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Xyloglucans in different types of cotton (*Gossypium* sp.) cells

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SUMMARY

The different xyloglucans obtained from the cell walls of cotton fibres, cotton leaves, suspension-cultured cotton cells, and from the culture medium of the latter cells have been characterized by methylation and ^{13}C-NMR. The proportions of the constituent monosaccharides are quite similar—glucose:xylose:galactose:fucose (2·4–2·0:1:0·5–0·3:0·3–0·1) and the xyloglucans all have a main chain of β-1,4-linked D-glucopyranosyl residues to which α-xylopyranosyl-, 2-*O*-β-galactopyranosyl-α-xylopyranosyl-, and 2-*O*-α-fucopyranosyl-2-*O*-β-galactopyranosyl-α-xylopyranosyl- residues are attached at C(*O*)6. An oligosaccharide, which was tentatively identified as the xyloglucan nonasaccharide XG_9 ($Glc_4Xyl_3GalFuc$), was isolated from the culture medium of suspension-cultured cells.

Key-words: cell-wall polysaccharides, cotton, *Gossypium* sp., structural analysis, xyloglucan.

INTRODUCTION

Xyloglucans (fucogalactoxyloglucans) have been recognized in the primary cell wall of plants, particularly dicotyledons, but also some monocotyledons and gymnosperms (Darvill *et al.* 1980; Fry 1989). In fact the xyloglucans studied were often obtained as secretion products in the culture medium of suspension-cultured cells, or sometimes from the walls of such cells. More rarely xyloglucans have been obtained from plant tissues grown *in vivo*. It is often stated that such xyloglucans in the suspension medium resemble those in the walls of cultured cells or normally-grown plant tissue (Darvill *et al.* 1980). This is probably true for the main structural features, but one can expect differences in molecular weight and distribution of the various side chains depending upon the function of the xyloglucan and the stage of development of the source plant tissue. Besides being a structural component of primary cell walls, xyloglucans have been attributed diverse roles, e.g. the control of cell expansion, growth substance effects and as a carbohydrate reserve (Fry 1989).

It was suspected that xyloglucans or xyloglucan metabolism could play a role in the control of cotton fibre elongation and it has been shown that xyloglucan deposition is restricted to the elongation phase of cotton fibre development (Delmer *et al.* 1985). As

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a first step it appeared appropriate to compare the xyloglucans from different cotton cell types. The structure of the xyloglucan from the cell walls of cotton fibres has already been reported (Buchala & Meier 1985; Hayashi & Delmer 1988) and the presence of xyloglucan in the suspension culture medium has been established (Buchala *et al.* 1987). Results are now presented for the structures of xyloglucans isolated from the cell walls of cotton fibres (differentiated, elongating cells), cotton leaves (differentiated), suspension-cultured cotton cells (undifferentiated, essentially spherical), and from the culture medium of the latter. It is also shown that oligosaccharides, which may derive from the xyloglucan in the cell wall of suspension-cultured cotton cells, are present in the culture medium.

MATERIALS AND METHODS

Plant tissues

Cotton plants (*Gossypium hirsutum* L. cv. Stoneville No. 406 and *Gossypium arboreum* L.) were grown as described previously (Jaquet *et al.* 1982). Mature green leaves and cotton fruits at about 20 days post-anthesis were harvested and the tissues immediately frozen at −80°C until required. Suspension cultures of cells derived from the meristem of cotton (*G. hirsutum*) shoot tips were maintained as described elsewhere (Grossman *et al.* 1982) with transfer every 7 days.

Radiolabelling of cell walls

Small cultures (6 ml) of the cotton cells were fed with 50 μCi of [U-^{14}C]sucrose (Amersham, UK). After 10 days the cells (50%) were transferred to fresh culture medium containing 50 μCi of [U-^{14}C]sucrose and after a further 10 days the cells were decanted. The cells were thoroughly washed with fresh culture medium and transferred to culture medium containing unlabelled sucrose (90 mM).

Isolation of polysaccharide material

The cultures were filtered through glass fibre paper (Whatman GF/A) and the filtrate was reduced in volume by evaporation *in vacuo*. The polysaccharides (ECP) were isolated by precipitation with ethanol, dialysis and freeze-drying. Cotton fibres were extracted with water followed by 24% KOH and the polysaccharides isolated as described by Fry (1988). Fresh cotton leaves were immediately treated with boiling 80% methanol and successively extracted with the same solvent (12 × 30 min) followed by 80% ethanol (4 × 30 min) and acetone (1 ×); the material was dried at 40°C and finely ground. The material was then successively treated with hot 2% EDTA (1 h, pH 6·8), 10% sodium chlorite (12 h, pH 4), 4% KOH containing 0·5% $NaBH_4$ (12 h) and finally 24% KOH containing 0·5% $NaBH_4$ (12 h) and the polysaccharides solubilized were isolated as described by Fry (1988).

Fractionation of polysaccharide material

Polysaccharides were fractionated on a column of QAE-Sephadex (Pharmacia, Uppsala, Sweden) using imidazole buffer (10 mM, pH 7) as described by York *et al.* (1986) and the xyloglucan-rich, neutral material was further fractionated on a column of fibrous cellulose (Whatman CF 11). The fraction eluted with water was discarded, the xyloglucan was eluted with 4% KOH and recovered as described above.

Sugar analysis

Polysaccharides were hydrolysed with sulphuric acid (see Fry 1988) and the sugars liberated were determined by GLC of their acetylated aldononitrile derivatives (Varma *et al.* 1973) on columns (15 m × 0·53 mm) of DB-225 (J. & W. Scientific) using a Hewlett-Packard 5890A chromatograph equipped with a flame ionization detector. Hydrolysates were routinely examined by PC on Schleicher & Schüll No. 2043b paper using ethyl acetate–pyridine–water (8:2:1 v/v) and detection with alkaline silver nitrate. Oligosaccharides in enzymatic hydrolysates were examined by HPLC on a HPIC-AS6A anion-exchange column (25 cm × 4 mm) coupled to a pulsed amperometric detector (Dionex, Sunnyvale, USA). The oligosaccharides were eluted in a gradient of sodium acetate (0–100 mM) in 100 mM NaOH at a flow rate of 1 ml min^{-1} and fractions of 0·5 ml were taken for further analyses.

Radioactivity in monosaccharides or oligosaccharides was measured by liquid scintillation counting or by scanning chromatograms (Linear Analyser LB282, Berthold, Wildbad, Germany).

NMR

The ^{13}C-NMR spectra of xyloglucans (*c.* 30 mg) were recorded at 30°C and 90 MHz (*c.* 70 000 transients) with broad band decoupling on a Bruker AM 300WB spectrometer using D_2O as solvent and DMSO as external standard. Signals were assigned on the basis of spectra of standard polysaccharides and reported values (Joseleau & Chambat 1984).

Enzymatic hydrolysis

Samples (*c.* 2 mg) of polysaccharide were dissolved in ammonium acetate buffer (1 ml, 50 mM, pH 5) and incubated with Driselase (Fluka AG, Buchs, Switzerland) or with a mixture of α-amylase (pig pancreas) and β-amylase (barley) at pH 6·8 in 50 mM phosphate buffer. The enzymes were deactivated by heating at 100°C for 5 min and hydrolysates were examined by PC with propan-1-ol:ethyl acetate:water (7:1:2 v/v) or by HPLC.

Methylation analysis

Samples of polysaccharide (5 mg) were methylated as described by Harris *et al.* (1984), but using lithium methylsulphinylmethanide, and each product was depolymerized using the formic acid/sulphuric acid method (Jansson *et al.* 1976). The methyl sugars were converted into their peracetylated alditol (Jansson *et al.* 1976) or aldononitrile (Tanner & Morrison 1984) derivatives and subjected to GLC–MS using a Hewlett-Packard 5890A gas chromatograph, equipped with a column of DB-225 (25 m × 0·33 mm), coupled to a 5970B mass spectrometer. Derivatives were identified by comparison of their retention times and spectra with data from the appropriate standard compounds. The peak areas obtained from the total ion chromatograms were corrected by using effective carbon response factors to give the molar ratios.

RESULTS AND DISCUSSION

Xyloglucans were found in all of the different cotton tissues that were examined. The presence of xyloglucans containing fucosyl residues in polysaccharide mixtures soluble in D_2O or DMSO-d_6 can be readily established by ^{13}C-NMR due to the typical signal

Table 1. Composition of the xyloglucans

	Molar ratio			
	Glucose	Xylose	Galactose	Fucose
Cotton fibre	2·4	1	0·3	0·1
Cotton leaf	1·4	1	0·4	0·2
Cultured cells*	1·7	1	0·3	0·2
Culture medium	2	1	0·5	0·3

*Small amounts of arabinose were also detected.

at *c.* 16 ppm assigned to the methyl group of the 6-deoxysugar fucose. So long as the absence of starch could be confirmed by attempted hydrolysis with a mixture of α- and β-amylase, analysis for glucose in acid hydrolysates of the polysaccharide mixtures, obtained by the sequential extraction procedure, indicated that little xyloglucan was solubilized until high concentrations, typically 24% KOH, of alkali were used. The water-soluble polysaccharides in the 24% KOH fractions, and the cotton ECP were fractionated by a combination of anion-exchange chromatography (York *et al.* 1986) and affinity-binding to cellulose powder (Aspinall *et al.* 1969) to yield four xyloglucans with typical (Darvill *et al.* 1980) sugar compositions (see Table 1) which gave the characteristic coloration with KI_3 (Kooiman 1960) and which were free of starch contamination. Fractionation may be specific for certain structural features and lead to loss of important structural information. Thus, no attempts were made to sub-fractionate the xyloglucans in the hope that global analysis would be sufficient to distinguish different cell types.

Treatment of the xyloglucans with the enzyme preparation Driselase from *Irpex lacteus*, which contains a multitude of *exo*- and *endo*-hydrolase activities but which lacks α-xylosidase activity (Fry 1988), gave the expected monosaccharide constituents, the oligosaccharide isoprimeverose (6-*O*-α-xylopyranosyl-glucose) and small amounts of unidentified higher oligosaccharides.

The ^{13}C-NMR spectra of the xyloglucans from leaf cultured cells and ECP were found to be very similar and certain signals could be assigned on the basis of reported values (Joseleau & Chambat 1984). The well-defined C1 anomeric signals at about 104 ppm, 99·3 ppm and 99·7 ppm were tentatively attributed to terminal β-galactopyranosyl-, internal and terminal α-xylopyranosyl residues respectively. A less-well-resolved group of signals centred at about 103 ppm was assigned to C1 of β-glucopyranosyl residues indicating structural complexity. It was also possible to distinguish signals at about 80 ppm, 70 ppm and 62 ppm from the C2 of substituted α-xylopyranosyl-, and C6 of substituted and free β-glucopyranosyl residues respectively. The high field signal at 16·4 ppm was attributed to C6 of fucopyranosyl residues.

The xyloglucans were permethylated (Harris *et al.* 1984) and hydrolysed (Jansson *et al.* 1976), and the resulting methyl sugars were converted into their peracetylated aldononitrile (Tanner & Morrison 1984) or glycitol (Jansson *et al.* 1976) derivatives which were analysed by GLC–MS. The following sugars were obtained and identified from each xyloglucan: 2,3,4-tri-*O*-methylfucose, 2,3,4-tri-*O*-methylxylose, 3,4-di-*O*-methylxylose, 2,3,4,6-tetra-*O*-methylgalactose, 3,4,6-tri-*O*-methylgalactose, 2,3,6-tri-*O*-methylglucose and 2,3-di-*O*-methylglucose. The molar ratios of these derivatives are given in Table 2.

Table 2. Methyl sugars obtained upon hydrolysis of the permethylated xyloglucans

	Molar ratio*			
	Fibre	Leaf	Cells	ECP
2,3,4-tri-*O*-methylfucose	0·1	0·3	0·2	0·45
2,3,4-tri-*O*-methylxylose	1·5	0·8	1	2
3,4-di-*O*-methylxylose	0·8*	0·7	0·8	0·6
2,3,4,6-tetra-*O*-methylgalactose	0·2	0·45	0·3	0·25
3,4,6-tri-*O*-methylgalactose	0·3	0·5	0·2	0·5
2,3,6-tri-*O*-methylglucose	1	1	1	1
2,3-di-*O*-methylglucose	2·2	1·3	1·7	2·4

*Eventual contamination of 3,4-di-*O*-methylxylose by 2,3-di-*O*-methylxylose would not be detected with the peracetylated glycitol acetate derivatives used for the analysis of the fibre xyloglucan.

In addition small amounts of 2,3-di-*O*-methylxylose, probably derived from contaminating xylan, were detected in all of the samples and traces of 2,4,6-tri-*O*-methylglucose were detected in the sample from cotton fibre. The latter derived from β-1,3-glucan (Huwyler *et al.* 1978) which could not be eliminated by the chromatographic methods described above.

Taking into account the difficulties in quantitative analysis of methyl sugars, there is a fairly good agreement between the number of methyl derivates corresponding to the putative side chains and branch points of the main glucan chain. These values and the ^{13}C-NMR spectra confirm that the xyloglucans have a main chain of β-1,4-linked glucopyranosyl residues substituted at C(O)6 by α-xylopyranosyl, 2-*O*-β-galactopyranosyl-α-xylopyranosyl-, and 2-*O*-α-fucopyranosyl-2-*O*-β-galactopyranosyl-α-xylopyranosyl side chains. The proportions of the various methyl derivatives show that the xyloglucans have similar structures, but do not permit conclusions concerning the distribution of the side chains. However, the ^{13}C-NMR for the ECP xyloglucan was simpler and had better resolved signals than those obtained for the xyloglucans from leaf and suspension-cultured cells, suggesting a more regular, repeating structure. This is also reflected in the higher proportion of terminal xylosyl residues. In addition the proportion of glucose in the cotton fibre xyloglucan, indicates a less substituted glucan main chain. Whether a somewhat lower and more regular substitution of the xyloglucan backbone is typical for fully elongated cells remains to be demonstrated. Other differences were also observed (Table 2), particularly in the number of side chains constituted by single xylosyl residues, but there is no evident relationship between the structural features of the xyloglucans and the cell type studied. Polysaccharide composition is known to vary with stage of development (cf. Buchala *et al.* 1989) and culture age. With the different types of tissue studied it was impossible to obtain a unified age or stage of development. It can also be seen (Table 2) that the xyloglucan from the ECP was not identical to the xyloglucan from the cell walls of the suspension-cultured cells suggesting that modifications may occur in the culture medium after secretion.

This was investigated by incubating the cells twice with [^{14}C]sucrose in order to uniformly label the cell-wall polysaccharides. The cells were then washed free of labelled low molecular sugars and transferred to culture medium containing unlabelled sucrose. The polysaccharides in the cell walls, the ECP, and the low molecular weight sugars in

the culture medium were examined and found to be labelled. Treatment of the ECP with Driselase gave labelled monosaccharides and a labelled oligosaccharide identical to isoprimeverose on HPLC. This shows that the xyloglucan in the ECP had been labelled. The low molecular weight sugars in the culture medium were examined by HPLC and the radioactivity measured in the eluent. Most of the radioactivity was found in fractions corresponding to mono- and disaccharides, but a small amount of radioactivity was also detected in a component which was chromatographically identical to the xyloglucan nonasaccharide XG_9. The material was isolated and the monosaccharide composition determined and found to be close to the expected values, but contaminated with a small amount of arabinose. It was concluded that the oligosaccharide was principally XG_9. The same nonasaccharide was found in the culture medium of suspension-cultured spinach cells (Fry 1986) where it appears to be a stable end product (Baydoun & Fry 1989). Whether the XG_9 is produced by hydrolysis of ECP or cell-wall xyloglucan is not known (Fry 1988). Attempts to find a similar oligosaccharide in cotton fibres grown *in vitro* were unsuccessful.

The anti-auxin effect of XG_9, described by York *et al.* (1984) and confirmed by McDougal & Fry (1988), requires the 1,2-linked fucose residue whereas some other structural features are less important, e.g. terminal xylose (McDougal & Fry 1989). Augur *et al.* (1992) have also shown that XG_{11}, an undecasaccharide with two α-fucosyl residues, is more efficient than XG_9. Some xyloglucan oligosaccharides, with or without fucosyl residues, also stimulate growth and activate carbohydrase or transglycosylase activities (Joseleau *et al.* 1992; Lorences *et al.* 1992). Thus, taking into consideration the low physiological concentration (10^{-8}–10^{-9} M) necessary for such effects and the complexity of their action, it is clear that global structural analysis of the cell-wall xyloglucans of different types of cells will only yield information of limited use. A better approach would involve following the eventual changes in the structure of xyloglucan (or other polysaccharides) of a single cell type during differentiation. Work is being carried out in this direction.

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