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Carbohydrate Research 277 (1995) 291–311

CARBOHYDRATE RESEARCH

Metabolism of xyloglucan generates xylose-deficient oligosaccharide subunits of this polysaccharide in etiolated peas ☆

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Received 31 March 1995; accepted 26 May 1995

Abstract

Oligosaccharide subunits of xyloglucan were isolated from the stems and roots of etiolated pea plants and structurally characterized. The two most abundant subunits of pea xyloglucan are the well-known nonasaccharide, XXFG, and heptasaccharide, XXXG. In addition, significant amounts of oligosaccharides that have not previously been reported to be subunits of pea xyloglucan were detected, including a decasaccharide, XLFG, two octasaccharides, XLXG and XXLG, a pentasaccharide, XXG, and a trisaccharide, XG. Several novel oligosaccharide subunits, including the octasaccharide, GXFG, and the hexasaccharide, GXXG, were also found.

Xyloglucan oligosaccharides generated by treatment of intact pea stem cell walls were compared to oligosaccharides generated by endoglucanase treatment of xyloglucan polysaccharides obtained by subsequent alkali extraction of the same cell walls. The results suggest that the xyloglucan in etiolated pea stems is distributed between at least two domains, one of which is distinguished by its enzyme accessibility. We further hypothesize that the chemical modification of a xyloglucan during cell-wall maturation depends on its physical environment (i.e., the domain in which it resides). For example, only the endoglucanase-released material, representing the

☆ This article is number XL in the series entitled The Structure of Plant Cell Walls.

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SSDI 0008-6215(95)00220-0

enzyme-accessible xyloglucan domain, contains significant amounts of the two unusual oligosaccharide subunits, GXXG and GXFG, both of which have a nonreducing terminal glucosyl residue. This structure may be generated during cell-wall maturation by the sequential action of an endolytic enzyme (such as xyloglucan endotransglycosylase or endoglucanase) and an α-xylosidase.

Keywords: Xyloglucan; Oligosaccccharide; Pea; Cell wall; Endoglucanase; α-Xylosidase

1. Introduction

Xyloglucans are hypothesized to play an important role in controlling plant cell-wall elongation, thereby affecting plant morphogenesis and growth [1]. Xyloglucans readily bond to cellulose [2] and are thought to form noncovalent crosslinks between cellulose microfibrils, thereby limiting the expansion of the cell wall [3]. It has been suggested that the cleavage of crosslinking xyloglucan molecules by endolytic enzymes is necessary for cell enlargement during growth [4]. Furthermore, some of the xyloglucan oligosaccharides released by endolytic enzymes have biological activity and are capable of inhibiting [5] or promoting [6] cell growth.

The capacity of xyloglucans to act as structural elements of the cell wall depends on the identity and distribution of their side chains. Xyloglucan is transported from its site of synthesis (the Golgi) to the cell wall where it is incorporated into the cellulose–xyloglucan network [4]. Side chains on the xyloglucan cellulosic backbone are necessary to maintain its solubility during this transport process. The specific effects of the side chain substitution pattern on the binding of xyloglucans to cellulose microfibrils during the assembly process are not well understood. It has been suggested, for example, that a "flat" backbone conformation is favored in the vicinity of a fucosylated side chain, facilitating the initial cellulose-binding step [7]. Furthermore, it has been proposed that xyloglucan crosslinks must be enzymatically cleaved for the primary cell wall to expand during growth. The susceptibility of specific glucosyl residues in a xyloglucan molecule to cleavage by such endolytic enzymes depends on the local side chain substitution pattern [8,9]. Thus, the side chain substitution patterns of xyloglucans are likely to have profound effects on their transport, the assembly of the xyloglucan–cellulose network, and the subsequent metabolism of xyloglucans within the plant's primary cell wall.

The biological activities of oligosaccharide fragments of xyloglucans also depend on the structures of their side chains. For example, it has been shown that removal of the fucosyl residue of XXFG destroys its activity as growth inhibitor [10]. Fucosyl residues are not necessary for the growth-promoting activity exhibited by xyloglucan oligosaccharides such as XLLG and XXXG [6]. The growth-promoting effects of oligosaccharides such as XXXG may stem in part from their ability to act as allosteric activators of endoglucanases in the cell wall [11]. These oligosaccharides may also act as acceptor substrates for xyloglucan endotransglycosylase, XET, an enzyme that is proposed to break and reform glycosidic bonds of the xyloglucan crosslinks between cellulose microfibrils [12]. The increase in XET-acceptor substrate concentration that accompanies the addition of these oligosaccharides may increase the rate of XET-catalyzed reactions,

accelerating the cleavage of xyloglucan crosslinks in the cell wall. The growth-promoting oligosaccharides may also act by competing with cellulose-bound xyloglucan molecules as acceptor substrates for XET, further loosening the cell wall by decreasing the probability that crosslinks will be re-formed by the glycosyl-transfer reaction. Based on experiments with small xyloglucan oligosaccharides [13], it has been suggested that removal of a single α-D-Xyl*p* residue at or near the nonreducing end of a xyloglucan can make the xyloglucan unsuitable to act as an acceptor substrate for XET, thus preventing crosslinks that were cleaved by XET from re-forming. However, in order to understand such complex relationships between the enzymes that facilitate cell-wall expansion and their physiological substrates and effectors, it is necessary to further analyze changes in the chemical structures of xyloglucans that are likely to occur during this process.

The structural analysis of xyloglucans has generally been approached by first treating them with an endo-(1 → 4)-β-glucanase in order to cleave the unbranched (1 → 4)-β-D-glucosyl residues of the backbone. The xyloglucan oligosaccharide subunits generated by this enzyme are then purified and characterized [8,9,14–17] using a combination of techniques, including nuclear magnetic resonance (NMR) spectroscopy, fast-atom bombardment mass spectrometry (FABMS), and chemical analyses. The chemical shifts of resonances in the NMR spectra of the borohydride-reduced forms of these oligosaccharides (i.e., oligoglycosyl alditols) have been correlated to the locations and identities of specific structures in these molecules, facilitating their rapid and accurate identification by one-dimensional ^{1}H NMR spectroscopy [9,14–17].

Xyloglucans are routinely solubilized from purified cell walls by treatment with strong alkali (e.g., 4 N KOH). Once extracted from the cell wall, the xyloglucan is normally soluble in aqueous buffer at the pH optimum for the fungal endoglucanase, permitting the enzyme-catalyzed release of oligosaccharide subunits. Alternatively, xyloglucan oligosaccharides can be released directly from purified, depectinated primary cell walls by treatment with the fungal endoglucanase. However, enzyme treatment does not result in complete removal of xyloglucan from the cell wall, and minor differences between the enzyme-released and alkali-released fractions have been observed [18]. This result is consistent with a cell-wall structure in which xyloglucans crosslink cellulose microfibrils, wherein part of the xyloglucan is tightly associated with cellulose microfibrils, and the remaining xyloglucan spans the intervening space [1,4,19,20]. This model predicts that the crosslinking region of the xyloglucan molecule must be accessible to enzymes in order for cell-wall expansion to occur, and that oligosaccharide subunits of the enzyme-accessible xyloglucan should be released by treatment of the intact cell wall with endoglucanase. Some of the enzyme-inaccessible xyloglucan can be released by subsequent treatment with strong alkali. We now report the structural analysis of the xyloglucan oligosaccharides generated by this sequential extraction method. Differences in the oligosaccharide compositions of the enzyme- and alkali-extracted fractions may reflect preferential binding of a xyloglucan domain to cellulose due to a favorable local side-chain substitution pattern, and may also reflect changes in xyloglucan structure that accompany the metabolism of xyloglucans during cell-wall expansion. We discuss the implications of these differences with respect to cell wall structure and growth.

2. Experimental

Isolation of cell walls.—Whole roots and stem sections (apical 2 cm of the third internode) were harvested from 10-day-old etiolated peas (*Pisum sativum*) and immediately frozen in liquid nitrogen. The frozen tissue was suspended in 100 mM potassium phosphate buffer, pH 7, containing 5 mM $Na_2S_2O_5$ and immediately homogenized with a Waring blender. The homogenized tissue was collected by filtration through sintered glass, washed twice more with the same buffer, suspended in aq 0.5% SDS (100 mL) containing 5 mM $Na_2S_2O_5$ and stirred at 4°C for 24 h. The suspension was then filtered through sintered glass, and the residue was washed with 500 mM phosphate buffer (3 × 100 mL) and resuspended in 500 mM phosphate buffer. The tissues were fully disrupted by rapid depressurization using a Parr bomb [21]. Pigments were removed by sequential washing of the disrupted material with phosphate buffer (3 × 100 mL), distilled water (3 × 100 mL), 1:1 methanol–chloroform (2 × 100 mL) and acetone (3 × 100 mL). The residue, composed of cell walls and starch, was air dried. Starch was removed from the cell-wall preparation by treatment of the air-dried residue (1 g) with amylase (type IIA, Sigma, 48 h at 20°C) in 100 mM phosphate buffer, pH 7 (100 mL), containing 0.01% thimerosal. Starch-free cell walls were then washed with deionized water (5 × 100 mL) and dried with acetone.

EPG treatment.—The cell walls were partially depectinated by treatment with a highly purified endopolygalacturonase (EPG) from *Aspergillus niger* [22]. Cell walls were suspended in 50 mM sodium acetate, pH 5.2 (100 mL/g cell walls), containing 0.02% thimerosal. EPG (25 units/g cell walls) was added and the suspension was incubated overnight at room temperature. Residual cell walls were collected by centrifugation at 10,000*g* for 30 min.

EG treatment.—The EPG-treated cell walls (1 g) were incubated (24 h, room temperature) in 10 mM sodium acetate, pH 5.2, 100 mL, containing 0.02% thimerosal and 20 units of EG (β-1,4-endoglucanase, EC 3.2.1.4, sold as *Trichoderma reesei* "cellulase" by Megazyme Australia, Inc.). The enzyme-solubilized material was collected by centrifugation (10,000*g* for 30 min), and the residue was re-treated with an additional 20 units of EG for 48 h. The solubilized materials were pooled, and the anionic components were removed by anion-exchange chromatography on a Q-Sepharose column (60 mL bed volume) equilibrated with 10 mM imidazole · HCl, pH 7. The neutral (nonbound) fraction was concentrated under vacuum and desalted on a Sephadex G-10 column to yield the mixture of xyloglucan oligosaccharides referred to as EG-OXG (~ 17 mg/g cell walls).

Chemical extraction.—The residue obtained after EG-extraction of 1 g of EPG-treated cell walls was washed extensively with water, dried with acetone and sequentially extracted with: *(i)* 0.5 M sodium carbonate containing 0.2% sodium borohydride (200 mL/g EG-residue, 8 h, 4°C); *(ii)* 0.5 M sodium carbonate containing 0.2% sodium borohydride (200 mL/g EG-residue, overnight, room temperature); *(iii)* 4% potassium hydroxide containing 0.2% sodium borohydride (200 mL/g EG-residue, overnight, room temp); *(iv)* 24% potassium hydroxide containing 0.2% sodium borohydride (200 mL/g EG-residue, overnight, room temp). The 24% potassium hydroxide extract was adjusted to pH 6 with M acetic acid and dialyzed against water (2 days) and then against

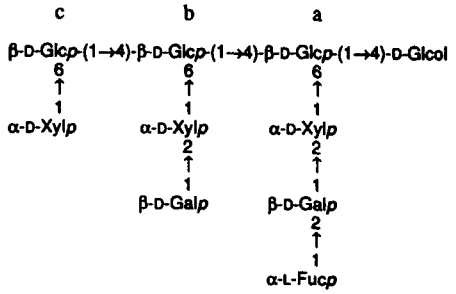
10 mM imidazole · HCl, pH 7 (2 days). Anionic components were removed from the retentate by anion-exchange chromatography on a Q-Sepharose column equilibrated with 10 mM imidazole · HCl buffer, pH 7. The neutral (nonbound) material was desalted on Sephadex G-10. The hexose-containing fractions were pooled, lyophilized, dissolved in acetate buffer, and treated with EG (1 unit/mg polysaccharide in 10 mM sodium acetate buffer, pH 5.2, containing 0.02% thimerosal, room temp for 96 h). The digest was passed through a small Q-Sepharose column (10 mL bed volume) equilibrated with 10 mM imidazole · HCl buffer, pH 7. The nonbound material was desalted on Sephadex G-10. This desalted mixture of xyloglucan oligosaccharides (KOH-OXG, ~10 mg/g cell walls) was lyophilized prior to being characterized.

Purification of xyloglucan oligoglycosyl alditols.—Oligosaccharides in the EG-OXG and KOH-OXG fractions were partially separated according to molecular weight by high-resolution Bio-Gel P-2 chromatography [16]. P-2 fractions were pooled as indicated in Figs 2 and 3, and each pool was reduced with sodium borohydride (2 mL of 10 mg/mL $NaBH_4$ in M NH_4OH, 1 h) to generate oligoglycosyl alditol derivatives of the oligosaccharides. Excess borohydride was quenched with glacial acetic acid, the quenched reaction mixtures passed through Dowex 50 (H^+, 2 mL) to remove sodium ions, and residual borate was removed by coevaporation with methanol. The resulting oligoglycosyl alditols were separated by reversed-phase chromatography on a Phenomenex Sperisorb ODS(1) column (250 × 4.6 mm) and detected by monitoring the refractive index of the eluant. Oligoglycosyl alditols containing 5–10 residues were resolved using 10% methanol as the mobile phase, and oligoglycosyl alditols containing fewer than five residues were separated in 5% methanol.

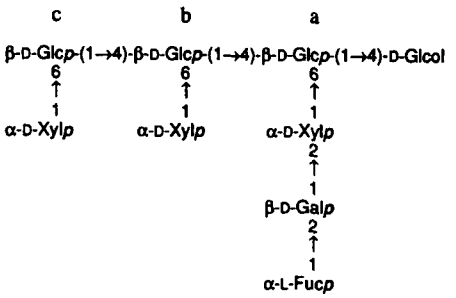
NMR spectroscopy.—Hydroxyl protons of the oligoglycosyl alditols (0.2–2.0 mg) were exchanged with deuterons, samples were dissolved in D_2O (0.5 mL), and NMR spectra were recorded with a Bruker AM 500 NMR spectrometer. Chemical shifts are reported relative to internal acetone at δ 2.225.

FAB-mass spectrometry.—FAB-mass spectra were recorded with a VG analytical ZAB-SE mass spectrometer operating at low resolution (1:1000) with an accelerating voltage of 8 kV. Underivatized oligoglycosyl alditols (1 μL of a ~10 μg/μL solution in H_2O) were mixed on the probe tip with 3-amino-1,2-propanediol (2 μL, Aldrich) for negative-ion FABMS. In addition, oligoglycosyl alditols (100 μg) were per-*O*-acetylated prior to positive-ion FABMS with a mixture of trifluoroacetic anhydride and acetic acid [23]. Each per-*O*-acetylated oligoglycosyl alditol (1 μL of a 10 μg/mL solution in CH_3OH) was mixed on the probe tip with 1-thioglycerol (3-mercapto-1,2-propanediol, 2 μL, Aldrich). The nominal masses reported herein were calculated from the observed monoisotopic exact masses of resolved isotopomers or from the chemical masses of unresolved high-mass ion clusters using the CARBOMASS software developed in this laboratory [24].

Nomenclature for XG oligoglycosyl alditols.—The sequence of xyloglucan oligosaccharides are expressed as a series of uppercase letters, each of which represent the branching pattern of an individual β-D-Glc*p* residue in the backbone [25]. Each code letter represents the entire substructure, including the backbone β-D-Glc*p* residue and its pendant side chain(s). For example, the letter "G" represents a β-D-Glc*p* or reducing D-Glc residue with no side chains attached, and the letter "X" represents a β-D-Glc*p*



XLFGol



XXFGol

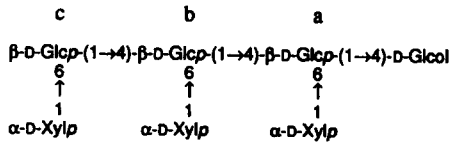
Fig. 1. Chemical structures and succinct names of the xyloglucan oligoglycosyl alditols derived from pea cell walls. The lower case letters above the structures are used to specify individual residues of the oligoglycosyl alditols. A description of this nomenclature is given at the end of the Experimental section.

residue with a terminal α-D-Xyl*p* residue attached at O-6. The structure of a xyloglucan oligosaccharide is thus written by starting at the nonreducing end and sequentially listing, from left to right, the code letters that represent the patterns of side-chain substitution for the β-D-Glc*p* residues in the backbone (Fig. 1). Note that glucitol residues in oligoglycosyl alditols are represented by the code "Gol".

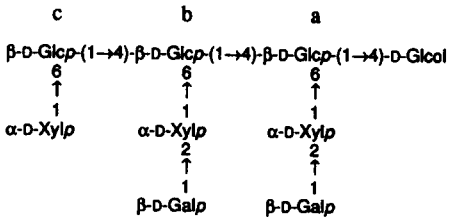
Specific residues within a xyloglucan oligoglycosyl alditol are designated with superscript lowercase letters. These superscripts reflect the position of each residue vis-a-vis the D-glucitol moiety. The order of backbone β-D-Glc*p* residues (starting with the nonreducing terminus and progressing toward the β-D-Glc*p* linked to O-4 of the glucitol) is thus $Glc^c \rightarrow Glc^b \rightarrow Glc^a \rightarrow$ Glcol. Specific side-chain residues are indicated by using the superscript letter of the β-D-Glc*p* residue to which the side chain is attached, as illustrated in Fig. 1.

3. Results

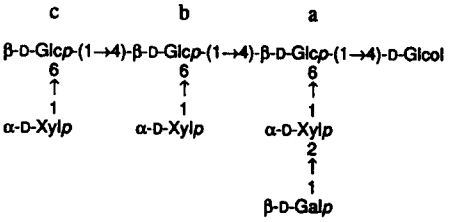
Xyloglucan extraction.—A two-step extraction procedure, in which xyloglucan was sequentially extracted with endoglucanase (EG) and strong alkali, was used to obtain



XXXGol



XLLGol

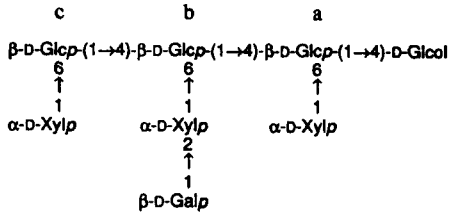


XXLGol

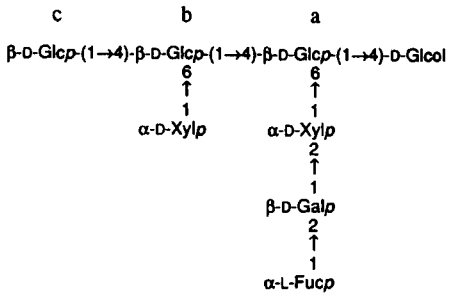
Fig. 1 (continued).

xyloglucan oligosaccharides from the stems and roots of etiolated pea plants. Cell walls prepared from these tissues were first treated with endopolygalacturonase (EPG) to remove pectic components. Subsequent EG treatment released approximately 6% of the EPG-pretreated cell walls. The EG-released material consisted of a mixture of neutral xyloglucan oligosaccharides (EG-OXG, ~ 70%) and anionic components (~ 30%) that were removed by ion-exchange chromatography on Q-Sepharose. The EG-OXG fraction was not completely resolved from salts during subsequent gel-permeation chromatography on G-10, as approximately 60% of this neutral fraction consisted of glucose and cellobiose, the products expected to form when cellulose is treated with EG. Similar results were obtained regardless of whether the cell walls were from roots or from stems.

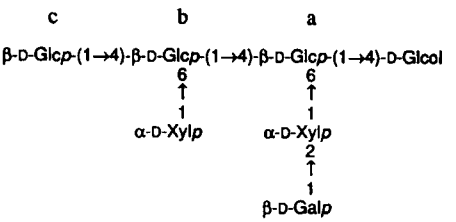
The residue from the EG-treatment was sequentially extracted with sodium carbonate, 4% KOH, and finally with 24% KOH. Approximately 1% of the residual walls was solubilized by 24% KOH. Ninety-five percent of this material consisted of neutral polysaccharides which were not bound to Q-Sephadex and which were completely resolved from salts during gel-permeation chromatography. These neutral polysaccha-



XLXGol



GXFGol

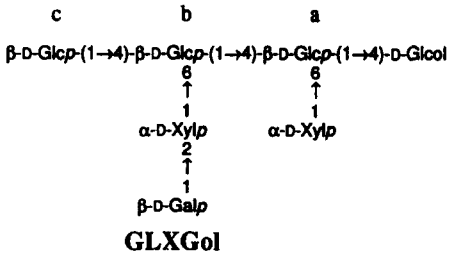


GXLGol

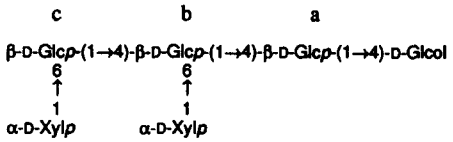
Fig. 1 (continued).

rides, consisting mostly of xyloglucans, were treated with EG to generate xyloglucan oligosaccharides, providing a fraction labeled KOH-OXG that could be directly compared with the EG-released, neutral oligosaccharide fraction EG-OXG. It is likely that some xyloglucan remained in the cell-wall residue after 24% KOH extraction, but this residue was not analyzed.

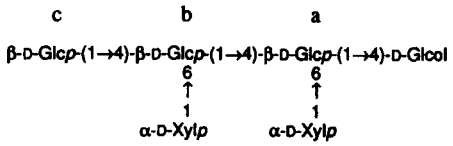
Bio-Gel P-2 chromatography.—The various xyloglucan oligosaccharide mixtures were initially resolved according to their degrees of polymerization by gel-permeation chromatography on a high-resolution Bio-Gel P-2 column. The P-2 elution profile of EG-OXG obtained from stems [Fig. 2(A)] reveals that it is a complex mixture of oligosaccharides. Glycosyl composition analysis of the material eluted at the void



GLXGol



XXGGol

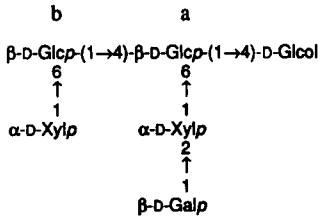


GXXGol

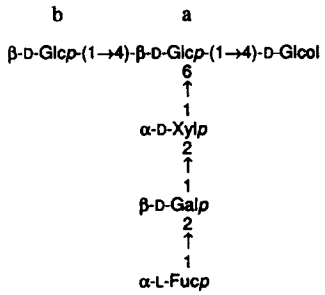
Fig. 1 (continued).

volume of the column indicated that its main component is probably a xylan or arabinoxylan. No further analyses were carried out on this fraction. Oligosaccharide fractions that were partially included in the Bio-Gel P-2 matrix are labeled according to the dp of their dominant component(s). For example, the first of these partially included fractions (labeled "12–20") is a heterogeneous mixture of xyloglucan oligosaccharides (dp 12–20) whose structures were not further analyzed. The glycosyl compositions of fractions labeled 10–3 indicate that they are composed predominantly (> 80%) of xyloglucan oligosaccharides. These oligosaccharides are the primary focus of this paper and were characterized by HPLC and spectroscopic analysis (see below). The major components of fractions labeled 2 and 1 were established as cellobiose and glucose, respectively, by comparison of their ^{1}H NMR spectra with those of authentic standards.

Differences in the size-distribution of oligosaccharides in the KOH-OXG and EG-OXG fractions from pea stems can be seen in the Bio-Gel P-2 profiles for these two fractions (Fig. 2). In addition, Bio-Gel P-2 chromatography of stem KOH-OXG resulted in peaks that are much sharper than those obtained for stem EG-OXG, indicating that the KOH-OXG fraction contains fewer oligosaccharide components than EG-OXG. The P-2 profiles of EG-OXG and KOH-OXG obtained from roots [Fig. 3(A) and 3(B)] are



XLGol



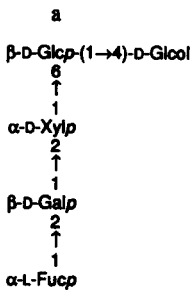
GFGol

Fig. 1 (continued).

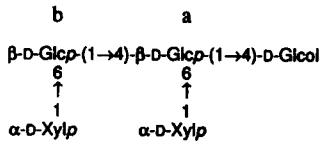
similar to those of the corresponding oligosaccharide fractions obtained from stems [Fig. 2(A) and 2(B)].

Reversed-phase chromatography.—Individual Bio-Gel P-2 fractions were reduced with sodium borohydride and further separated by reversed-phase HPLC, allowing them to be characterized by ^{1}H NMR spectroscopy and FABMS (see below). The identities and amounts of the oligosaccharide components of the various cell wall extracts, their degrees of polymerization, and the HPLC retention times of their oligoglycosyl alditol derivatives on ODS are summarized in Table 1.

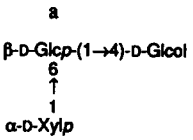
^{1}H NMR spectroscopy.—The HPLC-purified oligoglycosyl alditols were characterized by 1D ^{1}H NMR. Previously described xyloglucan oligoglycosyl alditols (i.e., XLFGol, XXFGol, XXLGol, XLXGol, XXXGol, XXGol and XGol) were readily identified by comparison of their ^{1}H NMR spectra with authentic standards. The chemical shifts of the anomeric resonances of these oligosaccharides were in good agreement ($\pm$0.005 ppm) with those published [8,9,14–17] for the standard compounds. However, the chemical shifts of several of these anomeric resonances are temperature-dependent, and therefore minor differences between the previously published and newly measured chemical shifts were observed because the spectra were recorded at slightly different temperatures. The anomeric resonances of novel xyloglucan oligoglycosyl alditols were assigned on the basis of previously deduced correlations between the chemical shifts of these resonances and characteristic structural features [8,9,14–17]. Chemical structures of the novel oligoglycosyl alditols were proposed on the basis of



FGol



XXGol



XGol

Fig. 1 (continued).

these assignments and substantiated by fast-atom bombardment analyses (see below), which confirmed the glycosyl composition and glycosyl sequence of the oligoglycosyl alditols.

Several of the novel oligoglycosyl alditols (e.g., GXXGol, GXLGol and GXFGol) lack an α-Xyl residue at C-6 of Glc^c (i.e., the β-Glc residue at the nonreducing end of the cellulosic backbone). The structures of these xylose-deficient oligoglycosyl alditols were deduced by comparing their ^1H NMR spectra to those of the analogous oligoglycosyl alditols having a full complement of α-Xyl residues. Diagnostic effects were observed in the NMR spectra of pairs of oligoglycosyl alditols (e.g., XXFGol versus GXFGol and XXXGol versus GXXGol, Table 2 and Fig. 4) in which one member of the pair lacks an α-Xyl^c residue. For example, the NMR spectrum of the oligoglycosyl alditol lacking an α-Xyl*p* residue at C-6 of Glc^c contains no resonance at δ 4.94 and the anomeric resonance of Glc^c of these xylose-deficient oligoglycosyl alditols is shifted upfield by approximately 0.03 ppm compared with the Glc^c H-1 resonance of the corresponding oligoglycosyl alditol with a full complement of α-Xyl residues. The

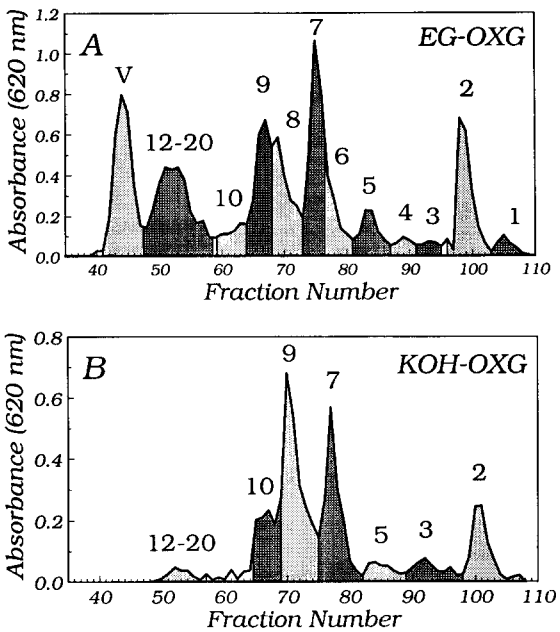


Fig. 2. Biogel P-2 profiles of xyloglucan oligosaccharides obtained from etiolated pea stems. (A) EG-OXG, oligosaccharides directly generated by EG-treatment of isolated stem cell walls. (B) KOH-OXG, oligosaccharides generated by EG-treatment of the 4 M KOH-solubilized xyloglucan. Pooled fractions are labeled according to the dp of their dominant component.

chemical shift of Glc^b H-1 is shifted very slightly upfield (< 0.01 ppm) when Xyl^c is absent, but the H-1 chemical shifts of the remaining residues are not significantly affected by the absence of Xyl^c.

One of the novel pea xyloglucan oligoglycosyl alditols, XXGGol, lacks the α-Xyl residue that is usually found at C-6 of Glc^a (i.e., the β-Glc residue linked directly to the alditol). The ^1H NMR spectrum of XXGGol (Table 2) exhibits several differences compared with that of XXXGol, which contains an α-Xyl^a residue, but is otherwise structurally identical to XXGGol. The presence of only one resonance in the region δ 4.95–4.97 of the spectrum of XXGGol (compared with two signals in this region for XXXGol) is consistent with the absence in XXGGol of either Xyl^a or Xyl^b. A resonance at δ 4.94 in the spectra of both oligoglycosyl alditols indicates that both have a terminal α-Xyl residue at C-6 of Glc^c (see above). The anomeric resonances of Glc^a and Glc^b in the spectrum of XXGGol are both shifted upfield relative to the corresponding resonances in XXXGol. This shielding effect is more pronounced for Glc^a ($\Delta\delta = 0.035$) than for Glc^b ($\Delta\delta = 0.021$), indicating that the "missing" α-Xyl residue in XXGGol is indeed Xyl^a.

Fast-atom bombardment mass spectrometry.—The glycosyl sequence of several of the xylose-deficient oligoglycosyl alditols were confirmed by negative-mode FABMS of the underivatized oligoglycosyl alditols and positive-mode FABMS of their per-*O*-acetylated derivatives. For example, the negative-ion FAB spectrum of GXFGol in-

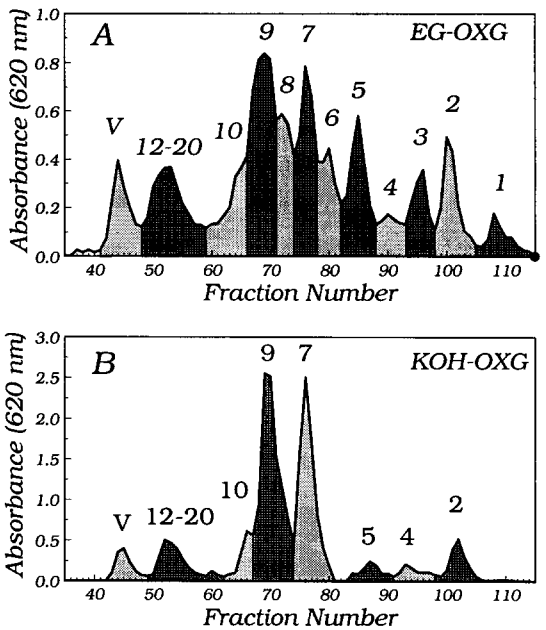


Fig. 3. Biogel P-2 profiles of xyloglucan oligosaccharides obtained from etiolated pea roots. (A) EG-OXG, oligosaccharides generated by EG of isolated root cell walls. (B) KOH-OXG, oligosaccharides generated by EG treatment of 4 M KOH-solubilized xyloglucan. Pooled fractions are labeled according to the dp of their dominant component.

cludes an abundant $[M-H]^-$ ion at m/z 1239 ($FucGalXyl_2Glc_3Glcol$), along with fragment ions [26] at m/z 783 (Y_5^-, FucGalXylGlcGlcol) and m/z 1077 (Y_7^-, $FucGalXyl_2Glc_2Glcol$), indicating that Glc^a bears the FucGalXyl side chain and Glc^b bears a terminal Xyl residue. The negative-ion FAB spectrum of GXXGol includes an $[M-H]^-$ ion at m/z 931 (Xyl_2Glc_3Glcol), along with a fragment ion at m/z 769 (Y_5^-, Xyl_2Glc_2Glcol), indicating that Glc^a and Glc^b each bear a terminal Xyl residue. Thus, GXFGol and GXXGol each contain only two Xyl residues, which are attached to Glc^a and Glc^b, with no xylosyl substituent on Glc^c.

The structure of XXGGol was confirmed by positive-ion FABMS of the per-*O*-acetylated derivative. The pseudomolecular $[M+NH_4]^+$ ion at m/z 1748 confirms the glycosyl composition Xyl_2Glc_3Glcol. Single cleavage B^+ ions [14] at m/z 547 XylGlc and 1051 Xyl_2Glc_2 indicate that Glc^c and Glc^b each bear a Xyl substituent. Low-abundance ions were detected at m/z 793 and 835, corresponding to the composition $XylGlc_2$. However, the m/z 835 ion was even less abundant than the m/z 793 ion, indicating [14] that the m/z 835 and m/z 793 ions result from two cleavage events, and are not due to the absence of a Xyl residue on either Glc^c or Glc^b. The single-cleavage B_5^+ ion at m/z 1339 Xyl_2Glc_3 indicates that one of the Glc residues does not bear a Xyl substituent. Together, these data indicate that the Glc residue without a side chain is Glc^a, confirming the structure XXGGol deduced by NMR analysis. The structure of GFGol was confirmed by similar mass spectral analysis.

Table 1
Oligosaccharide subunit compositions [a] of pea xyloglucan fractions

Oligosaccharide	dp [b]	HPLC Retention [c]	Stems		Roots	
			EG-OXG	KOH-OXG	EG-OXG	KOH-OXG
XLFG	10	21.2	12	8	7	6
XXFG	9	23.7	22	33	14	37
GXFG	9	7.9	4	— [d]	4	— [d]
XLXG + XXLG	8	10.4	14	8	9	4
GXLG	7	3.8	— [d]	— [d]	2	— [d]
GLXG	7	5.1	— [d]	— [d]	1	— [d]
XXXG	7	9.4	25	33	11	38
XXGG	6	4.9	— [d]	— [d]	1	— [d]
GXXG	6	4.4	13	— [d]	5	— [d]
XLG	6	4.8	— [d]	— [d]	5	— [d]
GFG	6	5.1	— [d]	— [d]	1	— [d]
FG	5	5.6	— [d]	— [d]	1	— [d]
XXG	5	NA [e]	10	6	20	6
XG	3	NA [e]	— [d]	12	15	10

[a] Normalized mol%, calculated by colorimetric analysis (anthrone) of P-2 fraction and relative refractive index of HPLC fractions.
[b] Oligosaccharides were initially separated according to dp by Bio-Gel P-2 chromatography (see Figs 1 and 2).
[c] Retention time (min) of oligoglycosyl alditol derivative on Spherisorb ODS1 column eluted with 10% methanol at 1.0 mL/min.
[d] Not detected.
[e] NA indicates not applicable, because HPLC of these two derivatives were run under different conditions.

Comparison of EG-OXG and KOH-OXG.—The chemical structure of the portion of pea xyloglucan that can be extracted by EG is significantly different from the portion that requires strong alkali for solubilization, as indicated by analysis of the oligosaccharide compositions of the EG-OXG and KOH-OXG fractions (Table 1). The commonly occurring nonasaccharide XXFG and heptasaccharide XXXG were found in both EG-OXG and KOH-OXG from both stem and roots, along with significant amounts of decasaccharide, XLFG, octasaccharides, XLXG and XXLG, and pentasaccharide, XXG. However, the xylose-deficient oligosaccharides, GXFG, GXLG, XXGG, and GXXG were found only in the EG-solubilized fractions, and not in the KOH-solubilized fractions.

Differences were also found when xyloglucan oligosaccharide extracts from etiolated stems were compared with those from roots. For example, root EG-OXG contained higher levels of the quantitatively minor xylose-deficient oligosaccharides GXLG and XXGG than did stem EG-OXG. A much higher percentage of the pentamer XXG was found in root EG-OXG. However, it should be noted that in other harvests the amount of XXG was similar (~10 mol%) in stem EG-OXG and root EG-OXG. In addition, significant variability in the amounts of the xylose-deficient oligosaccharides was observed, but in all cases the xylose-deficient oligosaccharides were found only in the EG-released material.

Table 2
Anomeric proton resonances [d] of pea XG oligoglycosyl aldtiols

Oligoglycosyl alditol	Residue								
	Glc [a]	Glc [b]	Glc [c]	Gal [a]	Gal [b]	Xyl [a]	Xyl [b]	Xyl [c]	Fuc [a]
XLFGol	4.631	4.525	4.533	4.620	4.549	5.130	5.178	4.943	5.257
XXFGol	4.620	4.539	4.548	4.620	—	5.134	4.953	4.942	5.256
GXFGol	4.619[e]	4.534	4.517	4.622[e]	—	5.136	4.953	—	5.257
XXLGol	4.621	4.562	4.551	4.555	—	5.169	4.959	4.942	—
GXLGol	4.620	4.558	4.515	4.558	—	5.165	4.955	—	—
XLGol	4.622	4.542	—	4.556	—	5.171	4.941	—	—
XLXGol	4.640	4.582	4.533	—	4.556[f]	4.951[f]	5.179	4.942	—
GLXGol	4.640	4.572	4.506	—	4.556	4.953	5.178	—	—
XXXGol	4.631	4.588	4.551	—	—	4.955	4.960	4.941	—
GXXGol	4.632	4.583	4.516	—	—	4.957	4.960	—	—
XXGGol	4.596[g]	4.567[g]	4.554	—	—	—	4.957	4.941	—
XXGol	4.632	4.568	—	—	—	4.958	4.942	—	—
XGol	4.614	—	—	—	—	4.934	—	—	—
GFGol	4.619	4.483	—	4.619	—	5.134	—	—	5.256
FGol	4.582[e]	—	—	4.617[e]	—	5.125	—	—	5.274

[a,b,c] These superscripts indicate the position of the residue vis-a-vis the alditol end.
[d] Chemical shifts (δ) in ppm relative to internal acetone (δ 2.225).
[e] The assignments of these pairs of resonances may be reversed.
[f] The Gal[b] and Xyl[a] H-1 resonances of XLXGol were obscured by much larger resonances of XXLGol, and so the chemical shifts of these two resonances in the spectrum of the standard XLXGol sample [16] are indicated.
[g] These two resonances were assigned on the basis of 2D TOCSY analysis of XXGGol prepared from tobacco xyloglucan (data not shown).

Are the xylose-deficient oligosaccharides genuine components of the cell wall?—The observed differences between EG- and KOH-extracted xyloglucans may have important implications vis-a-vis the processes of plant cell-wall growth and development (see Discussion). Therefore, it is critical to determine whether these differences reflect structural differences in the primary cell wall rather than artifacts of the extraction procedure. Experiments were performed to rule out the possibility that xylose-deficient oligosaccharides such as the octasaccharide GXFG and hexasaccharide GXXG were formed during the extraction procedure by degradation of the commonly occurring oligosaccharide subunits such as the nonasaccharide XXFG and heptasaccharide XXXG. An α-xylosidase capable of catalyzing the degradation of these substrates has been isolated from growing pea tissues [27]. Therefore, it was necessary to determine whether this enzyme activity was present at any stage of the extraction procedure.

The possibility of contaminating α-xylosidase activity in the fungal EG used to prepare the EG-OXG fractions was ruled out because no xylose-deficient oligosaccharides were found in the KOH-OXG fractions, which were generated by treatment of the KOH-solubilized polysaccharides with the fungal endoglucanase. Nevertheless, this conclusion was confirmed by incubating the oligoglycosyl alditol XXXGol with the EG under conditions comparable to those used for preparation of EG-OXG from cell walls. When the EG-treated XXXGol was analyzed by HPLC, a single peak corresponding to

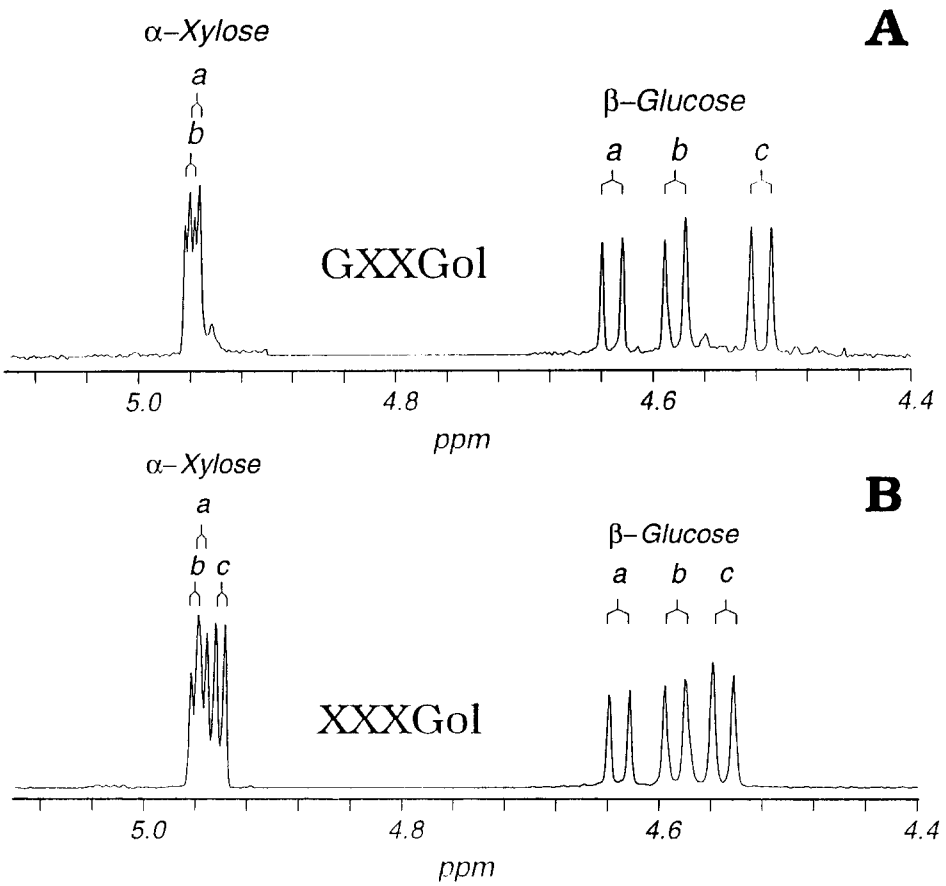


Fig. 4. Anomeric resonances in the ^{1}H NMR spectra of (A) GXXGol and (B) XXXGol from etiolated pea stems. The lower-case letters indicate the location of each residue vis-a-vis the alditol (see Experimental). GXXGol does not have an α-Xyl residue at C-6 of Glc^c, and, therefore, its ^{1}H NMR spectrum (A) does not have an α-Xyl^c H-1 signal at δ 4.940 that is present in the spectrum of XXXGol (B). In addition, the anomeric resonance of Glc^c in GXXGol is 0.035 ppm upfield relative to the Glc^c H-1 resonance in XXXGol. The GXXGol preparation contains ~ 15% XXGol as a contaminant. The HDO resonance and its sidebands were zeroed for clarity.

XXXGol (Table 1) was detected at 9.3 min, confirming that the EG preparation did not contain detectable amounts of α-xylosidase. The α-amylase and endopolygalacturonase preparations were analyzed using xyloglucan oligosaccharide substrates under conditions comparable to those used for cell-wall extraction (see Experimental). These analyses indicated that the α-amylase and endopolygalacturonase were also free of α-xylosidase activity.

The α-xylosidase found in growing pea tissues acts only at the nonreducing end of the xyloglucan backbone [27] and, therefore, can only release significant amounts of xylose when the xyloglucan has been already cleaved into fragments by an endolytic enzyme, such as EG. In order to determine whether residual α-xylosidase activity was

present in the cell wall during or after the EG-extraction step that generates the potential substrates for the α-xylosidase, the oligoglycosyl alditol XXXGol was incubated with pea-stem cell walls that had been prepared as if they were to be treated with EG. The oligoglycosyl alditol was then recovered from the cell-wall preparation by centrifugation, and the supernatant was analyzed by HPLC. No degradation products of XXXGol were detected, indicating that the pea cell wall did not contain detectable α-xylosidase activity at the time when its oligosaccharide substrates were being generated by EG.

Finally, the possibility that α-xylosidase could have generated the novel oligosaccharides during the process of cell-wall preparation was checked by comparing the following four methods: *(i)* tissue was harvested and extracted according to the standard method (see Experimental); *(ii)* the tissue was harvested and extracted according to the standard method, except that the final treatment with chloroform–methanol was omitted; *(iii)* the tissue was harvested and immediately frozen in liquid nitrogen, suspended in boiling ethanol for 20 min and then extracted according to the standard method; *(iv)* tissues were harvested and extracted according to the standard method, except tissues were not frozen in liquid nitrogen, all the extractions were performed at room temperature, and no chloroform–methanol treatment was performed. The cell walls prepared by these methods were treated with EG to extract xyloglucan oligosaccharides. If degradation of xyloglucan oligosaccharides had occurred during cell-wall preparation, treatment of the frozen tissue with boiling ethanol would most likely decrease the level of degradation while failure to chill the tissue and/or treat with denaturing solvents would probably increase the amount of degradation. However, the method of cell-wall preparation made no significant difference in the amount of GXLGol that was detected by HPLC analysis of the extracts. We conclude that GXLG and other xylose-deficient oligosaccharides (Table 1) are genuine components of the xyloglucan in the primary cell wall of peas.

4. Discussion

Previous studies [4] have concluded that pea xyloglucan is composed primarily of an alternating sequence of nonasaccharide, XXFG, and heptasaccharide, XXXG. However, we have also detected significant amounts of other oligosaccharide subunits of pea xyloglucan, including a decamer XXFG, two octamers XLXG and XXLG, a pentamer XXG, and a trimer XG. In addition, pea xyloglucan contains several novel, xylose-deficient oligosaccharides, such as GXFG and GXXGol. These results indicate that pea xyloglucan has a more complex structure than previously recognized.

Significant differences exist in the oligosaccharide subunit composition of the EG-extractable xyloglucan compared with that of the xyloglucan, whose extraction requires concentrated alkali. Most notably, xylose-deficient oligosaccharides, GXFG, GXXG, and XXGG, are present exclusively in the EG-extractable fraction, suggesting that this EG-accessible portion of xyloglucan is modified by plant enzymes during the development of the cell wall. The remaining xyloglucan, part of which can be extracted with concentrated alkali, does not appear to be modified by plant α-xylosidase(s), perhaps because its close association with cellulose limits its accessibility. The existence of an EG- and α-xylosidase accessible xyloglucan domain is consistent with the theory

[4,19] that xyloglucan forms load-bearing crosslinks between cellulose microfibrils in the growing cell wall, and that these crosslinks form a dynamic structure that is enzymatically modified during the growth process.

The xylose-deficient oligosaccharides detected in the EG-OXG fractions could have been released from the nonreducing ends of xyloglucans that had been enzymatically processed during cell wall development (Fig. 5). Plant endoglucanases [4] may facilitate cell-wall elongation by hydrolyzing xyloglucan crosslinks between cellulose microfibrils. The action of such endolytic enzymes on xyloglucans would generate nonreducing ends that are substrates for glycosidases found in the plant cell wall, including an α-xylosidase that selectively cleaves the glycosidic bonds of α-Xyl residues attached to C-6 of β-Glc residues at the nonreducing end of a xyloglucan chain [27]. Thus, the sequential action of a plant endoglucanase and an α-xylosidase would generate xyloglucan molecules that are xylose-deficient at their nonreducing termini. This, in turn, would result in the release of xylose-deficient oligosaccharides when the cell walls are extracted with fungal EG.

The generation of xylose-deficient oligosaccharides could also involve EG-catalyzed hydrolysis at a site where two unbranched (4-linked) β-Glc residues are adjacent in the xyloglucan backbone [Fig. 5(A) and (B)]. Such sites could theoretically be generated during the initial biosynthesis of the xyloglucan in the Golgi. Alternatively, the adjacent unbranched glucosyl residues may result from the action of a xyloglucan endotransglycosylase (XET) [28], an enzyme, found in the cell walls of growing plant tissues, that cleaves xyloglucans at the mid-chain and transfers the newly formed (potentially reducing) terminus to the nonreducing end of another xyloglucan chain. XET-catalyzed transfer of one xyloglucan chain to the xylose-deficient (nonreducing) terminus of another, modified xyloglucan chain [Fig. 5(A)] would produce a β-(1 → 4)-linkage between two unbranched glucosyl residues in the backbone. The available evidence indicates that the fungal EG specifically hydrolyzes the glycosidic bonds of unbranched (4-linked) β-Glc residues and cannot hydrolyze the glycosidic bonds of branched (4 → 6-linked) β-Glc residues. Therefore, the structure of the oligosaccharide products formed by this mechanism would depend on which of the two adjacent, EG-susceptible glycosidic bonds is hydrolyzed [Fig. 5(B)]. That is, oligosaccharide products that are xylose-deficient at the nonreducing end would arise by hydrolysis of the glycosidic bond between the unbranched β-Glc residues [Site A, Fig. 5(B)], and products that are xylose-deficient at the reducing end would arise by hydrolysis of the other EG-susceptible glycosidic bond [Site B, Fig. 5(B)].

Oligosaccharides that are xylose-deficient at the nonreducing end, such as GXXG, were found in much greater amounts than oligosaccharides that are xylose-deficient at the reducing end, such as XXGG (Table 1). This suggests that either *(i)* the fungal EG preferentially hydrolyzes the glycosidic bond at Site A, between the two unbranched β-Glc residues or *(ii)* internal sites having adjacent, unbranched (4-linked) β-Glc residues are rare in pea xyloglucan, and the majority of the xylose-deficient oligosaccharides are released from α-xylosidase-modified nonreducing ends of polymeric xyloglucan within the wall. Further investigation is required to determine whether such internal, xylose-deficient sites exist in pea xyloglucan and, if so, whether the fungal EG prefers site A or site B.

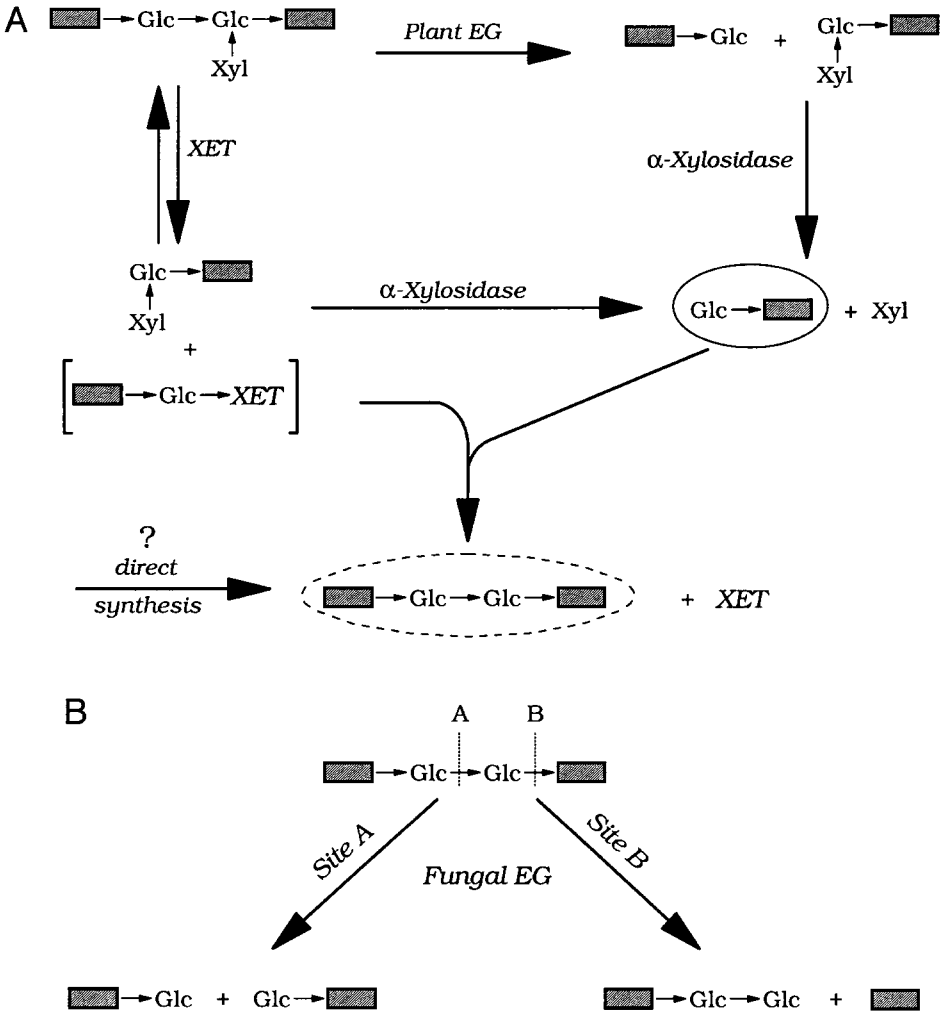


Fig. 5. (A) Possible in vivo pathways leading to xylose-deficient oligosaccharide fragments of pea xyloglucan. Only the two glucosyl residues that are connected by an EG-susceptible bond are shown, with the remainder of the xyloglucan depicted as a rectangle. Endogenous plant endoglucanase (EG) and/or xyloglucan endotransglycosylases (XET) can generate nonreducing ends that are potential substrates for the plant's α-xylosidase. The modified nonreducing end (circled with solid line) may give rise to the xylose-deficient oligosaccharides GXFG, GXLG, GLXG and GXXG, that are recovered following fungal EG treatment. Furthermore, if the modified nonreducing end is an acceptor substrate for XET, then sites (circled with dashed line) having two, adjacent nonbranched β-Glc residues could be formed by a transglycosylation reaction. Hydrolysis of such sites during the fungal EG could also release xylose-deficient oligosaccharides. (B) EG-catalyzed hydrolysis at sites containing two adjacent, unbranched β-Glc residues generates different xylose-deficient oligosaccharides, depending on which glycosidic bond is cleaved. Several oligosaccharides, namely GXFG, GXLG, GLXG, and GXXG, that would, by this scheme, arise from hydrolysis at site A were detected, but of the oligosaccharides that could arise from hydrolysis at site B, only XXGG was detected.

The α-xylosidase found in growing cell walls might regulate the activity of the XET by changing the concentration of XET acceptor substrates, because the presence of xylose-containing side chains at or near the nonreducing terminus appears to be necessary for xyloglucan oligosaccharides to be good acceptor substrates for the XET [13]. However, the extent to which the absence of a single α-Xyl residue at the nonreducing terminus of a xyloglucan polysaccharide may reduce its ability to act as an acceptor substrate is not known, because the requirement for α-xylosyl residues at the nonreducing terminus has only been demonstrated [13] for small oligosaccharides, having just three glucosyl residues in the backbone. If α-xylosidase-modified nonreducing ends of polymeric xyloglucan are competent acceptor substrates for XET, then the detection of XXGG, an oligosaccharide that is xylose-deficient at the reducing end, may indeed be the manifestation of an XET-catalyzed transfer reaction, as suggested above. It is unlikely that XXGG is derived from sites with adjacent, unbranched β-Glc residues that are directly formed during xyloglucan synthesis with the Golgi, because no such xylose-deficient oligosaccharides are detected in the KOH-OXG fraction that represents an enzyme-inaccessible portion of the xyloglucan. Clearly, further analysis of the substrate specificity XET is required to understand the etiology of oligosaccharides such as XXGG and to unambiguously demonstrate whether the α-xylosidase plays a role in the regulation of XET. Furthermore, analysis of residual xyloglucan not extracted by EG and KOH may provide evidence for the existence of other xyloglucan domains not described in this paper.

Acknowledgements

This research is supported by United States Department of Energy (DOE) grant DE-FG05-93ER20115 (to AD), and by the DOE-funded (DE-FG05-93ER20097) Center for Plant and Microbial Complex Carbohydrates. R.G. wishes to thank the Ministerio Español de Educación y Ciencia and the Fullbright Commission of the United States for a Fullbright grant funding his stay at the CCRC.

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