (2003) Isolation and characterization of *O*-acetylated glucomannans from aspen and birch wood. *Carbohydrate Research*, **338**, 525–534.

Thimm, J.C., Burritt, D.J., Sims, I.M., Newman, R.H., Ducker, W.A., Melton, L.D. (2002) Celery (*Apium graveolens*) parenchyma cell walls: cell walls with minimal xyloglucan. *Physiologia Plantarum*, **116**, 164–171.

Thomas, J.R., Darvill, A.G., Albersheim, P. (1989) The structure of plant cell walls XXIV. Isolation and structural characterization of the pectic polysaccharide rhamnogalacturonan II from walls of suspension-cultured rice cells. *Plant Physiology*, **185**, 261–277.

Thompson, D.S. (2008) Space and time in the plant cell wall: relationships between cell type, cell wall rheology and cell function. *Annals of Botany*, **101**, 203–211.

Thompson, J.E., Fry, S.C. (2000) Evidence for covalent linkage between xyloglucan and acidic pectins in suspension-cultured rose cells. *Planta*, **211**, 275–286.

Thornber, J.P., Northcote, D.H. (1962) Changes in the chemical composition of a cambial cell during its differentiation into xylem and phloem tissues in trees. 3. Xylan, glucomannan and α-cellulose fractions. *Biochemical Journal*, **82**, 340–346.

Ulvskov, P., Wium, H., Bruce, D., *et al.* (2005) Biophysical consequences of remodeling the neutral side chains of rhamnogalacturonan I in tubers of transgenic potatoes. *Planta*, **220**, 609–620.

Wada, S., Ray, P.M. (1978) Matrix polysaccharides of oat coleoptile cell walls. *Phytochemistry*, **17**, 923–931.

Waeghe, T.J., Darvill, A.G., McNeil, M., Albersheim, P. (1983) Determination by methylation analysis of the glycosyl-linkage compositions of microgram quantities of complex carbohydrates. *Carbohydrate Research*, **123**, 281–304.

Wende, G., Fry, S.C. (1996) 2-*O*-β-D-Xylopyranosyl-(5-*O*-feruloyl)-L-arabinose, a widespread component of grass cell walls. *Phytochemistry*, **44**, 1019–1030.

Willför, S., Sundberg, A., Pranivich, A., Holmbom, B. (2005) Polysaccharides in some industrially important hardwood species. *Wood Science and Technology*, **39**, 601–617.

Woodward, J.R., Fincher, G.B., Stone, B.A. (1983) Water-soluble (1→3),(1→4)-β-D-glucans from barley (*Hordeum vulgare*) endosperm. II. Fine structure. *Carbohydrate Polymers*, **3**, 207–225.

Zandleven, J., Beldman, G., Bosveld, M., Benen, J., Voragen, A. (2005) Mode of action of xylogalacturonan hydrolase towards xylogalacturonan and xylogalacturonan oligosaccharides. *Biochemical Journal*, **387**, 719–725.

Zhang, Z.Q., Pierce, M.L., Mort, A.J. (2007) Changes in homogalacturonans and enzymes degrading them during cotton cotyledon expansion. *Phytochemistry*, **68**, 1094–1103.

Zykwinska, A.W., Ralet M-C.J., Garnier, C.D., Thibault J-F.J. (2005) Evidence for in vitro binding of pectin side chains to cellulose. *Plant Physiology*, **139**, 397–407.