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Note

N-Glycan structures of glycoproteins in suspension-cultured *Arabidopsis thaliana* T87 cells

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Abstract Suspension-cultured plant cells are feasible bioreactors for the production of pharmaceutical proteins. Post-translational modification, such as glycosylation, is important for *in vivo* biological and physiological roles of proteins. However, little is known about the effect of suspension-culture conditions on protein *N*-glycosylation. In this study, we studied the *N*-glycan structures of soluble and endogenous glycoproteins at different growth stages of suspension-cultured *Arabidopsis thaliana* T87 cells. At the late growth stage, the amount of *N*-glycans with terminal *N*-acetylglucosamine (GlcNAc) decreased, whereas that of *N*-glycans with fucose and xylose increased. This indicates that *N*-glycans have more plant-specific sugar residues as the cultivation reaches the late or end stage of plant growth.

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

Recombinant DNA technology enabled advances in heterologous protein production in various host cells, such as chicken, sheep, mammalian, insect, silkworm, and plant cells. Attention has been focused on plants as a protein factory, because the plant system is low in cost, lacks animal pathogens, and capable of post-translational modifications (Xu et al. 2011). Suspension-cultured plant cells have been traditionally used for the production of plant-derived natural compounds. Thus, pharmaceutical protein production has been attempted in suspension-cultured plant cells (Basaran and Rodríguez-Cerezo 2008; Karg and Kallio 2009; Hellwing et al. 2004). Compared with whole plants, the plant cell culture system has some advantages, such as inexpensive chemicals for their culture, controllable culture conditions, and reproducible environmental parameters (Xu et al. 2011). These advantages allow suspension-cultured cells to meet the regulatory requirements for the production of pharmaceutical proteins and to work as an environmentally friendly bioreactor. Recently, β-glucocerebrosidase produced by plant suspension-cultured cells has become the most advanced biopharmaceutical for commercialization (Aviezer et al. 2009; Shaaltiel et al. 2007).

Most pharmaceutical proteins are post-translationally modified. Glycosylation is a critical protein modification process for expression of biological and physiological activities in the cell. *N*-Glycosylation, i.e., modification at an asparagine residue, has been extensively studied and shown to be diversified among eukaryotes (Brooks 2004; Pattison and Amtmann 2009; Rayon et al. 1998; Tomiya et al. 2004; Wilson 2002). Therefore, heterologously expressed proteins are modified under a host-specific system. In plants, the typical *N*-glycan structure is the core structure $Man_3GlcNAc_2$ (Man, mannose; GlcNAc, *N*-acetylglucosamine) with β(1,2)-xylose (Xyl) and α(1,3)-fucose (Fuc), termed M3FX here (Figure 1). However, mammalian *N*-glycans generally have α(1,6)-Fuc residues linked to $Man_3GlcNAc_2$ instead, but not have β(1,2)-Xyl. Plant-specific sugar residues have been suggested to be potentially immunogenic or allergenic (Altmann 2007; Bencúrová et al. 2004; Jin et al. 2008). Previously, we examined *N*-glycan structures derived from endogenous and soluble proteins of suspension-cultured cells of tobacco (*Nicotiana tabacum*) BY2 (Misaki et al. 2001; Palacpac et al. 1999) and *A. thaliana* MM2d (Fujiyama et al. 2007), and showed that M3FX is the predominant structure. However, little is

Abbreviations: Fuc, Fucose; GlcNAc, *N*-Acetyl-glucosamine; HPLC, High performance liquid chromatography; MALDI-TOF MS, Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; Man, Mannose; PA, 2-Pyridylamine; RP, Reverse phase; SF, Size fractionation; Xyl, Xylose.

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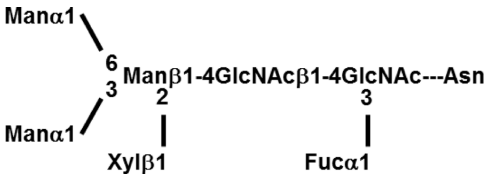

Figure 1. Schematic representation of plant-specific *N*-glycan.

known about the effect of culture conditions on the *N*-glycosylation profile.

In this study, we determined the *N*-glycan structures of endogenous and soluble glycoproteins at different growth stages of suspension-cultured *A. thaliana* T87 cells. While *A. thaliana* T87 cell has photosynthetic performance under light illumination, tobacco BY2 and *A. thaliana* MM2d cells do not have active plastids. The effect of growth stages on the *N*-glycosylation of suspension-cultured *A. thaliana* T87 cells was discussed.

The *A. thaliana* suspension-cultured cell strain T87 (Axelos et al. 1992) was cultured in a modified MS medium (Nagata et al. 1992) with constant agitation at 120 rpm and 22°C under a 16-h-light/8-h-dark photoperiod. Four milliliters of the suspension culture was transferred into 95 ml of fresh medium at one-week intervals. The cells were harvested on days 3, 5, and 7, and ground with a mortar and pestle in the presence of liquid N_2. The thawed cell homogenate was centrifuged at 12,000 rpm for 15 min at 4°C. The resulting supernatant was used as the solution of soluble and endogenous glycoproteins for structural analysis. The analysis of *N*-glycan structures used a combination of reversed-phase (RP-) and size-fractionation (SF-) HPLCs, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS), and exoglucosidase digestion (Misaki et al. 2003). Briefly, sugar chains released from glycoproteins by hydrazinolysis were *N*-acetylated and purified. The *N*-glycans were labeled with 2-pyridylamine (PA, Hase et al. 1978) and excess PA was removed using cellulose microcrystalline (MERCK) (Shimizu et al. 2001). PA-labeled *N*-glycans (PA-glycans) were fractionated by RP- and SF-HPLCs using Cosmosil 5C18-AR (Nacalai Tesque, Kyoto, Japan) and Asahipak NH2P-50 4E (Showa Denko, Tokyo, Japan) columns by monitoring the intensity at excitation and emission wavelengths of 310 nm and 380 nm, respectively. The molecular masses of the PA-glycans were determined by MALDI-TOF MS with an Autoflex mass spectrometer (Bruker Daltonics, Bremen, Germany) using 2,5-dihydroxybenzoic acid (Sigma, St. Louis, MO) as the matrix. On the basis of the deduced structure determined by MALDI-TOF MS, the PA-glycans were digested with α-mannosidase (almond, Sigma) and *N*-acetylglucosaminidase (*Diplococcus pneumoniae*, Roche, Mannheim, Germany), as described previously (Misaki et al. 2001; Palacpac et al. 1999), and

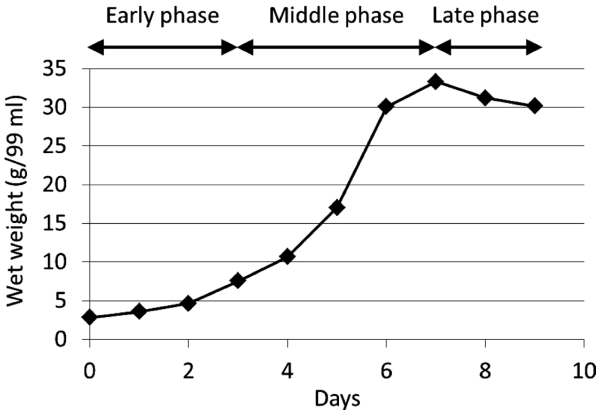

Figure 2. Growth curve of *A. thaliana* T87 cells. This growth curve shows an early phase (days 1–3), a middle phase (days 3–7), and a late phase (days 7–9).

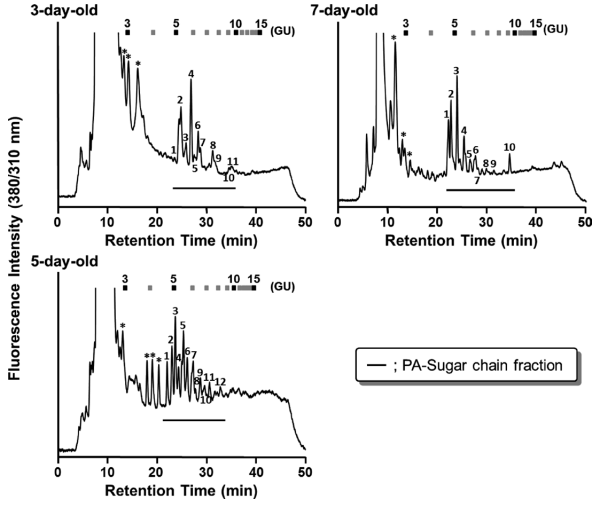

Figure 3. *N*-Glycan analysis of 3-, 5-, and 7-day-old *A. thaliana* T87 cells. Total *N*-glycans prepared from glycoproteins in each cell and labeled with PA were analyzed by RP-HPLC using a C_{18} column. Asterisks indicate non-PA-glycan derivatives. Numbers at the top represent the elution positions of glucose units (GU) based on the elution times of PA-isomalto-oligosaccharides with degrees of polymerization from 3 to 15.

analyzed by SF- or RP-HPLC. The elution positions of the *N*-glycan in the supernatant were compared with those of authentic PA-glycans (Takara Biomedicals, Shiga, Japan). Other plant-specific *N*-glycans were prepared from horseradish peroxidase and tobacco suspension-cultured BY2 cells (Misaki et al. 2001).

We examined the growth of the cell suspension of *A. thaliana* T87 under light illumination. The growth curve shows an early phase on days 1–3 followed by a middle phase on days 3–7 and a late phase on days 7–9 (Figure 2). Therefore, in this study, we divided the cell growth of suspension culture into three phases: early, middle, and late phases. When the cells were transferred to a fresh medium, the wet weight of the cells was 2.85 g/99 ml. After 7 days, the wet weight reached 33.3 g with an

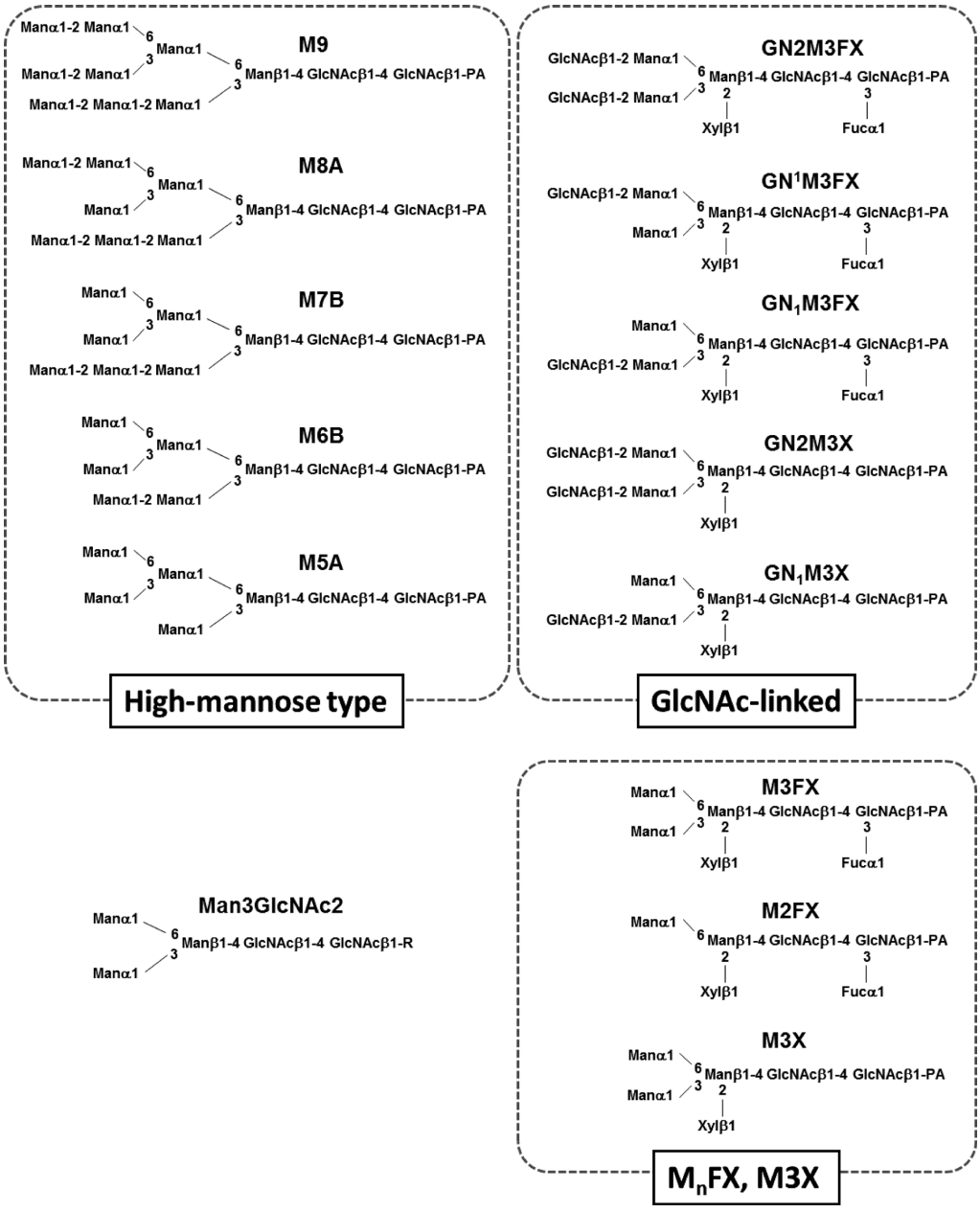

Figure 4. Schematic representation of *N*-glycans and their abbreviations detected in *A. thaliana* T87 cells and used in this study. High-mannose type, GlcNAc-linked, and M_nFX, and M3X were categorized.

approximately 11-fold increase. *A. thaliana* T87 cells grown for 3, 5, and 7 days were used to extract soluble glycoproteins.

PA-labeled *N*-glycans were prepared from soluble glycoproteins, purified, and characterized by a combination of RP- and SF-HPLCs. The molecular mass of the PA-glycan corresponding to each peak was determined using MALDI-TOF MS and compared with the calculated mass of authentic PA-glycans, suggesting a possible structure. From the deduced structure, the PA-glycan was digested with *α*-mannosidase and *N*-acetylglucosaminidase, and the digested PA-glycan was further examined using HPLC to compare its elution position with that of authentic PA-glycan. MALDI-TOF MS and exoglycosidase digestion showed that the peaks marked by bars contained *N*-glycans and those marked by asterisks contained no *N*-glycans (Figure 3).

PA derivatives prepared from *A. thaliana* T87 cells grown for 3, 5, and 7 days were fractionated by RP-HPLC (Figure 3): 3 days, Peak Nos. 1–11; 5 days, Peak Nos. 1–12; 7 days, Peak Nos. 1–10. To separate the same retention structures detected in RP-HPLC, each of the collected fractions was rechromatographed by SF-HPLC (data not shown) and the resultant fractions were denoted as a-d. The structures of the *N*-linked sugar chains from *A. thaliana* T87 cells are summarized in

Table 1. Relative amounts of PA-glycan structures detected in *A. thaliana* T87 cells

N-Glycan structure	Day 3		Day 5		Day 7	
	Peaks	Ratio (%)	Peaks	Ratio (%)	Peaks	Ratio (%)
High-mannose type						
M9A	2-b	3.4	4-a	5	2-d	1.5
M8A	1-c	12.3	3-a	10.3	1-d	7.7
M7B	4-b	1.5	6-b	2.9	—	—
M6A	5-b	1.7	7-b	4.3	4-b	0.6
M5A	8-c	1.5	9-c	0.9	—	—
GlcNAc-linked						
GN2M3FX	3-a	30.3	5-a	44.5	2-b, 3-c	42.3
GN^1M3FX	5-a	9.1	6-a, 7-a	16.2	4-a	3.6
GN_1M3FX	1-b	12	2-b	4.7	1-b	18
GN2M3X	10-a	0.9	—	—	8-a	1.3
GN_1M3X	8-b	6	9-b	1	6-b	4.3
MnFX, M3X						
M3FX	1-a	19.2	2-a	9.4	2-a	19.1
M2FX	—	—	—	—	3-a	0.9
M3X	8-a	2.1	9-a	0.8	6-a	0.7

The relative ratio of structures was calculated on the basis of the peak area in the SF-HPLC profile. Peaks indicate the PA-glycans collected by RP- and SF-HPLCs.

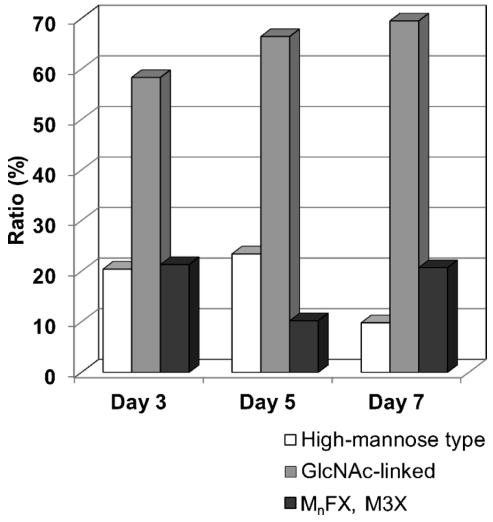

Figure 5. Relative amounts of high-mannose type, GlcNAc-linked, and M_nFX and M3X. On the basis of their structures, the ratio of each type shown in Figure 4 is summarized.

Table 1 and Figure 4.

The structural analysis of *N*-glycans of endogenous and soluble glycoproteins showed that *N*-glycosylation is dependent on the growth stage of the cell culture. At the early phase, the high-mannose-type glycan content was 20.4% of the total glycan, which increased to 23.4% at the middle phase, and then dropped down to 9.8% at the late phase (Figure 5). The amount of GlcNAc-linked glycans increased from 58.3% to 69.5%. In contrast, the amount of *N*-glycans with α1,3-Fuc and β1,2-Xyl changed and reached 90.3% at the late phase, though that of the most typical plant-type glycan, M3FX, was 19.1%. Throughout the growth, the most predominant structure was GN2M3FX: early phase, 30.3%; middle phase, 44.5%; and late phase, 42.3% (Table 1). However, the second most predominant structures were M3FX in the early phase, GN^1M3FX in the middle phase, and M3FX in the late phase. This *N*-glycosylation profile is quite different from that of *A. thaliana* MM2d (Fujiyama et al. 2007), where M3FX was 70% of the total glycan.

Growth conditions and developmental stage affect the *N*-glycosylation of endogenous and soluble proteins in tobacco leaves (Elbers et al. 2001). The ratio of high-mannose-type *N*-glycans to other types showed no marked change with temperature or light intensity. However, senescence affected the glycosylation profile and so the terminal GlcNAc-free *N*-glycan content increased in the late stage. In the suspension-cultured cells, glycans at the late stage of growth showed significant reduction in terminal GlcNAc residue content, but a larger increase in Fuc and Xyl residue content, suggesting that *N*-glycan maturation proceeded. The glycosylation patterns in BY2 cells were also growth-phase-dependent (Yin et al. 2009).

For mammalian and insect expression systems, culture conditions affected the glycosylation of recombinant proteins (Pacis et al. 2011; Joosten and Shuler 2003). Although Xu et al. (2011) reviewed the improvement of the medium and process to increase the production of recombinant proteins in plant cells, no study of the control of *N*-glycosylation has yet been reported. Monosaccharide in the culture medium is a key factor in altering the *N*-glycosylation profile of endogenous or secreted proteins (Becerra-Arteaga and Shuler 2007). Likewise, the supplementation of the culture medium controls the extent of *N*-glycosylation. It is also necessary to study environmental parameters that affect the production and *N*-glycosylation profile of recombinant

proteins in plant suspension-cultured cells. Light illumination is one of such environmental parameters. In this study, we examined the *N*-glycans of soluble and endogenous glycoproteins at different growth stages of *Arabidopsis* cells cultured under light illumination. To discuss the effects of light illumination on protein *N*-glycosylation in suspension-cultured cells, an *N*-glycan analysis of the soluble and endogenous proteins from plant cells cultured in the dark is now in progress.

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