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Structural Characterization of Physiologically Active Polysaccharides from Natural Products (*Arabidopsis*)

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Abstract To determine the functions of specific cell wall polysaccharides, polysaccharides of three mutants, *mur3-1*, *mur3-2*, and *mur3-3*, obtained from *Arabidopsis* wild type, underwent structural characterization. Upon sequential separation of pectins (RG-I and RG-II) and cross-linking glycans (xyloglucan, XG), only XG was affected by the *mur3* mutation. Wild-type XG contained a considerable amount of fucose, whereas the fucose level in *mur3* XGs was less than 20% that of wild type. Further analysis of XGs by matrix-assisted laser-induced/ionization time-of-flight (MALDI-TOF) mass spectrometry indicated that *mur3* lines considerably or completely lost the fucosylated XG oligosaccharides such as XXFG and XLFG and the double-galactosylated oligosaccharide XLLG. ^{1}H-NMR spectroscopic analyses of the XG oligosaccharides from *mur3-3* plant revealed the absence of fucose and a galactose level in the galactosylated side chain that was reduced by 40% compared to that of *Arabidopsis* wild-type plant. In contrast, 85% less fucose and a slight loss of galactose were observed in the *mur3-1* and *mur3-2* lines which show normal growth habit. Of the three *Arabidopsis mur3* lines studied here, *mur3-3* is disrupted by a T-DNA insertion in the exon of *MUR3* which encodes XG-specific galactosyltransferase, and exhibits slight dwarfism. These results indicated that the T-DNA insertion at the *MUR3* locus did not induce the complete loss of galactose in XG, and that galactose, rather than fucose, in the XG side chains made a major contribution to overall wall strength.

Keywords: structure, polysaccharide, *Arabidopsis*, *mur3*. MALDI-TOF, NMR

Introduction

The cell walls of higher plants determine the boundaries of the plant form, protect against pathogen invasion, and provide mechanical strength to the plant body (1, 2). Cell walls comprise primarily cellulose microfibrils, cross-linking glycans (also referred to as hemicelluloses), pectic polysaccharides and small amounts of structural proteins (2). Compared with other biological macromolecules, little is known about the synthesis and assembly of plant cell walls. The main components of pectic material are homogalacturonans, and the highly branched rhamnogalacturonans I and II (RG-I and RG-II). The major cross-linking glycan in the primary cell wall of most of higher plants is xyloglucan (XG). XGs have a backbone composed of β-D-glucopyranosyl 1, 4-linked residues. In most dicotyledonous plants, the glucosyl residues are substituted at C-6 with α-D-Xyl*p*(1 → 6)- (X), β-D-Gal*p*-(1 → 2)-α-D-Xyl*p*(1 → 6)- (L), or α-L-Fuc*p*(1 → 2)-β-D-Gal*p*-(1 → 2)-α-D-Xyl*p*(1 → 6)- (F) side chains (3, 4). The β-D-Gal*p* residues in these side chains are often O-acetylated (5). However, other types of XG structure have been found in some species (6). XGs fragmented by treatment with endo-(1 → 4)-β-glucanase (EC 3.2.1.4) yield several oligosaccharides which have the basic unit XXXG (6-8). In most of higher plants, this basic unit is more complex, consisting of octa- (XXLG or XLXG), nona- (XLLG, XXFG), and deca-saccharides (XLFG) (6).

XGs are believed to interact non-covalently with cellulose and thereby form a crosslinked network in the primary wall (9, 10). The formation of this network is likely to be important for the structural integrity of the walls. Levy *et al.* (11) have reported that a key component in the association between cellulose and XG is the presence of the L-fucose-containing trisaccharide side-chain. Computer modeling of the XG structure has predicted that fucose-containing XGs adopt spatial conformations more favorable for cellulose binding than nonfucosylated XG. Fucosylated XGs bind cellulose *in vitro* at a 2-fold higher rate than do nonfucosylated XGs (12). However, XG fucosylation is not absolutely required for the formation of cellulose-XG networks (6). Fucose-containing XG is also thought to play a role in the regulation of plant growth. Several studies have shown that XG-derived oligosaccharides, called oligosaccharins, act as inhibitors of auxin-stimulated elongation of pea epicotyls (13). The biological activity of these oligosaccharins depends on the presence of terminal L-fucose (14).

To determine the functions of specific cell wall polysaccharides, *Arabidopsis* mutants with altered monosaccharide composition of their cell wall material have been isolated (15). Three of these mutant lines (*mur1*, *mur2*, and *mur3*) exhibit significantly decreased fucose content in cell wall polymers (15). The *mur1* plants are defective in the interconversion of GDP-D-mannose to GDP-L-fucose (16), which leads to the absence of fucose in glycoproteins, pectins, and XG (17). Whereas *mur1* mutants are completely deficient in cell wall L-fucose levels and slightly dwarfed, *mur3* mutants have approximately a 50% reduction in total cell wall L-fucose content and have a normal growth habit (15). In this paper, we report the structural characterization of polysaccharides of *mur3* mutants obtained from *Arabidopsis thaliana* wild type.

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Materials and Methods

Plant material and growth conditions *Arabidopsis* seeds from wild-type (ecotype Columbia), *mur3-1* and *mur3-2* [mutagenized by ethylmethanesufonate (EMS)], and *mur3-3* (mutagenized by T-DNA insertion) lines were kindly supplied by Dr. W.S. York, CCRC, Univ. of Georgia, USA. All seeds had been back-crossed at least four times (15). Surface-sterilized seeds were chilled in water for 3 days to synchronize the germination response. Soil-grown plants were grown in growth chambers (16 hr of light, 22°C). *Arabidopsis* wild type, *mur3-1*, *mur3-2*, and *mur3-3* plants were grown in potting soil (Fafard 3B; Fafard, Inc., Anderson, SC, USA) in a controlled environmental chamber with a 14-hr-light, 19°C, and 10-hr-dark, 15°C, cycle. The rosette leaves were harvested from 4-week-old plants and stored at -80°C.

Cell wall preparations Alcohol insoluble residue (AIR) was prepared by grinding frozen tissue to a powder under liquid nitrogen. The ground tissue was suspended in 80% (v/v) ethanol (approximately 20 mL/g tissue) and further disrupted using a Polytron homogenizer. AIR was collected on nylon mesh and washed with aqueous 80% (v/v) ethanol and then with absolute ethanol. The washed AIR was suspended in methanol:chloroform (1:1, v/v), stirred for 1 hr at room temperature (RT), collected by filtration through Whatman paper, washed with acetone, and finally air dried.

Partial depectination of AIR AIR was suspended in 10 mL of 50 mM sodium acetate (pH 5, NaOAc) containing 0.01% (w/v) thimerosal. Endo-polygalacturonase (EPG, 5 units) from *Aspergillus niger* and pectin methylesterase (PME, 5 units) from *Aspergillus oryzae* (supplied by Novozymes A/S, Bagsvaerd, Denmark) were added. The suspension was incubated for 24 hr at 24°C in a shaking incubator. The suspensions were filtered, and the solid residues were treated a second time with EPG and PME. The supernatants after enzyme treatment were fractionated by size-exclusion chromatography on a Superdex-75 HR10/30 column (1.3×30 cm; Amersham Biosciences, Uppsala, Sweden) equilibrated at 0.6 mL/min in 50 mM ammonium formate buffer (pH 5.2). The separations were monitored using an HP 1037A Refractive Index-Detector (Hewlett-Packard, Palo Alto, CA, USA) at 30°C. The high and intermediate Mw fractions were pooled, dialyzed and lyophilized to give Fr. P-I and Fr. P-II.

Xyloglucan endoglucanase treatment The partially depectinated AIR was suspended in 10 mL of 20 mM NaOAc (pH 5) containing 0.01%(w/v) thimerosal. XG endoglucanase (XEG) (10 units, supplied by Novozymes A/S) was added (18), and the suspensions were incubated at 24°C for 24 hr in a shaking incubator and then filtered. The filtrate containing XG oligosaccharides was further purified on a Q-Sepharose column (1×5 cm, Amersham Biosciences) eluted with 50 mM ammonium formate, pH 6.4, at a flow rate of 0.1 mL/min. Unabsorbed fractions containing the oligosaccharides (Fr. XN) were pooled and lyophilized several times to remove volatile ammonium formate salts.

Alkali treatment The insoluble residues remaining after XEG treatment were suspended in 10 mL of 1 N KOH and stirred at RT for 24 hr. The suspensions were filtered, and the insoluble residues were then suspended in 10 mL of 4 N KOH. The suspensions were stirred for 24 hr at RT and then filtered. The filtrate was adjusted to pH 5 with glacial acetic acid and dialyzed (3,500 Mw cutoff tubing, Spectrum Laboratories, Rancho Dominguez, CA, USA) against six changes of deionized water over 2 days. The retentates were lyophilized and then digested with XEG (as described above) to generate KOH-solubilized XG oligosaccharides (Fr. K2N). To completely remove the salts, Fr. K2N was further applied to a C-18 cartridge (Supelclean LC-18 SPE tube; Supelco, Bellefonte, PA, USA), which was then washed with water (10 mL) to remove salts. The oligosaccharides were then eluted from the cartridge with 10 mL of aqueous 25%(v/v) methanol. The eluant was concentrated under vacuum and lyophilized.

Reduction of the oligosaccharides The XG oligosaccharides in Fr. XN and Fr. K2N were converted to the corresponding oligoglycosyl alditol derivatives by reduction with $NaBH_4$ (10 mg/mL in 1 M NH_4OH, 20 mL). After being incubated for 1 hr at RT, the solution was chilled (0°C), and the remaining borohydride was converted to borate by dropwise addition of acetic acid. The solvent was evaporated and the residue was desalted on a C-18 cartridge as described above.

Analysis of sugar composition The sugar composition of the polysaccharide samples was determined by gas chromatography (GC) analysis of their alditol acetates. Samples were hydrolyzed with 2 M trifluoroacetic acid for 1.5 hr at 121°C, converted into the corresponding alditol acetates (19, 20), and analyzed by GC at 60°C for 1 min, 60°C→220°C (30/min), 220°C for 12 min, 220°C→250°C (8°C/min), and 250°C for 15 min, using a Hewlett-Packard HP 6890 GC equipped with an SP-2330 capillary column (0.25 μm film thickness, 0.32 mm i.d. × 30 m, Supelco). Molar ratios were calculated from the peak areas and response factors on a flame ionization detector (FID).

Matrix-assisted laser-induced/ionization time-of-flight mass spectrometry (MALDI-TOF MS) MALDI-TOF mass spectra were recorded using a Hewlett-Packard LDI 1700 XP spectrometer operated at an accelerating voltage of 30 kV, an extractor voltage of 9 kV, and a source pressure of approx. 8×10^{-7} Torr (21). The matrix for oligosaccharide analysis was prepared by mixing (1:1 v/v) 2,5-dihydroxbenzoic acid (DHB, 0.2 M) and 1-hydroxy-isoquinoline (HIC, 0.06 M), both in 50% aqueous acetonitrile.

^{1}H-NMR spectroscopy Solutions of XG oligosaccharides in D_2O (0.6 mL, 99.9%; Cambridge Isotope Laboratories, Andover, MA, USA) were analyzed at 25°C using Inova spectrometers (Varian Medical Systems, Palo Alto, CA, USA) operating at 500 and 600 MHz. Five diagnostic regions in the anomeric region of the spectra (Fig. 3. and Table 3) were integrated: (a) δ 5.26, H1 of all *a*-Fuc*p* residues, (b) δ 5.17, H1 of *a*-Xyl*p* residues bearing a

terminal β-Gal*p* residue at O2, (c) δ 5.13, H1 of *a*-Xyl*p* residues bearing either a terminal β-Gal*p* residue or an *a*-Fuc*p*-(1→2)-β-Gal*p* disaccharide at O2, (d) δ 4.94-4.96, H1 of terminal *a*-Xyl*p* residues, and (e) δ 1.25, H6 of all *a*-Fuc*p* residue. The spectra of oligosaccharides from Fr. K2N do not contain any resonances corresponding to *O*-acetylated side chains because of alkali treatment.

Results and Discussion

Glycosyl compositions of polysaccharides from cell walls of *Arabidopsis* wild type and *mur3* lines A cell wall fractionation experiment was undertaken to determine the effect of the *mur3* mutation on each L-fucose-containing cell wall polymer (XG, RG-I, and RG-II). Upon initial separation of pectins and cross-linking glycans, only the latter class of polymers was affected by the *mur3* mutation (Fig. 1). Because the initial fractionation was not sufficient to purify specific polysaccharides to complete homogeneity, XG oligosaccharides were purified from the alkali-soluble fractions *via* XEG digestion followed by a column chromatography using Q-Sepharose. The monosaccharide compositions were determined by GC. Wild-type XG contained a considerable amount of fucose, whereas only small amounts (less than 20% of wild type) were present in *mur3* XG (Table 1). The relative amount of galactose was almost the same in K2N fractions of wild type and the three *mur3* lines, but showed a loss of 30-50% of the substitution in XN fractions from *mur3* lines. These data shown in Table 1 also indicated that the missing fucose residues were not replaced by other monosaccharides.

Identification of XG oligosaccharides in XG fractions of *Arabidopsis* wild type and *mur3* lines by MALDI-TOF Further analysis of XGs of wild-type and *mur3* lines by MALDI-TOF supported the results from the glycosyl composition analysis. XEG-generated XG fragments underwent mass determination and were assigned using the classification system of Fry *et al.* (7) to XXXG, XXLG (or XLXG), XXFG, and XLFG that contained either zero or one *O*-acetyl group. These oligosaccharides are composed of XXXG-type building units (22, 23). Table 2 illustrates the relative abundance of each

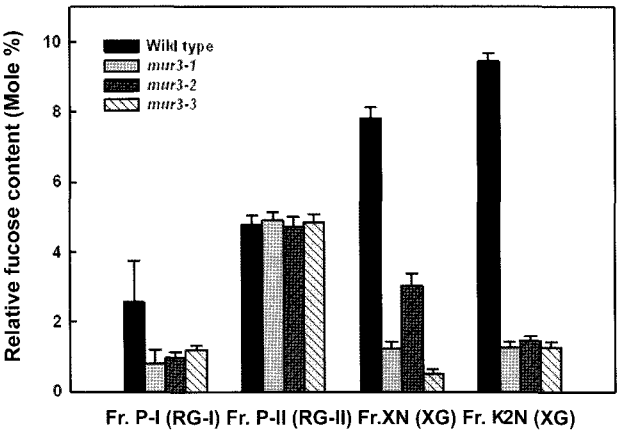

Fig. 1. Fucose content of cell wall polysaccharides in wild type and *mur3* plants. Error bars represent SE from four samples.

XG oligosaccharide. The major difference between wild type and *mur3* lines was the abundance of *m/z* 1,394 and *m/z* 1,556 ions in *mur3* lines, corresponding to the $(M+Na)^+$ ions of the fucosylated XG oligosaccharides XXFG and XLFG, respectively. Especially, XXFG and XLFG were not detected in XN and K2N fractions of *mur3-3* (Fig. 2). The *mur3* samples also contained comparable quantities of the galactosylated oligosaccharides XXLG or XLXG (*m/z* 1,247). However, the *mur3-1* and *mur3-2* samples showed only a trace amount of the double-galactosylated oligosaccharide XLLG (*m/z* 1,410), while the XLLG oligosaccharide was not detected uniquely in *mur3-3* among the three *mur3* lines (Fig. 2).

An *Arabidopsis* mutant, called *mur3*, was selected on the basis of the under-representation of fucose in cell wall polymers (15), and it specifically affected the XG trisaccharide side-group structure. *MUR3* encodes the galactosyltransferase specifically responsible for the first step in the formation of the α-L-Fuc*p*(1→2)-β-D-Gal*p*- side group (24). The *mur3* mutation eliminates the entire disaccharide extension from the first xylose in the instance of leaf-derived XG. L-Fucose- or L-galactose-containing XG fragments are believed to serve as signal molecules regulating extension growth (13, 17). Furthermore, XG fucosylation is thought to facilitate binding of XG to

Table 1. Glycosyl compositions of oligosaccharide fractions released by XEG from the XGs of *Arabidopsis* wild type and its mutants (Mole%)

Glycosyl Residue[1)]	Fr. XN				Fr. K2N			
	Wild type	*mur3-1*	*mur3-2*	*mur3-3*	Wild type	*mur3-1*	*mur3-2*	*mur3-3*
Fuc	7.8	1.3	3.0	0.5	9.6	1.3	1.5	1.3
Ara	1.8	1.4	1.8	2.3	2.5	2.8	3.1	7.2
Xyl	34.6	42.3	38.9	37.5	29.6	31.5	36.3	35.9
Man	-	-	-	-	8.9	9.8	7.7	7.0
Gal	10.7	5.2	7.2	5.7	11.9	10.1	9.3	8.3
Glc	45.1	49.8	49.1	54.0	37.5	44.5	42.1	40.3
	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

[1)]Molar ratio, neutral sugars determined by GC of alditol acetates, and expressed as anhydrosugars.

Table. 2. Distribution of oligomeric units in XN and K2N fractions from leaves of *Arabidopsis* wild-type, *mur3-1*, *mur3-2*, and *mur3-3*

Structure	Oligomer	Relative Abundance			
		Wild-type	*mur3-1*	*mur3-2*	*mur3-3*
X X G-G-G-G X	XXXG	++++	++++	++++	++++
L X X G-G-G-G X	XXLG	+ (*)	+	+	ND[1)]
X X G-G-G-G X L	XLXG	+ (*)	+	+	++
L-F X X G-G-G-G X	XXFG	+++ (*)	+- (*)	+- (*)	ND
L X X G-G-G-G X L	XLLG	+ (*)	+-	+-	ND
L-F X X G-G-G-G X L	XLFG	+++ (*)	+- (*)	+- (*)	ND

++++; High, +; low, +-; trace, (*); existence of acetylated galactose residue.
[1)]Not detected.

cellulose by straightening the glucan backbone, thereby contributing to wall strength and integrity (11, 12). However, *mur3* plants, except for *mur3-3*, have a growth habit and wall strength that is indistinguishable from wild type under a variety of growth conditions, which argues against a role of fucosylated XGs in establishing a strong cellulose–XG network or as a source of growth-regulating signal molecules *in vivo*.

Analysis of fucose and galactose content in XG oligosaccharides from wild type and *mur3* lines of *Arabidopsis* by ^{1}H-NMR spectroscopy A structural reporter approach was used to determine the fucose content of *Arabidopsis* XG oligosaccharides. The enzymatically depectinated AIR was treated with XEG and then with 4 M KOH. XEG treatment of AIR hydrolyzes the 'enzyme-accessible domain' of XG, thereby generating a mixture of *O*-acetylated XG oligosaccharides. Subsequent treatment with 4 M KOH solubilizes much of the remaining XG as a high MW polysaccharide with no *O*-acetyl substituents, which are hydrolyzed under the alkaline extraction conditions. XEG treatment of the alkali-soluble XG generates a mixture of oligosaccharides. Figure 3 illustrates the application of this structural reporter method, showing examples of ^{1}H-NMR spectra of KOH-extracted XG oligosaccharides from 4.8 to 5.3 ppm. The amount of fucose present per unit of oligosaccharide in wild type and *mur3* lines of *Arabidopsis* was determined by ^{1}H-NMR spectroscopy (Table 4). The amount of fucose present in XG was markedly decreased in *mur3* mutants of *Arabidopsis* and especially was not detected at all in the *mur3-3* line (Table 4). In contrast, the galactose content of

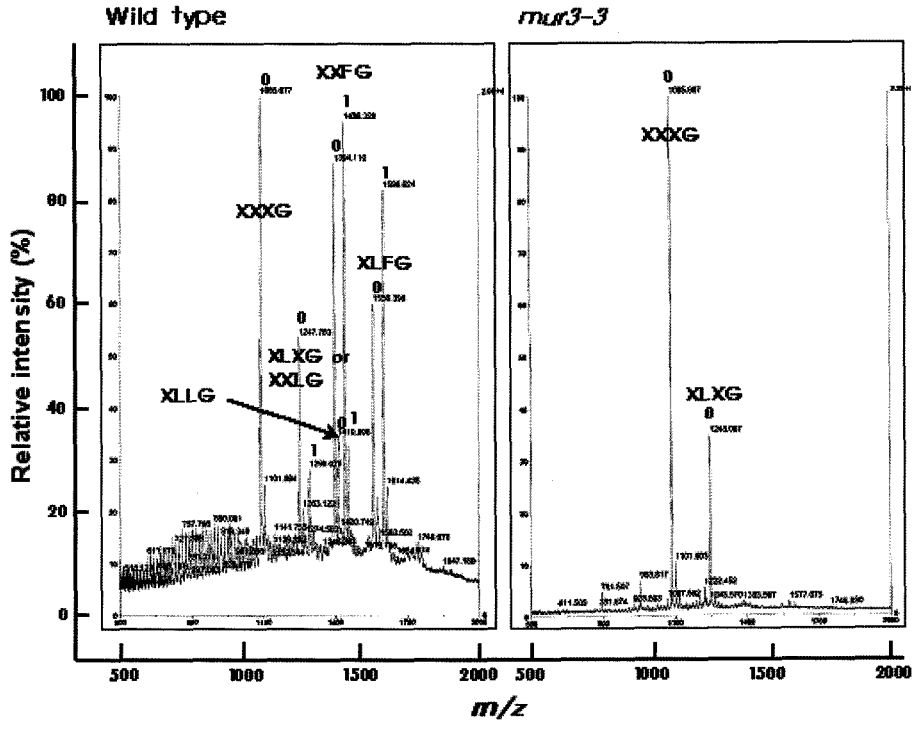

Fig. 2. MALDI-TOF mass spectra of XN fractions obtained from wild type and *mur3-3* xyloglucans. The nomenclature of XG oligosaccharides is illustrated in Table 2. XG-type units corresponding to the observed *m/z* are indicated for individual signals. The numbers 0 and 1 indicate the number of O-acetyl substituents for each oligosaccharide.

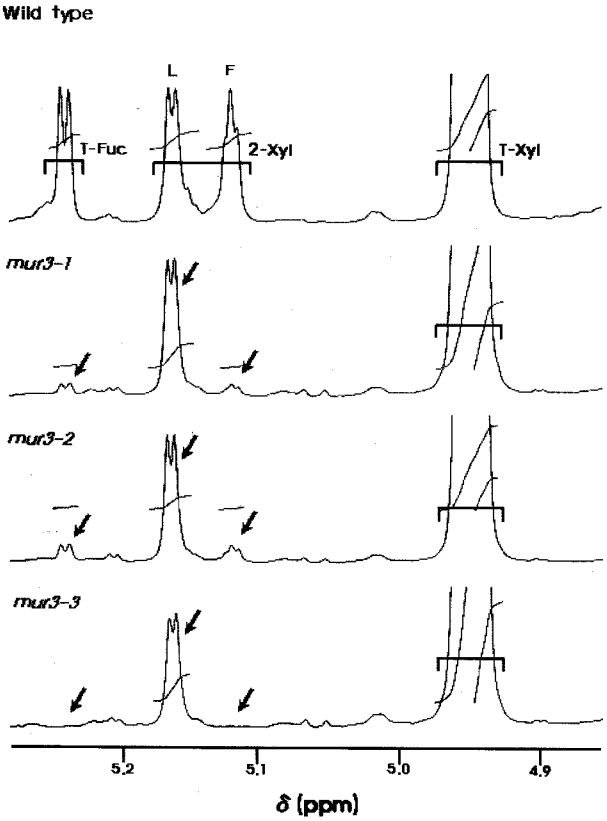

Fig. 3. Partial ^{1}H-NMR spectra of reduced XG oligosaccharides prepared from wild-type and *mur3* xyloglucans. XG oligosaccharides prepared from the 4 N KOH extract from rosette leaves of wild-type *Arabidopsis* and *mur3* lines were also reduced. ^{1}H-NMR spectra of all reduced products were recorded at 500 MHz. Nomenclature of XG oligosaccharides and their side chains are illustrated in Table 2. T-Fuc means non-reducing terminal fucose and 2-Xyl is 2-linked xylose. The arrow indicates the diagnostic region.

XG was slightly decreased in *mur3-1* and *mur3-2* lines, but was decreased to 60% that of *Arabidopsis* wild type in the *mur3-3* line.

Glycosyl composition analysis indicated that the cell walls prepared from leaves of the *mur3* mutant contained about 80% less fucose than wild-type walls did (Table 1). Comparable amounts of fucose were present in RG-I and II isolated from the AIR of wild-type and *mur3* plants (Fig. 1). However, no fucose was detected by ^{1}H-NMR spectroscopic analyses of the XG oligosaccharides in the leaves of the *mur3-3* plant. The level of fucose in the XG in *mur3-1* and *mur3-2* plants was less than 15% of that in wild-type plants (Table 4). The *mur3-1* and *mur3-2* mutants are induced by EMS treatment and have a normal growth habit. However, the *mur3-3* mutant is disrupted by a T-DNA insertion in the exon of *MUR3* which encodes XG-specific galactosyltransferase (24) and is slightly dwarfed. The results of the present study have indicated that this T-DNA insertion at the *MUR3* locus does not induce the complete loss of galactose in XGs, but that the reduced galactose level in the *mur3-3* mutant leads to the complete elimination of fucose residues. It is known that *mur2* is impaired in XG-specific fucosyltransferase, which prevents XG fucosylation (25). Remarkably, the growth habits of *mur2* and *mur3* (EMS-induced mutants) plants are indistinguishable from those of wild type, despite such radical alteration of their fundamental crosslinking polymers (15). The tensile strengths of *mur2* and *mur3* floral stems are also comparable with wild type (24, 25). Thus, if a fucosylated trisaccharide acts to facilitate normal growth

Table 4. Side-chain analysis of XGs in K2N fractions from genetically modified *Arabidopsis*

Line	Side chain per subunit		
	Xyl	GalXyl	FucGalXyl
Wild type	2.13	0.45	0.42
mur3-1	2.59	0.37	0.03
mur3-2	2.54	0.40	0.06
mur3-3	2.72	0.28	ND[1)]

XG was extracted from depectinated cell wall preparations of wild type, *mur3-1*, *mur3-2*, and *mur3-3* rosette leaves, and the relative proportions of the various XG side chains in each extract were determined by ^{1}H-NMR spectroscopy. Data were normalized by setting the total number of side chains per oligosaccharide subunit to 3, consistent with the 'XXXG-type' structure (23) of *Arabidopsis* XG. *O*-Acetyl substituents were hydrolyzed by 4 N KOH treatment.
[1)]Not detected.

Table 3. Diagnostic ^{1}H NMR assignments for the oligoglycosyl alditols in K2N fraction from genetically modified *Arabidopsis*

^{1}H NMR assignments	Related XG oligomer	δ ^{1}H (ppm)			
		Wild type	*mur3-1*	*mur3-2*	*mur3-3*
t-α-Fuc H-1	XXFG*ol*/XLFG*ol*	5.257	5.256	5.256	ND[1)]
2-linked α-Xyl in L side chain H-1	XLXG*ol*/XLFG*ol* XXLG*ol*	5.175 5.165	5.175 5.165	5.175 5.164	5.175 ND
2-linked α-Xyl in F side chain H-1	XXFG*ol* XLFG*ol*	5.134 5.127	5.132 5.127	5.133 5.127	ND ND
t-α-Xyl H-1	X**X**XG*ol* XX**X**G*ol* **X**XXG*ol*	4.958 4.952 4.940	4.959 4.952 4.940	4.959 4.951 4.939	4.958 4.951 4.939
Fuc H-6	XXFG*ol*/XLFG*ol*	1.255	1.256	1.256	ND

[1)]Not detected.

or to increase the tensile strength of the wall during growth, then this functional property can be replaced by the degree of galactosylation. Pena *et al.* (26) reported that the galactose residues of XG were essential to maintain the mechanical strength of the primary cell walls in *Arabidopsis* during growth. In this study, *mur3-3*, which shows slight dwarfism, exhibited an absence of fucose and considerable loss of galactose in XG. By comparing the structural characteristics of *mur2* and *mur3*, we postulate that galactose-containing side chains of XG make a major contribution to overall wall strength, whereas XG fucosylation plays only a comparatively minor role.

Acknowledgments

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