Annals of Botany **96**: 91–99, 2005
doi:10.1093/aob/mci153, available online at www.aob.oupjournals.org

Widespread Occurrence of a Covalent Linkage Between Xyloglucan and Acidic Polysaccharides in Suspension-cultured Angiosperm Cells

ZOË A. POPPER and STEPHEN C. FRY*

The Edinburgh Cell Wall Group, Institute of Molecular Plant Sciences, School of Biological Sciences, The University of Edinburgh, Daniel Rutherford Building, The King's Buildings, Mayfield Road, Edinburgh EH9 3JH, UK

Received: 19 January 2005 Returned for revision: 17 February 2005 Accepted: 3 March 2005 Published electronically: 18 April 2005

- *Background and Aims* Covalent linkages between xyloglucan and rhamnogalacturonan-I (RG-I) have been reported in the primary cell walls of cultured *Rosa* cells and may contribute to wall architecture. This study investigated whether this chemical feature is general to angiosperms or whether *Rosa* is unusual.
- *Methods* Xyloglucan was alkali-extracted from the walls of L-[1-^{3}H]arabinose-fed suspension-cultured cells of *Arabidopsis*, sycamore, rose, tomato, spinach, maize and barley. The polysaccharide was precipitated with 50 % ethanol and subjected to anion-exchange chromatography in 8 M urea. Eluted fractions were Driselase-digested, yielding [^{3}H]isoprimeverose (diagnostic of [^{3}H]xyloglucan). The *Arabidopsis* cells were also fed [6-^{14}C]glucuronic acid, and radiolabelled pectins were extracted with ammonium oxalate.
- *Key Results* [^{3}H]Xyloglucan was detected in acidic (galacturonate-containing) as well as non-anionic polysaccharide fractions. The proportion of the [^{3}H]isoprimeverose units that were in anionic fractions was: *Arabidopsis*, 45 %; sycamore, 60 %; rose, 44 %; tomato, 75 %; spinach, 70 %; maize, 50 %; barley, 70 %. In *Arabidopsis* cultures fed D-[6-^{14}C]glucuronate, 20 % of the (*galacturonate*-^{14}C)-labelled pectins were found to hydrogen-bond to cellulose, a characteristic normally restricted to hemicelluloses such as xyloglucan.
- *Conclusions* Alkali-stable, anionic complexes of xyloglucan (reported in the case of *Rosa* to be xyloglucan–RG-I covalent complexes) are widespread in the cell walls of angiosperms, including gramineous monocots.

Key words: Dicots, gramineous monocots, pectin, cell wall (primary), xyloglucan–pectin linkage, hemicellulose, pectin, rhamnogalacturonan.

INTRODUCTION

Xyloglucan and pectins are quantitatively and dynamically important primary cell wall (PCW) polymers. Xyloglucan, a neutral hemicellulose, has a widespread occurrence in land plant PCWs (Popper and Fry, 2003), typically contributing 20–25 % of the dry weight of the dicot PCW (Keegstra *et al.*, 1973) and 2–5 % of the dry weight of the monocot PCW (Fry, 1989*a*; Hayashi, 1989). It is thought to function in cell enlargement by serving as a substrate for xyloglucan endotransglucosylase (XET) activity, which is able to cut and rejoin intermicrofibrillar xyloglucan chains (Fry *et al.*, 1992; Nishitani and Tominaga, 1992; Thompson *et al.*, 1997; Thompson and Fry, 2001). Pectins are acidic polysaccharides, rich in α-D-galacturonate (GalA) residues. There are three major pectic domains: homogalacturonan and the rhamnogalacturonans RG-I and RG-II (O'Neill *et al.*, 1990; Ridley *et al.*, 2001). Pectins constitute about 35 % of the dry weight of dicot PCWs (Fry, 2000) and are major targets for a variety of hydrolytic enzymes both endogenous and pathogen/herbivore-derived [examples include endopolygalacturonase (EPG), pectin methyl esterase, rhamnogalacturonase and arabinanase]. A covalent linkage between xyloglucan and pectin would be likely to make a major contribution to cell wall structure and metabolism. A better understanding of the occurrence of this linkage is therefore necessary for attempts to model the PCW and to explain and manipulate plant growth.

The occurrence of covalent xyloglucan–pectin linkages within the dicot PCW was first proposed in the PCW-model of Albersheim and co-workers (Keegstra *et al.*, 1973). They proposed that the reducing terminus of xyloglucan is glycosidically linked to what would today be termed an RG-I side-chain. Evidence for these linkages was centred on the products released after EPG digestion of *Acer* PCWs. EPG solubilized approx. 50 % of the total PCW 'pectin' (as defined by the presence of uronic acid residues) and approx. 10 % of the 'xyloglucan' [as defined by the presence of xylose (Xyl) residues]; a large proportion (approx. 70 %) of this Xyl-containing polysaccharide bound to the anion-exchanger, DEAE-Sephadex, and co-eluted with 'pectin', suggesting a bond between xyloglucan (which is neutral) and an acidic pectin (Talmadge *et al.*, 1973). EPG-digested PCWs released a further proportion of the wall (approx. 16 %) on extraction with 8 M urea or with 0·5 M NaOH containing 100 mM $NaBH_4$, or on treatment with endo-β-glucanase. The solubilized wall material was shown to contain sugar residues thought to be characteristic of xyloglucan and pectins (Bauer *et al.*, 1973). In the 1973 model (Keegstra *et al.*, 1973) Xyl residues were not distinguished from 2-*O*-methylxylose and it was assumed that all Xyl was in xyloglucan and all uronic acid residues were in pectin. Later research showed that Xyl (and/or 2-*O*-methylxylose) and uronic acid residues could be derived from a single species of polymer [such as glucuronoarabinoxylan (GAX) or RG-II] without necessarily implying the existence of a xyloglucan–RG-I complex (Spellman *et al.*, 1983;

---

* For correspondence. E-mail S.Fry@ed.ac.uk

Prade *et al.*, 1999). Many PCW models now place xyloglucan and RG-I in independent networks (McCann and Roberts, 1991).

However, recent evidence supports the existence of xyloglucan–RG-I conjugates. Thompson and Fry (2000) found approx. 30 % of xyloglucan (extracted from non-lignified suspension-cultured rose cells by 6 M NaOH) to be covalently bonded to anionic material, as judged by high-voltage electrophoresis and by anion-exchange chromatography. The anion-associated xyloglucan did not lose its charge after treatment with 8 M urea, 6 M NaOH or proteinase. Femenia *et al.* (1999) reported the presence of 'pectic-xylan–xyloglucan' complexes in alkali extracts from lignified cauliflower stems. This is also compatible with xyloglucan–pectin covalent complexes, although the possibility that lignin was responsible for the cross-linking of these two polysaccharides cannot be excluded. Enzymic digestion of the complex gave evidence consistent with hemicellulose–pectin complexes. Endo-xylanase and EPG treatments each (separately) caused a decrease in the apparent M_r of the pectin, xyloglucan and xylan components. Abdel-Massih *et al.* (2003) extracted nascent pectin from particulate enzyme preparations (from etiolated pea shoots) incubated with UDP-[U-^{14}C]galactose. The nascent [^{14}C]pectin had a strong affinity for paper (a characteristic of xyloglucan; Fry *et al.*, 1992). The paper-binding ability of the [^{14}C]pectin was greatly reduced by treatment with endo-1,4-β-glucanase, an observation which is consistent with the presence of xyloglucan in the radioactive paper-binding complex. Further support for a linkage between xyloglucan and RG-I has recently been presented by Vidal *et al.* (2003) who, similarly to Thompson and Fry (2000), observed strong binding of xyloglucans to an anion-exchange resin and subsequent co-elution with highly acidic RGs under high salt conditions.

In the present paper we report evidence for the presence of alkali-stable xyloglucan–pectin linkages in non-lignified suspension-cultured cells of all angiosperm cultures tested, both dicots and monocots

MATERIALS AND METHODS

Cell cultures

Cell-suspension cultures of rose (*Rosa* sp., 'Paul's Scarlet'), maize (*Zea mays* L., 'Black Mexican Sweetcorn'), barley (*Hordeum vulgare* L.), tomato (*Lycopersicon esculentum* Mill.) and sycamore (*Acer pseudoplatanus* L.) were grown under constant dim illumination (10 μmol m^{-2} s^{-1}) on an orbital shaker at 25 °C. *Arabidopsis thaliana* (L.) Heynh. 'Erecta' and spinach (*Spinacia oleracea* L. 'Monstrous Viroflay') cells were grown under constant moderate illumination (25 μmol m^{-2} s^{-1}) on an orbital shaker at 135 r.p.m.

Rose cells were sub-cultured every two weeks by 10-fold dilution into fresh medium as described by Fry and Street (1980). Barley and *Arabidopsis* cells were sub-cultured weekly by 10-fold dilution into fresh medium. *Arabidopsis* medium was adapted from May and Leaver (1993) with the modification that the sole carbon source used was 2 % (w/v) glycerol instead of 3 % (w/v) glucose (Glc). The medium used for barley and maize was as described by Kerr and Fry (2003). *Acer* was maintained as described by Talmadge *et al.* (1973) and the spinach culture was maintained in Murashige and Skoog medium (Sigma, M5524) as described by Fry (1982). The tomato culture was maintained as described by Aldington and Fry (1994). Rose, tomato and sycamore were maintained as 55-mL cultures, and *Arabidopsis*, spinach, maize and barley as 220-mL cultures.

Radiolabelling of hemicelluloses in cell cultures

Radioactive arabinose (L-[1-^{3}H]Ara; 148 MBq μmol^{-1}; synthesized by the method of Evans *et al.*, 1974; filter sterilized) was added at 3·7 MBq per 55 mL cell suspension culture 7 d after sub-culturing, when the cultures were in the logarithmic growth stage (Thompson and Fry, 1997). L-[1-^{3}H]Ara labels Ara and Xyl residues within PCW polysaccharides and therefore labels the Xyl moiety of isoprimeverose units in xyloglucan. The culture was incubated for 8 h then filtered through a polypropylene frit in an empty PolyPrep column (BioRad Inc.) under vacuum, and washed with 4 × 5 mL deionised water. The washed cells were used immediately for hemicellulose extraction.

Radiolabelling of uronic acid residues in cell cultures

Radioactive glucuronic acid (D-[6-^{14}C]GlcA; 2·0 MBq μmol^{-1}, synthesized by the method of Sowden, 1952) was added at 1 MBq per 5 mL of *Arabidopsis* cell-suspension culture. The cells had been transferred 3 d previously from a medium containing 2 % (w/v) Glc as sole carbon source to a fresh medium containing 2 % (w/v) glycerol as sole carbon source; this pre-treatment appeared to promote GlcA uptake. The cells took up approx. 40 % of the ^{14}C over 8 h. Feeding of D-[6-^{14}C]GlcA labels GalA residues (and therefore pectins) and also GlcA residues within PCW polysaccharides, but not the neutral hexose or pentose residues (Feingold and Avigad, 1980; Brown and Fry, 1993). The cells were collected and washed as described above.

Buffers

Unless otherwise stated, all buffer solutions were pyridinium acetate (PyOAc), chosen for its volatility. Concentrations quoted refer to the sum of acetate + acetic acid; for example '11 mM PyOAc buffer, pH 4·7' was 11 mM acetic acid adjusted to pH 4·7 by the addition of pyridine.

Dialysis

Polysaccharide samples were dialysed against running tap water for 2 d, then against 11 mM PyOAc, pH 4·7, containing 0·5 % w/v chlorobutanol.

Polysaccharide analysis

Driselase digestion was performed as described by Fry (2000). Driselase digests xyloglucan to isoprimeverose, glucose, galactose and fucose. Radioactive digestion products were separated by paper chromatography in solvent systems 1, 2 and 3 and assayed for radioactivity.

Paper chromatography and electrophoresis

Whatman No.1 chromatography paper was used for analytical paper chromatography and electrophoresis. Solvent systems used were: (1) ethyl acetate/pyridine/water (9 : 3 : 2, v/v/v) for 20 h (Thompson and Fry, 1997); (2) butan-1-ol/acetic acid/water (12 : 3: 5, v/v/v) for 16 h; and (3) 80 % (w/w) phenol for 48 h. ^{3}H-labelled Driselase-digestion products had non-radioactive internal markers of isoprimeverose, Xyl, xylobiose (Xyl$_2$) and Ara. After separation in solvent system 1, which gives the best separation of isoprimeverose, Xyl, Xyl$_2$, Ara and Driselase-indigestible material (which remained at the origin), internal markers were stained with dilute aniline hydrogen-phthalate, which does not appreciably affect the efficiency of scintillation counting (Kerr and Fry, 2003). The appropriate portion of the chromatogram was then cut and assayed for ^{3}H.

Paper electrophoresis (3 kV for 70 min) was carried out in a white-spirit-cooled apparatus as described by Fry (2000). The electrode-buffer was acetic acid/pyridine/water, (10 : 1 : 189, by volume, pH 3·5) and the paper was wetted with half-strength electrode-buffer. This electrophoretic method resolves GlcA, GalA and aldobiouronic acids (Fry *et al.*, 2001); 4-*O*-methyl-glucuronate (MeGlcA) migrates close to GlcA but well resolved from GalA (Popper and Fry, 2003).

Extraction of ^{3}H-labelled hemicelluloses

^{3}H-labelled cells (7 g fresh weight) were suspended in 70 mL 6 M NaOH containing 1 % (w/v) NaBH$_4$ and left shaking at 37 °C for 24 h. This treatment was shown by Edelmann and Fry (1992) to extract essentially all the xyloglucan from the walls of cell-suspension cultures. The alkali extract was filtered through a polypropylene frit (in an empty 'PolyPrep' column; BioRad), then acidified by the addition of acetic acid (0·5 volumes, added slowly with stirring on ice), dialysed, freeze-dried, and re-dissolved in 60 mL of 11 mM acetate buffer, pH 4·7. This preparation is referred to as ^{3}H-hemicellulose.

Fractionation of neutral and anionic xyloglucan

The ^{3}H-hemicellulose solution was enriched in xyloglucan content by precipitation in 50 % ethanol and the pellet was re-dissolved in 11 mM PyOAc containing 8 M urea (pH 5·3) and fractionated on the basis of charge on Q-Sepharose FastFlow [10 mL bed volume; pre-treated with 2 M sodium acetate (adjusted to pH 7·0 with acetic acid) and equilibrated with 11 mM acetate buffer, pH 5·3, containing 8 M urea]. Neutral polysaccharides were eluted with 8 M urea in 11 mM buffer. Acidic polysaccharides were then eluted with 8 M urea in a step-gradient of buffer (25 mL each of 11, 22, 44, 88, 175, 350, 525, 700, 875, 1050, 1225, 1400 mM acetate, pH 5·3, followed by 2 M sodium acetate, pH 7·0 and finally 1 M NaOH). Each fraction was dialysed, a portion of it was assayed for urea (Coulombe and Favreau, 1963) to confirm that this had been removed by dialysis, and the remainder was Driselase-digested (Thompson and Fry, 1997) to find its ^{3}H-polymer composition.

Extraction of ^{14}C-labelled pectins

^{14}C-labelled cells were washed in 6 × 20 mL 0·5 % chlorobutanol (each wash was for 1 h at room temperature with gentle mixing) before extraction in 0·18 M ammonium oxalate (pH 3·7 at 80 °C, for 2 h). The extract (referred to as ^{14}C-pectin) was neutralized, dialysed against running tap water, dried and re-dissolved in pyridine/acetic acid/water (1 : 1: 98 by volume, approx. pH 4·7) containing 0.5 % chlorobutanol.

Cellulose-binding assay

Solutions (1·25 mL) of the ^{14}C-pectin were incubated for 2 d with gentle mixing in a tube containing a 5 × 25-mm piece of Whatman 3MM paper (=cellulose). Before and 2 d after the addition of the paper, 50 μL of the solution was assayed for radioactivity. A further 400 μL of ^{14}C-polymer solution was dried, hydrolysed (in 2 M TFA, 120 °C, 1 h), and electrophoresed: the radioactive spot co-migrating with external marker GalA was assayed for ^{14}C.

Scintillation counting

Aqueous solutions of ^{3}H-sugars (eluted from the chromatography paper) and of ^{3}H-polysaccharides were mixed with 10 volumes of water-miscible scintillation fluid ('OptiPhase HighSafe 3'; Wallac Oy, Turku, Finland). Strips of chromatography paper were assayed for ^{14}C (without elution) after addition of 2 mL of water-immiscible scintillation fluid ('OptiScint'; Wallac).

RESULTS

Radio-labelling of PCW polysaccharides

To facilitate sensitive detection of xyloglucan, we radiolabelled the wall polysaccharides of cell cultures in the pentose residues by feeding L-[1-^{3}H]Ara *in vivo*. All rapidly growing 7-d cell-suspension cultures tested (spinach, *Arabidopsis*, tomato, rose, sycamore, maize and barley) took up >80 % of the exogenous [^{3}H]Ara within 1 h. This is comparable to the reported behaviour of rose and maize cultures (Edelmann and Fry, 1992; Thompson and Fry, 1997; Kerr and Fry, 2003). The cells incorporated the ^{3}H into both [^{3}H]Ara and [^{3}H]Xyl residues of polymers, indicating that 4-epimerisation readily occurred, as reported before (Feingold and Avigad, 1980). Thus, the Xyl residue of isoprimeverose [Xyl-α-(1→6)-Glc] became radiolabelled. Isoprimeverose is the Driselase digestion-product indicative of the presence of xyloglucan. Other radiolabelled Driselase-digestion products generated included Xyl and xylobiose [Xyl-β-(1→4)-Xyl], both of which are largely derived from xylans; and Ara itself, which is derived from several diverse polysaccharides and glycoproteins. Ara residues are labelled to a higher specific activity than Xyl (Wende and Fry, 1997).

Ethanol precipitation

Wall-bound ^{3}H-hemicelluloses were alkali-extracted from the cultures after 8 h of radio-labelling. To enrich the hemicellulose extract in xyloglucan prior to anion-exchange chromatography, we precipitated the [^{3}H]xyloglucan with 50 % (v/v) ethanol as described by Thompson and Fry (1997). When the ethanol-*soluble* material was Driselase-digested, the major ^{3}H-labelled product (40–80 % of the total ethanol-soluble radioactivity) in *Arabidopsis*, spinach, tomato, sycamore, maize and rose was [^{3}H]Ara (data not shown). The remainder of the ethanol-soluble ^{3}H-labelled material was Driselase-indigestible; [^{3}H]isoprimeverose and [^{3}H]xylobiose were present in only trace quantities (less than 1 % of the total ethanol-soluble radioactivity). This showed that the majority of the [^{3}H]xyloglucan had been precipitated by the 50 % ethanol.

Evidence for anionic ^{3}H-hemicelluloses

The 50 % ethanol-*precipitated* material (xyloglucan-enriched hemicellulose) was subjected to anion-exchange chromatography in the presence of 8 M urea. This led to the elution of only 7–16 % of the ^{3}H in the neutral pool (fractions 1 and 2; eluted with 11 mM PyOAc, which is the same concentration as the loading buffer); in all species, the majority of the ^{3}H was eluted as acidic material [fractions 6–13 (175–1400 mM PyOAc), 14 (2 M sodium acetate, pH 7·0) and 15 (1 M NaOH)] (Fig. 1). Less than 1 % of the total radioactivity was eluted in fractions 3–5.

The anionic ^{3}H-hemicellulose from spinach and tomato cultures was found to be eluted in two peaks (Fig. 1): fraction 11 (1050 mM PyOAc) and fraction 14 (2 M sodium acetate, pH 7·0). However, anionic ^{3}H-hemicellulose extracted from *Arabidopsis* differed in its elution pattern, with a greater proportion being eluted in the more anionic fraction 14. Anionic ^{3}H-hemicellulose from maize was eluted in two major peaks, both of which were less anionic than in spinach, *Arabidopsis* and tomato. That from rose was eluted in one anionic peak. The ^{3}H-elution patterns of xyloglucan-enriched hemicellulose were similar to those observed in a replicate experiment in which the cells were fed one-quarter of the amount of radioactivity (data not shown).

Evidence for anionic ^{3}H-xyloglucans

The 50 %-ethanol-precipitable anionic ^{3}H-polysaccharides shown in Fig. 1 could have included GAXs, RGs and arabinogalactan-proteins (AGPs) as well as xyloglucans. To distinguish specific hemicelluloses, we applied Driselase digestion.

A proportion of each appreciably radiolabelled fraction, from 1 (neutral) to 15 (increasingly anionic), was Driselase-digested. Digestion products were separated by paper chromatography in solvent systems 1, 2 and 3. Exact co-chromatography of a peak of ^{3}H with a non-radioactive, stainable internal marker of isoprimeverose (in each of the three solvent systems) confirmed that all cultures tested contained anionic [^{3}H]xyloglucan. This is illustrated for *Arabidopsis* (Fig. 2); the other five species tested gave similar results (data not shown).

The data in Fig. 2 and many related experiments (not shown) were quantified so that the elution profile of [^{3}H]xyloglucan could be determined (Fig. 3). Between 30 % (in *Arabidopsis*) and 55 % (in rose and spinach) of the [^{3}H]isoprimeverose-yielding polymer (=[^{3}H]xyloglucan) was eluted in the neutral fraction (Fig. 3). In a subsequent experiment, a value of 70 % was obtained for barley (data not shown).

The remaining 30–70 % of the [^{3}H]xyloglucan was eluted in anionic fractions. The distribution of [^{3}H]xyloglucan between the various anionic fractions differed between species, *Arabidopsis* [^{3}H]xyloglucan being particularly highly anionic.

Elution of pectins and xylans

The following observations permitted conclusions to be drawn about the acidic polysaccharides that were associated with the [^{3}H]xyloglucans in the anionic fractions.

Driselase-generated [^{3}H]Xyl and [^{3}H]Xyl$_2$, which are indicative of [^{3}H]xylans, indicated that the xylans were eluted mainly in the moderately anionic fractions (Fig. 3). Elution of [^{3}H]Xyl- and [^{3}H]Xyl$_2$-yielding polymers approximately mirrored each other, as expected if they arise from the same polysaccharide, but their elution did not closely mirror that of [^{3}H]isoprimeverose, supporting the conclusion that xylans and xyloglucans were not tightly associated with each other.

[^{3}H]Ara is generated by Driselase from both RG-I and arabinoxylans, and was thus expected to be eluted mainly in the anionic fractions, as observed (Fig. 3). In tomato, approx. 10 % of the [^{3}H]Ara-yielding polymer was eluted in the neutral fraction, probably representing the Ara-rich xyloglucans that are characteristic of the Solanaceae (Eda and Kato, 1978; Akiyama and Kato, 1982; York *et al.*, 1996). In most species the highest amount of [^{3}H]Ara-yielding polymer was eluted with 525–1225 mM PyOAc in fractions 8–12 (Fig. 2). However, in spinach and *Arabidopsis* [^{3}H]Ara-yielding polymers were more abundant in later fractions, suggesting that they were more acidic. Staining of paper chromatograms with aniline hydrogen-phthalate showed that Driselase had also released non-radioactive GalA (indicating the presence of pectins) from the polymers in fractions 11–14.

Driselase-resistant ^{3}H-polymers (Fig. 3) cannot be classified precisely. On acid hydrolysis they yield mainly [^{3}H]Ara, and they probably include AGPs (which largely resist Driselase). Between 5 and 18 % of the Driselase-indigestible material was eluted in the neutral fractions. However, the greatest proportion of Driselase-indigestible material was eluted in acidic fractions; in particular, in *Arabidopsis*, 45 % of it was eluted in fraction 14 (1400 mM PyOAc; Fig. 3).

Cellulose-binding of ^{14}C-labelled pectins

^{14}C-Pectins were extracted from *Arabidopsis* cultures after 8 h in the presence of D-[6-^{14}C]GlcA, which radiolabels only the uronate residues (Brown and Fry, 1993). Hot ammonium oxalate was used to extract, relatively

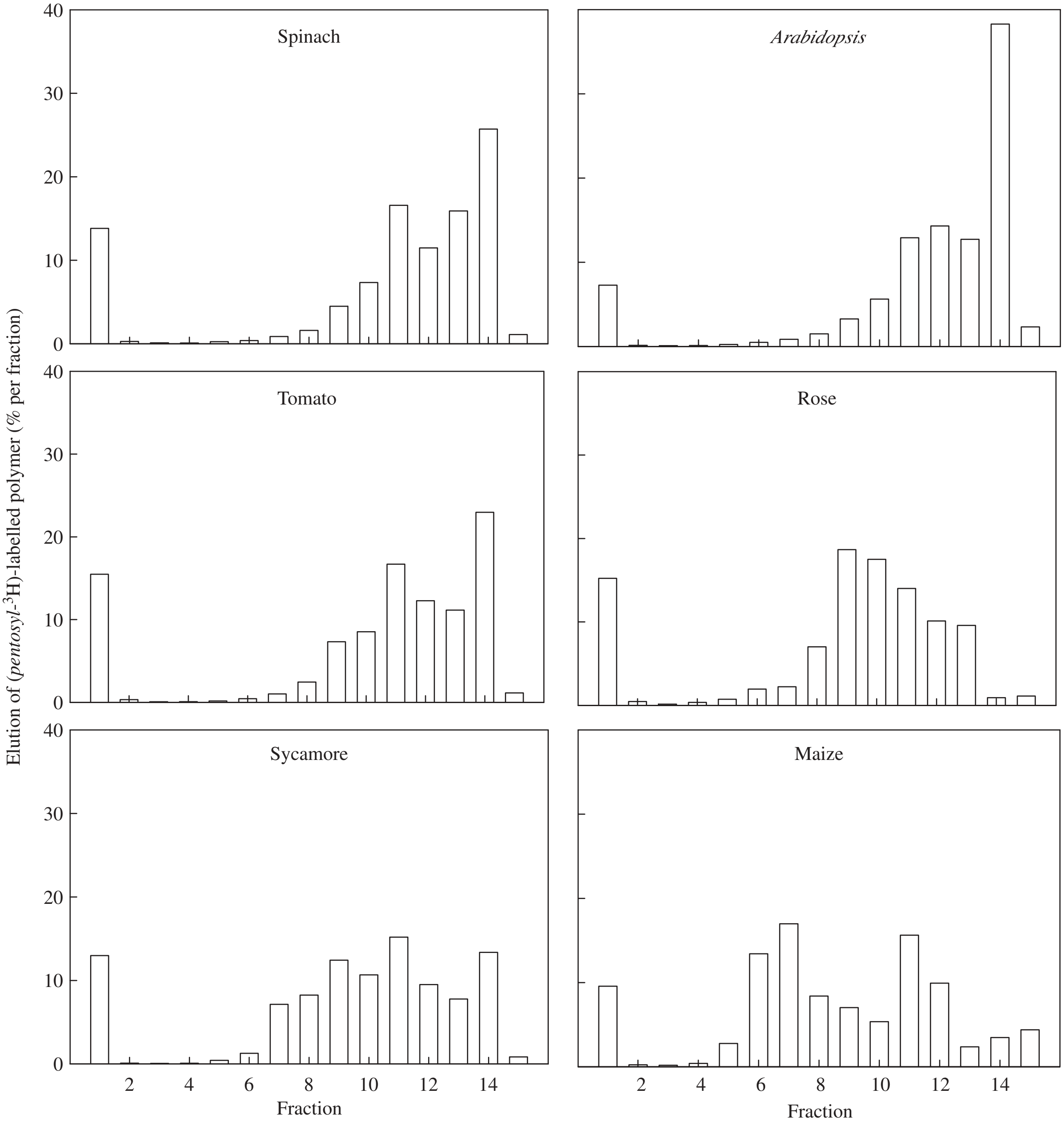

FIG. 1. Behaviour of (*pentosyl*-^{3}H)-labelled, xyloglucan-enriched hemicellulose, from various cell-suspension cultures, during anion-exchange chromatography. ^{3}H-Hemicellulose was extracted with 6 M NaOH, neutralized, and precipitated with 50 % ethanol; the insoluble material was subjected to anion-exchange chromatography on Q-Sepharose FastFlow with a gradient of acetate buffer.

specifically, the pectins. Xyloglucans and other hemicelluloses are not efficiently extracted until 6 M NaOH at 37 °C is used (Edelmann and Fry, 1992). The extracted (*uronate*-^{14}C)-labelled pectins were assayed for their ability to bind to cellulose (paper) from aqueous solution, a characteristic of hemicelluloses. No drying step was included, as drying appears to promote non-specific adsorption of many polysaccharides to paper.

A substantial proportion (21 %) of the total ^{14}C in the oxalate extract was able to bind to paper, suggesting that the pectins were attached to another wall component that has strong paper-binding ability. Acid hydrolysis of the ^{14}C-pectins, before and after paper binding, showed that 20 % of the [^{14}C]GalA-yielding polymer (i.e. [^{14}C]pectin) in this extract had bound to paper. Additionally, approx. 10 % of the [^{14}C]aldobiouronic acid-yielding material in the extract had bound to paper.

DISCUSSION

Xyloglucan is a neutral polysaccharide (Fry, 1989*a*; Hayashi, 1989), composed of a β-(1→4)-D-glucan backbone

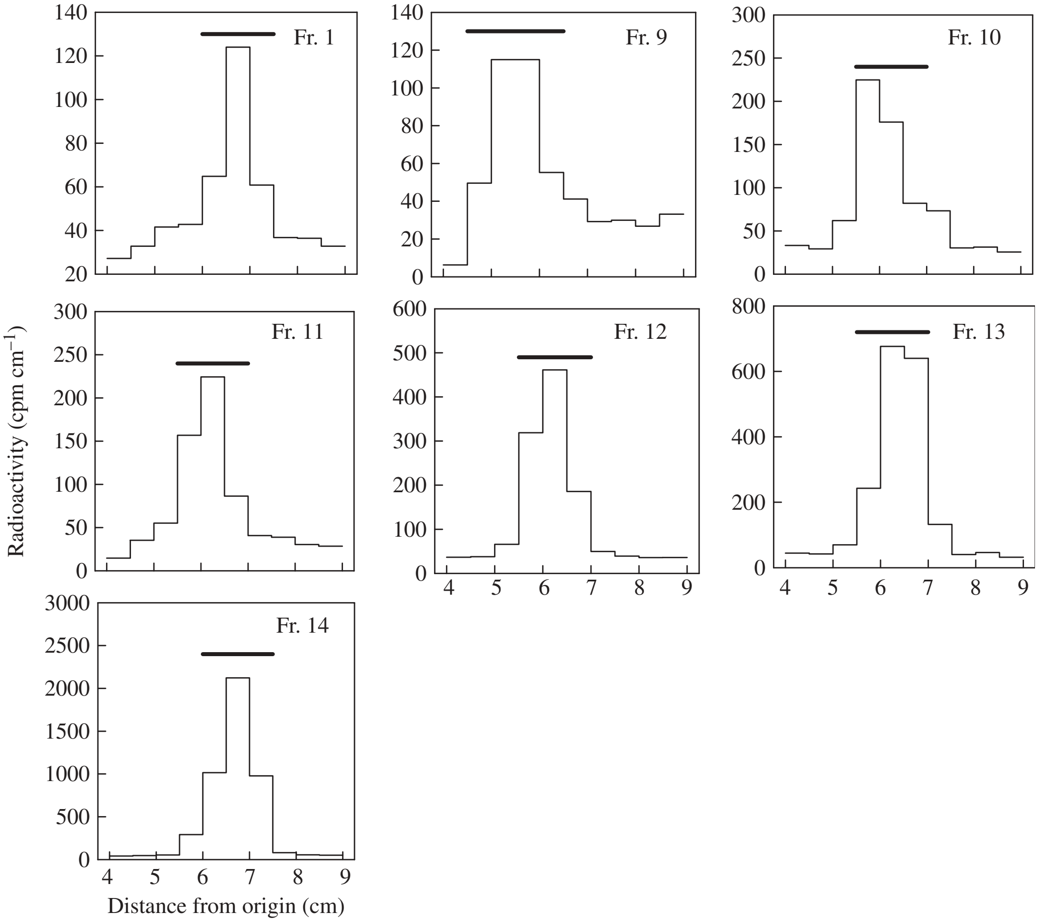

FIG. 2. Paper chromatography of Driselase-digestion products of the Q-Sepharose fractions of the (*pentosyl*-^{3}H)-labelled, xyloglucan-enriched hemicellulose extracted from a 7-d-old *Arabidopsis* culture. The fractions (Fr.) were selected from those shown in Fig. 1 (*Arabidopsis*). The paper chromatogram was developed in solvent system 2 and the non-radioactive internal marker of authentic isoprimeverose (indicated by the dark line) was stained with dilute aniline hydrogen-phthalate. The chromatogram was then cut into strips, which were assayed for radioactivity. The figure shows only the relevant zone (4–9 cm from the origin); the solvent front typically ran approx. 45 cm past the origin in solvent system 2. R_F values of major Driselase digestion-products of this solvent system are: isoprimeverose, 0·195; xylobiose, 0·21; galacturonic acid, 0·23; galactose, 0·27; glucose, 0·285; mannose, 0·325; arabinose, 0·35; xylose, 0·375. Additional chromatograms, in solvent systems 1 and 3, confirmed the identity of isoprimeverose (data not included).

substituted with α-D-Xyl, β-D-Gal, and in some cases α-L-Fuc and/or α-L-Ara. Widely accepted PCW models propose at least two independent polymer networks: a cellulose–xyloglucan network held together by hydrogen-bonds, and a pectic network held together by Ca^{2+} bridges. In onion epidermal cell walls, both the pectic and the cellulose–xyloglucan networks have a preferred orientation (Chen *et al*., 1997), which is suggestive of communication between the two networks. A high-M_r complex of xyloglucan linked to another polysaccharide, in particular another abundant PCW polymer such as pectin, would be expected to play a more effective tethering role in PCW architecture (Fry, 1989*b*; Kerr and Fry, 2003) than lower-M_r free xyloglucans. The widespread taxonomic distribution of the xyloglucan–pectin linkage, reported here, suggests its importance to angiosperm PCW structure and function. Elucidation of the chemical structure of the linkage and its mode of synthesis are likely to promote our understanding of, and in future enable manipulation of, important physical properties of PCWs.

The extracted anionic [^{3}H]xyloglucan is unlikely to be ester-bonded to pectin. Very mild conditions of NaOH hydrolysis (1 M NaOH at 20 °C) break all detectable ester bonds within 24 h, whereas we extracted anionic [^{3}H]xyloglucan with 6 M NaOH at 37 °C. This suggests the existence of a highly alkali-stable bond between xyloglucan and an acidic PCW polymer.

Kerr and Fry (2003) found that in cultured maize cells, the average M_r of pulse-labelled intraprotoplasmic [^{3}H]xyloglucans increased up to 30 min after labelling but prior to secretion. The increase in *average* M_r of the ^{3}H-polymers was too large to be entirely ascribed to on-going (NDP-sugar-dependent) chain elongation within the Golgi cisternae (which could theoretically account for at most a doubling of the average M_r of the [^{3}H]xyloglucans; see fig. 10 of Kerr and Fry, 2003), so it is likely that the increase was due to the post-synthetic bonding of [^{3}H]xyloglucans to each other or to other (non-radioactive) polymers. Their bonding to RGs could account for the widespread formation of 'anionic xyloglucan', reported here.

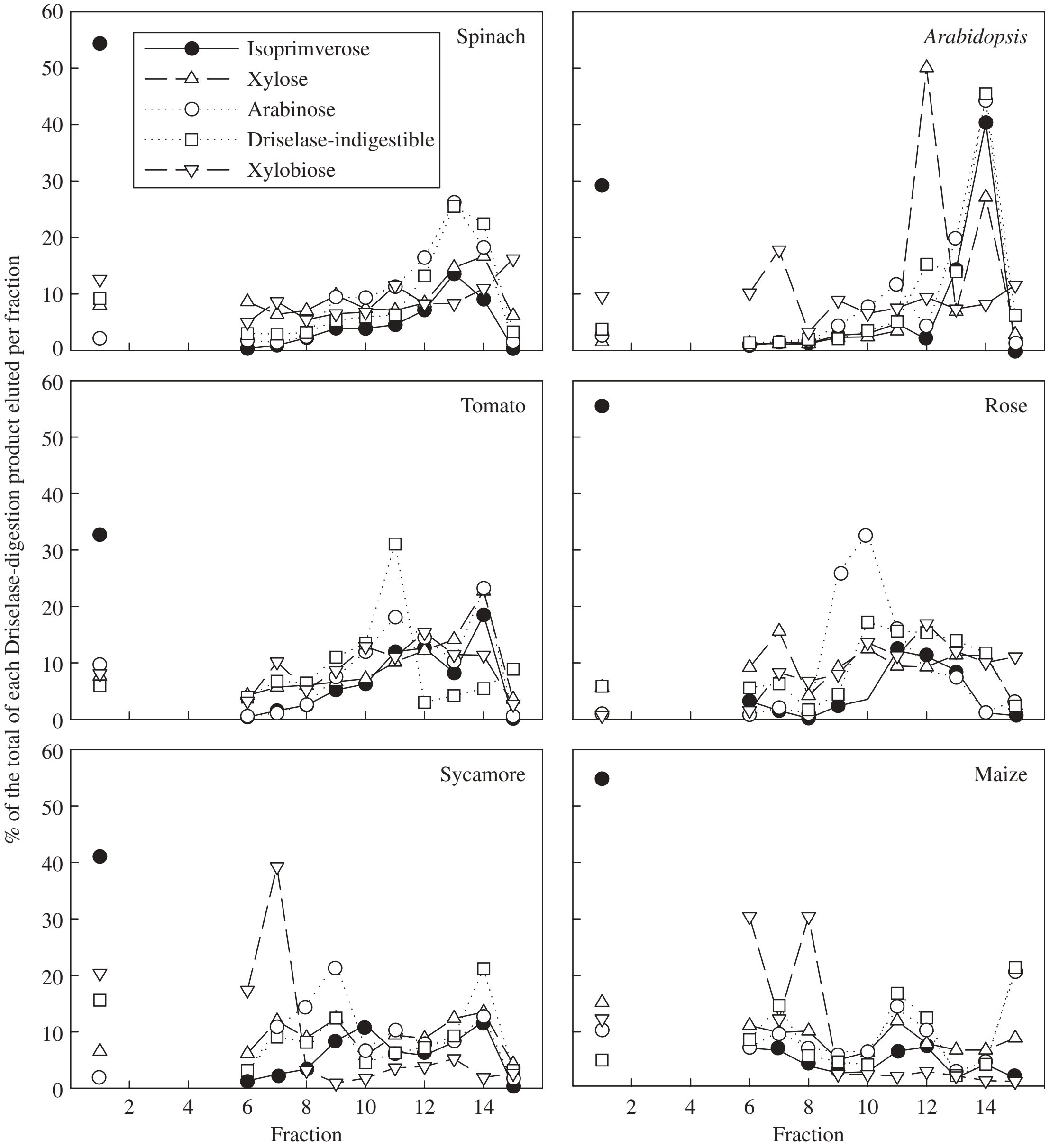

Fig. 3. Anion-exchange chromatography profiles of polymers containing individual (*pentosyl*-^{3}H)-labelled residues. The Q-Sepharose fractions reported in Fig. 1 were Driselase-digested and the radioactive products (isoprimeverose, xylobiose, Xyl, Ara and Driselase-indigestible material) were separated by paper chromatography in solvent system 1 and assayed by scintillation-counting. Fractions 2–5 contained little radioactivity and were therefore not analysed.

It has also been reported that nascent pectin extracted from pea stems is complexed with xyloglucan (Abdel-Massih *et al*., 2003), supporting this conclusion. In agreement with the observations of Abdel-Massih *et al*. (2003), the cellulose- (paper-) binding ability of hot-oxalate-extracted [*GalA*-^{14}C]pectin, noted in the present work on *Arabidopsis* cells, indicates an unexpected ability of some pectins to bind to cellulose. Paper-binding is not a characteristic of pectins (particularly if the sample is not *dried* on to the paper). It is therefore more likely that the [*GalA*-^{14}C]-pectin, solubilized by hot oxalate, was attached to, and able to bind to paper via, a hemicellulose (such as xyloglucan) that does have strong paper-binding ability. We probably underestimated the proportion of pectin that was hemicellulose-linked in this way, since the extractant used (hot oxalate) would not efficiently break the hemicellulose–cellulose hydrogen-bonds that may have been present *in muro*. Some hemicelluloses (especially xylans and glucuronomannans) contain GlcA and/or MeGlcA residues, which would be radioactive in the [^{14}C]GlcA-fed cells; such hemicelluloses would also show up as paper-binding ^{14}C-polymers. However, it is unlikely that these hemicelluloses would have been extracted from the cell walls by hot oxalate. We verified that the ^{14}C-residues present in the paper-binding polymers reported here were indeed GalA (diagnostic of pectins), not GlcA or MeGlcA. Thus, we conclude that some of the pectin in *Arabidopsis* PCWs was strongly (probably covalently) linked to a cellulose-binding hemicellulose (e.g. xyloglucan).

We investigated the existence of a xyloglucan–pectin linkage in a wide variety of angiosperm cell-suspension cultures chosen to represent the diversity within angiosperm PCW structure. The major hemicellulose in Poales (gramineous monocots) is GAX, not xyloglucan, and the PCWs of Poales (represented here by maize and barley) often contain only about 20 % of the xyloglucan present in non-gramineous monocot and dicot PCWs (Fry, 1989*a*; Hayashi, 1989). They also contain high concentrations of feruloylated GAXs (present but not feruloylated in dicots) and have lower galacturonan contents than dicot PCWs (Shibuya *et al*., 1983; Jarvis *et al*., 1988). Additionally, PCWs of plants within the Poales contain mixed-linkage glucan, a hemicellulose absent from all other angiosperm PCWs (Nevins *et al*., 1978; Smith and Harris, 1999; Popper and Fry, 2003). PCWs of the Centrospermae (represented by spinach) are rich in ferulic acid, which in these dicots forms esters with Ara and Gal residues of pectic polysaccharides (Fry, 1982; Micard *et al*., 1997). It is therefore differently linked from the ferulic acid found in gramineous monocots. Xyloglucans so far extracted from different sources show some differences. The majority of xyloglucans extracted from both gymnosperms and angiosperms are reported to have a backbone consisting of repeat units of XXXG, whereas xyloglucans extracted from solanaceous plants (represented by tomato) have two, instead of three, consecutive branched Glc residues, which alternate with two unsubstituted Glc residues (XXGG; Vincken *et al*., 1997). [For an explanation of the abbreviated nomenclature of xyloglucan oligosaccharides (XXXG etc.), see Fry *et al.* (1993).] Additionally, xyloglucans isolated from the Solanaceae differ from those of other dicots in the branching of their xylosyl substituents; XXXG and XXFG are absent (Vincken *et al*., 1997) and Xyl residues are 2-*O*-substituted predominantly with α-L-Ara and β-D-Gal (York *et al*., 1996). Xyloglucan extracted from Poales also differs in composition, containing less Xyl, Gal and Fuc than typical dicot xyloglucan (Hayashi, 1989; McDougall and Fry, 1994; Vincken *et al*., 1997).

It is interesting that despite the wide variability in xyloglucan structure within angiosperms we found that in all angiosperms tested a substantial proportion of the xyloglucan was attached to an acidic polymer. It is possible that the structure of the acidic polymer is highly variable. RG-I is known to vary in methylation and/or acetylation of the GalA residues in its backbone (Perrone *et al*., 2002) as well as in the degree of substitution with arabinan and galactan side-chains (McNeil *et al*., 1982; Lerouge *et al*., 1993). It seems remarkable that a linkage between xyloglucans and an acidic polysaccharide, probably RG-I, appears to be conserved among angiosperms. This suggests that the linkage is important to PCW integrity and function.

Xyloglucan is likely to have a major functional role in gramineous monocot PCWs since the xyloglucan-modifying enzyme XTH extracted from grass tissues has a high XET specific activity (Fry *et al*., 1992). This is especially significant since gramineous monocot PCWs are widely reported to contain very much less xyloglucan than do dicot PCWs (Bacic *et al*., 1988; Smith and Harris, 1995). However, maize xyloglucan has a much higher molecular weight than that of dicot xyloglucan (Kerr and Fry, 2003). This may partially account for the survival of gramineous monocots despite their low xyloglucan content. Anionic xyloglucan was readily detected in suspension-cultured cells of gramineous monocots (maize and barley) as well as in those of dicots.

In conclusion, we obtained evidence for anionic xyloglucan in all angiosperm PCWs investigated despite wide variation in their xyloglucan structures as well as differing overall PCW compositions. This indicates that the xyloglucan–pectin linkage is evolutionarily conserved and may be required for effective PCW structure and function.

ACKNOWLEDGEMENTS

We thank Professor Peter Dominy (University of Glasgow) and Dr Antonio Encina (University of León) for the kind provision of *Arabidopsis* and barley cell suspension cultures, respectively. We thank the UK BBSRC for funding this project.

LITERATURE CITED

Abdel-Massih RM, Baydoun EA-H, Brett CT. 2003. *In-vitro* biosynthesis of 1,4-β-galactan attached to a pectin–xyloglucan complex in pea. *Planta* **216**: 503–511.

Akiyama Y, Kato K. 1982. An arabinoxyloglucan from extracellular polysaccharides of suspension-cultured tobacco cells. *Phytochemistry* **21**: 2112–2114.

Aldington S, Fry SC. 1994. Rhamnogalacturonan II—a biologically active fragment. *Journal of Experimental Botany* **45**: 287–293.

Bacic A, Harris PJ, Stone BA. 1988. Structure and function of plant cell walls. In: Preiss J, ed. *The biochemistry of plants. Vol. 14. Carbohydrates*. San Diego: Academic Press, 297–371.

Bauer WD, Talmadge K, Keegstra K, Albersheim P. 1973. The structure of plant cell walls. II. The hemicellulose of the walls of suspension-cultured sycamore cells. *Plant Physiology* **51**: 174–187.

Brown JA, Fry SC. 1993. Novel *O*-D-galacturonoyl esters in the pectic polysaccharides of suspension-cultured plant cells. *Plant Physiology* **103**: 993–999.

Chen L, Reginald HW, McCann MC. 1997. Investigation of macromolecular orientation in dry and hydrated walls of single onion epidermal cells by FTIR microspectroscopy. *Journal of Molecular Structure* **408**: 257–260.

Coulombe JJ, Favreau L. 1963. A new simple semimicro method for colorimetric determination of urea. *Clinical Chemistry* **9**: 102–108.

Eda S, Kato K. 1978. An arabinoxyloglucan isolated from the midrib of the leaves of *Nicotiana tabacum*. *Agricultural and Biological Chemistry* **42**: 351–357.

Edelmann HG, Fry SC. 1992. Factors that affect the extraction of xyloglucan from the primary cell walls of suspension-cultured rose cells. *Carbohydrate Research* **228**: 423–431.

Evans EA, Sheppard HC, Turner JC, Warrell DC. 1974. A new approach to specific labelling of organic compounds with tritium: catalysed exchange in solution with tritium gas. *Journal of Labelled Compounds* **10**: 569–587.

Feingold DS, Avigad G. 1980. Sugar nucleotide transformations in plants. In: Preiss J, ed. *The Biochemistry of Plants*. New York: Academic Press, 101–170.

Femenia A, Rigby NM, Selvendran RR, Waldron KW. 1999. Investigation of the occurrence of pectic-xylan–xyloglucan complexes in cell walls of cauliflower stem tissues. *Carbohydrate Research* **39**: 151–164.

Fry SC. 1982. Phenolic components of the primary cell wall: feruloylated disaccharide of D-galactose and L-arabinose from spinach polysaccharide. *Biochemical Journal* **203**: 493–504.

Fry SC. 1989*a*. The structure and functions of xyloglucan. *Journal of Experimental Botany* **40**: 1–11.

Fry SC. 1989*b*. Cellulases, hemicelluloses and auxin-stimulated growth: a possible relationship. *Plant Physiology* **75**: 532–536.

Fry SC. 2000. *The growing plant cell wall: chemical and metabolic analysis*. (Reprint edn). Caldwell, NJ: The Blackburn Press.

Fry SC, Street HE. 1980. Gibberellin-sensitive suspension cultures. *Plant Physiology* **65**: 472–477.

Fry SC, Dumville JC, Miller JG. 2001. Fingerprinting of polysaccharides attacked by hydroxyl radicals *in vitro* and in the cell walls of ripening pear fruit. *Biochemical Journal* **357**: 729–735.

Fry SC, Smith RC, Renwick KF, Martin DJ, Hodge SK, Matthews KJ. 1992. Xyloglucan endotransglycosylase, a new wall-loosening enzyme activity from plants. *Biochemical Journal* **282**: 821–828.

Fry SC, York WS, Albersheim P, Darvill A, Hayashi T, Joseleau J-P, *et al.* 1993. An unambiguous nomenclature for xyloglucan-derived oligosaccharides. *Physiologia Plantarum* **89**: 1–3.

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

Jarvis MC, Forsyth W, Duncan HJ. 1988. A survey of the pectic content of non-lignified monocot cell walls. *Plant Physiology* **88**: 309–314.

Keegstra K, Talmadge KW, Bauer WD, Albersheim P. 1973. The structure of plant cell walls. III. A model of the walls of suspension-cultured sycamore cells based on the interconnections of the macromolecular components. *Plant Physiology* **51**: 188–196.

Kerr EM, Fry SC. 2003. Pre-formed xyloglucans and xylans increase in molecular weight in three distinct compartments of a maize cell-suspension culture. *Planta* **217**: 327–339.

Lerouge P, O'Neill MA, Darvill AG, Albersheim P. 1993. Structural characterization of endo-glycanase-generated oligo glycosyl side-chains if rhamnogalacturonan-I. *Carbohydrate Research* **243**: 359–371.

May MJ, Leaver CJ. 1993. Oxidative stimulation of glutathione synthesis in *Arabidopsis thaliana* suspension cultures. *Plant Physiology* **103**: 621–627.

McCann MC, Roberts K. 1991. Architecture of the primary cell wall. In: Lloyd CW, ed. *The cytoskeletal basis of plant growth and form*. London: Academic Press, 109–128.

McDougall GJ, Fry SC. 1994. Fucosylated xyloglucan in the suspension-cultured cells of the gramineous monocotyledon, *Festuca arundinacea*. *Journal of Plant Physiology* **143**: 591–595.

McNeil M, Darvill AG, Albersheim P. 1982. Structure of plant cell walls. 12. Identification of seven differently linked glycosyl residues attached to the *O*-4 of the 2,4-linked L-rhamnosyl residues of rhamnogalacturonan-I. *Plant Physiology* **70**: 1586–1591.

Micard V, Grabber JH, Ralph J, Renard CMGC, Thibault J-F. 1997. Dehydrodiferulic acids from sugar-beet pulp. *Phytochemistry* **44**: 1365–1368.

Nevins DJ, Yamamoto R, Huber DJ. 1978. Cell wall β-D-glucans of five grass species. *Phytochemistry* **17**: 1503–1505.

Nishitani K, Tominaga R. 1992. Endo-xyloglucan transferase, a novel class of glycosyl; transferase that catalyzes transfer of a segment of xyloglucan molecule to another xyloglucan molecule. *Journal of Biological Chemistry* **267**: 21058–21064.

O'Neill MA, Albersheim P, Darvill A. 1990. The pectic polysaccharides of primary cell walls. In: Dey DM, ed. *Methods in plant biochemistry. Volume 2*. London: Academic Press, 415–441.

Perrone P, Hewage CM, Thomson AR, Bailey K, Sadler IH, Fry SC. 2002. Patterns of methyl and *O*-acetyl esterification in spinach pectins: new complexity. *Phytochemistry* **60**: 67–77.

Popper ZA, Fry SC. 2003. Primary cell wall composition of bryophytes and charophytes. *Annals of Botany* **91**: 1–12.

Prade RA, Zhan D, Ayoubi P, Mort AJ. 1999. Pectins, pectinases and plant–microbe interactions. In: Harding SE, ed. *Biotechnology and genetic engineering reviews*. Andover: Intercept, 361–391.

Ridley BL, O'Neill MA, Mohnen D. 2001. Pectins: structure, biosynthesis, and oligogalacturonide-related signalling. *Phytochemistry* **57**: 929–967.

Shibuya N, Misaki A, Iwasaki T. 1983. The structure of arabinoxylan and arabinoglucuronoxylan isolated from rice endosperm cell walls. *Agricultural and Biological Chemistry* **47**: 2223–2230.

Smith BG, Harris PJ. 1999. The polysaccharide composition of Poales cell wall: Poaceae cell walls are not unique. *Biochemical Systematics and Ecology* **27**: 33–53.

Smith BG, Harris PJ. 1995. Polysaccharide composition of unlignified cell walls of pineapple [*Ananas comosus* (L.) Merr.] fruit. *Plant Physiology*. **107**: 1399–1409.

Sowden JC. 1952. 6-C^{14}-D-Glucose and 6-C^{14}-D-glucuronolactone. *Journal of the American Chemical Society* **74**: 4377–4379.

Spellman MW, McNeill M, Darvill AG, Albersheim P, Dell A. 1983. Characterization of a structurally complex heptasaccharide isolated from the pectic polysaccharide rhamnogalacturonan II. *Carbohydrate Research* **122**: 131–153.

Talmadge K, Keegstra K, Bauer WD, Albersheim P. 1973. The structure of plant cell walls. I. The macromolecular components of the walls of suspension-cultured sycamore cells with a detailed analysis of the pectic polysaccharides. *Plant Physiology* **51**: 158–173.

Thompson JE, Fry SC. 1997. Trimming and solubilisation of xyloglucan after deposition in the walls of cultured rose cells. *Journal of Experimental Botany* **48**: 297–305.

Thompson JE, Fry SC. 2000. Evidence for covalent linkage between xyloglucan and acidic pectins in suspension-cultured rose cells. *Planta* **211**: 275–286.

Thompson JE, Fry SC. 2001. Restructuring of wall-bound xyloglucan by transglycosylation in living plant cells. *Plant Journal* **26**: 23–34.

Thompson JE, Smith RC, Fry SC. 1997. Xyloglucan undergoes interpolymeric transglycosylation during binding to the plant cell wall *in vivo*: evidence from ^{13}C/^{3}H dual labelling and isopycnic centrifugation in caesium trifluoroacetate. *Biochemical Journal* **327**: 699–708.

Vidal S, Williams P, Doco T, Moutounet M, Pellerin P. 2003. The polysaccharides of red wine: total fractionation and characterization. *Carbohydrate Polymers* **54**: 439–447.

Vincken JP, York WS, Beldman G, Voragen AGJ. 1997. Two general branching patterns of xyloglucan, XXXG and XXGG. *Plant Physiology* **114**: 9–13.

Wende G, Fry SC. 1997. 2-*O*-β-D-Xylopyranosyl-(5-*O*-feruloyl)-L-arabinose, a widespread component of grass cell walls. *Phytochemistry* **44**: 1019–1030.

York WS, Kolli VSK, Orlando R, Albersheim P, Darvill AG. 1996. The structures of arabinoxyloglucans produced by solanaceous plants. *Carbohydrate Research* **285**: 99–128.