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Characterization of extracellular β(1,4)-xylan backbone O-substituted by arabinogalactans type II in a plant cell suspension

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In this paper we demonstrate for the first time the existence of an ether linkage between arabinogalactans type II (AGII) and a β(1,4)-xylan backbone. This polysaccharide fraction was isolated by ethanolic precipitation from a culture medium of Silene alba cells suspension. A combination of chromatographic methods, the use of a pure specific xylanase and the determination of the reducing end, has enabled us to establish the orientation of the linkage. The results were confirmed by methylation analysis assisted by mass spectrometry and by ^{13}C-NMR spectroscopy. The proposed xylan structure is a β(1,4)-xylan backbone carrying AGII side chains of different sizes which are interspersed along the xylan chain. Such a result may be considered as an additional argument for supporting the importance of AGII in plant cell biology.

INTRODUCTION

The coexistence of arabinogalactans and xylans in the same fractions has been attributed to either contamination by cofractionation (Keegstra *et al.*, 1973; Wilkie, 1979; Stevenson *et al.*, 1986) or substitution of xylans by short side chains such as Gal→5Ara and Gal→4Xyl→2Ara (Buchala, 1973; McNeil *et al.*, 1984). These polysaccharides were mostly found in the plant cell walls and among the excretion products of suspension-cultured plant cells (Aspinall *et al.*, 1969; Wilkie, 1979; McNeil *et al.*, 1979, 1984; Akiyama & Kato, 1982; Stevenson *et al.*, 1986).

Arabinogalactans II (AGII), characterized by 3-O-, 6-O- and 3-, 6-O-linked β-galactose, are known to be structurally involved, with pectic polysaccharides, proteoglycans and glycoproteins found in cell walls and in the culture media of plant cell suspensions (Keegstra *et al.*, 1973; Fincher *et al.*, 1983; Showalter & Varner, 1989). They also occur in some plasma membrane glycoproteins of plant cells (Norman *et al.*, 1990; Knox *et al.*, 1991; Levy & Staehelin, 1992). Whatever their origin, plant xylans have the common structural feature of a β(1,4)-linked xylose backbone in some cases substituted at 2-O- or/and 3-O- by sidechains. The substituents are acetyl groups, terminal monosaccharides (Ara→, GlcA→, 4-O-Me-GlcA→) and oligosaccharides (Buchala, 1973; Wilkie, 1979; McNeil *et al.*, 1984; Reicher *et al.*, 1984; Hoffmann *et al.*, 1992).

It is known that arabinoxylans (Wilkie, 1979; McNeil *et al.*, 1984), some pectic polysaccharides (Fry, 1982, 1983; Rombouts & Thibault, 1986) and xyloglucans (Ishii & Hiroi, 1990) are esterified on their terminal non-reducing arabinose, galactose or xylose by coumaric and/or ferulic acids which are known to be able to oxidatively cross-link xylans (Wilkie, 1979; Ishii, 1991) and pectic side chains (Guillon *et al.*, 1989; Guillon & Thibault, 1990). Furthermore, it was hypothesised that highly branched arabino-(3,6)galactans may connect both rhamnogalacturonan to hydroxyproline-rich wall proteins (Keegstra *et al.*, 1973) and xyloglucans to homogalacturonans (Chambat *et al.*, 1984). In addition, xylans hydrogen bond to cellulose (Fry, 1986).

The aim of the present work is to specify the link between AGII and xylans in order to clarify the features of these compounds. For this purpose, a xylan fraction mainly composed of 4-O-linked (56·7 mol %) and 2,4-O-linked xylosyl residues (11·3%) together with (3/6)-O-linked galactose (16·2%) (Solo Kwan & Morvan, 1991), was hydrolysed by a 39 kDa xylanase (Debeire *et al.*, 1990). Analysis of both oligomeric fractions and the residual polymeric fraction (RF-X) in the hydrolysate indicated a junction between arabinogalactans II and the xylan backbone. ^{13}C-NMR spectroscopy and methylation

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analysis, as well as the identification of sugar residue at the reducing end, have allowed us to identify this linkage.

EXPERIMENTAL

Materials

Suspension-cultured *Silene alba* (Miller) E.H.L. Krause cells were obtained according to the procedure of Dubois & Bouriquet (1973). Polysaccharides were isolated from the culture medium (2·5 l) of 14-day-old cells as described previously (Solo Kwan & Morvan, 1991). The fraction eluted with 0·1 M NaCl (SF0·1) was used.

Chromatographic methods

Size exclusion chromatography (SEC)
Bio-Gel P-2 (105 × 2·7 cm, Bio-Rad, USA), Bio-Gel P-6 (60 × 1·5 cm) and Sephacryl S-200 HR columns (114 × 2·2 cm; Pharmacia IBF, Sweden) were equilibrated in deionized water. The calibration of S-200 HR column was carried out using lactose (Lac) and dextran sulfate standards of different molecular masses (300–200 kDa, $D_{300-200}$; 40 kDa, D_{40} and 10 kDa, D_{10}) according to their K_{av} ($\approx V_e - V_0$ versus $V_t - V_0$).

Ion-exchange chromatography (AEC)
Dowex 50 × 8 (15 × 2·5 cm) and Dowex 1 × 2 (15 × 2 cm) columns were used as described by Debeire *et al.* (1990). Semi-hydrophobic anion-exchange chromatography (SHAEC) was carried out on a Spherosil LS DEA column (10 × 2 cm; Pharmacia/IBF, Sweden) equilibrated in 50 mM sodium acetate, pH 6·6. The column was eluted with the same buffer (60 ml) followed by a step gradient of NaCl (0–0·2 M and 0·2–0·5 M); 1·7 ml fractions were collected.

HPLC analysis
A Lichrosorb AX-W column (10 μm, 250 × 4·5 cm; Merck, Germany) was used under the following conditions: H_2O at 10 mM KH_2PO_4/15 min at 1 ml/min, isocratic step 5 min and increased up to 25 mM KH_2PO_4 over 10 min; fractions of 0·5 ml were collected. The gradient profile was established with a conductimetric detector (Milton Roy, USA), by using KH_2PO_4 standards for calibration. The determination of the main fractions was made by comparing their relative retention times to those of oligosaccharidic standards with known structures (2b and 3b–c; Debeire *et al.*, 1990).

Elution on liquid chromatography was monitored at 206 nm with Uvicord S II (Pharmacia LKB, Sweden) detector and via colorimetric assays of carbohydrate.

Thin layer chromatography (TLC)
TLC analysis was performed on silica gel 60 plates (20 × 20 cm; Merck, Germany) which were developed in butan-1-ol/acetic acid/water (2/1/1, v/v). The carbohydrate detection was achieved with 0·1% (w/v) orcinol in 20% sulfuric acid and the plates were maintained for 5–10 min at 100°C. For amino acid analysis by TLC, 1 mg (weighed) of RF-X was hydrolysed with 5·6 N HCl at 100°C for 24 h. After co-evaporating acid with methanol, 20 and 30 μg were deposited on TLC plates. Amino acids were detected by spraying the plates with ninhydrine reagent (0·1%, w/v, in acetone with 1 ml glacial acetic acid). The plates were dried in warm air with a hair-dryer until colour development. Suitable carbohydrate and/or amino acid standards were used.

Gas liquid chromatography (GLC) analysis
GLC analysis was performed on a Delsi 300 chromatograph equipped with a CP Sil 5CB capillary column (60 m × 0·32 mm, 0·11 μm; Chrompack, The Netherlands) and a FID detector. The column temperature was 120–240°C with a temperature gradient of 2°C/min and nitrogen at 0·45 MPa was used as the carrier gas. Analysis was recorded on a CR-4A computing integrator (Shimadzu, Japan).

Analytical methods

Total sugars were measured by the phenol–sulfuric acid method (Dubois *et al.*, 1956) and uronic acids by the *m*-hydroxybiphenyl–sulfuric acid method (Blumenkrantz & Asboe-Hansen, 1973) using D-Xyl and D-GlcA as standards. Carbohydrate values were corrected according to the Montreuil & Spik method (1963). The monosaccharide composition of the polysaccharide fractions was determined by GLC analysis (see above) as either (i) trimethylsilylated methylglycosides (TMSG) obtained after methanolysis (1 M methanol/HCl, 16 h, 80°C) or hydrolysis (4 M trifluoroacetic acid, 100°C, 2 h) followed by methanolysis (0·5 M methanol/HCl, 3 h, 80°C) and trimethylsilylation (pyridine:BSTFA 1% TMCS, v/v, Pierce, USA) according to Montreuil *et al.*, (1986); or (ii) alditol acetates (AA) obtained after hydrolysis by 4 M trifluoroacetic acid (TFA) (2 h, 100°C), reduction with $NaBH_4/NH_4OH$ and peracetylation (Jones & Albersheim, 1972). The total monosaccharide composition was expressed by combining uronic acid values obtained from trimethylsilylation and neutral sugars from peracetylation. Protein contents were assayed by the Lowry method (Lowry *et al.*, 1951).

Methylation and mass spectrometry analysis

Methylation and mass spectrometric analysis were respectively carried out according to Paz Parente *et al.* (1985) and Jansson *et al.* (1976). The partially methylated monosaccharides were reduced by $NaBD_4/NH_4OH$ and acetylated. Following this, analysis of partially methylated alditol acetates (PMAA) was performed by GLC/EI-MS under the following conditions: Delsi DI 700 apparatus equipped with a DB-1

capillary column (25 m × 0·2 mm, 0·12 μm; J&W Scientific, USA), temperature program from 80 to 180°C/3°C/min to 240°C at 6°C/min for 10 min. Mass spectra were recorded on a coupled Riber 10–10 mass spectrometer using an electron energy of 70 eV and an ionization current of 0·2 mA. Calculations were carried out on a Girdel 300 apparatus according to the above GLC analysis. Data were carried via response factors (Sweet *et al.*, 1975).

^{13}C-NMR analysis of RF-X

The ^{13}C-NMR spectra of samples in D_2O solution (7 mg/0·5 ml) were recorded on a Bruker AM 400 spectrometer (100·62 MHz) at 80°C using a 5 mm dual probe. Chemical shifts (ppm) were measured relative to dimethylsulfoxide (DMSO, 39·6 ppm) as an internal standard and are reported as values relative to tetramethylsilane (TMS).

Enzymatic hydrolysis of SF0·1

33 mg of SF0·1 dissolved in 0·05 M sodium acetate buffer pH 5·8, were hydrolysed by 47 U (783 nkat) of xylanase purified from *Clostridium thermolacticum*, as described previously (Debeire *et al.*, 1990). Hydrolysis was stopped by heating (boiling water bath 5 min). 3 vol. of absolute ethanol (abs EtOH) were added to the hydrolysate and stored at 4°C overnight. The supernatant as well as the pellet were further fractionated. The yield of the fractions obtained was expressed relative to the weight percentage (w/w ≈ w %) of SF0·1.

Alkaline cleavage of O-glycosidic linkages

3 mg of RF-X, previously reduced with $NaBD_4$/NH_4OH, was saponified with 0·5 N NaOH (pH ≈10) overnight at 40°C. After neutralization by acetic acid at pH 6·5–7·0, 3 vol. abs EtOH were added and the mixture was maintained at 4°C overnight. Centrifugation yielded a pellet and a supernatant whose alditol acetate derivatives were both analysed by GLC.

Characterization of glycosyl residues at the reducing end

Analysis was conducted by GLC-MS analysis of alditol acetate derivatives after two distinct kinds of preliminary reduction with $NaBD_4$/NaOH and $NaBD_4$/NH_4OH followed by hydrolysis in 2 N TFA for 2 h at 100°C.

RESULTS AND DISCUSSION

Heterogeneity of the fraction SF0·1

Since two fractions, I and II, were eluted in water from the S-200 HR column SF0·1 was clearly heterogeneous (Fig. 1A). From their molar compositions (Table 1), I was a fraction consisting of arabinogalactan II (Ara: 16·7%; Gal: 32·9% and uronic acids: 8·0%) and xylans (Xyl: 38·0%), and II contained xylan (Xyl: 74·2% and uronic acids: 8·4%). Fraction I was as acidic as fraction II. Each represented 50 w% of SF0·1.

Further examination of SF0·1 fractions on a hydrophobic anion–exchange column (Spherosil DEA) eluting with a (0→0·2 M NaCl gradient), showed that fraction I primarily consisted of a material I-1, having a similar Ara/Xyl/Gal composition to fraction I (Table 1), whereas fraction II separated into four fractions (Fig. 1B) which had slightly different sugar compositions, especially with regard to 4-OMe-GlcA (Table 1). No significant carbohydrate fraction was obtained via the second gradient (0·2→0·5 M NaCl). Thus, the compositions of fractions I and II suggested that the

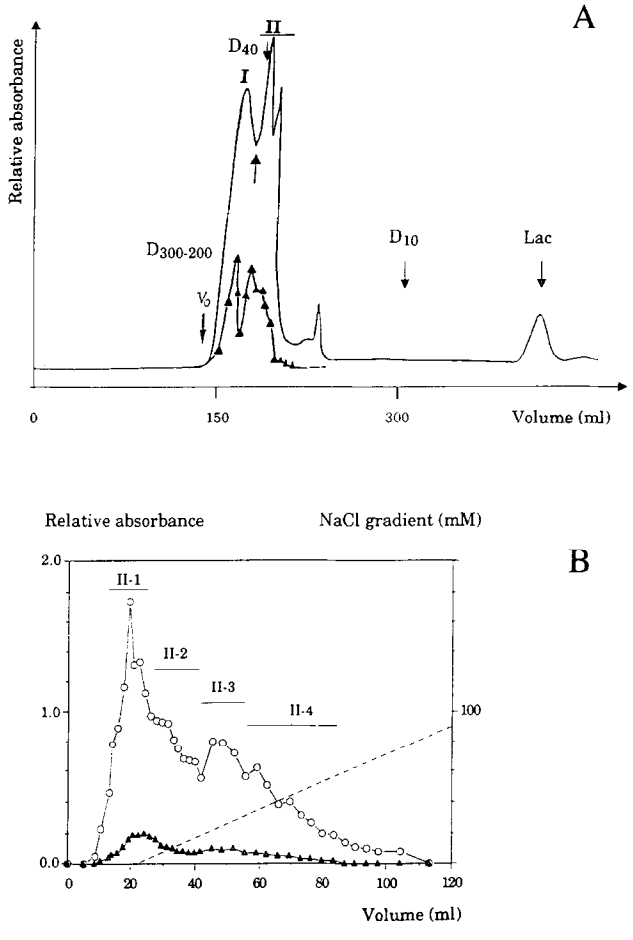


Fig. 1. Chromatography of SF0·1 and its fractions. (A) SEC analysis of the fraction SF0·1 on Sephacryl S-200 HR. Amplitudes are the relative intensity of absorbance at 206 nm (—); ↑, elution position of RF-X; Vl_o, void volume of the column. ↓, relative elution positions of dextran sulfate standards ($D_{300-200}$, D_{40} and D_{10}) and lactose (Lac). (B) (SHAEC) analysis of fraction II on Spherosil LS DEA. (– – –), NaCl gradient from 0 to 0·15 M in 25 mM AcONa buffer, pH 6·6. Curves are expressed as relative absorbance at 480 nm (—○—, total sugar) and at 520 nm (—▲—, uronic acid).

Table 1. Monosaccharide composition of SF0·1 subfractions obtained on Sephacryl HR S-200 and on Spherosil DEA columns

Fractions (w %)[b]	Rha	Fuc	Ara	Xyl	Man	Glc	Gal	GalA	GlcA	4-OMe GlcA
Molar composition (molar %)[a]										
SF0·1 (100·0)	1·1	0·2	11·3	62·4	2·1	1·9	14·6	nd	1·6	4·8
I (50·0)	1·7	nd	16·7	38·0	1·3	1·4	32·9	nd	2·7	5·3
II (50·0)	1·1	tr	4·1	74·2	2·6	2·0	7·6	nd	1·2	7·2
Molar %[c]										
I-1 (29·8)	2·5	nd	16·5	34·4	0·8	3·2	34·8	nd	2·4	5·4
II-1 (17·0)	0·3	nd	3·1	75·9	3·4	5·0	6·6	nd	tr	5·3
II-2 (11·0)	0·3	nd	0·8	74·8	nd	2·1	8·0	nd	nd	14·2
II-3 (7·0)	nd	nd	2·5	79·7	0·7	3·6	7·2	nd	tr	6·3
II-4 (6·0)	0·6	nd	2·5	80·0	0·5	1·2	5·9	nd	0·8	8·5

[a]Values obtained by combining GLC data of alditol acetates and trimethylsilylated methylglycosides (TMSG).
[b]Weight percentage relative to SF0.1.
[c]Values of TMSG derivatives.
nd, not detected; tr, trace.

former was composed of AGII and xylans, and the latter was enriched with 4-O-methylglucuronoxylan of heterogeneous molecular sizes.

Since SF0·1 represents xylan and AGII populations, the use of a specific endoxylanase will shed light on the possible co-occurrence of arabinogalactans II in the xylan fractions, particularly fraction I. Indeed, assuming that these two different polysaccharides are co-eluted, the hydrolysis of all xylans should not change the elution position of AGII polymers on the S-200 HR column. In addition, this fraction should not contain xylose at the reducing end. Conversely, the change of elution position of the residual polymers having xylose at the reducing end, and the occurrence of oligosaccharides containing both xylans and AGII, should support the hypothesis of a covalent linkage between AGII and xylans.

Characterization of the fraction SF0·1

After hydrolysis of SF0·1 by endoxylanase and EtOH precipitation, we obtained two fractions; one in the supernatant (S_3; 54·0 w % of SF0·1) containing acidic and neutral oligomers and the other in the pellet (P_3; 46·0 w %) containing the resistant polysaccharides.

1. Oligosaccharide analysis

Oligosaccharides in the supernatant were fractionated by anion-exchange chromatography (Dowex)/SEC (P-2, P-6)/TLC and HPLC (see Fig. 2). The elution on Bio-Gel P-2 was monitored by TLC analysis using appropriate neutral (NS: X2-5 with DP 2-5) and acidic oligosaccharide standards (AS: 2b, 4-O-Methyl-GlcA-tetraxylose and 3c-b, 4-O-Methyl-GlcA-pentaxylose) which had, respectively, DP of 5 and 6 (Debeire *et al.*, 1990).

The supernatant S_3 gave an acidic fraction (AF: 36·7 w %) and a neutral fraction (NF: 11·7 w %) by ion-exchange chromatography (see Fig. 2). TLC analysis of

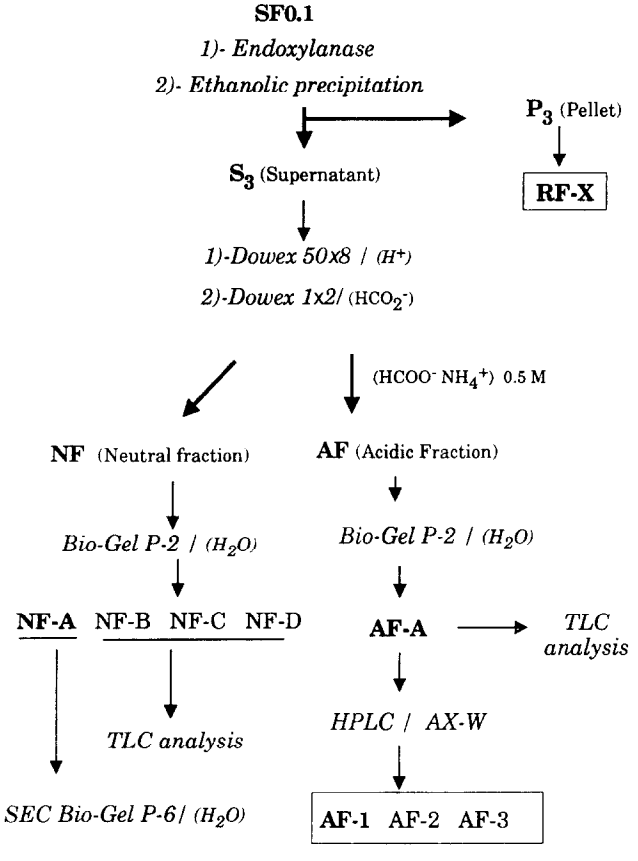


Fig. 2. Diagram of oligosaccharide determination in the supernatant of SF0·1 hydrolysed by 39 kDa xylanase. NF-A D, fractions of NF; and AF-A, AF-1, -2, -3, fractions of AF.

the AF fractions eluting from Bio-Gel P-2 indicates that it contained larger oligosaccharides in aliquots 28–32 (Fig. 3A), and two major smaller oligosaccharides (from aliquot 32) having the same reference front (R_f) as standards 2b and 3b-c. In an attempt to confirm this initial result, the AF-A fraction (23·4 w %; Ara: 1·2 mol %; Xyl:

87·6; Man: 1·4; Glc: 4·5; Gal: 2·9; and 4-O-MeGlcA: 2·4) corresponding to pooled aliquots (28–38), was further characterized by HPLC on a weak anion exchange column using the same standards (Fig. 3B). Since the major fraction AF-1 (14·8 w %) was mainly composed of xylose (83·2 mol %) and 4-O-MeGlcA (16·8 mol %), an average DP of 6 was estimated. It corresponded approximately to those of the two smaller oligosaccharides. The fact that AF-1 and R_f had the same HPLC elution and R_f as the standards also, showed it consisted of small acidic oligosaccharides similar to the standards. In addition, the linkage composition of AF-A (Table 2) gave an average DP of 5·6 (corresponding to the ratio: total xylose residues/2-O-plus 3-O-Me-Xyl) confirming that the DP ranged between 5 to 6.

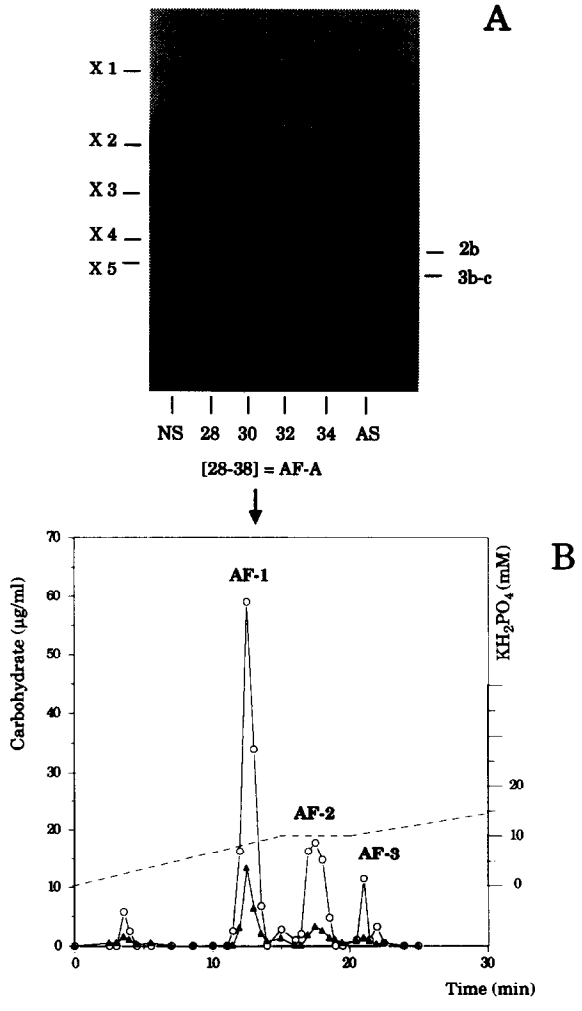


Fig. 3. Chromatographic identification of acidic oligosaccharides. (A) TLC analysis of AF elution on Bio-Gel P-2 (computerized picture of TLC chromatogram; 28, 30, 32 and 34 denote the position number of eluted aliquots; NS, neutral standards (X2-5); X1, xylose; AS, acidic standards, 2b [4-O-MeGlcAxylotetrose] and 3b-c [4-O-MeGlcAxylopentoses]). (B) HPLC analysis of AF-A on AX-W column (concentration expressed in μg/ml of Xyl and GlcA equivalents; arabic numerals represent the number of uronic acid equivalent, AF-1 = 1 uronosyl residue, AF-2 = 2, ...). —○—, total carbohydrate; —▲—, uronic acid; and — — —, gradient of KH_2PO_4.

Table 2. Methylation analysis of the main acidic subfraction AF-A

Methyl ethers[a] PMMA	Proposed linkage[b]	Relative molar %[c]
2,3,4-Me_3-Rha	1- or T-Rha→	0·4
2,3,4-Me_3-Xylp	T-Xyl*p*	4·3
2,3-Me_2-Xyl	→4-	75·0
2-Me-Xyl	→3,4-	3·3
3-Me-Xyl	→2,4-	14·0
2,3,4,6-Me_4-Galp	T-Gal*p*→	0·7
2,4,6-Me_3-Galp	→3-	0·4
2,3,4-Me_3-Galp	→6-	0·5
2,4-Me_2-Galp	→3,6-	0·4
2,3,6-Me_3-Glc	→4-	1·0

[a]2,3,4-Me_3-Rha denotes 1,5-di-O-acetyl-2,3,4-tri-O-methyl-rhamnitol, etcetera.
[b]T-Xyl*p*→, →3-Gal denote, respectively, terminal non-reducing xylopyranose end and 3-O-linked galactose, ectcetera.
[c]Values expressed relative to effective carbone response (e.c.r.) (Sweet *et al.*, 1975).

Furthermore, it was observed that other glycosyl residues (Ara, Gal, Glc and Man) also occurred in the AF-A fraction. Permethylation analysis revealed that galactosyl residues were mainly 3-O-, 6-O and 3-, 6-O-linked, indicating the occurrence of AGII (Table 2). Since these neutral glycosyl residues were not detected in the AF-1 fraction, the AGII-containing carbohydrates should be oligosaccharides of higher molecular mass or containing more uronic acids, and probably correspond to the minor fractions such as AF-2, AF-3 (Fig. 3B). They may explain the difference of 8·6 w % between AF-A and AF-1.

Therefore, analysis of the acidic oligosaccharides showed that they corresponded to a α-4-O-methylglucurono(1→2)-xylotetrose (2b) and a mixture of two isomers of *a*-4-O-methylglucurono(1→2)xylopentose (3b-c). According to Debeire *et al.* (1990), their structures are as follows:

β-Xyl(1→4)β-Xyl(1→4)β-Xyl(1→4)β-Xyl(1 **2b**
2
↑
1
α-4-O-MeGlcA

β-Xyl(1→4)β-Xyl(1→4)β-Xyl(1→4)β-Xyl(1→4)β-Xyl(1
2 2
↑ ↑
1 or 1
α-4-O-MeGlcA
⇓ ⇓
(3b) (3c)

As follows for the neutral fraction NF, its SEC analysis on Bio-Gel P-2 (Fig. 4A) indicated that it consisted of four neutral fractions: NF-A (4·9 w %), -B

(2·6 w%), -C (3·4 w%) and -D (0·5 w%). No uronic acid was detected in these materials (Table 3). Further identification by TLC analysis (Fig. 4B) with the neutral oligosaccharide standards, demonstrated that NF-B, -C and -D were, respectively, identified as a β-(1,4)-xylotriose, a β-(1,4)xylobiose and xylose mixed with an unknown compound (☆). The latter were quantitatively insignificant. In addition, the focus of monosaccharides (Ara, Gal, Glc and Man) on the NF-A fraction (Table 3) meant that these compounds, especially Ara and Gal, might belong to AGII-containing oligosaccharides. To this end, the analysis of NF-A on a Bio-Gel P-6 column revealed two main sugar fractions, respectively A (≈60%) with K_{av} [0·20–0·47] and B (≈40%) with K_{av} [0·48–0·56] whose only sugar residue at their reducing ends (RE) was xylose. They were made of Ara/(Xyl; Re)/Man/Glc/Gal, respectively 4·5 mol%/(70·7; 7·5)/1·7/4·7/10·9 for A and 2·1/(59·0; 22·9)/0·5/4·0/11·5 for B.

According to these data, the average DP of fraction A is about 12·5–13·3, and that of B is about 4·2–4·4. These DP values are in accordance with the elution profile from the P-6 column. Thus, the recovery of xylose, arabinose and galactose in these neutral fractions, demonstrated that NF-A contained neutral high molecular weight oligosaccharides originating from complex xylan. Such data cannot but enhance the suggestion of the involvement of AGII in some oligosaccharidic fractions.

2. Characterization of the residual polysaccharide, RF-X

Recovering only xylose at the reducing end (Table 3; Fig. 5) supports the view that RF-X had a xylan backbone. The fact that no difference was obtained by the two reduction methods used (see Experimental), indicated that there is no alkali-labile O-glycosidic linkage between sugar residues and other compounds (proteins, peptides, ...). In addition, proteins or amino acids were not evidenced by protein assay and TLC analysis respectively, after hydrolysis of RF-X (data not shown). The determination of sugar residues at the reducing end

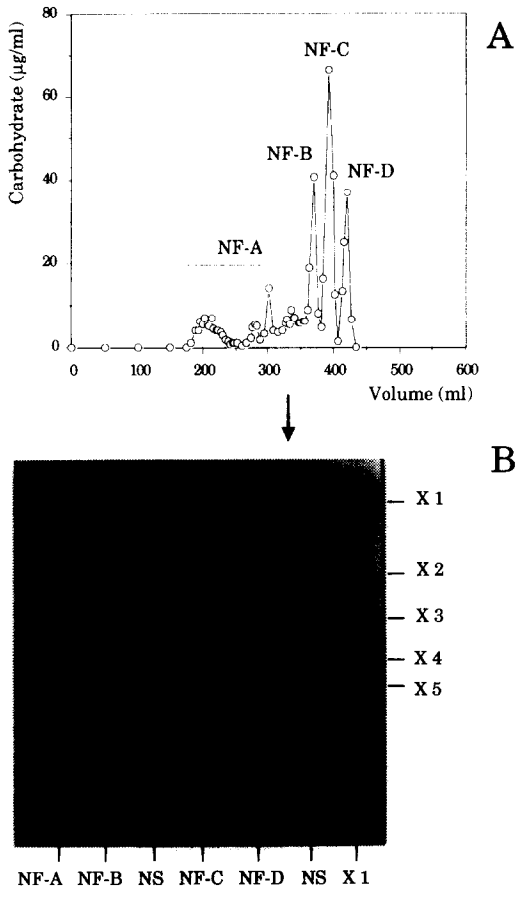


Fig. 4. Chromatographic identification of neutral oligoxylans. (A) Chromatogram of the neutral fraction NF on Bio-Gel P-2 (μg/ml of Xyl equivalent. (B) TLC analysis of NF subfractions (NF-A, -B, -C and -D): computerized picture of photo of TLC chromatogram, NS, neutral xylan standards (X2–5 = DP2–5); X1, Xyl; ☆: unknown compound.

(RE) by GLC/EI-MS permitted the estimation of its average DP (Table 3) expressed either as total Xyl vs RE (minimum DP) or as Xyl + Ara + (4-OMe)-GlcA/RE (maximum DP). A minimum DP of 24 and a maximum DP of 85 were estimated.

Since it is known that most arabinogalactans II

Table 3. Composition of neutral subfractions and of the residual fraction purified on Sephacryl HR S-200 column. Identification of the monosaccharides located at the reducing end of the polysaccharides in the RF-X

	Molar composistion (molar %)[a]									
Fractions (w%)[b]	Rha	Fuc	Ara	Xyl	Man	Glc	Gal	GalA	GlcA	4-OMe GlcA
NF (11·7)	nd	nd	9·8	83·8	1·3	2·7	2·4	nd	nd	nd
NF-A (4·9)	nd	nd	5·1	72·3	5·7	5·8	11·1	nd	nd	nd
RF-X (41·3)	2·0	nd	26·0	12·0	2·2	1·2	51·6	nd	3·5	1·0
RE[c]				0·5						

[a]Values obtained by combining GLC data for alditol acetates and trimethylsilylated methylglycosides (TMSG).
[b]Weight percentage relative to SF0·1.
[c]Sugar residue at the reducing end (RE).
nd, not detected.

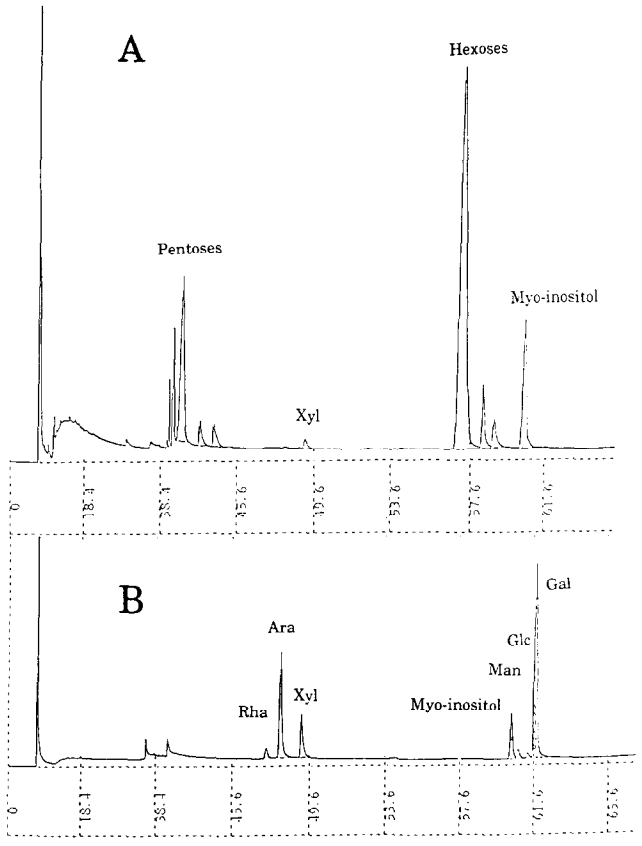


Fig. 5. GLC determination of the composition of RF-X. (A) Composition of the reducing end: reduction with $NaBD_4$, hydrolysis and acetylation. (B) Total composition of RF-X: reduction with $NaBD_4$, hydrolysis reduction with $NaBH_4$ and acetylation.

consist of galactose, arabinose and GlcA (Fincher *et al.*, 1983), the actual molecular mass is between the two limits 3,500–13,000 Da. The majority of the material would be expected to be retained on the S-200 HR column (Fig. 1A). Nevertheless, RF-X was eluted at a position ($K_{av} \approx 0{\cdot}13$) marked by an arrow in Fig. 1A. It was situated between fractions I ($K_{av} \approx 0{\cdot}10$) and II ($K_{av} \approx 0{\cdot}16$), corresponding to an approximate molecular mass of 40 kDa ($K_{av} \approx 0{\cdot}15$) according to calibration with dextran sulfate standards (Fig. 1A).

Moreover, upon alkaline hydrolysis followed by ethanol addition, RF-X yielded two fractions, ethanolic pellet and supernatant, containing the same monosaccharides characterizing both xylan and AGII (data not shown). Thus, the only explanation for the slight SEC retention of RF-X ($K_{av} \approx 0{\cdot}13$) is the integration of the AGII into the structure of the xylan molecules. This is consistent with previous results relevant to the AGII-containing high oligosaccharides (NF-A). At this stage, it was clear that AGII were covalently linked to xylans. Furthermore, the linkage composition of RF-X clearly indicated the substitution at O-2 of one-third of xylose, following the ratio of 4-O-linked plus 2,4-O-linked xylosyl residues over 2-O- substituted xylose (Table 4). The minimal DP of homoxylan was approxi-

Table 4. Methylation analysis of RF-X

Methyl ethers[a] PMMA	Proposed linkage[b]	Relative molar %[c]
2,3,4-Me_3-Rha	1- or T-Rha→	1·1
2,3,5-Me_3-Araf	T-Ara*f*→	5·7
3,5-Me_2-Araf	→2-	0·3
2,5-Me_2-Araf	→3-	0·5
2,3-Me_2-Ara	→5-	12·6
2-Me-Ara	→3,5-	0·2
2,3,4-Me_3-Xylp	T-Xyl*p*→	1·7
2,3-Me_2-Xyl	→4-	10·2
2-Me-Xyl	→3,4-	0·3
3-Me-Xyl	→2,4-	5·0
2,3,4,6-Me_4-Galp	T-Gal*p*→	10·6
2,4,6-Me_3-Galp	→3-	9·0
2,3,4-Me_3-Galp	→6-	10·3
2,4-Me_2-Galp	→3,6-	24·3
2,3,4,6-Me_4-Manp	T-Man*p*	0·2
2,3,6-Me_3-Man	→4-	3·1
2,3-Me_2-Man	→6,4-	1·8
3,6-Me_2-Man	→2,4-	0·6
2,3,6-Me_3-Glc	→4-	2·6
2,3-Me_2-Glc	→6,4-	0·5

[a] 2,3,4-Me_3-Rha denotes 1,5-di-O-acetyl-2,3,4-tri-O-methyl-rhamnitol, etcetera.
[b] T-Ara*f* →, → 2-Ara*f* denote, respectively, terminal non-reducing arabinose end and 2-O-linked arabinose, etcetera.
[c] Values expressed relative to effective carbone response (e.c.r.) (Sweet *et al.*, 1975).

mately 10. One-third of arabinose was attached to the terminal non-reducing end, and two-thirds were 5-O-linked, indicating arabinan side-chains. Galactose was found in 3-, 6- and 3, 6-O-linked positions which reflects a typical highly substituted AGII. Finally 4- and 4, 6-O-linked glucose and mannose may attest to the presence of free or linked glucan or (gluco)mannan.

^{13}C-NMR analysis of RF-X confirmed to the occurrence of xylans and AGII. The former was distinguished by the following signals: C-1 at 102·2 ppm and C-5 at 65·7 ppm (T-βXyl→), 63·5 ppm (→4-Xyl), 63·4 ppm (→2,4-βXyl) and 63·0 ppm (→2,3,4-βXyl) according to the data of previous experiments (Debeire *et al.*, 1990; Hoffmann *et al.*, 1992). The detection of the terminal non-reducing xylose (T-βXyl→) confirmed the oligosaccharidic nature of the xylan, even though C-1 of the reducing end was not observed because of its high DP (≥ 10) and the low level present. Moreover, a low signal at 59·9 ppm may correspond to C-5 of the α anomeric form of the reducing end, whereas the signal of its β form was not recognised among other C-5 signals (63·3–63·6 ppm) of xylosyl residues.

As regards AGII, they were defined by the C-1 signals of galactose and arabinose as follows: 109·6 ppm (T-α-Ara→), 108·1 ppm (→5-α-Ara), 104·1 ppm (T-β-Gal→), 103·9 ppm (→3-β-Gal), 103·8 ppm (→6-β-Gal) and 103·2 ppm (→3,6-β-Gal) (Joseleau *et al.*, 1977; Cartier

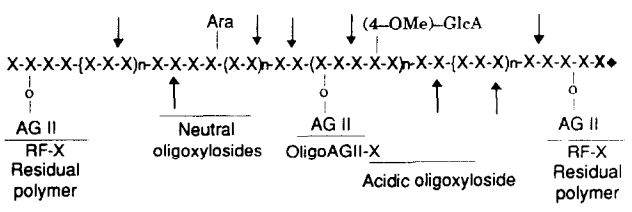
et al., 1987; Hervé Du Penhoat *et al.*, 1987; Seymour *et al.*, 1990; Saulnier *et al.*, 1992). The occurrence of terminal rhamnose at the non-reducing end was indicated by signals at 98·1 ppm (C-1) and 17·1 ppm (C-6). Finally, a low signal at 127–128 ppm might be attributed to the C-1 of phenolic compounds, ferulic or diferulic, and thus might suggest the participation of ferulic or diferulic acids.

CONCLUSION

According to our present results, RF-X was produced by the hydrolysis of fraction I, while most oligosaccharides, particularly acidic and neutral low oligosaccharides, mainly originated from fraction II. Moreover, AGII moieties were bound to xylan oligomers by O-ether linkages forming AGII-bearing xylans (AGII-X), whereas O-ester linkages might be implicated in cross-linking AGII-X fractions *via* phenolic acids carried by AGII and xylans. These aromatic compounds may be oxidized by extracellular peroxidases to give diferulic acids (Fry, 1982; Guillon & Thibault, 1990; Ishii, 1991). The absence of galacturonic acid implies the pectic origin of AGII, and the linkage between pectic polysaccharides and xylans, as earlier suggested (Nishitani & Nevins, 1989; Selvendran & King, 1989), should not be considered. The occurrence of proteins, peptides or amino acids was not detected in the RF-X hydrolysate (≈30 mg/ml) by TLC analysis up to deposited amounts of 30 μg.

Furthermore, the following results: (i) recovery of AGII in both oligosaccharidic fractions (AF-A and NF-A) and the residual fraction RF-X; and (ii) the discovery of xylose as a single reducing end after hydrolysis of SF0·1 by homogeneous enzyme, allowed us to propose a structural model of the xylan substrate named 'Arabinogalactan II-bearing xylan (AGII-X)': The following scheme represented the action of the xylanase on this extracellular AGII-X.

As a result, arabinogalactans II were neither specific to pectic polysaccharides nor proteins, but they are involved in the structure of large wall components. By substituting several wall constituents (pectins, proteins and xylans), AGII made these polymers more soluble



X: xylose residues; X◆: reducing end; Ara:arabinose residues; (4-OMe)-GlcA: 4-O-methylglucuronic acid; n: repetition number; AGII: arabinogalactans type II; AGII-X, arabinogalactan II–xylan; →: endoxylanase accessibility; ò: covalent linkages such as oside–oside; O-glycosidic linkage.

and then, facilitated their cell wall transit. Thus, these results propose that wall solubility or fluidity is generated by the structural involvement of AGII-containing polymers. Furthermore, the finding of AGII in other plant polysaccharides may be in agreement with many works that have argued the biological properties of AGII-containing polymers, such as the internalization of extracellular oligosaccharide signal molecules *via* plasma membrane-associated arabinogalactan-rich glycoproteins (Norman *et al.*, 1990) and/or *via* pectic mediators of cell wall porosity (Baron-Epel *et al.*, 1988).

Finally, in the light of our results and the previous work, the structural implications of arabinogalactans II in plant cell wall may be considered to be their contribution to the affinity (ligand, epitope) and size exclusion properties of the plant cell wall within the framework of the wall porosity.

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