

Characterization of *N*-Glycans from Arabidopsis. Application to a Fucose-Deficient Mutant¹

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The structures of glycans *N*-linked to Arabidopsis proteins have been fully identified. From immuno- and affinodetections on blots, chromatography, nuclear magnetic resonance, and glycosidase sequencing data, we show that Arabidopsis proteins are *N*-glycosylated by high-mannose-type *N*-glycans from Man₅GlcNAc₂ to Man₉GlcNAc₂, and by xylose- and fucose (Fuc)-containing oligosaccharides. However, complex biantennary structures containing the terminal Lewis a epitope recently reported in the literature (A.-C. Fitchette-Lainé, V. Gomord, M. Cabanes, J.-C. Michalski, M. Saint Macary, B. Foucher, B. Cavalier, C. Hawes, P. Lerouge, and L. Faye [1997] Plant J 12: 1411–1417) were not detected. A similar study was done on the Arabidopsis *mur1* mutant, which is affected in the biosynthesis of L-Fuc. In this mutant, one-third of the Fuc residues of the xyloglucan has been reported to be replaced by L-galactose (Gal) (E. Zablackis, W.S. York, M. Pauly, S. Hantus, W.D. Reiter, C.C.S. Chapple, P. Albersheim, and A. Darvill [1996] Science 272: 1808–1810). *N*-linked glycans from the mutant were identified and their structures were compared with those isolated from the wild-type plants. In about 95% of all *N*-linked glycans from the *mur1* plant, L-Fuc residues were absent and were not replaced by another monosaccharide. However, in the remaining 5%, L-Fuc was found to be replaced by a hexose residue. From nuclear magnetic resonance and mass spectrometry data of the *mur1* *N*-glycans, and by analogy with data reported on *mur1* xyloglucan, this subpopulation of *N*-linked glycans was proposed to be L-Gal-containing *N*-glycans resulting from the replacement of L-Fuc by L-Gal.

Mutants have been widely used for biochemical studies of *N*-glycosylation in yeast and mammals. Although a rapidly increasing number of mutants are available in several

plant species and particularly in Arabidopsis, only one of them presents a clearly identified mutation that affects the biosynthesis of *N*-linked glycans. This Arabidopsis *cgl* mutant lacks the *N*-acetyl glucosaminyltransferase I and is unable to synthesize complex-type *N*-glycans (von Schaeuwen et al., 1993). Another Arabidopsis mutant, *mur1*, does not synthesize L-Fuc in the leaves (Reiter et al., 1993). This mutant was found to be affected in the gene encoding for a GDP-D-Man-4,6-dehydratase, a key enzyme in the biosynthesis of L-Fuc (Bonin et al., 1997). The structure of cell wall polysaccharides from plants carrying the *mur1* mutation has been investigated recently (Zablackis et al., 1996) and compared with cell wall polysaccharides from wild-type plants (Zablackis et al., 1995). This study has shown that in the xyloglucan of this Fuc-deficient mutant, L-Fuc is replaced by L-Gal, a monosaccharide structurally similar to L-Fuc, without affecting the biological activity of Fuc-containing oligosaccharides derived from these polymers.

L-Fuc is a monosaccharide that is found not only in primary cell wall polysaccharides but also in the plant *N*-linked oligosaccharides. Consequently, the study of *N*-glycosylation in the *mur1* mutant could provide information on the biosynthesis and roles of glycans *N*-linked to proteins when L-Fuc is not available in Arabidopsis. In plants the *N*-glycosylation of proteins starts by the transfer in the ER of the oligosaccharide precursor Glc₃Man₉GlcNAc₂, which is subsequently modified during the transport of the glycoprotein by glycosidases and glycosyltransferases located in the ER, the Golgi apparatus, and the vacuole. These enzymes convert the precursor to high-mannose-type *N*-glycans ranging from Man₉GlcNAc₂ to Man₅GlcNAc₂ and eventually to complex-type *N*-glycans having an α (1,3)-Fuc and/or a β (1,2)-Xyl residue (for review, see Lerouge et al., 1998). Recently, some complex *N*-linked glycans have been described as having two antennae containing a Lewis a epitope that is usually found on cell-surface mammalian glycoconjugates (Fitchette-Lainé et al., 1997; Melo et al., 1997). These structures result from the

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Abbreviations: CID-MS-MS, collision-induced dissociation MS; HPAEC, high pH anion-exchange chromatography; HPAEC-PAD, HPAEC coupled to pulsed amperometric detection; PA, pyridylamino derivative; PNGase A, peptide *N*-glycosidase A.

elongation of the intermediate complex-type *N*-glycan by action of $\alpha(1,4)$ -fucosyltransferase and $\beta(1,3)$ -galactosyltransferase. Complex-type *N*-glycans have been described as being *N*-linked to extracellular glycoproteins. In contrast, vacuolar glycoproteins are *N*-glycosylated by modified oligosaccharides having only an $\alpha(1,3)$ -Fuc and/or a $\beta(1,2)$ -Xyl residue linked to the core $\text{Man}_3\text{GlcNAc}_2$ and are named paucimannosidic-type *N*-glycans (Lerouge et al., 1998). These oligosaccharides arise from the elimination from complex-type *N*-glycans of terminal residues in the vacuole (Vitale and Chrispeels, 1984).

We report in this paper the first complete structural analysis, to our knowledge, of *N*-linked glycans isolated from leaves of *Arabidopsis*. The strategy developed in the first part of our study was applied to the *N*-glycosylation analysis of proteins isolated from the *mur1* mutant to analyze the effects of the absence of L-Fuc on the structure and the biosynthesis of *N*-linked oligosaccharides. We show that in wild-type *Arabidopsis*, the proteins are *N*-glycosylated by high-mannose-type *N*-glycans and by Xyl- and Fuc-containing *N*-glycans previously described in other plants. Complex biantennary structures containing terminal Lewis a epitopes were not detected. Furthermore, as found previously in the cell wall xyloglucan (Zablackis et al., 1996), the analytical data suggest a partial but less prominent replacement of L-Fuc by L-Gal in the core of the *N*-glycans in the *mur1* mutant.

MATERIALS AND METHODS

Enzymes, Chemicals, and Plant Material

PNGase A from almond was purchased from Boehringer-Mannheim. Pepsin and β -*N*-acetylglucosaminidase from Jack bean were from Sigma. BioGel P4 and AG50W-X2 were from Bio-Rad. C_{18} Bond Elut cartridges were from Varian (Sugarland, TX). 2-aminopyridine (99% purity) was from Aldrich.

Wild-type *Arabidopsis* and mutant plants were grown under long-day conditions in the greenhouse, and leaf material was harvested from plants in early bolting stages.

SDS-PAGE and Western-Blot Analyses

Proteins isolated from leaves (as described in the next paragraph) were resolved by SDS-PAGE in a homogeneous 15% polyacrylamide gel under reducing conditions according to the method of Laemmli (1970), and electrophoretically transferred to nitrocellulose membrane. Affinodetection using the concanavalin A/peroxidase method (Faye and Chrispeels, 1985) and immunodetection with antibodies directed against Fuc- or Xyl-containing plant *N*-glycans were carried out, respectively, as described in Faye et al. (1993).

Preparation of *N*-Linked Glycans from Protein Extracts Isolated from Wild-Type and *mur1* Plants

Protein extracts from *Arabidopsis* were obtained by homogenizing 100 g of freeze-dried leaf material in 2 L of 50

mm Hepes (pH 7.5) and 2 mM sodium bisulfite containing 0.1% SDS. Insoluble material was eliminated by centrifugation at 4400g for 15 min at 4°C. A second protein extraction was performed using the pellet. The supernatants were pooled and filtered through Miracloth (Calbiochem). Proteins were precipitated at 0°C overnight with 12.5% TCA (final concentration), and the pellet was then washed three times with 90% (v/v) acetone. The proteins were digested with pepsin in 30 mL of 0.01 N HCl, pH 2.2, at 37°C for 48 h. Pepsin (20 mg) was dissolved in 1 mL of buffer and one-half of the enzyme solution was added at 0 and 24 h, respectively. The pepsin digest was neutralized with NaOH, heated at 100°C for 5 min, and lyophilized. The sample was desalted on a BioGel P4 column (70 × 2.6 cm) equilibrated with 0.1 N acetic acid. A portion of each fraction was assayed for monosaccharide composition. Glycopeptide fractions were pooled and then deglycosylated with 1 milliunit of PNGase A in a 100 mM sodium acetate buffer (pH 5.0) overnight at 37°C (Tomiyama et al., 1987). *N*-glycans were purified by successive elutions through a AG50W-X2 column (5 × 1 cm) and a C_{18} Bond Elut cartridge. Complex-type *N*-glycans were separated from high-mannose-type oligosaccharides by affinity chromatography on a concanavalin A-Sepharose 4B column as described in Montreuil et al. (1986).

Fluorescent Derivatization of *N*-Glycans with 2-Aminopyridine

Freeze-dried *N*-linked oligosaccharides were coupled to 2-aminopyridine as described previously by Hase et al. (1984). PA oligosaccharides were separated from the excess of reagent by gel permeation on a BioGel P4 column (40 × 1.6 cm) equilibrated in 50 mM ammonium acetate buffer, pH 6.0.

HPAEC Analysis

Chromatography of *N*-linked glycans was achieved by HPAEC-PAD. Analytical HPAEC-PAD was performed on a DX500 system equipped with a GP 40 gradient pump, an ED 40 detector, and a CarboPac PA1 column (250 × 4.6 mm, Dionex, Sunnyvale, CA). Analysis of the monosaccharide composition was carried out by hydrolysis of the glycan fraction with 2 N trifluoroacetic acid at 100°C for 2 h, followed by analysis of the resulting monosaccharides using 16 mM NaOH at 1 mL min⁻¹ as described in Hardy (1989). Elution of the high-mannose-type *N*-glycans was carried out using a linear gradient from 0 to 100 mM NaOAc in 100 mM NaOH at 1 mL min⁻¹ over 30 min.

HPAEC of PA oligosaccharides was achieved by dissolving the sample in 0.1 N NaOH and elution on a CarboPac PA1 column using a gradient from 0 to 100 mM NaOAc in 100 mM NaOH at 1 mL min⁻¹ over 60 min. PA oligosaccharides were detected using a FL 2000 system (Spectra-Physics, San Jose, CA) fluorescence detector with excitation and emission wavelengths at 320 and 400 nm, respectively. PA oligosaccharides were collected and desalted by gel filtration on a Sephadex G-10 column (30 × 1 cm) equilibrated and eluted in 50 mM sodium acetate, pH 6.0.

Reverse-Phase HPLC

A liquid chromatograph equipped with a SFM-25 fluorescence spectrophotometer (Kontron, Milan, Italy) was used. PA oligosaccharides were separated on a Spherisorb ODS2 C₁₈ column (250 × 4.6 mm, Prolabo, Creteil, France). Elution of PA oligosaccharides was performed at a flow rate of 1 mL min⁻¹ at 55°C using solvent A (10 mM sodium phosphate buffer, pH 3.8), and solvent B (10 mM sodium phosphate buffer, pH 3.8, 0.5% 1-butanol) (Tomiya et al., 1988). After injection of the sample, the ratio of solvent B to A increased with a linear gradient from 20:80 to 50:50 over 60 min. PA oligosaccharides were detected by fluorescence using excitation and emission wavelengths of 320 and 400 nm, respectively.

β-N-Acetylglucosaminidase Digestion of PA Oligosaccharides

PA oligosaccharides were isolated by HPAEC and then digested with 1 unit of β-N-acetylglucosaminidase in a 50 mM sodium acetate buffer, pH 5.0, at 25°C. The digest was analyzed by HPAEC with fluorescence detection after 1 and 18 h of incubation time.

NMR

¹H-NMR was performed at 600 MHz on a spectrometer (model DMX600, Bruker, Billerica, MA). The spectrum was recorded at 297 K in ²H oxide (²H₂O 99.97). Chemical shifts were expressed in parts per million downfield from internal tetraethylsilane and with an ²H water presaturation sequence.

Electrospray MS

The electrospray ionization mass experiments were carried out on a triple-quadrupole mass spectrometer (Quattro II, Micromass, Manchester, UK). Samples were dissolved in water:methanol:acetic acid (49:49:2) at a concentration of approximately 50 pmol μL⁻¹ and the solution was infused into the electrospray ion source by a syringe pump (Harvard Apparatus, South Natick, MA). Optimization of the parameters was performed both in the MS and MS-MS mode using (Glc)n-PA. Typically, best conditions were defined for a cone voltage of about 60 V, a collision energy range between 30 and 40 eV, and an Ar gas pressure at 4.0 mTorr. Spectra were recorded at a scan speed of 10 s and were smoothed 1 time.

RESULTS

Western-Blot Analysis of Protein Extracts from Wild-Type and *mur1* Arabidopsis Plants

A first analysis of the N-linked glycans associated to proteins isolated from leaves of the wild-type and the *mur1* mutant of Arabidopsis was obtained by affino- and immunodetections on blots using specific probes. We used antibodies of well-established specificities for the different oli-

gosaccharide epitopes that are found in plant N-glycans, i.e. the β(1,2)-Xyl residue and the α(1,3)-Fuc residue linked to the chitobiose unit (Faye et al., 1993). Glycoproteins from wild-type plants were detected on western blots by affino-detection using concanavalin A (Faye and Chrispeels, 1985) (Fig. 1B, lane 1), as well as by immunoblotting with antibodies directed against the β(1,2)-Xyl residue (Fig. 1C, lane 1) or directed against the α(1,3) Fuc residue (Fig. 1D, lane 1) linked to the core Man₃GlcNAc₂ (Faye et al., 1993). Similar results were obtained on the protein extract from *mur1* plants, except that no protein was detected by the antibodies specific for the α(1,3)-Fuc residue linked to the proximal glucosamine unit (Fig. 1D, lane 2). Some differences in the detection level of some glycoproteins (see the glycoprotein band at 35 kD) were observed, but the identity of these glycoproteins was not investigated further. Protein extracts from both the wild-type and *mur1* plants were also analyzed by immunoblotting with antibodies directed against Lewis a epitopes (data not shown). In contrast to results obtained in other plants, only very weak signals were detected in both extracts, confirming that such epitopes are almost completely absent in Arabidopsis leaves, as previously reported in Fitchette-Lainé et al. (1997). As a consequence, these results indicate that Arabidopsis leaf proteins are N-glycosylated by both high-mannose and paucimannosidic or complex-type N-glycans of limited size (no Lewis a-containing N-glycans). Similar results were obtained with the *mur1* mutant, except for the absence of detection of the α(1,3)-Fuc epitope as expected from the previous results on this mutant (Reiter et al., 1993).

Structural Identification of N-glycans Synthesized by Arabidopsis Wild-type and *mur1* Plants

The complete structural analysis of N-linked glycans associated with proteins from leaves of wild-type Arabidopsis and *mur1* plants was performed to investigate whether the partial replacement of L-Fuc by L-Gal observed in the cell wall xyloglucan (Zablackis et al., 1996) also

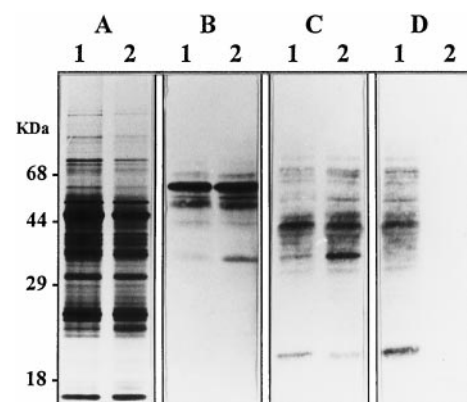


Figure 1. SDS-PAGE and western-blot analysis of protein extracts from leaves of wild-type Arabidopsis (lanes 1) or *mur1* mutant (lanes 2). A, Silver staining; B, affino-detection with concanavalin A; C, immunodetection with antibodies specific for β(1,2)-Xyl-containing plant N-glycans; D, immunodetection with antibodies specific for α(1,3)-Fuc-containing plant N-glycans.

occurs in the glycoproteins of the *mur1* mutant. Since the *N*-glycosylation of proteins in the wild-type *Arabidopsis* has not yet, to our knowledge, been investigated in detail, *N*-linked glycans from both the wild-type and the Fuc-deficient mutant were analyzed. After TCA precipitation proteins from both wild-type and *mur1* leaves were digested with pepsin. The resulting glycopeptides were isolated by size-exclusion chromatography on a BioGel P4 column and the fraction corresponding to glycopeptides from the wild-type and the *mur1* plants were further treated with PNGase A. PNGase A from sweet almond is able to release both high-mannose-type and modified plant *N*-glycans presenting or not presenting an $\alpha(1,3)$ -Fuc residue linked to the proximal glucosamine unit (Taga et al., 1984). The reducing *N*-linked oligosaccharides released from the wild-type *Arabidopsis* and *mur1* leaves were fractionated by affinity chromatography on a concanavalin A-Sepharose 4B column to separate paucimannosidic and complex-type *N*-glycans (concanavalin A⁻ fraction) from high-mannose-type *N*-glycans (concanavalin A⁺ fraction). The HPAEC-PAD profile of the concanavalin A⁺ fraction isolated from the wild-type plants (Fig. 2) shows a mixture of high-mannose-type *N*-glycans, which were identified as structures from Man₅GlcNAc₂ to Man₉GlcNAc₂ (Man-5 to Man-9, see Fig. 3) by comparison of their retention times in HPAEC-PAD chromatography with standard oligosaccharides from porcine thyroglobulin as previously described (Rayon et al., 1996). The high-mannose-type *N*-glycans isolated from the *mur1* plants were found to be identical and in the same ratio as those of the wild-type plants (data not shown).

The *N*-glycans contained in the concanavalin A⁻ fraction from both the wild-type and the *mur1* plants were analyzed by HPAEC-PAD. The profiles were composed of a complex mixture of unresolved peaks. As a consequence, these oligosaccharides were reductively aminated with

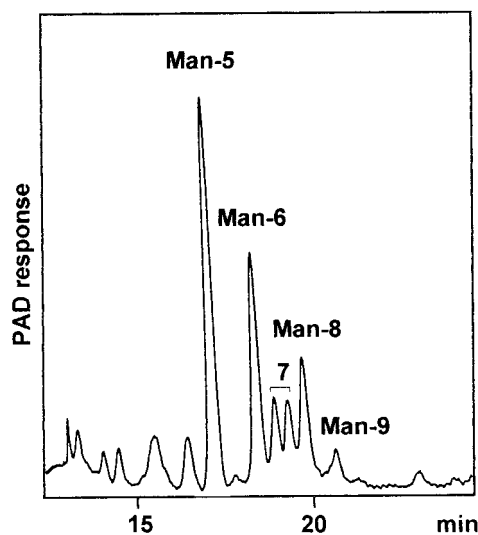


Figure 2. HPAEC-PAD profile of high-mannose-type *N*-glycans Man₅GlcNAc₂ to Man₉GlcNAc₂ (Man-5 to Man-9) isolated from wild-type *Arabidopsis* leaves. Corresponding structures are represented in Figure 3.

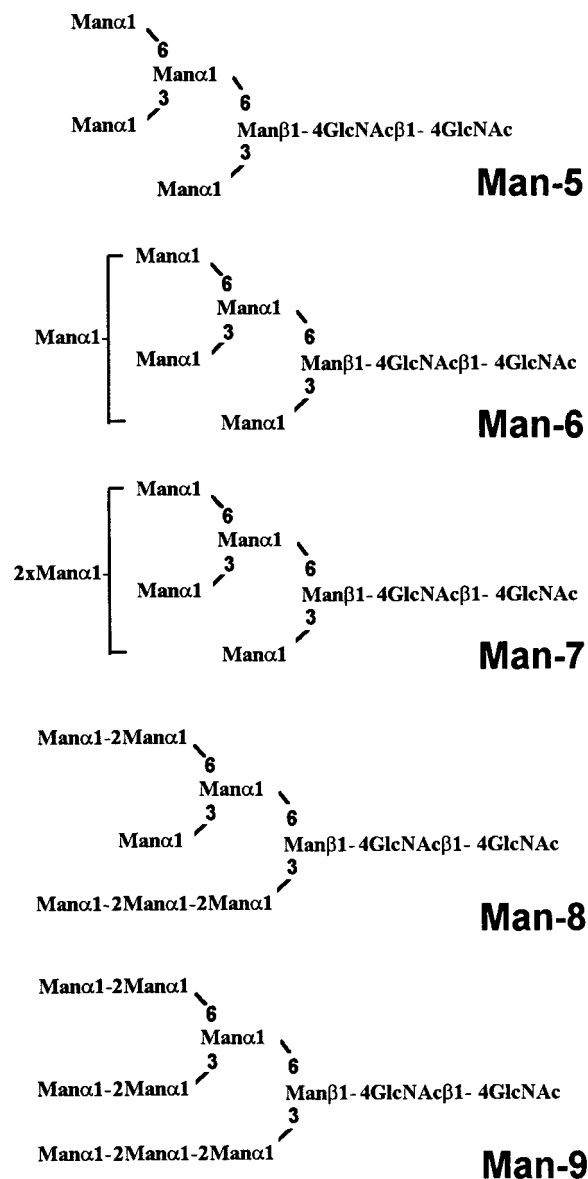


Figure 3. Abbreviations and structures of high-mannose-type *N*-glycans Man₅GlcNAc₂ to Man₉GlcNAc₂ (Man-5 to Man-9) isolated from wild-type *Arabidopsis* leaves.

2-aminopyridine, and the resulting PA oligosaccharides were then analyzed with HPAEC using a fluorescence detector. Under these conditions all labeled glycans from both preparations were found to be well resolved (Fig. 4). The elution profile of the PA oligosaccharides obtained from wild-type plants showed the presence of six major compounds (Fig. 4A). The structures of these oligosaccharides were identified by comparison with standard glycans and by glycosidase sequencing. Compounds a through h were collected separately. Glycans a and e were identified as the PA derivatives of Man₃XylFucGlcNAc₂ (M₃XFGN₂, Fig. 5) and Man₃XylGlcNAc₂ (M₃XGN₂, Fig. 5) oligosaccharides, respectively, by comparison of the retention time with reference standards. The structures of the other oligosaccharides were further determined after digestion with

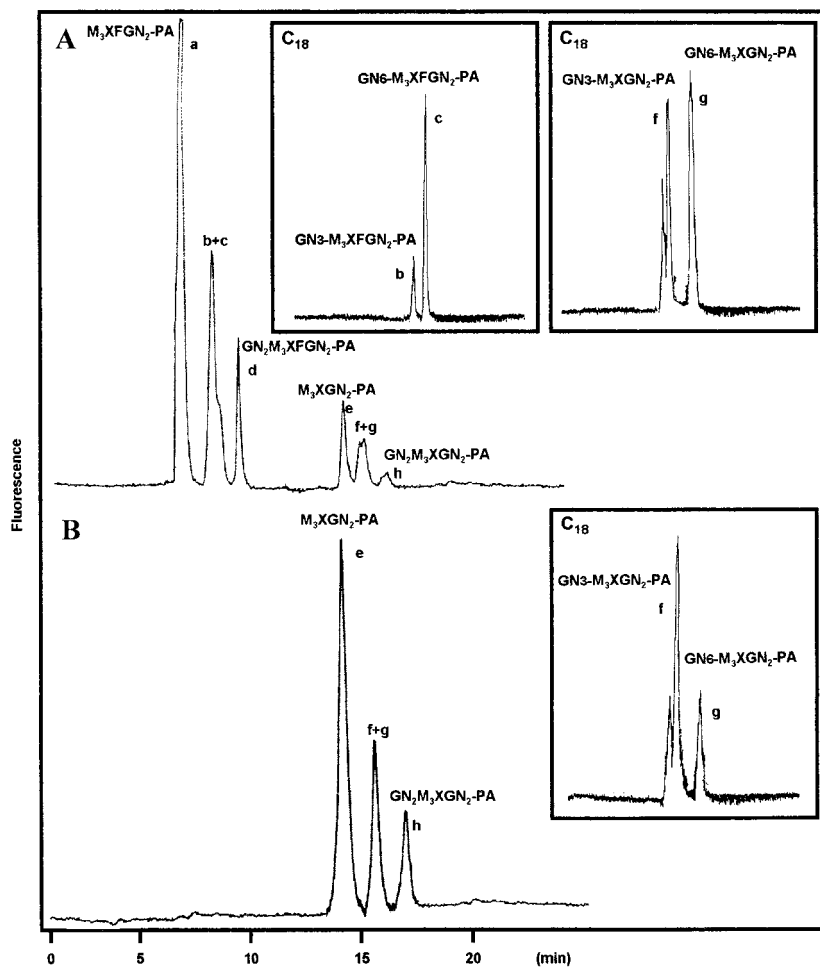


Figure 4. HPAEC profiles of the PA derivatives of paucimannosidic and complex-type *N*-glycans. *N*-glycans isolated from wild-type Arabidopsis (A) and *mur1* mutant (B) leaves. The chromatograms presented in the boxed insets correspond to the C_{18} reverse-phase profiles of peaks b+c and f+g purified by HPAEC. Abbreviations refer to *N*-linked glycans represented in Figure 5.

β -*N*-acetylglucosaminidase, an enzyme that specifically releases terminal β -GlcNAc residues. The material collected in peak d was found to be progressively converted to compounds b+c and then to compound a during β -*N*-acetylglucosaminidase treatment (Fig. 6). β -*N*-acetylglucosaminidase action on the material in peak b+c released a single β -GlcNAc residue, leading to compound a. As a consequence, compounds b+c and d were identified as Xyl and Fuc-containing oligosaccharides bearing one or two terminal glucosamine residues, respectively. Peaks f+g and h were analyzed in the same manner and were found to correspond to Xyl-containing glycans bearing one and two terminal glucosamine residues, respectively. Peaks b+c and f+g were subjected to a second chromatography analysis using a reverse-phase C_{18} column. In this case, both fractions were shown to be split further into two components (Fig. 4A, including boxed insets). PA derivatives of plant complex-type *N*-glycans bearing a single terminal GlcNAc residue have been described to be efficiently separated on a reverse-phase column. The isomers having the glucosamine unit associated to the mannose arm $\alpha(1,3)$ -linked to the β -mannose residue (GN3-M₃XFGN₂ and GN3-M₃XGN₂) are eluted sooner in these conditions than *N*-linked glycans having a terminal GlcNAc residue on the $\alpha(1,6)$ -antennae (Takahashi et al.,

1986; Hayashi et al., 1990). In conclusion, paucimannosidic and complex-type oligosaccharides *N*-linked to glycoproteins from Arabidopsis can be classified into two groups: Xyl- and Fuc-containing oligosaccharides having zero, one, or two terminal glucosamine residues (M₃XFGN₂ to GN₂M₃XFGN₂, Fig. 5) and analogous nonfucosylated structures (M₃XGN₂ to GN₂M₃XGN₂, Fig. 5).

Paucimannosidic and complex-type *N*-glycans isolated from the *mur1* plants were submitted to the same analysis. The nonfucosylated PA structures e to h (Fig. 4B), identical to those of the minor group found in the wild-type extract, were detected and identified using the same approach as M₃XGN₂ to GN₂M₃XGN₂ oligosaccharides represented in Figure 5. To investigate the presence in the pool of *mur1* *N*-glycans of minor oligosaccharides co-migrating with the xylosylated oligosaccharides and containing an additional hexose residue, the PA derivatives of complex and paucimannosidic-type *N*-glycans were then analyzed by electrospray MS (Fig. 7A). Three major (M+H)⁺ ions at $m/z = 1121, 1324, \text{ and } 1527$ were assigned to the major xylosylated PA oligosaccharides M₃XGN₂ to GN₂M₃XGN₂ identified by chromatography. However, a second family of ions was also detected at $m/z = 1283, 1486, \text{ and } 1689$, having an additional 162 mass units. These ions were not detected in the wild type (data not shown) and were esti-

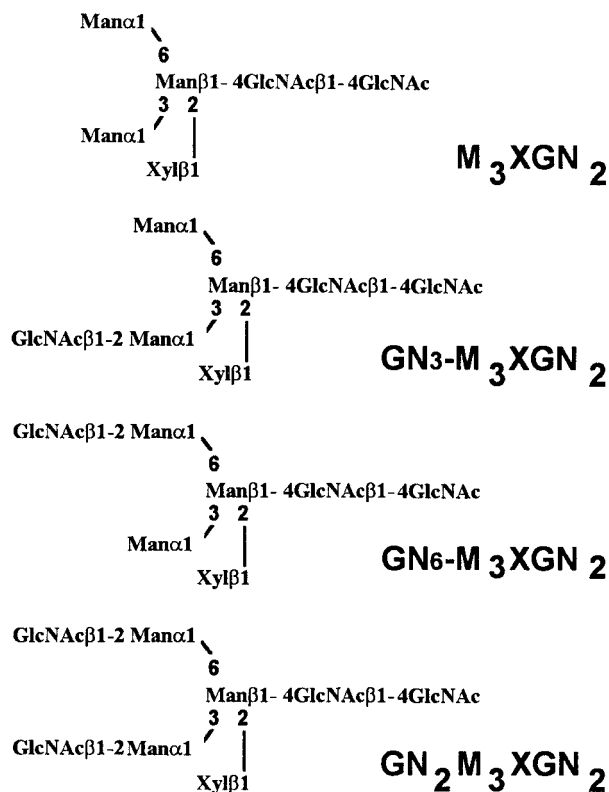


Figure 5. Abbreviations and structures of paucimannosidic and complex-type *N*-glycans isolated from *Arabidopsis*.

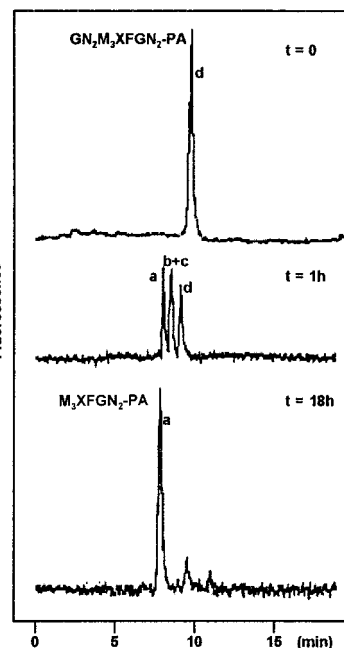
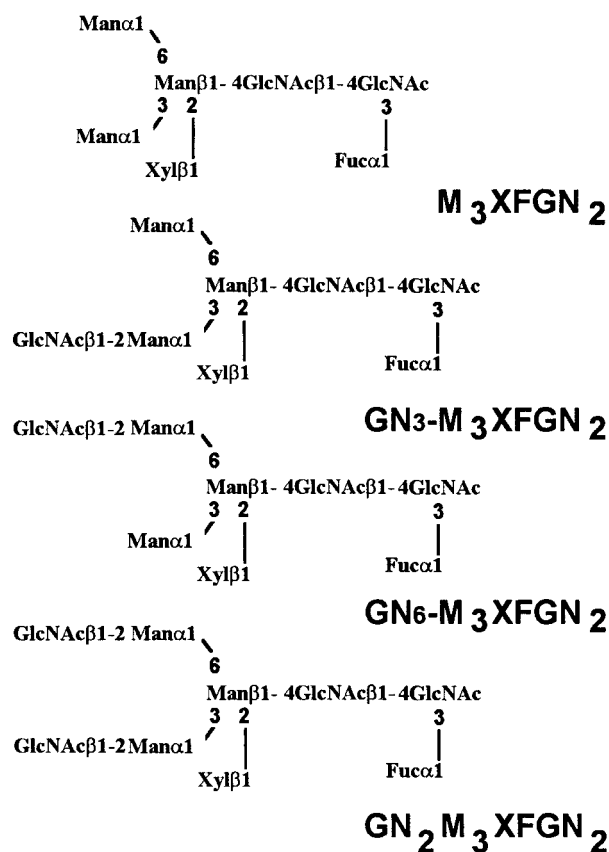


Figure 6. HPAEC profiles of the β -*N*-acetylglucosaminidase digest of compound d after different incubation times. Peak a, PA derivative of M_3XFGN_2 ; peak b+c, PA derivatives of $GN_6-M_3XFGN_2$ and $GN_3-M_3XFGN_2$ (see Fig. 5).

ated to represent about 5% to 10% of the total oligosaccharides (Fig. 7A). These ions were supposed to correspond to $(M+H)^+$ ions of the PA *L*-Gal-containing *N*-glycans $M_3XGalGN_2$ to $GN_2M_3XGalGN_2$ represented in Figure 8. To demonstrate that these ions arise from the replacement of *L*-Fuc by *L*-Gal, the most abundant $(M+H)^+$ at $m/z = 1283$ was submitted to a CID-MS-MS analysis (Fig. 7B). This MS analysis allows the observation of fragment ions specifically arising from the cleavage of glycosidic linkages of the selected $(M+H)^+$ ion of PA oligosaccharides. As a consequence, some information on the oligosaccharide sequence can be deduced from the CID-MS-MS spectrum (Gu et al., 1994). In the CID-MS-MS of the ion at $m/z = 1283$, the successive loss of three mannose residues and one Xyl residue from the nonreducing end is observed. Furthermore, the detection of the fragment ion at $m/z = 462$ is strong evidence that the additional hexose unit is linked to the proximal GlcNAc residue (Fig. 7B). This fragmentation pattern is consistent with data reported in the literature on fragmentation of PA *N*-glycans either having or not having an additional residue linked to the proximal glucosamine residue (Gu et al., 1994). The mixture of labeled glycans was then analyzed by 1H -NMR. In addition to the H-1 signal of the β -Xyl at 4.43 ppm, the NMR spectrum shows, in the 4.5 to 5.2 ppm region, four signals that were assigned to the H-1 of mannose residues of the different *N*-glycans (Fig. 9). In addition to these signals, a minor doublet at 5.422 ppm ($J = 3.5$ Hz) was assigned to the H-1 of the α anomer of the additional hexose residue detected by MS. The chemical shift of this signal is in agreement with NMR data on terminal α -Galp residues (Zablackis et al., 1996).

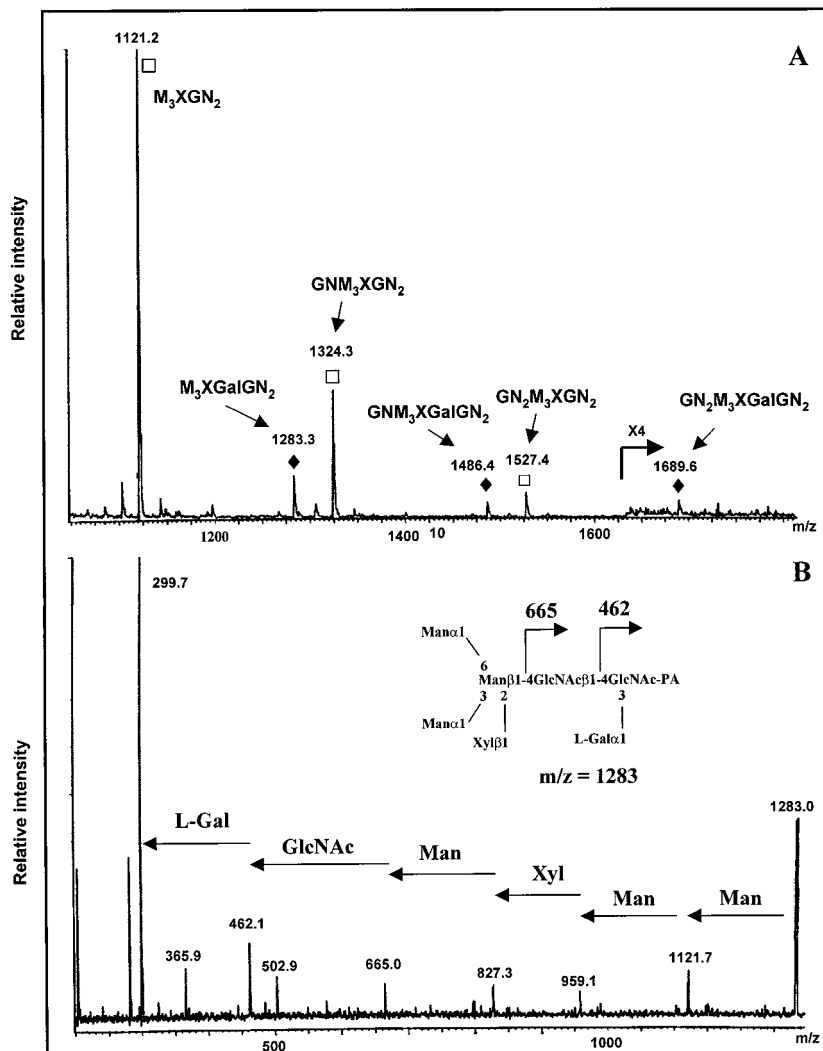


Figure 7. MS analysis of the *mur1* N-glycans. A, Electrospray mass spectrum of the PA derivatives of the paucimannosidic and complex-type N-glycans isolated from the *mur1* mutant. □, (M+H)⁺ ions of the PA derivatives of M₃XGN₂, GNM₃XGN₂, and GN₂M₃XGN₂ (see Fig. 5). ♦, (M+H)⁺ ions of the PA derivatives of the L-Gal-containing oligosaccharides M₃XGalGN₂, GNM₃XGalGN₂, and GN₂M₃XGalGN₂ (see Fig. 8). B, CID-MS-MS of the (M+H)⁺ ion of the PA derivative of M₃XGalGN₂ (*m/z* = 1283).

The amount of L-Gal containing N-glycans was estimated to represent about 5% by integration of the H-1 signal of L-Gal to H-1 signal of Man-4 (Fig. 9, signals a and b).

Taken together, these results indicate that the second family of N-linked glycans detected in the *mur1* mutant contains an α -Gal linked to the proximal GlcNAc residue. The presence of Gal in the *mur1* N-glycan preparation was confirmed by sugar composition analysis. However, due to the low amount of material, the absolute configuration of the Gal and its location on the GlcNAc were not determined. However, since L-Fuc was found to be replaced by L-Gal in the *mur1* cell wall xyloglucan, the MS and NMR data strongly suggest that the same process occurs in the N-glycans of this mutant. As a consequence, the partial replacement of L-Fuc by L-Gal leads to the formation of plant N-glycans with a novel core structure represented in Figure 8. Thus, we conclude that the *mur1* mutation results in the accumulation of major nonfucosylated structures (95%) and minor L-Gal-containing-oligosaccharides (5%) without affecting, in a detectable manner, the processing of N-linked glycans.

DISCUSSION

The structures of N-linked glycans associated to proteins isolated from Arabidopsis have been elucidated by affinity- and immunodetection on blots with specific probes, as well as by structural identification of reducing oligosaccharides released from glycoproteins by treatment with a specific peptide N-glycosidase. High-mannose-type N-glycans from Man-5 to Man-9 have been identified. Furthermore, paucimannosidic and complex-type N-glycans having an α (1,3)-Fuc, a β (1,2)-Xyl residues have been fully identified. However, the total N-glycosylation in Arabidopsis presents a lower diversity than observed in other plants (Takahashi et al., 1986; Fitchette-Lainé et al., 1997). No complex-type N-glycans containing terminal Lewis a epitopes have been detected, indicating that these glycans either are not synthesized in Arabidopsis plants, or they are present only in very low amounts in this plant or only in some tissues.

N-linked glycans associated to proteins from the Fuc-deficient *mur1* mutant have been isolated and identified using the same approach as was developed for the wild-

type plants. This study has shown that mostly nonfucosylated oligosaccharides are *N*-linked to proteins of the *mur1* mutant. No modification in the processing of the *N*-glycans was observed in the mutant. Major nonfucosylated oligosaccharide structures have been identified, bearing zero, one, or two terminal glucosamine residues in a ratio similar to that found in wild-type plants. This result confirms that the addition of the $\alpha(1,3)$ -Fuc residue is a late event in the processing of plant *N*-linked glycans. As a consequence, the absence of L-Fuc transfer does not disturb the biosynthesis of these oligosaccharides. In addition to the nonfucosylated oligosaccharides, a second family of oligosaccharides was detected by MS in the total protein extract from the *mur1* plants. From analytical data these oligosaccharides, representing about 5% of the *N*-glycans, were found to have an additional hexose residue (α -galactose from NMR data) linked to the proximal glucosamine residue. Since the chitobiose unit of *N*-glycans in plants and other organisms has been reported so far as only substituted by Fuc residues, such data strongly suggest that the minor population of *N*-glycans, identified by MS, result from the *mur1* mutation. By analogy with previously reported data on the *mur1* xyloglucan (Zablackis et al., 1996), we propose that the minor 5% subpopulation of *N*-glycans found in this mutant are new plant *N*-glycans having $\alpha(1,3)$ -L-Gal linked to the proximal glucosamine and resulting from the replacement of L-Fuc by L-Gal.

The replacement of one-third of the terminal L-Fuc residues in the xyloglucan by L-Gal, a monosaccharide structurally related to L-Fuc, indicates that the $\alpha(1,2)$ -fucosyl-

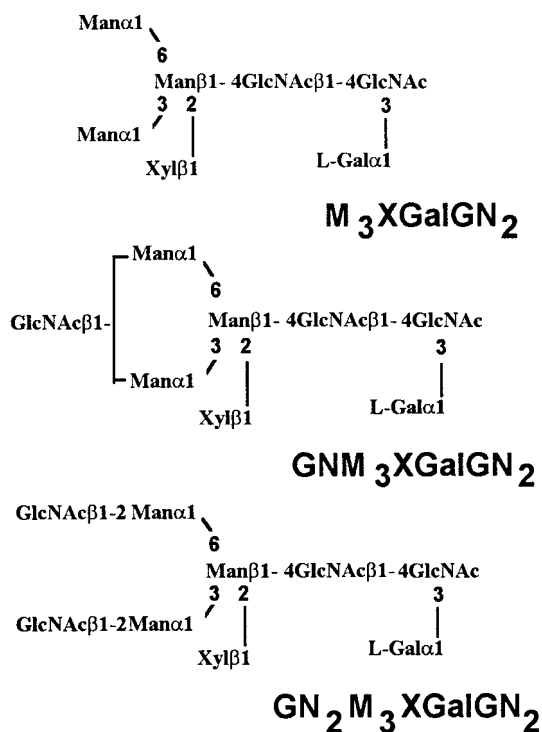


Figure 8. Abbreviations and structures of L-Gal-containing paucimanosidic and complex-type *N*-glycans isolated from the *mur1* mutant.

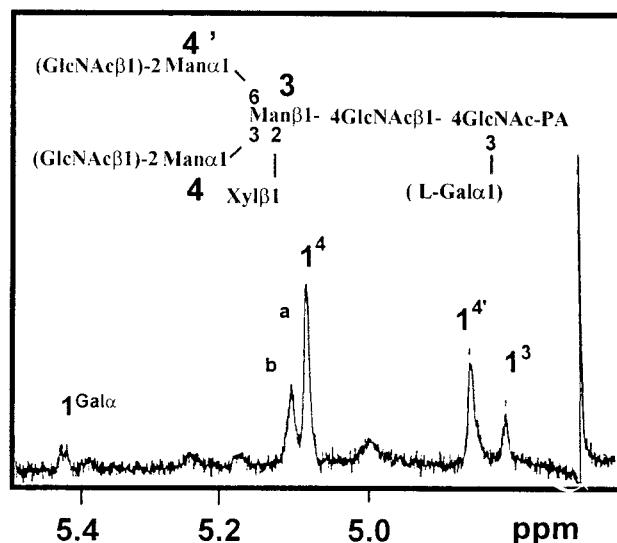


Figure 9. 1H -NMR spectrum of the mixture of PA derivatives of the paucimanosidic and complex-type *N*-glycans isolated from the *mur1* mutant leaves. Numbers and letters refer to the corresponding protons (for example, 1^4 = signal of H-1 proton of residue 4). A, H1 signal of the nonsubstituted Man 4 of M_3XGN_2 and $GN_6-M_3XGN_2$. B, H1 signal of substituted Man 4 of $GN_3-M_3XGN_2$ and $GN_2M_3XGN_2$.

transferase involved in the xyloglucan biosynthesis is able to transfer L-Gal from GDP-L-Gal to the xyloglucan backbone in the absence of GDP-L-Fuc. This phenomenon also seems to occur with the $\alpha(1,3)$ -fucosyltransferase involved in *N*-linked glycan biosynthesis, which is localized in the same compartment as the xyloglucan $\alpha(1,2)$ -fucosyltransferase, i.e. the *trans* cisternae of the Golgi apparatus (Driouich et al., 1993; Fitchette-Lainé et al., 1994). However, the difference in level of L-Gal incorporation suggests that the two fucosyltransferases show different efficiencies in the replacement of L-Fuc by L-Gal.

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