Fractionation of carbohydrates in *Arabidopsis* root cell walls shows that three radial swelling loci are specifically involved in cellulose production

Liangcai Peng[1,2], Charles H. Hocart[1], John W. Redmond[2], Richard E. Williamson[1]

[1]Plant Cell Biology Group, Research School of Biological Sciences, Institute of Advanced Studies, Australian National University, PO Box 475, Canberra, ACT 2601, Australia
[2]Plant Microbe Interaction Group, Research School of Biological Sciences, Institute of Advanced Studies, Australian National University, PO Box 475, Canberra, ACT 2601, Australia

Abstract. Three non-allelic radial swelling mutants (*rsw1*, *rsw2* and *rsw3*) of *Arabidopsis thaliana* L. Heynh. were shown to be specifically impaired in cellulose production. Fractionation methods that identify, characterise and quantify some of the major cell wall polysaccharides in small quantities of seedlings demonstrated that changes in the production of cellulose are much more pronounced than changes in the production of non-cellulosic polysaccharides. A crude cell wall pellet was sequentially extracted with chloroform methanol (to recover lipids), dimethyl sulphoxide (starch), ammonium oxalate (pectins) and alkali (hemicelluloses). Crystalline cellulose remained insoluble through subsequent treatments with an acetic/nitric acid mixture and with trifluoroacetic acid. Cetyltrimethylammonium bromide precipitation resolved neutral and acidic polymers in the fractions, and precipitation behaviour, monosaccharide composition and glycosidic linkage patterns identified the major polysaccharides. The deduced composition of the walls of wild-type seedlings and the structure and solubility properties of the major polymers were broadly typical of other dicots. The three temperature-sensitive, radial swelling mutants produced less cellulose in their roots than the wild type when grown at their restrictive temperature (31 °C). There were no significant differences at 21 °C where no radial swelling occurs. The limited changes seen in the monosaccharide compositions, glycosidic linkage patterns and quantities of non-cellulosic polysaccharides support the view that the *RSW1*, *RSW2* and *RSW3* genes are specifically involved in cellulose synthesis. Reduced deposition of cellulose was accompanied by increased accumulation of starch.

Key words: *Arabidopsis* (cellulose, mutants) – Carbohydrate fractionation – Cellulose synthesis – Cell walls – Mutant (*Arabidopsis*)

Introduction

Research on cell wall polysaccharides seeks to understand several issues including the structure of the polysaccharides, their synthesis and any subsequent modification, their assembly into the wall and their contribution to wall properties. The structures of many polysaccharides and the broad structure of the plant cell wall are relatively well documented (Carpita and Gibeaut 1993) but progress in the other areas remains limited. In particular, enzymology has had few successes in purifying synthases or in providing a detailed picture of biosynthetic mechanisms.

Mutational analysis has the proven ability to make well-defined changes in carbohydrates in microbial cell walls. The mutants illuminate wall structure-function relationships and identify genes involved in carbohydrate synthesis and modification (Cannon and Anderson 1991; Cid et al. 1995; Lussier et al. 1997). Some of the powerful methods available in *Arabidopsis* genetics have recently been used to study plant cell walls. Mutants with cell walls altered by single gene changes illuminate many structural and functional effects of changing a single polymer (Baskin et al. 1992; Reiter et al. 1993; Potikha and Delmer 1995; Reiter et al. 1997; Turner and Somerville 1997; Arioli et al. 1998a; Nicol et al. 1998; Taylor et al. 1999). The genes involved encode GDP-D-mannose-4,6-dehydratase (Bonin et al. 1997), a putative membrane-bound endo-1,4-β-glucanase (Nicol et al. 1998) and cellulose synthase catalytic subunits (Arioli et al. 1998a; Taylor et al. 1999). The number of candidate genes is rapidly increasing as bioinformatic tools to recognise genes encoding glycosyl transferases and other enzymes improve (Saxena et al. 1995; Campbell et al. 1997) and large-scale genome and cDNA sequencing delivers more sequence data for analysis (Cutler and Somerville 1997; Saxena and Brown 1997; Arioli et al. 1998b).

Mutants have been selected for resistance to herbicides that inhibit cellulose synthesis (Heim et al. 1989; Heim et al. 1990), for changes in non-cellulosic polysaccharide composition (Reiter et al. 1993; Reiter et al.

1997) and for morphological or microscopic abnormalities likely to indicate cell wall changes that can be confirmed by subsequent chemical analyses. Reduced cellulose causes radial swelling (Baskin et al. 1992; Arioli et al. 1998a), reduced birefringent retardation (Potikha and Delmer 1995) and xylem collapse (Turner and Somerville 1997; Taylor et al. 1999) in *Arabidopsis* and brittle culms in barley (Kokubo et al. 1989). Powerful genetics must, however, be combined with analytical methods to identify changed cell wall structure and composition. To date, however, only Zablackis et al. (1995) have thoroughly analysed *Arabidopsis* leaf cell walls and analyses of any mutant are less detailed. Such details are likely to prove important, however, in assigning clear functions to the many genes likely to be involved in cell wall production.

In this paper we (i) extend and validate published methods (Heim et al. 1991) to separate, characterise and quantify the major cell wall carbohydrates of *Arabidopsis* seedling roots and (ii) use those methods to examine the polysaccharides in roots of three temperature-sensitive radial swelling mutants. Reduced cellulose production in shoots of the *rsw1* mutant has been briefly reported (Arioli et al. 1998a) and we will report elsewhere further details of the readily extracted β-1,4-glucan that accumulates in shoots. In this study, we focus on roots (which do not accumulate the glucan) and extend that analysis to non-cellulosic polysaccharides and to the polysaccharides of two other, non-allelic mutants (*rsw2* and *rsw3*) that show a similar temperature-sensitive, radial swelling phenotype (Baskin et al. 1992). Cellulose production is more severely impaired in the roots of all three mutants than is the production of non-cellulosic polysaccharides, leading to the conclusion that all three genes are specifically involved in cellulose synthesis. That finding acquires additional significance with the recognition that RSW1 (Arioli et al. 1998a) and RSW2 (D.R. Lane, J.E. Burn and R.E. Williamson; personal communication) encode unrelated proteins, as will *RSW3* given the absence of genes encoding proteins related to either RSW1 or RSW2 in its chromosomal region (J.E. Burn, R.E. Williamson; personal communication).

Materials and methods

Plant material. Seedlings of wild-type Columbia and of backcrossed *rsw1*, *rsw2*, *rsw3* (Baskin et al. 1992) mutants of *Arabidopsis thaliana* (L.) Heynh. were grown under continuous light (90 μmol m^{-2} s^{-1}) for either 7 d at 21 °C or for 2 d at 21 °C and 5 d at 31 °C on vertical agar plates. The medium contained 1% glucose (Baskin et al. 1992). Methylation analysis was carried out on fractions prepared from about 5000 seedlings that were not separated into roots and shoots. All other experiments were conducted on three replicate batches of 100–150 roots.

Fractionation. The fractionation scheme (Fig. 1) was developed from that used by Heim et al. (1991) with *Arabidopsis* which is similar to that Carpita (1984) used with *Zea*. Separated roots were freeze-dried to constant weight and ground in a mortar and pestle with 3 ml of cold potassium phosphate buffer (0.5 M, pH 7.0). The combined homogenate after two buffer rinses (2 ml each) was
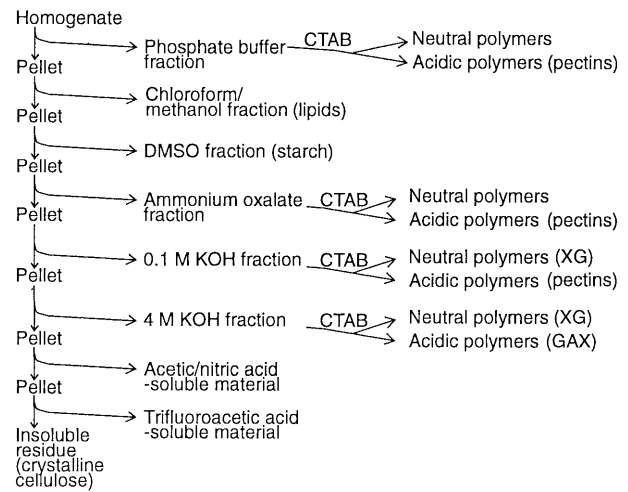

Fig. 1. Flow chart for the fractionation scheme showing the major polymers recovered in each fraction or sub-fraction. *GAX*, glucuronoarabinoxylan; *XG*, xyloglucan

centrifuged at 2100 *g* for 10 min. The pellet after washing (2 × 2 ml buffer and 2 × 2 ml distilled water) formed the crude cell wall fraction and the combined supernatants formed the phosphate buffer fraction. Walls were stirred with chloroform/methanol (1:1, v/v; 2 × 3 ml, 40 °C, 1 h), methanol (2 ml, 40 °C, 30 min), acetone (2 ml, 30 min) and water (2 × 3 ml) with the whole procedure repeated in the case of whole seedlings. Combined supernatants were dried in a nitrogen stream. The pellet was successively extracted: (i) with dimethyl sulfoxide (DMSO)/water (9:1, v/v, overnight under nitrogen), washed with DMSO/water (2 × 3 ml) and with water (3 × 3 ml); (ii) with ammonium oxalate (0.5%, 3 ml, 100 °C, 1 h) and washed with water (2 × 3 ml); (iii) with 0.1 M KOH containing 1 mg ml^{-1} sodium borohydride (3 ml, 25 °C, 1 h) repeated once for roots and twice for whole seedlings with a final H$_2$O wash (2 ml); (iv) with 4 M KOH containing 1 mg ml^{-1} sodium borohydride (3 ml, 25 °C, 1 h) repeated once for roots, twice for shoots. The pellet was boiled with intermittent stirring in acetic acid-nitric acid-water (8:1:2, by vol.; 3 ml, 1 h) (Updegraff 1969), washed twice with water and the combined supernatants diluted with 5 ml water. The remaining pellet was digested for 1 h with 2 M trifluoroacetic acid (TFA) in a sealed tube heated to 120 °C in an autoclave. The final pellet was pure cellulose giving only glucose on hydrolysis. Combined supernatants from each stage were dialysed and lyophilised after neutralisation with acetic acid or KOH where appropriate. Anionic polymers (pectins and glucuronoarabinoxylans) were precipitated overnight at 37 °C from fractions containing 1–2% carbohydrates after mixing with an equal volume of 1–2% cetyltrimethylammonium bromide (CTAB) in 30 mM Na$_2$SO$_4$. The pellet from centrifugation for 10 min at 2100*g* was redissolved in 3 ml of 5 M Na$_2$SO$_4$, freeze-dried, mixed with 80% ethanol and the acidic polymers collected by centrifugation at 2100*g* for 10 min. Ethanol precipitation was repeated to remove more CTAB before dialysis and storage at 4 °C. Neutral polymers in the original CTAB supernatant were lyophilised and resuspended in 3 ml of 80 % ethanol. The resulting pellet was resuspended in 2 ml distilled water and dialysed.

Acid hydrolysis. Polysaccharides (2–20 μg) dissolved in 100 μl of 2 M TFA were heated in a sealed tube for 1 h at 120 °C in an autoclave. *Myo*-inositol (10 μg) was added as the internal standard for GC-MS analysis. The supernatant (2100*g* for 5 min) was dried under vacuum at 45 °C to remove TFA. Cellulose (10–100 μg of dried sample) was dissolved in 100 μl of 72% (w/w) H$_2$SO$_4$ at room temperature for ≥1 h, mixed with 2 ml of water and hydrolysed at 120 °C as described for TFA. The sample was neutralised with

Ba(OH)$_2$ and BaCO$_3$ and the supernatant (10 min, 2100g) was analysed. Carbohydrates were quantified in terms of nanomoles total monosaccharides determined by colorimetric assay in the case of uronic acids or by GC-MS in other cases. Colorimetric assays were made separately on extracts prepared from three replicate samples of seedlings whereas GC-MS determinations were made on a single sample pooled from those three independent extracts. Total carbohydrates were estimated by colorimetric assays where amounts were small or compositional data was not required.

Derivatisation of neutral monosaccharides to alditol acetates. A freshly prepared solution of NaBH$_4$ (100 µl; 10 mg ml^{-1} in 2 M aqueous NH$_3$) was added to each sample. Samples were capped, mixed well and incubated at 60 °C for 2 h. Excess NaBH$_4$ was decomposed by adding acetone (50 µl). The sample was then dried under a stream of nitrogen at 40 °C. Acetic acid (20 µl), ethyl acetate (100 µl) and acetic anhydride (300 µl) were added to the dry, reduced sample. After mixing well, perchloric acid (70%, 20 µl) was added and the solution mixed again. After 5 min cooling on ice, water (1 ml) was added, followed by 1-methylimidazole (20 µl; Blakeney et al. 1983). After mixing, the sample was allowed to stand for 5 min. Dichloromethane (200 µl) was added, mixed gently, and centrifuged for phase separation. The lower phase was removed with a glass pipette and stored at −20 °C in a screw-capped glass vial until analysed by GC-MS.

Methylation analysis. Polysaccharide samples (2–20 µg) in glass bottles were dried overnight at 60 °C in a vacuum oven and dissolved, under nitrogen, in DMSO (200 µl), with heating at 45 °C for the minimum time necessary. Finely powdered dry sodium hydroxide (10 mg) was added to the solution in DMSO under nitrogen at room temperature. Immediately, the sample mixture was sealed tightly with a cap, mixed well and heated at 30 °C for 1 h, then left for a further 1 h with intermittent vortexing. For DMSO-insoluble polysaccharides such as cellulose and starch, methyl iodide (10 µl) was added prior to heating at 30 °C. After intermittent vortexing for 1 h, methyl iodide (200 µl) was added dropwise, under nitrogen, over 30 s, and then the mixture was capped again, mixed well and heated at 30 °C for 1 h with intermittent vortexing. After methylation, the mixture was extracted with chloroform-methanol (1 ml; 2:1, v/v), and then washed with water (1 ml). The lower phase was collected by using a glass pipette, and evaporated in a 45 °C water bath under a stream of nitrogen (Needs and Selvendran 1993). The residue was hydrolysed with 2 M TFA, reduced with NaBD$_4$ and acetylated (as described above), prior to GC–MS.

Preparation of standards. Partially methylated alditol acetate standards were prepared by the method of Doares et al. (1991) modified by directly using NaOH as the base instead of potassium hydride. Approximately 20-mg individual samples of each of methyl-α-D-glucopyranoside, methyl-β-D-galactopyranoside, methyl-α-D-mannopyranoside, methyl-6-deoxy-α-L-galactopyranoside, methyl-α-D-rhammnopyranoside, methyl-β-D-xylopyranoside and methyl-β-D-arabinopyranoside were dried at 60 °C overnight under vacuum and dissolved in DMSO (400 µl) under nitrogen. Finely powdered NaOH (20 mg) was added to each sample, and immediately vortexed for 10 min under nitrogen. After adding methyl iodide (100 µl), the reaction solution was mixed and heated at 30 °C for another 10 min. The sample was then acetylated by addition of 1-methylimidazole (200 µl), followed by acetic anhydride (2 ml) with further mixing. After 10 min at room temperature, water (5 ml) was mixed to decompose excess acetic anhydride. After cooling to room temperature, each sample was extracted with dichloromethane (2 × 1 ml) by briefly centrifuging for phase separation. The extracts were combined in a single glass tube, and then washed with water (5 × 3 ml). The dichloromethane layer was evaporated in a stream of nitrogen at 40 °C, and the oily residue was then hydrolysed with 2 M TFA, reduced with NaBD$_4$ and acetylated, as described above, prior to GC–MS. Partially methylated alditol acetates were identified by relative retention time

and mass spectra. Partially methylated alditol acetates of pentoses in the furanose ring form were generated by methylation of D-xylose and D-arabinose samples.

Gas chromatography–mass spectrometry. Samples were injected (0.2–0.8 µl injection volume) via an autosampler onto a fused-silica capillary column coated with a 70% cyanopropyl polysil-phenylene-siloxane bonded phase (SGE Pty, Melbourne; BPX70, 12 m long, 0.22 mm i.d., thickness 0.25 µm) which was eluted with He (inlet pressure 15 psi) directly into the ion source of a Fisons MD800 GC–MS (injection port 250 °C; interface 250 °C; source 200 °C). The column was temperature-programmed from 80 °C (hold 2 min) to 170 °C at 30 °C/min and then to 240 °C (hold 10 min) at 8 °C/min for alditol acetates or to 240 °C (hold 4 min) at 3 °C/min for partially methylated alditol acetates. The mass spectrometer was operated in the electron impact ionisation mode with an ionisation energy of 70 eV. Mass spectra were acquired with full scans in 0.45 s from 50 to 250 u for alditol acetates or from 40 to 450 u for partially methylated alditol acetates.

Colorimetric assays. Total carbohydrates were analysed by the phenol/H$_2$SO$_4$ method (Dubois et al. 1956), hexoses by the anthrone/H$_2$SO$_4$ method (Dische 1962), pentoses by the orcinol/HCl method (Dische 1962) and uronic acids by the *m*-hydroxybiphenyl/H$_2$SO$_4$ method (Blumenkrantz and Asboe-Hansen 1973). Cellulose was solubilised in 67% H$_2$SO$_4$ (25 °C, 1 h with shaking) prior to colorimetric assay.

Paper chromatography. Chromatograms were developed in descending mode over 18 h with butanol, acetic acid, water (12:3:5, by vol.) and then over 16 h with ethyl acetate, pyridine, water (8:2:1, by vol.). Monosaccharides were visualised by staining with AgNO$_3$/NaOH (Churms 1982) and fixed with 25% (v/v) Ilfospeed paper fixer.

Estimating total polysaccharides. Acetic/nitric acid insoluble cellulose was measured directly in three replicate samples by means of the anthrone/H$_2$SO$_4$ method. The quantities of pectins and hemicelluloses could not be measured directly but were estimated as follows: pectins as total monosaccharides in the ammonium oxalate fraction plus total monosaccharides in the 0.1 M KOH fraction × 0.7 plus total monosaccharides in the CTAB pellet from the phosphate buffer fraction; hemicelluloses as total monosaccharides in the 4 M KOH fraction plus total monosaccharides in the 0.1 M KOH fraction × 0.3. The multipliers of 0.3 and 0.7 for CTAB supernatant and pellet, respectively, from the 0.1 M KOH fraction are based on the distribution of total monosaccharides in the wild type. Values for total monosaccharides are the sum of values determined by the *m*-hydroxybiphenyl method (with three replicates) in the case of uronic acids and by GC-MS of single pooled samples in all other cases. Starch was estimated as total hexoses in the DMSO fraction measured with the anthrone/H$_2$SO$_4$ assay.

Enzyme digestion. Ammonium oxalate and 4 M KOH fractions were digested with endo-β-1,4-glucanase (endo-cellulase, EC 3.2.1.4; from *Trichoderma*; Megazyme International Ireland, Wicklow, Ireland). Carbohydrate samples in sodium acetate (50 mM, pH 4.7, 400 µl) were digested with 0.125 U ml^{-1} of enzyme for 48 h at 37 °C. Supernatants (14 000g, 20 min) were lyophilised and analysed for glucose by GC/MS without acid hydrolysis.

Statistical analysis. Glucose as cellulose and uronic acids in the ammonium oxalate fraction were analysed for one-way analysis of variance (ANOVA) using InStat for Macintosh (v1.12, GraphPad Software).

Results

The fractionation methods were validated using roots of wild-type seedlings grown at 21 °C before being applied

Table 1. Monosaccharide[a] composition (nmol mg^{-1} dry weight) of primary fractions from roots of *Arabidopsis* wild-type seedlings grown at 21 °C

	UroA	Glc	Gal	Man	Xyl	Ara	Rha	Fuc	Total
PO$_4$ buffer	47	21	10	4	9	10	7	1	109
Chloroform/methanol	0	11	3	3	2	1	1	0	21
DMSO	0	22	3	0	3	4	1	0	33
Ammonium oxalate	144	18	18	3	16	34	12	4	249
0.1 M KOH	30	5	18	0	10	13	5	3	84
4 M KOH	18	40	22	10	67	30	3	9	199
HAc/HNO$_3$-soluble	0	8	1	2	3	2	3	1	20
TFA-soluble	0	29	0	3	9	3	0	0	44
TFA-insoluble	0	483	0	0	0	0	0	0	483
Total	239	637	75	25	119	97	32	18	1242
Totals as %	19	51	6	2	10	8	3	1	100

[a]UroA, uronic acids; Glc, glucose; Gal, galactose; Man, mannose; Xyl, xylose; Ara, arabinose; Rha, rhamnose; Fuc, fucose; HAc acetic acid

to the radial swelling mutants. The distribution of the major cell wall polysaccharides among the fractions was deduced from monosaccharide composition (determined by GC–MS and *m*-hydroxybiphenyl assay of uronic acids), from charge (deduced by CTAB precipitation) and from linkage patterns (determined by methylation analysis).

Polysaccharides in wild-type roots. Seven-day-old seedlings comprise expanded cotyledons with a short hypocotyl and an unbranched root. Table 1 shows the monosaccharide analyses of the fractions before CTAB sub-fractionation.

Pectins were identified as anionic polymers that are rich in galacturonic acid and show many linkages characteristic of known pectins. The phosphate buffer, ammonium oxalate and 0.1 M KOH fractions of roots and shoots were all rich in a uronic acid (Table 1) which co-migrated with galacturonic acid on paper chromatograms. Later fractions (4 M KOH and acetic/nitric acid soluble) lacked galacturonic acid on paper chromatograms and had little rhamnose, features consistent with pectins having been extensively extracted. Cetyltrimethylammonium bromide precipitated about 85% of polysaccharides from the phosphate buffer fraction, 100% from the ammonium oxalate fraction and 70% from the 0.1 M KOH fraction. Galacturonic acid formed over half of the monosaccharides in the ammonium oxalate fraction and in the anionic polysaccharides precipitated with CTAB from the phosphate buffer fraction (Fig. 2A). Other monosaccharides (mainly glucose, galactose, arabinose and xylose) individually comprised less than 12% of total monosaccharides. Methylation analysis of an ammonium oxalate fraction prepared from whole seedlings (Fig. 3A) showed partially methylated alditol acetates corresponding to t-Rha[1], 2-Rha, 2,4-Rha, t-Gal*p*, t-Ara*f*, t-Ara*p*, 5-Ara*f*,

t-Xyl*p*, 4-Gal*p*, 4-Glc*p* and t-Glc*p*/t-Man*p* based on relative retention time and mass spectra. (The abundant 1,4-linked galacturonic acid present in pectins was not assayed by the methods used as the carboxyl group was not reduced before methylation; Carpita and Shea 1989.) Such compositions and linkages are typical of pectins, including those of *Arabidopsis* leaves (Zablackis et al. 1995). No attempts were made to resolve the pectins further (cf. Zablackis et al. 1995).

Xylose is abundant in dicot hemicelluloses such as xyloglucan (XG) and glucuronoarabinoxylan (GAX). The 4 M KOH fractions were rich in xylose (Table 1) and methylation analysis of the 4 M KOH fraction from whole seedlings after centrifugation at 14 000*g* for 1 h showed (Fig. 3E) the t-Xyl*p*, 2-Xyl*p*, 4-*Xyl*p*, 4-Glc*p*, 4,6-Glc*p*, t-Fuc, 2-Gal*p* and t-Gal*p* consistent with XG, 4-Man*p* but not 4,6-Man*p* consistent with glucomannan, and 4-Xyl*p* consistent with GAX (Zablackis et al. 1995). Neither 2-Xyl*p* nor 4-Xyl*p* were resolved by relative retention time but their mass spectra identified the presence of both in one peak. However, t-Glc*p* and t-Man*p* could not be unequivocally identified, because they show the same mass spectrum and relative retention time so that either or both may be present. A small peak with relative retention time appropriate for the t-Ara*f* expected for GAX was seen but its mass spectrum did not identify it conclusively.

The presence of GAX and XG inferred from linkage analysis was confirmed by charge fractionation with CTAB (Fig. 2B). The glucose:xylose ratio in the neutral polymer supernatant resembled the typical 4:3 ratio of XG (Fry 1988; Zablackis et al. 1995). Fucose:xylose ratios were broadly appropriate for XG. The small amounts of arabinose and the very small amounts of mannose in the supernatant presumably came from polymers other than XG. The high levels of xylose, arabinose and uronic acids in the CTAB pellet (Fig. 2B) were consistent with the presence of GAX (Fry 1988; Zablackis et al. 1995).

Glucose comprised about 97% of the material that was insoluble in acetic/nitric acids (the sum of the TFA-soluble and -insoluble material in Table 1) and methylation analysis showed 4-Glc with relatively low levels of t-Glc (Fig. 3D). When the residue from the TFA

---

[1]Results are discussed in terms of the linkage deduced from each partially methylated alditol acetate. The linkage precedes the conventional 3 letter abbreviation for monosaccharides (see footnote to Table 1) so that, for example, 4-Glc denotes 4-linked glucose, t-Glc terminal glucose and so on with pyranose and furanose ring forms indicated by *p* and *f*, respectively.
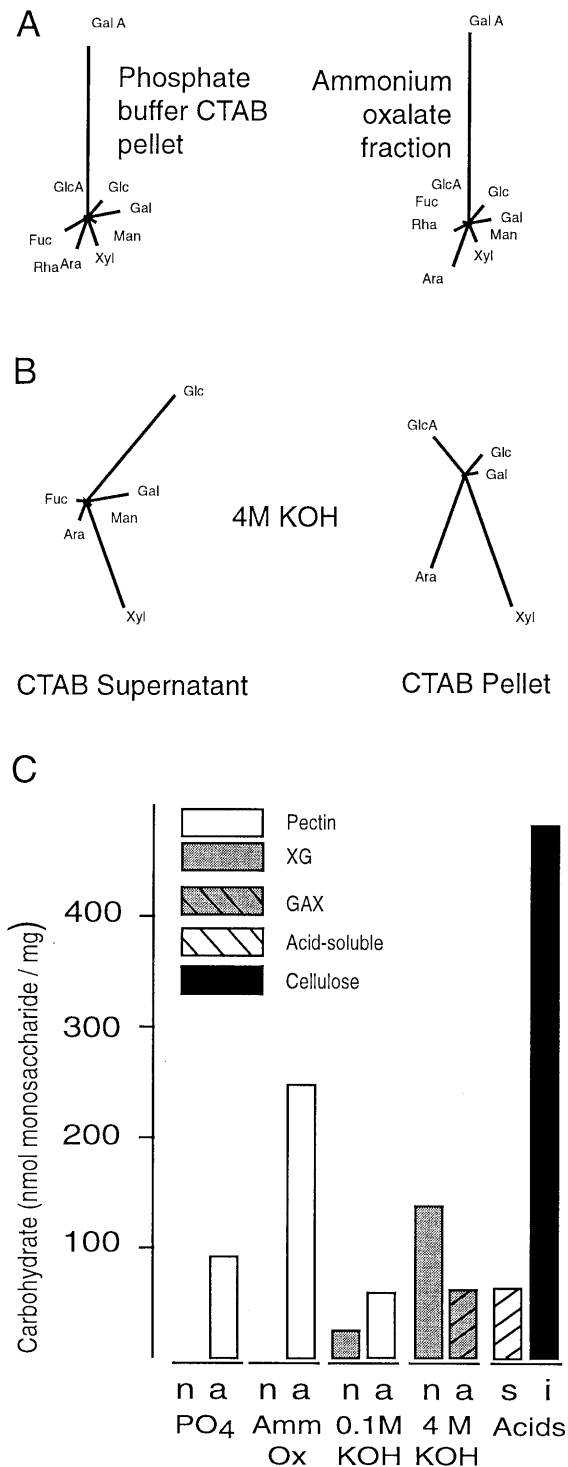
Fig. 2A–C. Identification by monosaccharide composition of the major polysaccharide classes and a summary of their distribution during the fractionation of roots of wild-type *Arabidopsis* grown at 21 °C. **A, B** Compositional analysis of the major cell wall polysaccharides and polysaccharide classes. Relative abundance of each monosaccharide (abbreviations in footnote to Table 1) is proportional to line length. **A** Pectins are recovered in the CTAB pellet from the phosphate buffer fraction, and with minimal neutral polysaccharides, in the whole ammonium oxalate fraction. **B** Xyloglucan occurs in the CTAB supernatant from the 4 M KOH fraction and GAX in the CTAB pellet from the 4 M KOH fraction. **C** Diagram summarising the total quantities of monosaccharides (nmol total monosaccharide mg^{-1} root dry weight) recovered after acid hydrolysis of polysaccharides from the various fractions and sub-fractions. The identities of the major polysaccharide classes in sub-fraction are indicated. *PO₄* phosphate buffer fraction; *Amm Ox*, ammonium oxalate fraction; *Acids*, acetic/nitric acid followed by TFA. Subfractions: *n*, neutral; *a*, anionic; *s*, soluble; *i*, insoluble

3H and 3D), consistent with shorter chains in the acid-soluble material than in the acid-insoluble material.

Some minor components that were not pursued in this study include: the glucose-rich, neutral polymers that comprise about 15% of total carbohydrates in the phosphate buffer fraction; the non-starch material in the DMSO fraction; the material in the chloroform/methanol fraction where the abundant galactose in the shoot probably originates from galactolipids of the chloroplast fragments (Douce and Joyard 1980) that visibly contaminate the crude cell wall fraction; the main neutral polymer in the 0.1 M KOH fraction which monosaccharide analyses suggest may be XG; the source of the 4-Man$p$ seen in the methylation analysis of the 4 M KOH fraction.

From these results, the amounts of monosaccharides recovered in particular fractions can be reconstructed and assigned to different polysaccharides to give an overall indication of the effectiveness of the fractionation scheme (Fig. 2C).

Changes in the mutants. To compare the three non-allelic radial swelling mutants with the wild type, seeds were grown for either 7 d at 21 °C or for 2 d at 21 °C followed by 5 d at 31 °C. The 2 d at 21 °C before seedlings are transferred to 31 °C promoted uniform germination and subsequent growth. The root had not emerged at day 2 so almost all cell walls harvested at day 7 would have been deposited at the restrictive temperature. Neither roots nor hypocotyls of the mutants were significantly shorter than those of the wild type when grown at 21 °C but both were shorter when grown at 31 °C. The mutant roots swell along their whole length whereas roots on mutant seedlings transferred at day 5 only swell sub-apically (Baskin et al. 1992). Dry weights were not affected in proportion to the severely reduced elongation growth but growth at 31 °C strongly favoured dry weight accumulation in the shoot at the expense of the root in all genotypes including the wild type (data not shown).

Monosaccharide composition and methylation analysis were used to compare the non-cellulosic polysaccharides of mutants and wild type. Mutant and

digestion was hydrolysed with 72% H_2SO_4 the only monosaccharide liberated was glucose (TFA-insoluble in Table 1). Small amounts of material were solubilised with the acetic/nitric acid mixture and rather larger amounts with TFA (Table 1). Glucose dominated both acid-soluble fractions but other monosaccharides were detectable although their linkages could not be identified with the samples analysed (Fig. 3H). The t-Glc:4-Glc ratio in the acid-soluble material was high compared to the ratio in the acid-insoluble material (compare Figs.
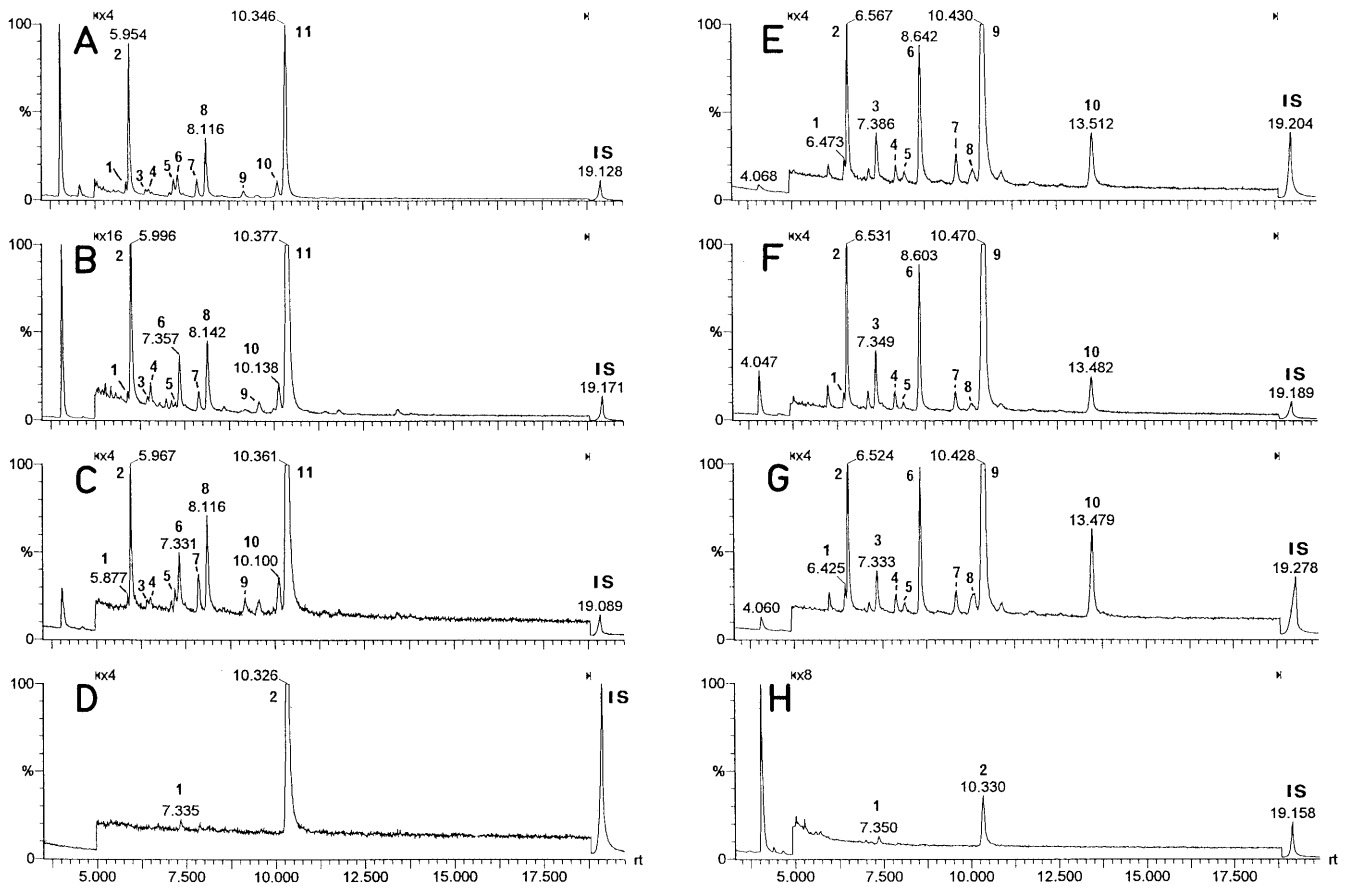
Fig. 3A–H. Total ion current from the GC–MS trace of partially methylated alditol acetates generated by methylation analysis of cell wall fractions prepared from whole seedlings of wild-type and mutant *Arabidopsis*. The total ion currents between 5 and 18 min have been magnified by a factors of 4 (**A, C, D, E, F,** and **G**), 8 (**H**) or 16 (**B**) as indicated at the top of the traces. Relative retention times (*rt*) were calculated against the internal standard (*IS*, *myo*-inositol). **A–C** Ammonium oxalate fractions of the wild type grown at 21 °C (CTAB precipitated) (**A**), *rsw2* grown at 31 °C (**B**) and *rsw3* grown at 31 °C (**C**). The numbered peaks present in each trace correspond to the following deduced linkages typical of pectins: *1*, t-Rha; *2*, t-Ara*f*; *3*, t-Ara*p*; *4*, t-Xyl*p*; *5*, 2-Rha; *6*, t-Glc*p* and/or t-Man*p*; *7*, t-Gal*p*; *8*, 5-Ara*f*; *9*, 2,4-Rha; *10*, 4-Gal*p*; *11*, 4-Glc*p*. **D** Acetic-nitric acid- insoluble fraction. The numbered peaks correspond to the following deduced linkages: *1*, t-Glc*p*; *2*, 4-Glc*p*. **E–G** Hemicelluloses in the supernatant of 4 M KOH fractions prepared from the wild type grown at 21 °C (**E**), *rsw2* grown at 31 °C (**F**), and *rsw3* grown at 31 °C (**G**). The numbered peaks in all three traces correspond to the following deduced linkages typical of XG, GAX and mannan: *1*, t-Fuc; *2*, t-Xyl*p*; *3*, t-Glc*p* and/or t-Man*p*; *4*, t-Gal*p*; *5*, 2-Ara*p* and 5-Ara*f*; *6*, 2-Xyl*p*; and 4-Xyl*p*; *7*, 4-Man*p*; *8*, 2-Gal*p*; *9*, 4-Glc*p*; *10*, 4,6-Glc*p*. **H** Acetic-nitric acid soluble material. Numbered peaks correspond to the following deduced linkages: *1*, t-Glc*p*; *2*, 4-Glc*p*. The ratio of t-Glc:4-Glc is much higher than in the acetic/nitric acid insoluble fraction (compare with **D**). Unnumbered peaks are non-carbohydrate according to their mass spectra

wild-type roots had similar monosaccharide compositions in their ammonium oxalate fractions (pectins; Fig. 4), their 4 M KOH fraction (hemicelluloses; Fig. 4), in their 0.1 M KOH fractions (pectins and hemicelluloses) and in their CTAB pellet (pectins) from the phosphate buffer fraction (data not shown). The same linkages were found in the polysaccharides in the ammonium oxalate (Fig. 3B,C) and 4 M KOH fractions (Fig. 3F,G) of the mutants grown at 31 °C as occur in polysaccharides in the equivalent wild-type fractions (Fig. 3E–H). Endo-*β*-1,4-glucanase did not release any free glucose from the ammonium oxalate and 4 M KOH extracts prepared from the roots of all genotypes whether grown at 21 or 31 °C.

The total quantities of nitric/acetic acid insoluble cellulose present in the root were measured by the anthrone/H_2SO_4 assay in three independently processed replicates (Table 2). All three mutants had significantly less cellulose than the wild type at 31 °C. Table 2 also shows the quantities of uronic acids measured by the *m*-hydroxybiphenyl method in the ammonium oxalate fraction of three independently processed replicates. This is the major pectin-containing fraction and Fig. 4 shows that uronic acids accounted for > 50% of its total monosaccharides. The quantities of uronic acids in the mutants were not significantly different from those in the wild type at either 21 or 31 °C. The total quantities of pectins in the mutants (estimated by the formula in *Materials and methods*) were expressed as a percentage of the quantities in the wild type grown at the same temperature (Fig. 5). Estimated total pectins were higher than wild-type values in *rsw1* and lower in *rsw2* (consistent with uronic acid measurements in the ammonium oxalate fraction) but the pectin changes were much less marked than those seen with cellulose. Estimates of total hemicellulose production showed little
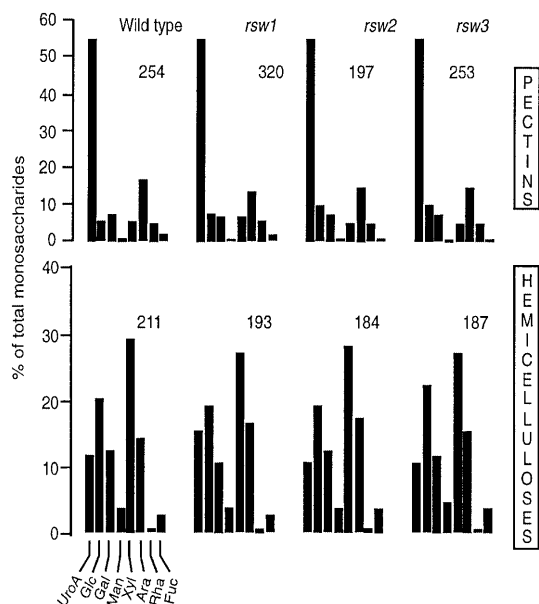
Fig. 4. Histograms comparing the monosaccharide composition of pectins in the ammonium oxalate fraction and hemicelluloses in the 4 M KOH fraction for the roots of wild-type *Arabidopsis* and three mutants grown at 31 °C. The number at the top right of each panel shows total monosaccharides (nmol mg^{-1} root dry weight) in the fraction

Fig. 5. Estimated total production of the major polysaccharide classes in mutant roots of *Arabidopsis* (nmol monosaccharide mg^{-1} dry weight) expressed as a percentage of the production by roots of wild-type (*WT*) seedlings grown at the same temperature. Reductions in cellulose production in the mutants exceed reductions in other polysaccharide classes and are more pronounced at 31 than at 21 °C

change in the mutants so that their hemicellulose: cellulose ratio when grown at 31 °C (0.70 in *rsw1*, 0.87 in *rsw2* and 0.78 in *rsw3*) greatly exceeded the ratio in the wild type (0.44).

Increased starch production. Significantly increased starch was detected in all mutants but most notably with *rsw1* (37 ± 2 nmol hexose mg^{-1} in the wild type at 31 °C; 126 ± 6 nmol mg^{-1} in *rsw1*; 66 ± 4 nmol mg^{-1} in *rsw2*; 77 ± 4 nmol mg^{-1} in *rsw3*; measured as hexose in the DMSO fraction by the anthrone/H$_2$SO$_4$ assay).

Discussion

The fractionation method. We fractionated the major polysaccharides of wild-type seedlings of *Arabidopsis* by sequential selective extraction steps followed by charge-based-subfractionation. Heim et al. (1991) used the selective extraction steps to follow ^{14}C-incorporation into *Arabidopsis* seedling cell walls. They did not, however, identify the major wall polymers or polymer
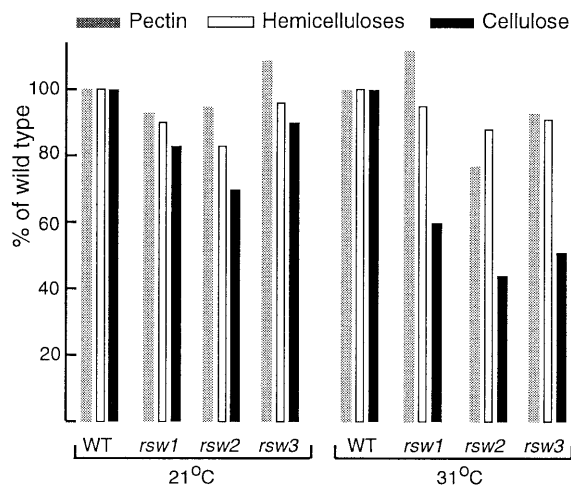
classes nor determine their distribution between fractions, data which we provide. Extending Heim et al.'s fractionation with CTAB resolves most polymers whose distributions overlap in the primary fractions as seen in Fig. 2C. After this refinement, our method provides more resolution of polysaccharides than provided by the TFA-soluble/TFA-insoluble fractionation (Reiter et al. 1993, 1997) but less than the scheme of Zablackis et al. (1995). It is relatively simple, applicable to small quantities of material and could probably be extended to resolve more polysaccharides if required. The TFA method will remain valuable for rapid quantitation and the more extensive fractionation will remain valuable where more detailed characterisation of mutants is required than can be provided by the other methods.

In the present method, pectins partition between the phosphate buffer, ammonium oxalate and 0.1 M KOH fractions and are precipitated with CTAB. Zablackis et al. (1995) recovered about half of the pectic polysaccharides from leaves in the phosphate buffer fraction whereas we recovered 23% of the total from roots. Hemicelluloses extract mainly in the 4 M KOH fraction, but some extract in the 0.1 M KOH fraction. Monosaccharide composition, methylation analysis and charge suggest that XG remains in the supernatant after CTAB precipitates GAX. The acetic/nitric acid mixture

Table 2. Quantities[a] of glucose as cellulose in the acid-insoluble fraction and of uronic acids as pectins in the ammonium oxalate fraction of roots of wild-type *Arabidopsis* and mutants grown at 21 and 31 °C

	Wild type		*rsw1*		*rsw2*		*rsw3*	
	21°	31°	21°	31°	21°	31°	21°	31°
Glucose	483 ± 25	526 ± 25	401 ± 15	315 ± 20	336 ± 22	234 ± 28	435 ± 36	270 ± 29
Uronic acid	144 ± 5	139 ± 9	145 ± 2	177 ± 7	147 ± 4	107 ± 8	146 ± 3	153 ± 6

[a]Quantities as nmol mg^{-1} dry weight measured by the anthrone/H$_2$SO$_4$ method for glucose and by the *m*-hydroxybiphenyl method for uronic acids. Values are mean ± SD for three replicate tissue samples processed independently

releases about 2–4% of the alkali-insoluble material and 2 M TFA releases a further 7–8%. Both acid-soluble fractions are rich in glucose but contain small amounts of other monosaccharides that may come from residual hemicelluloses particularly tightly bound to microfibrils (Hayashi 1989). Some β-1,4 glucan may be released from regions of the microfibril that are not fully crystalline. The final insoluble residue comprises only glucose, is unaffected by TFA, is soluble in 72% H_2SO_4 and contains 4-Glc, all features pointing to it being crystalline cellulose. The t-Glc:4-Glc ratio in the acetic-nitric acid soluble material is much higher than in the final insoluble material, a result consistent with the acetic/nitric acid mixture preferentially extracting short chains or creating them.

Changed carbohydrates in the mutants. Numerous genes are likely to be involved in wall formation, some with general regulatory roles, as well as synthases or other enzymes that act on only one polymer. The changes in wall composition that occur in mutants currently provide the best clues to gene function. The changes may also show whether the production of one polysaccharide can be reduced without activating compensatory mechanisms. Those mechanisms might, for example, increase production of other polymers to compensate for the missing polysaccharide or reduce production of other polysaccharides in line with the reduction shown by the directly affected polysaccharide.

We find that the roots of all three radial swelling mutants have significantly less cellulose than does the wild type when grown at 31 °C, the temperature at which the radial swelling phenotype is expressed (Baskin et al. 1992) but not at 21 °C. These changes in cellulose production are more marked than those we estimate for the production of matrix polysaccharides. There are no statistically significant differences in the quantities of uronic acids in the ammonium oxalate fractions of the mutants at either 21 or 31 °C. Galacturonic acid comprises >50% of the monosaccharides in this fraction which contains >60% of total pectins (Fig. 4). Estimates of total pectins (summing all monosaccharides, in the three sub-fractions) show a small excess in *rsw1* and a deficiency in *rsw2*. In terms of percentage changes, however, the changes in pectins are much less than the changes in cellulose production (Fig. 5). Changes in estimated total hemicellulose levels are even smaller. Taken together with the lack of clear changes in the composition (monosaccharide analyses) or glycosidic linkages (methylation analyses) in the pectins and hemicelluloses, we consider that this provides a strong case that the *RSW1*, *RSW2* and *RSW3* genes act specifically in cellulose synthesis.

The much smaller changes in other polysaccharide classes suggest that any compensatory production of other wall components is limited in extent. The limited compensatory changes in other polysaccharides are most striking with hemicelluloses, a class of polysaccharides whose members bind cellulose. The hemicellulose:cellulose ratio of 0.44 in the wild type rises in all mutants at 31 °C reaching 0.87 in *rsw2*. Edelmann and Fry also found that pea internodes did not reduce XG production when dichlorobenzonitrile inhibited cellulose synthesis (Edelmann and Fry 1992). They also noted that hemicelluloses do not extract more readily from cellulose-depleted walls, an effect they thought could reflect covalent linkage of XG into the wall by an enzyme such as xyloglucan endotransglycosidase.

Whereas production of matrix polysaccharides is little changed in mutants grown at 31 °C, substantial additional starch accumulates. Cellulose contains about 75% of total polymer glucose in wild-type roots grown at 21 and 31 °C and in mutant roots grown at 21 °C. This falls to just over 60% in all mutants at 31 °C and is accompanied by the percentage in starch rising from <5% to around 20%. Other changes in glucose distribution are small.

In conclusion, we have fractionated the cell walls of *Arabidopsis* seedling roots to identify and quantify their major carbohydrates. The changes in cell wall composition that occur in three, non-allelic radial swelling mutants point to the specific involvement of the *RSW1*, *RSW2* and *RSW3* genes in producing cellulose and to changes in other wall polysaccharides being relatively small. The lack of structural relationship between the three genes emphasises that combining genetic and polysaccharide analyses has much to offer in elucidating higher-plant cellulose synthesis.

References

Arioli T, Peng L, Betzner AS, Burn J, Wittke W, Herth W, Camilleri C, Höfte H, Plazinski J, Birch R, Cork A, Glover J, Redmond J, Williamson RE (1998a) Molecular analysis of cellulose biosynthesis in *Arabidopsis*. Science 279: 717–720

Arioli T, Burn JE, Betzner AS, Williamson RE (1998b) How many cellulose synthase-like gene products make cellulose? Trends Plant Sci 3: 165–166

Baskin TI, Betzner AS, Hoggart R, Cork A, Williamson RE (1992) Root morphology mutants in *Arabidopsis thaliana*. Aust J Plant Phys 19: 427–437

Blakeney AB, Harris PJ, Henry RJ, Stone BA (1983) A simple and rapid preparation of alditol acetates for monosaccharide analysis. Carbohyrdr Res 113: 291–299

Blumenkrantz N, Asboe-Hansen G (1973) New method for quantitative determination of uronic acids. Anal Bioch 54: 484–489

Bonin CP, Potter I, Vanzin GF, Reiter W-D (1997) The *MUR1* gene of *Arabidopsis thaliana* encodes an isoform of GDP-D-mannose-4,6-dehydratase, catalyzing the first step in the de novo synthesis of GDP-L-fucose. Proc Natl Acad Sci USA 94: 2085–2090

Campbell JA, Davies GJ, Bulone V, Henrissat B (1997) A classification of nucleotide-diphospho-sugar glycosyl transferases based on amino acid sequence similarities. Biochem J 326: 929–942

Cannon RE, Anderson SM (1991) Biogenesis of bacterial cellulose. Crit Rev Microbiol 17: 435–447

Carpita NC (1984) Fractionation of hemicelluloses from maize cell walls with increasing concentrations of alkali. Phytochemistry 23: 1089–1093

Carpita NC, Gibeaut DM (1993) Structural models of primary cell walls in flowering plants: consistency of molecular structure

with the physical properties of the walls during growth. Plant J 3: 1–30

Carpita NC, Shea EM (1989) Linkage structure of carbohydrates by gas chromatography–mass spectrometry (GC–MS) of partially methylated alditol acetates. In: Biermann CJ, McGinnis GD (eds) Analysis of carbohydrates by GLC and MS. CRC Press, Boca Raton, pp 157–216

Churms SC (1982). CRC handbook of chromatography. Carbohydrates, vol 1. CRC Press, Boca Raton, Florida

Cid VJ, Duran A, del Rey F, Snyder MP, Nombela C, Sanchez M (1995) Molecular basis of cell integrity and morphogenesis in *Saccharomyces cerevisiae*. Microbial Rev 59: 345–386

Cutler S, Somerville C (1997) Cellulose synthesis: cloning in silico. Curr Biol 7: R108–R111

Dische Z (1962) Color reactions of carbohydrates. In: Whistler RL, Wolfrom ML (eds) Methods in carbohydrate chemistry, vol 1. Academic Press, New York, pp 475–514

Doares SH, Albersheim P, Darvill AG (1991) An improved method for the preparation of standards for glycosyl-linkage analysis of complex carbohydrates. Carbohydr Res 210: 311–317

Douce R, Joyard J (1980) Plant galactolipids. In: Stumpf PK, Conn EE (eds) The biochemistry of plants. A comprehensive treatise, vol 4. Academic Press, New York, pp 321–362

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for determination of sugars and related substances. Anal Chem 28: 350–356

Edelmann HG, Fry SC (1992) Effect of cellulose synthesis inhibition on growth and the integration of xyloglucan into pea internode cell walls. Plant Physiol 100: 993–997

Fry SC (1988) The growing plant cell wall. Longman, Harlow

Hayashi T (1989) Xyloglucans in the primary cell wall. Annu Rev Plant Physiol Plant Mol Biol 40: 139–168

Heim DR, Roberts JL, Pike PD, Larrinua IM (1989) Mutation of a locus of *Arabidopsis thaliana* confers resistance to the herbicide isoxaben. Plant Physiol 90: 146–150

Heim DR, Roberts JL, Pike PD, Larrinua IM (1990) A second locus *Ixr* B1 in *Arabidopsis thaliana*, that confers resistance to the herbicide isoxaben. Plant Physiol 92: 858–861

Heim DR, Skomp JR, Waldron C, Larrinua IM (1991) Differential response to isoxaben of cellulose biosynthesis by wild-type and resistant strains of *Arabidopsis thaliana*. Pest Biochem Physiol 39: 93–99

Kokubo A, Kuraishi S, Sakurai N (1989) Culm strength of barley: correlations among maximum bending stress, cell wall dimensions and cellulose content. Plant Physiol 91: 876–882

Lussier M, White AM, Sheraton J, di Paolo T, Treadwell J, Southard SB, Horenstein CI, Chen-Weiner J, Ram AF, Kapteyn JC, Roemer TW, Vo DH, Bondoc DC, Hall J, Zhong WW, Sdicu AM, Davies J, Klis FM, Robbins PW, Bussey H (1997) Large scale identification genes involved in cell surface biosynthesis and architecture in *Saccharomyces cerevisiae*. Genetics 147: 435–450

Needs PW, Selvendran RR (1993) An improved methylation procedure for the analysis of complex polysaccharides including resistant starch and a critique of the factors which lead to undermethylation. Phytochem Anal 4: 210–216

Nicol F, His I, Jauneau A, Vernhettes S, Canut H, Höfte H (1998) A plasma membrane-bound putative endo-1,4-beta-D-glucanase is required for normal wall assembly and cell elongation in *Arabidopsis*. EMBO J 17: 5563–5576

Potikha T, Delmer DP (1995) A mutant of *Arabidopsis thaliana* displaying altered patterns of cellulose deposition. Plant J 7: 453–460

Reiter W-D, Chapple CCS, Somerville CR (1993) Altered growth and cell walls in a fucose-deficient mutant of *Arabidopsis*. Science 261: 1032–1035

Reiter W-D, Chapple C, Somerville CR (1997) Mutants of *Arabidopsis thaliana* with altered cell wall polysaccharide composition. Plant J 12: 335–345

Saxena IM, Brown RM (1997) Identification of cellulose synthase(s) in higher plants: sequence analysis of processive β-glycosyltransferases with the common motif 'D, D, D35Q(R,Q)XRW. Cellulose 4: 33–49

Saxena IM, Brown RM, Fevre M, Geremia RA, Henrissat B (1995) Multidomain architecture of β-glycosyl transferases: implications for mechanism of action. J Bacteriol 177: 1419–1424

Taylor NG, Scheible W-R, Cutler S, Somerville CR, Turner SR (1999) The *irregular xylem3* locus of *Arabidopsis* encodes a cellulose synthase required for secondary cell wall synthesis. Plant Cell 11: 769–780

Turner SR, Somerville CR (1997) Collapsed xylem phenotype of *Arabidopsis* identified mutants deficient in cellulose deposition in the secondary cell wall. Plant Cell 9: 689–701

Updegraff DM (1969) Semimicro determination of cellulose in biological materials. Anal Biochem 32: 420–424

Zablackis E, Huang J, Muller B, Darvill AG, Albersheim P (1995) Characterization of the cell wall polysaccharides of *Arabidopsis thaliana* leaves. Plant Physiol 107: 1129–1138