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Tissue Culture Note

N-linked glycan structures of glycoproteins in suspension-cultured *Arabidopsis thaliana* MM2d cells

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Abstract The oligosaccharide structures of total glycoproteins in suspension-cultured *Arabidopsis thaliana* MM2d cells are determined. The *N*-linked sugar chains released by hydrazinolysis were labeled with 2-aminopyridine, and analyzed by a combination of HPLC, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, and exoglucosidase digestion. The glycan structures determined were $GlcNAcMan_3FucXylGlcNAc_2$ (8.5%), $Man_3FucXylGlcNAc_2$ (70.0%), $Man_3XylGlcNAc_2$ (13.7%), and $GlcNAcMan_3XylGlcNAc_2$ (7.8%). All the glycan structures of glycoproteins from *Arabidopsis* MM2d cells used in this study contain typical plant bisecting either β1,2-xylose or α1,3-fucose residues, showing that suspension-cultured MM2d cells have a glycosylation potential similar to that of tobacco BY2 suspension-cultured cells.

Key words: *Arabidopsis thaliana*, glycoproteins, glycosylation, *N*-linked glycan structures, suspension-cultured cells.

Plant cells have been developed to produce many low molecular weight molecules, such as ingredients used in the food and pharmaceutical industries (Wu and Zhong 1999; Bisaria and Panda 1991; Jimenez-Aparicio and Gutierrez-Lopez 1999). Remarkable progress has been made in recent years in understanding basic plant metabolism, and in developing bioprocesses and operation bioreactors for plant cell culture (Shuler 1994; Taticek et al. 1994). The wide range of plant metabolites produced using large-scale plant cell bioreactors includes flavors, colorants, oils, sweeteners and antioxidants. For instance, the efficient production of ginseng metabolites derived from *Panax ginseng* culture, which are used as additives in liquor, has been intensively studied (Wu and Ho 1999; Palazon et al. 2003). Also, taxol and other taxanes have been produced from *Taxus brevifolia* and related plants, and their production has been commercialized (Zhong 2002).

Recently, recombinant DNA technology have paved the way for plant suspension-cultured cells to be used in pharmaceutical protein production (Hein et al. 1991; Horn et al. 2004; Fischer et al. 2004), because compared with whole plants, the plant cell culture system has some advantages, such as a low production cost, the use of cheaper chemicals for their culture, and the lack of animal pathogens (Hellwig et al. 2004). In particular, it is easier to control culture conditions for suspension-cultured cells.

Some pharmaceuticals are proteins that have been modified by posttranslational processes, such as glycosylation. The *N*-linked glycans of glycoproteins have physiologically and biologically important roles (Gomord and Faye 2004; Faye et al. 2005). The potential of glycosylation in vegetable plants has been examined (Wilson et al. 2001). However, the potential of glycosylation in various suspension-cultured plant cells has not yet been elucidated. We have elucidated the sugar chain analysis of glycoproteins in tobacco BY2 suspension cells (Palacpac et al. 1999). In this study, a suspension-cultured cell line MM2d established from the *Arabidopsis thaliana* ecotype Landsberg erecta (Menges and Murray 2002) was used for examining its glycosylation potential.

Arabidopsis thaliana MM2d cells were maintained in a modified MS plant salt mixture (MS plant salt mixture, Nippon Seiyaku, Japan), containing KH_2PO_4 0.2 g l^{-1}, *myo*-inositol 0.1 g l^{-1}, thiamine HCl 1 mg l^{-1}, 2,4-D 0.2 mg l^{-1}, and sucrose 30 g l^{-1}, and used for glycan analysis. *Arabidopsis* MM2d cells were cultivated in 1 L of medium using a Mitsuwa fermentor KMJ-2CA at 28°C for 10 days in the dark. Aseptic air was supplied through a sparger at 300 ml min^{-1}, and the cell suspension was agitated with an impeller at 150 rpm.

Cells harvested on day 11 were ground with a mortar

Abbreviations: MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; 2,4-D, 2,4-dichlorophenoxyacetic acid; PA, 2-aminopyridine; RP, reversed-phase; SF, size-fractionation

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and pestle in the presence of liquid N_2, and the thawed cell homogenate was centrifuged at 12,000 rpm for 15 min at 4°C. The resulting supernatant was used as a solution of total glycoproteins for structural analysis. The structures of *N*-linked sugar chains were analyzed by a combination of reversed-phase (RP-) and size-fractionation (SF-) HPLC, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS), and exoglucosidase digestion, as described previously (Misaki et al. 2003). Briefly, sugar chains were released from purified glycoproteins by hydrazinolysis. After *N*-acetylation with saturated sodium bicarbonate and acetic anhydride, the hydrazinolysate was desalted, and fractionated on a TSK gel Toyopearl HW-40 (Tosoh) column (Misaki et al. 2003). The oligosaccharides were labeled with 2-aminopyridine (PA, Kondo et al. 1990). PA-sugar chains were fractionated on a Hitachi HPLC apparatus and monitored at the excitation and emission wavelengths of 310 nm and 380 nm, respectively. The conditions for RP- and SF-HPLC using Cosmosil 5C18-AR (Nacalai Tesque, Kyoto, Japan) and Asahipak NH2P-50 4E (Showa Denko) columns were as described previously (Misaki et al. 2003).

For the exoglycosidase digestion of PA-sugar chains, *N*-acetylglucosaminidase (1 mU; *Diplococcus pneumoniae*, Roche, Germany) and α-mannosidase (10 mU; jackbean, Sigma, USA) were used as described previously (Misaki et al. 2003). The reactions were stopped by boiling the mixtures for 3 min. After centrifugation at 12,000 rpm for 10 min at 4°C, the supernatant was analyzed by SF-HPLC. The elution positions of the components of the supernatant were compared with the elution positions of authentic PA-sugar chains purchased from Takara Biomedicals.

The molecular masses of PA-sugar chains were determined by MALDI-TOF MS using an autoflex mass spectrometer (Bruker Daltonics).

PA-sugar chains from the *Arabidopsis thaliana* MM2d suspension-cultured cells were purified and characterized by a combination of RP- and SF-HPLCs (Figure 1). Figure 1A shows peaks of PA derivatives as analyzed by RP-HPLC. Other fractions eluted before peak 1 did not contain PA-labelled *N*-linked sugar chains. Each of the collected fractions (Nos. 1–8) was rechromatographed by SF-HPLC (Figure 1B). The structures of *N*-linked glycans are shown in Figure 2. MALDI-TOF MS and exoglycosidase digestion showed that peaks 1-a to 1-e, 2-a to 2-e, 3-b to 3-e, 4-a to 4-e, 5-a to 5-e, 6-b to 6-e, 7-a to 7-f, and 8-c to 8-e do not contain *N*-linked sugar chains.

The molecular mass of the *N*-glycan corresponding to peak 3-a (*m/z* 1290.14) agreed well with the calculated mass of $Man_3FucXylGlcNAc_2$-PA (M3FX; 1289.17 for $[M+Na]^+$). The elution position of this

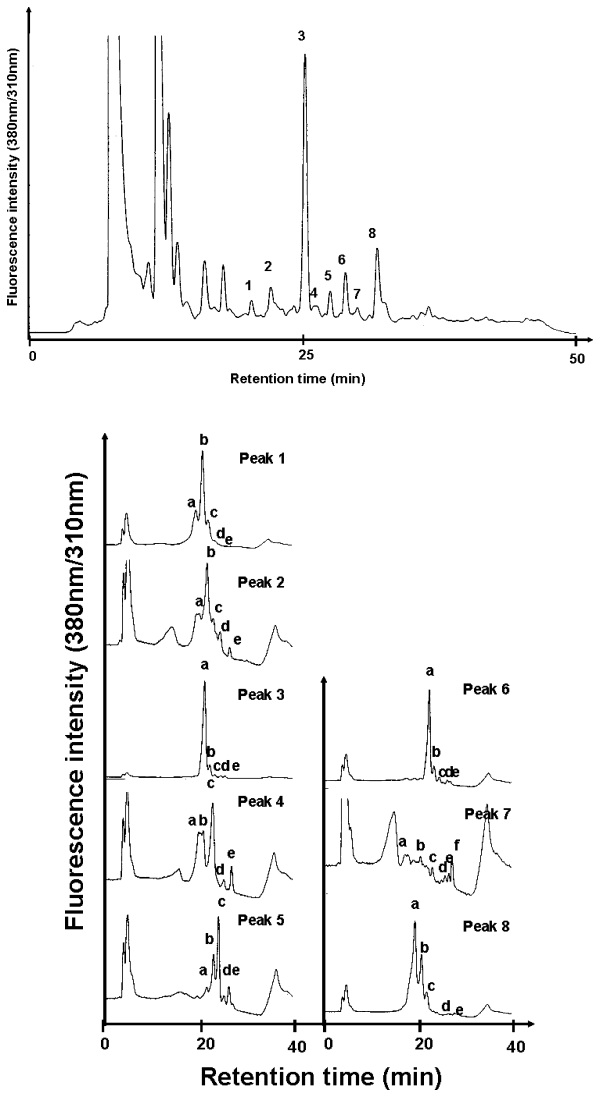

Figure 1. PA sugar chain derivatives from suspension-cultured *A. thaliana* MM2d cells. (A) RP-HPLC profile of PA-sugar chains eluted by increasing the acetonitrile concentration in 0.02% trifluoroacetic acid linearly from 0 to 6% for 40 min at a flow rate of 1.2 ml/min. 1–8, individual fractions collected and purified in SF-HPLC. (B) SF-HPLC patterns of collected fractions in (A). PA-sugar chains were eluted by increasing the water content in the water-acetonitrile mixture from 26 to 50% for 25 min at a flow rate of 0.7 ml/min. The excitation and emission wavelengths were 310 and 380 nm, respectively.

N-glycan on the RP-HPLC chromatogram was identical to that of M3FX prepared from horseradish peroxidase (Kurosaka et al. 1991; Takahashi et al. 1998). These results suggest that the structure of the glycan corresponding to peak 3-a should be Manα1,6(Manα1,3)(Xylβ1,2)Manβ1,4GlcNAcβ1,4(Fuc α1,3)GlcNAc-PA (M3FX), as shown in Figure 2.

The molecular mass of the *N*-glycan corresponding to peak 6-a (*m/z* 1492.58) agreed well with the calculated mass of $GlcNAcMan_3FucXylGlcNAc_2$-PA (GNM3FX; 1492.36 for $[M+Na]^+$). The elution position of this *N*-glycan on the RP-HPLC

Peak	Abbreviation	Structure
3-a	M3FX (70.0%)	Manα1 → 6, Manα1 → 3, Manβ1-4GlcNAcβ1-4GlcNAcβ1-PA, Xylβ1 at 2, Fucα1 at 3
6-a	GN^1M3FX (8.5%)	GlcNAcβ1-2Manα1 → 6, Manα1 → 3, Manβ1-4GlcNAcβ1-4GlcNAcβ1-PA, Xylβ1 at 2, Fucα1 at 3
8-a	M3X (13.7%)	Manα1 → 6, Manα1 → 3, Manβ1-4GlcNAcβ1-4GlcNAcβ1-PA, Xylβ1 at 2
8-a 8-b	GN_1M3X (7.8%)	Manα1 → 6, GlcNAcβ1-2Manα1 → 3, Manβ1-4GlcNAcβ1-4GlcNAcβ1-PA, Xylβ1 at 2

Figure 2. Structures of *N*-linked glycans obtained from suspension-cultured *A. thaliana* MM2d cells. Numbers in parentheses represent molar ratios.

chromatogram was identical to that of standard GlcNAcβ1,2Manα1,6(Manα1,3)(Xylβ1,2)Manβ1,4GlcNAcβ1,4(Fucα1,3)GlcNAc-PA (GN_1M3FX). The elution position on the RP-HPLC chromatogram of the *N*-acetylglucosaminidase-digested sugar chain was identical to that of $Man_3FucXylGlcNAc_2$-PA. These results suggest that the glycan structure corresponding to peak 6-a should be GlcNAcβ1,2Manα1,6(Manα1,3)(Xylβ1,2)Manβ1,4GlcNAcβ1,4(Fucα1,3)GlcNAc-PA (GN^1M3FX).

The MALDI-TOF MS analysis of oligosaccharides corresponding to peak 8-a showed that this peak contained two *N*-glycan species. The molecular masses of the *N*-glycans corresponding to the peak at *m*/*z* 1144.04 and 1347.28 agreed well with the calculated masses of $Man_3XylGlcNAc_2$-PA (M3X; 1143.03 for $[M+Na]^+$) and $GlcNAcMan_3XylGlcNAc_2$-PA (GNM3X; 1346.22 for $[M+Na]^+$), respectively. The elution positions of the putative M3X- and GNM3X-type *N*-glycans on the RP- and SF-HPLC chromatograms were identical to those of the sugar chains Manα1,6(Manα1,3)(Xylβ1,2)Manβ1,4GlcNAcβ1,4GlcNAc-PA (M3X) and Manα1,6(GlcNAcβ1,2Manα1,3)(Xylβ1,2)Manβ1,4GlcNAcβ1,4GlcNAc-PA (GN_1M3X), respectively. The GN_1M3X sugar chain was digested with *N*-acetylglucosaminidase, resulting in the fact that its elution positions on the SF- and RP-HPLC chromatograms were identical to those of $Man_3XylGlcNAc_2$-PA. These results suggest that the glycan structures corresponding to peak 8-a should be Manα1,6(Manα1,3)(Xylβ1,2)Manβ1,4GlcNAcβ1,4GlcNAc-PA (M3X) and Manα1,6(GlcNAcβ1,2Manα1,3)(Xylβ1,2)-Manβ1,4GlcNAcβ1,4GlcNAc-PA (GN_1M3X). As shown below, the GN_1M3X-type sugar chain was also contained in peak 8-b.

The molecular mass of the *N*-glycan containing the PA-sugar chain corresponding to peak 8-b (*m*/*z* 1347.28) agreed well with the calculated mass of $GlcNAcMan_3XylGlcNAc_2$-PA (GNM3X; 1346.22 for $[M+Na]^+$). The elution positions of the *N*-glycan on the RP- and SF-HPLC chromatograms were identical to those of the sugar chain Manα1,6(GlcNAcβ1,2-Manα1,3)(Xylβ1,2)Manβ1,4GlcNAcβ1,4GlcNAc-PA (GN_1M3X). The digestion of the sugar chains with *N*-acetylglucosaminidase resulted in elution positions on the RP- and SF-HPLC chromatograms identical to those of $Man_3XylGlcNAc_2$-PA. These results suggest that the glycan structure corresponding to peak 8-b should be Manα1,6(GlcNAcβ1,2Manα1,3)(Xylβ1,2)Manβ1,4GlcNAcβ1,4GlcNAc-PA (GN_1M3X).

The *N*-linked glycan structures of total glycoproteins prepared from *Arabidopsis* MM2d suspension-cultured cells were determined as $GlcNAcMan_3FucXylGlcNAc_2$ (8.5%), $Man_3FucXylGlcNAc_2$ (70.0%), $Man_3XylGlcNAc_2$ (13.7%), and $GlcNAcMan_3XylGlcNAc_2$ (7.8%), as shown in Figure 2 and Table 1. All the sugar chains have a plant-specific sugar residue, β1,2-xylose linked to the core $Man_3GlcNAc_2$. Plant-specific sugar residues, such as β1,2-xylose and α1,3-fucose, have been cause of concern because of their potential immunogenicity or allergenicity (Bencurova et al. 2004; Jin et al. 2006). Glycans without terminal *N*-acetylglucosamine residues account for 83% of the glycan structures. Terminal *N*-

Table 1. Comparison of *N*-linked glycan structures

	Abbreviation	Tobacco BY2	*Arabidopsis* MM2d	*Arabidopsis* plant	
$Man_9GlcNAc_2$	M9	—	—	3.0	
$Man_8GlcNAc_2$	M8	—	—	3.5	
$Man_7GlcNAc_2$	M7	—	—	2.7	26.1
$Man_6GlcNAc_2$	M6	—	—	6.1	
$Man_5GlcNAc_2$	M5	7.5	—	10.8	
$GlcNAc_2Man_3FucXylGlcNAc_2$	GN2M3FX	26.5	—	26.6	
$GlcNAcMan_3FucXylGlcNAc_2$	GN^1M3FX	21.7	8.5	15.0	
	GN_1M3FX	—	—		
$GlcNAc_2Man_3XylGlcNAc_2$	GN2M3X	—	—	1.4	
$GlcNAcMan_3XylGlcNAc_2$	GN^1M3X	—	—	1.5	
	GN_1M3X	—	7.8		
$GlcNAcMan_3GlcNAc_2$	GNM3	—	—	1.4	
$Man_3FucXylGlcNAc_2$	M3FX	41.0	70.0	26.4	
$Man_3XylGlcNAc_2$	M3X	3.3	13.7	1.6	

Data from Palacpac et al. 1999, and Strasser et al. 2004

acetylglucosamine residues of *N*-linked glycans at the non-reducing ends are possible substrates for membrane-bound *β*-*N*-acetylglucosaminidase involved in the protein *N*-glycan process (Altmann et al. 1995). Previously, we determined the *N*-linked glycan structures of total glycoproteins in the soluble fraction of suspension-cultured tobacco BY2 cells (Palacpac et al. 1999); M5 (7.5%), GN^1M3FX (21.7%), M3X (3.3%), M3FX (41.0%) and GN2M3FX (26.5%) as shown in Table 1. In tobacco BY2 cells, intermediate structures on the glycosylation process, such as M5, were observed. Sugar chain profile differed from the cell wall fraction from rice cultured cells to the soluble fraction (Maeda and Kimura, 2006). In this study, we prepared the soluble fraction from *Arabidopsis* MM2d cells and used for sugar chain analysis.

Here, we studied glycosylation in suspension-cultured cells derived from the *A. thaliana* ecotype Landsberg erecta. Because genome sequences from the *A. thaliana* ecotype Columbia have been established (The Arabidopsis Genome Initiative 2000), it is possible to clarify the molecular mechanism underlying glycosylation. A sugar chain analysis of glycoproteins from *A. thaliana* Columbia whole plants has been reported (Table 1, Strasser et al. 2004). Whole plants of *A. thaliana* Columbia have more high-mannose type sugars (26.1%), differing from that potential of *N*-linked protein glycosylation in *Arabidopsis* MM2d suspension-cultured cells. This difference might be derived from the status of the tissue differentiation. *A. thaliana* T87 suspension-cultured cells are derived from *A. thaliana* Columbia (Axelos et al. 1992). Currently, a sugar chain analysis in *A. thaliana* T87 suspension-cultured cells is under way.

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