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Production of mouse monoclonal antibody with galactose-extended sugar chain by suspension cultured tobacco BY2 cells expressing human β(1,4)-galactosyltransferase

Kazuhito Fujiyama [a,*], Aiko Furukawa [a], Atsuya Katsura [a], Ryo Misaki [a], Takeshi Omasa [b], Tatsuji Seki [a]

[a] *The International Center for Biotechnology, Osaka University, 2-1 Yamada-oka, Suita, Osaka 565-0871, Japan*
[b] *Department of Biotechnology, Graduate School of Engineering, Osaka University, 2-1 Yamada-oka, Suita, Osaka 565-0871, Japan*

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Abstract

Previously, we developed a transgenic tobacco BY2 cell line (GT6) in which glycosylation was modified by expressing human β(1,4)-galactosyltransferase (βGalT). In this study, we produced a mouse monoclonal antibody in GT6 cells, and determined the sugar chain structures of plant-produced antibodies. Galactose-extended *N*-linked glycans comprised 16.7%, and high-mannose-type and complex-type glycans comprised 38.5% and 35.0% of the total number of glycans, respectively. *N*-linked glycans with the plant-specific sugars β(1,2)-xylose and α(1,3)-fucose comprised 9.8%. The introduction of human βGalT into suspension cultured tobacco cells resulted in the production of recombinant proteins with galactose-extended glycans and decreased contents of β(1,2)-xylose and α(1,3)-fucose.

Keywords: Antibody; Sugar chain; Tobacco cell; Galactosyltransferase; Glycosylation

The *in planta* production of pharmaceutical proteins has been attempted [1–3]. Most pharmaceutical proteins are post-translationally modified by reactions such as the glycosylation and hydroxylation of amino acids. In particular, glycosylation at an asparagine residue of a polypeptide is sometimes important for biological activity, structural stability, and proteases sensitivity [1–3]. Antibodies have one *N*-glycosylation site at the Fc region of their heavy (H) chains. The glycan structure is critical for the functional and structural properties of antibodies [4–6]. Recently, the α(1,6)-fucose (Fuc) linked to the proximal *N*-acetylglucosamine (GlcNAc) of core Manβ1,4GlcNAcβ1,4GlcNAc ($Man_1GlcNAc_2$; Man mannose) affects antibody-dependent cellular cytotoxicity [7].

The *N*-linked glycan structures of plant glycoproteins differ from those of mammalian glycoproteins [8]. Plant glycan structures do not have complex-type β(1,4)-galactose (Gal) or sialic acid, whereas they do have β(1,2)-xylose (Xyl) and α(1,3)-Fuc linked to the core $Man_3GlcNAc_2$, such as in Man(α1–6)[Man(α1–3)][Xyl(β1–2)]Man(β1–4)GlcNAc(β1–4)[Fuc(α1–3)]GlcNAc (M3FX). These sugar chains with the plant-specific residues β(1,2)-Xyl and α(1,3)-Fuc have been shown to be potentially immunogenic or allergenic [9–13]. Structural analysis of sugar chains on antibodies produced in plants and suspension cultured cells showed that the recombinant antibodies carry plant-specific residues, similarly to endogenous proteins in host plants [14–19]. To produce stable and human-compatible glycosylated antibodies in plants, the extension of β(1,4)-Gal is necessary to further extend the sialic acid residue in complex-type glycans. Previously, we established a transgenic tobacco BY2 cell line (GT6) by expressing human β(1,4)-galactosyltransferase (GalT) [20] and showed

* Corresponding author. Fax: +81 6 6879 7454.
E-mail address: fujiyama@icb.osaka-u.ac.jp (K. Fujiyama).

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that the foreign protein produced in GT6 contained galactosylated glycans. Bakker et al. [21] also produced a mouse monoclonal antibody (mAb) in tobacco plants engineered to transfer β(1,4)-Gal and showed that the recombinant antibody was galactosylated. However, the glycan structures of the antibody contained β(1,4)-Gal, β(1,2)-Xyl, and α(1,3)-Fuc. Previously, we examined the glycan structures of a mAb produced by suspension cultured tobacco BY2 cells, and found that M3FX is predominant glycan structure [14]. In this study, we introduced mAb cDNA into GT6, and examined the potential of using GT6 cells for the production of galactosylated antibodies.

Materials and methods

Generation of GT6 cells expressing mouse IgG. A transgenic suspension cultured tobacco BY2 cell line, GT6, expressing human β(1,4)-GalT was maintained in modified Linsmaier and Skoog (mLS) medium [20].

The plasmid pGPTV-HPT-IgG [14] carrying the mouse heavy and light (L) chains was used to transform GT6 cells via *Agrobacterium tumefaciens* strain LBA4404 [22]. Transformants were selected and maintained in mLS medium containing antibiotics (250 mg/l carbenicillin sodium salt, 150 mg/l kanamycin, and 50 mg/l hygromycin B sulfate).

Preparation of N-linked glycan and analysis by high-performance liquid chromatography (HPLC). Using fully grown transgenic tobacco cells, a recombinant mAb was purified as described previously [14]. *N*-linked glycans were released from purified recombinant IgG (2 mg) by hydrazinolysis and fractionated on a TSK gel Toyopearl HW-40 (Tosoh) column [14]. The obtained oligosaccharides were pyridylaminated (PA) as described previously [14,23]. PA-sugar chains were purified by HPLC and monitored on the basis of the fluorescence intensity at 380 nm (excitation; 310 nm). For reversed-phase (RP)-HPLC, PA-sugar chains were eluted from a Cosmosil 5C18-AR column (Nacalai Tesque) by linearly increasing the acetonitrile concentration in 0.02% trifluoroacetic acid (TFA) from 0% to 6% for 40 min at a flow rate of 1.2 ml/min. For size-fractionation (SF)-HPLC using an Asahipak NH2P-50 4E column (Showa Denko), the PA-sugar chains were eluted by linearly increasing the water content of the water–acetonitrile mixture from 26% to 50% for 25 min at a flow rate of 0.7 ml/min.

Analysis of oligosaccharide structures. PA-sugar chains were digested with *N*-acetylglucosaminidase (GlcNAc-ase, 1 mU, *Diplococcus pneumoniae*, Roche), α-mannosidase (α-Man-ase, 10 mU, almond, Sigma), and β-galactosidase (β-Gal-ase, 200 mU, *D. pneumoniae*, Roche) as described previously [14,20,24]. The reactions were stopped by boiling the mixtures for 3 min. After centrifugation at 12,000 rpm for 10 min at 4 °C, the resulting supernatant was analyzed by SF-HPLC or RP-HPLC. Their elution positions were compared with the elution positions of authentic PA-sugar chains (M7B, M6B, M3B, and M2A) purchased from Takara Bio (Shiga).

The sugar chain Man(α1–6)[GlcNAc(β1–2)]Man(α1–3) Man(β1–4)GlcNAc(β1–4)GlcNAc (GN_1M3) was prepared from authentic M3B using recombinant human *N*-acetylglucosaminyltransferase I (GnT-I) [25] and the sugar chain of M3FX was prepared using horseradish peroxidase [26,27]. The molecular masses of PA-sugar chains were determined by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) using an autoflex mass spectrometer (Bruker Daltonics).

Results

GT6 cell line producing mAb

We expressed cDNAs encoding the H and L chains of the mouse antibody against RNaseA in suspension cultured tobacco BY2 cells genetically engineered by inserting human β(1,4)-GalT. Of the transformants obtained, clone 44 was selected because of its fast growth and used for further experiments.

The recombinant mAb produced by suspension cultured tobacco cells was purified using a Protein G column, and used for glycan structure analysis. The PA-sugar chains obtained from the recombinant mAb were fractionated and characterized by both RP- and SF-HPLCs (Fig. 1A and B). Fig. 1A shows several fractions of PA derivatives as analyzed by RP-HPLC. Then, each collected fraction (1–10) was subjected to SF-HPLC (Fig. 1B). The structures of the *N*-linked glycans obtained are shown in Fig. 2.

In this study, small 8 fractions, indicated by asterisks in Fig. 1B, were not analyzed. MALDI-TOF MS and exoglycosidase digestion showed that 27 fractions, underlined in Fig. 1B, as well as fractions eluted before peak 1 (0–16 min) in Fig. 1A, did not contain PA-labeled *N*-linked sugar chains.

Structural analysis of N-linked glycans from GT6-derived mAb

The molecular masses of compounds contained in other fractions were analyzed by MALDI-TOF MS. Each fraction was further examined by exoglycosidase digestion and HPLC.

The molecular mass of the *N*-glycan corresponding to the PA-sugar chain in fraction 3-1 (*m*/*z* 1289.81) agreed well with the calculated mass of $Man_3FucXylGlcNAc_2$-PA (1289.19 for $[M+Na]^+$). The elution position of fraction 3-1 on the RP-HPLC profile was identical to that of Man(α1–6)[Man(α1–3)][Xyl(β1–2)]Man(β1–4)GlcNAc(β1–4)[(Fuc(α1–3)]GlcNAc-PA (M3FX). These results suggest that the glycan in fraction 3-1 was M3FX, as shown in Fig. 2.

The molecular masses of the *N*-linked glycans corresponding to the PA-sugar chains in fractions 6-4 (*m*/*z* 1637.74), 7-4 (*m*/*z* 1475.67), and 10-1 (*m*/*z* 989.94) agreed well with the calculated masses of $Man_7GlcNAc_2$-PA (1637.50), $Man_6GlcNAc_2$-PA (1475.36), and $Man_3GlcNAc_2$-PA (989.94), respectively. The elution positions of the *N*-linked glycans on the RP-HPLC profile were identical to those of the authentic sugar chains M7B, M6B, and M3B (Fig. 2). The digestion of each glycan by α-Man-ase yielded a product with an elution position identical to that of $ManGlcNAc_2$-PA on the SF-HPLC profile. These results suggest that the glycans in fractions 6-4, 7-4, and 10-1 were M7B, M6B, and M3B, respectively (Fig. 2).

The molecular mass of the *N*-linked glycan corresponding to the PA-sugar chain in fraction 7-1 (*m*/*z* 1030.95) agreed well with the calculated mass of $GlcNAcMan_2GlcNAc_2$-PA (1029.99). Treatment of this glycan with GlcNAc-ase yielded a product with an elution position identical to that of $Man_2GlcNAc_2$-PA on the SF-HPLC

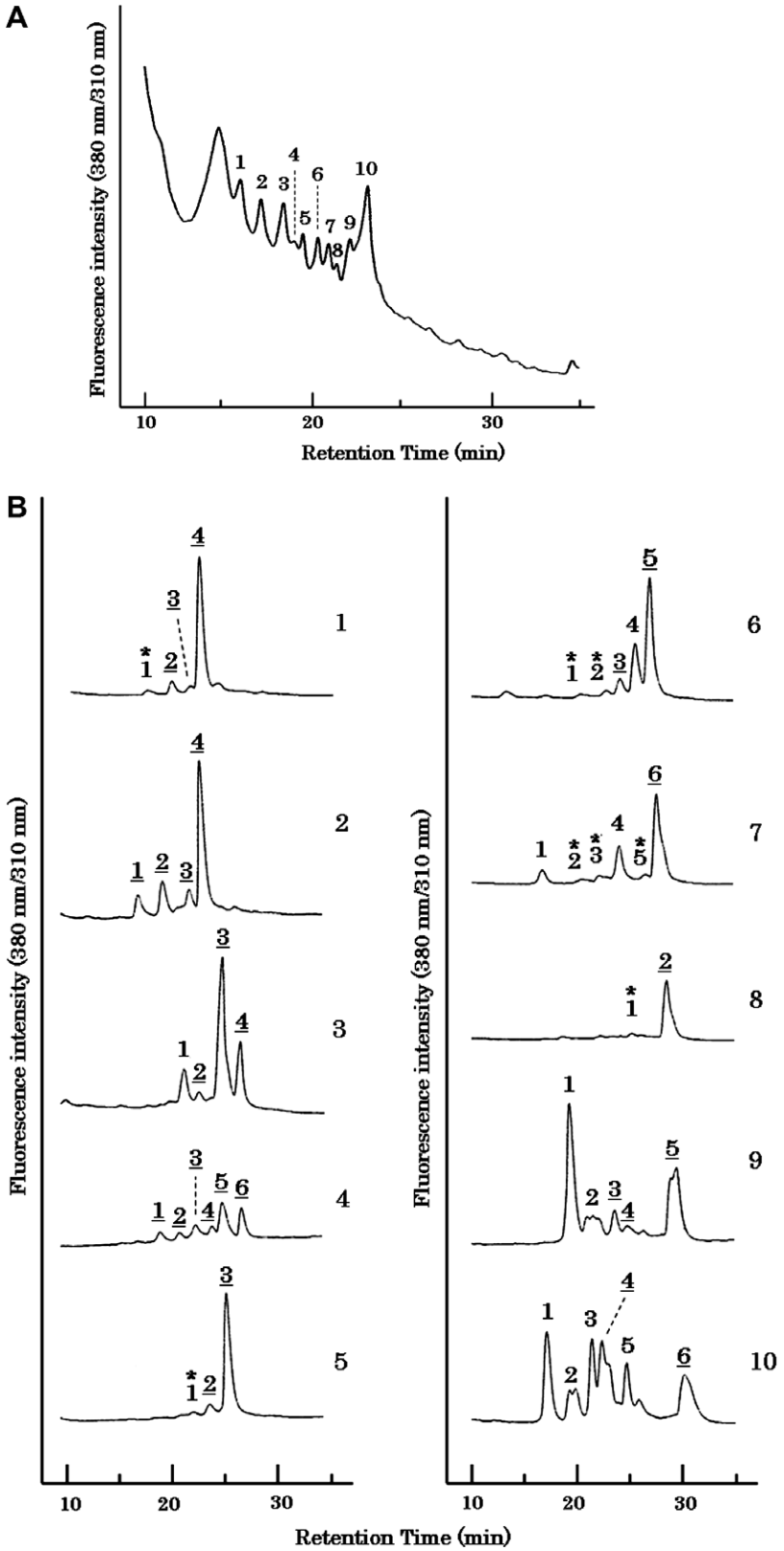


Fig. 1. PA derivatives from mAb produced from suspension cultured tobacco GT6 cells. (A) RP-HPLC profile of PA-sugar chains eluted by linearly increasing CH_3CN concentration in 0.02% trifluoroacetic acid from 0% to 6% for 40 min at flow rate of 1.2 ml/min. 1–10, individual fractions collected and purified in SF-HPLC. (B) SF-HPLC profiles of collected fractions in (A). PA-sugar chains were eluted by increasing the water content in the water–CH_3CN 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. Small 8 fractions, indicated by asterisks in (B), were not analyzed. MALDI-TOF MS and exoglycosidase digestion showed that 27 fractions, underlined in (B) did not contain PA-labeled *N*-linked sugar chains.

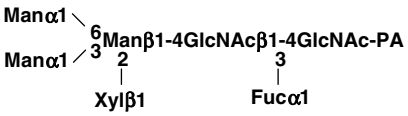
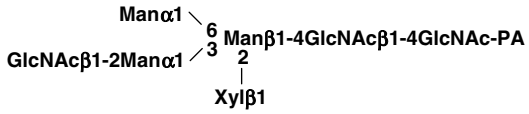
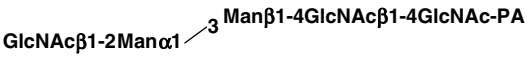
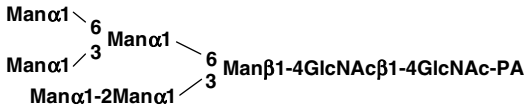
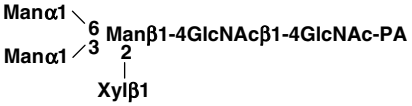
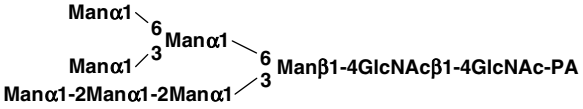
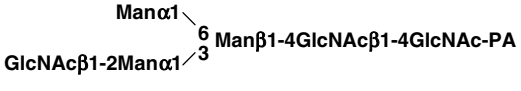
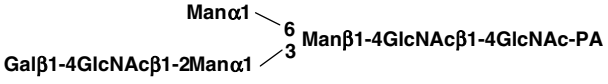
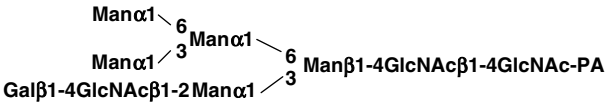
Peak name	Abbreviation	Structure
3-1	M3FX (1.1%)	
9-2	GN_1M3X (2.1%)	
7-1	GN_1M2 (2.1%)	
7-4	M6B (4.4%)	
10-2a	M3X (6.6%)	
6-4	M7B (6.8%)	
9-1	GN_1M3 (32.9%)	
10-1	M3B (22.3%)	
10-2b	M4B (5.0%)	
10-3	$GalGN_1M3$ (8.9%)	
10-5	$GalGN_1M5$ (7.8%)	

Fig. 2. Structures of *N*-linked glycans obtained from mAb produced by suspension cultured tobacco GT6 cells. Numbers in parentheses represent molar ratios.

profile. Moreover, the product derived from GlcNAc-ase digestion had the same elution position on the RP-HPLC profile as that of the authentic sugar chain Man(α1–3)Man(β1–4)GlcNAc(β1–4)GlcNAc-PA (M2A). These results suggest that the glycan in fraction 7-1 was GlcNAc(β1–2)Man(α1–3)Man(β1–4)GlcNAc(β1–4)GlcNAc-PA (GN_1M2).

The molecular mass of the *N*-linked glycan corresponding to the PA-sugar chain in fraction 9-1 (*m*/*z* 1192.99) matched the calculated mass of $GlcNAcMan_3GlcNAc_2$-PA or $GalGlcNAcMan_2GlcNAc_2$-PA (1192.13). The β-Gal-ase digestion of the *N*-glycan did not yield a product with a structure of $GlcNAcMan_2GlcNAc_2$-PA as deduced using the SF-HPLC profile. The elution position of the PA-sugar chain in fraction 9-1 on the RP-HPLC profile was identical to that of Man(α1–6)[GlcNAc(β1–2)Man(α1–3)]Man(β1–4)GlcNAc(β1–4)GlcNAc-PA (GN_1M3). The elution positions of the GlcNAc-ase-digested sugar chain on the SF- and RP-HPLC profiles were identical to those of the authentic M3 sugar chain (M3B). These results

suggest that the glycan in fraction 9-1 was GN_1M3 (Fig. 2).

The molecular mass of the *N*-linked glycan corresponding to the PA-sugar chain in fraction 9-2 (*m*/*z* 1324.61) agreed well with the calculated mass of $GlcNAcMan_3XylGlcNAc_2$-PA or $GalGlcNAcMan_2XylGlcNAc_2$-PA (1324.24). The elution position of the PA-sugar chain in fraction 9-2 on the RP-HPLC profile was identical to that of the GN_1M3X sugar chain Man(α1–6)[GlcNAc(β1–2)Man(α1–3)][Xyl(β1–2)]Manβ1,4GlcNAcβ1,4GlcNAc-PA prepared from proteins in the BY2 cells [28]. The β-Gal-ase digestion of the *N*-linked glycan did not yield a product with a possible structure of $GlcNAcMan_2XylGlcNAc_2$-PA, as analyzed by SF-HPLC. On the other hand, the digestion of the *N*-linked glycan with GlcNAc-ase yielded a product with an elution position identical to that of $Man_3XylGlcNAc_2$-PA, as analyzed by SF-HPLC. The elution position of this product was the same as that of authentic Man(α1–6)[Man(α1–3)][Xyl(β1–2)]Man(β1–4)GlcNAc(β1–4)GlcNAc-PA (M3X) on the RP-HPLC profile. These results suggest that the glycan in fraction 9-2 was GN_1M3X.

MALDI-TOF MS showed that peak 10-2 corresponded to two *N*-linked glycans, one with a molecular mass of 1122.00 and the other with a molecular mass of 1152.03. The molecular masses of these glycans were agreed well with the calculated masses of $Man_3XylGlcNAc_2$-PA (1121.05) and $Man_4GlcNAc_2$-PA (1151.08), respectively. The sugar chain was separated again by RP-HPLC, resulting in two peaks, namely, fraction 10-2a (6.6%) and fraction 10-2b (5.0%). The elution positions of fractions 10-2a and 10-2b on the RP-HPLC profile were identical to those of M3X and the authentic M4B sugar chain, respectively (Fig. 2). These results suggest that the two glycans in fraction 10-2 were M3X (10-2a), and M4B (10-2b).

The molecular mass of the *N*-linked glycan corresponding to the PA-sugar chain in fraction 10-3 (*m*/*z* 1355.04) agreed well with the calculated mass of $GalGlcNAcMan_3GlcNAc_2$-PA (1354.27). The elution position of this glycan on the RP-HPLC profile was identical to that of Man(α1–6)[Gal(β1–4)GlcNAc(β1–2)Man(α1–6)]Man(β1–4) GlcNAc(β1–4)GlcNAc-PA ($GalGN_1M3$) prepared from GT6 intracellular proteins [20]. The βGal-ase digestion of the *N*-linked glycan yielded a product with a possible structure of $GlcNAcMan_3GlcNAc_2$-PA as determined by SF-HPLC. The subsequent GlcNAc-ase digestion of the β-Gal-ase digestion product yielded $Man_3GlcNAc_2$-PA, as shown by SF-HPLC, whose elution position on the RP-HPLC profile was identical to that of M3B. These results suggest that the glycan corresponding to peak 10-3 was $GalGN_1M3$.

The molecular mass of the *N*-linked glycan corresponding to the PA-sugar chain corresponding to peak 10-5 (*m*/*z* 1678.91) agreed well with the calculated mass of $GalGlcNAcMan_5GlcNAc_2$-PA (GalGNM5; 1678.55). The elution position of the *N*-linked glycan on the RP-HPLC profile was identical to that of Man(α1–6)[Man(α1–3)]Man(α1–6)[Gal(β1–4)GlcNAc(β1–2)Man(α1–3)]Man(β1–4)GlcNAc(β1–4)GlcNAc-PA ($GalGN_1M5$) prepared from GT6 intracellular proteins [20]. The elution position of the β-Gal-ase-digested sugar chain on the SF-HPLC profile was identical to that of $GlcNAcMan_5GlcNAc_2$-PA (GNM5). The subsequent GlcNAc-ase digestion of the β-Gal-ase digestion product yielded a glycan whose elution position on the SF-HPLC profile was identical to that of Man(α1–6)[Man(α1–3)]Man(α1–6) [Man(α1–3)]Man(β1–4)GlcNAc(β1–4)GlcNAc-PA (M5A). These results suggest that the glycan corresponding to peak 10-5 was $GalGN_1M5$.

Discussion

In our previous study [28], we showed that the glycan structures of glycoproteins from tobacco BY2 cells are GN_1M3FX (21.7%), M3X (3.3%), M3FX (41.0%), and, GlcNAc(β1–2)Man(α1–6)[GlcNAc(β1–2)Man(α1–3)][Xyl(β1–2)]Man(β1–4)GlcNAc(β1–4)[Fuc(α1–3)]GlcNAc (GN_2M3FX, 26.5%) as shown in Table 1. The introduction of human β(1,4)-GalT into tobacco BY2 cells altered the glycosylation pathway, resulting in the fact that galactosylated glycans accounted for 47% of the total number of glycans, whereas plant-specific glycans with β(1,2)-Xyl and α(1,3)-Fuc accounted for only 7% [20].

A mAb was expressed in suspension cultured tobacco BY2 cells and its glycan structures were analyzed [14]. The high-mannose-type glycan was $Man_{5-6}GlcNAc_2$ (22.3%), whereas glycans bearing β(1,2)-Xyl and α(1,3)-Fuc were GN_1M3FX (24.4%), $GlcNAcMan_3XylGlcNAc_2$ (17.8%), M3FX (24.3%), and M3X (8.1%). A glycan analysis of the mAb produced in GT6 clone 44 cells indicates that the galactosylated glycans were $GalGN_1M5$ and $GalGN_1M3$. The predominant glycan was $GlcNAcMan_3GlcNAc_2$ (32.9%), which can be a substrate for β(1,4)-GalT. Glycans with β(1,2)-Xyl or α(1,3)-Fuc comprised 9.8% of the total number of glycans. Compared with the profile of the glycans from the total glycoproteins in GT6, the amount of galactosylated glycans decreased to 16.7%. Presently, we do not know the reason for this decrease. For the production of an antibody in a transgenic plant expressing human β(1,4)-GalT [21], structural analysis showed four groups of eight galactosylated glycans, namely: (1) $GalGlcNAc_2Man_3GlcNAc_2$, (2) $Gal_{1-2}GlcNAc_2FucXylMan_3GlcNAc_2$, (3) $GalGlcNAc_{1-2}XylMan_3GlcNAc_2$, and (4) $Gal_{1-2}GlcNAc_2FucMan_3GlcNAc_2$. Galactosylated sugars also have plant-specific core-bound Xyl and Fuc residues. Recently, a chimeric human GT was constructed and examined to determine whether glycosylation can be affected [29]. The chimeric β(1,4)-GalT consists of the transmembrane domain of *Arabidopsis thaliana* β(1,2)-xylosyltransferase and the catalytic domain of human β(1,4)-GalT. The expression of the chimeric human β(1,4)-GalT in tobacco plant cells markedly changed the glycosylation profile of recombinant mAb Fc and decreased the amount of plant-typical glycans containing β(1,2)-Xyl and α(1,3)-Fuc. Cox et al. genetically engineered

Table 1
Comparison of sugar chain structures

	BY2[28] (%)	IgG from BY2[14] (%)	GT6[20] (%)	IgG from GT6 (%)
$Man_8GlcNAc_2$	—	—	—	—
$Man_7GlcNAc_2$	—	—	25.2	6.8
$Man_6GlcNAc_2$	—	15.4	19.5	4.4
$Man_5GlcNAc_2$	7.5	6.9	1.4	—
$GlcNAcMan_5GlcNAc_2$	—	3.1	—	—
$GalGlcNAcMan_5GlcNAc_2$	—	—	35.5	7.8
$GlcNAcMan_3GlcNAc_2$	—	—	—	32.9
$GalGlcNAcMan_3GlcNAc_2$	—	—	11.8	8.9
$GlcNAc_2FucXylMan_3GlcNAc_2$	26.5	—	—	—
$GlcNAcFucXylMan_3GlcNAc_2$	21.7	17.8	—	—
$FucXylMan_3GlcNAc_2$	41.0	24.3	—	1.1
$XylMan_3GlcNAc_2$	3.3	8.1	6.6	6.6
$GlcNAcXylMan_3GlcNAc_2$	—	24.4	—	2.1
$Man_3GlcNAc_2$	—	—	—	22.3
$Man_4GlcNAc_2$	—	—	—	5.0
$GlcNAcMan_2GlcNAc_2$	—	—	—	2.1

28,14,20 refer to corresponding reference.

the aquatic plant *Lemna,* which is capable of producing mAbs with humanized glycan structures [30].

During the transportation of glycoproteins from ER through the Golgi apparatus, *N*-linked glycans are processed along the secretory pathway in the cell. Our data show that the H chain of the recombinant mAb was also glycosylated during its trafficking from ER to the Golgi apparatus. Although galactosylated structures accounted for 16.7% of the total number of glycans, the amount of paucimannose structures, such as $Man_3GlcNAc_2$, increased to 22.3%, and those of high-mannose-type structures decreased in the GT6-derived antibody compared with those in the BY2-derived mAb [14]. Though in this study, we selected mAb producer GT6 clone 44 for determination of glycan structure, glycan structures of other clones are not clear. Presently, it is difficult to achieve the complete galactosylation of the target recombinant proteins and to homogenize the glycans of the proteins.

The glycans of recombinant proteins, such as IgG, are critical for pharmacological and biological activities, and structural stability. For antibodies, the presence of Gal residues enhances the binding of IgG to FcRI [4,5]. β(1,4)-Galactosylation enables the subsequent sialylation of glycoproteins to complex-type glycans. Recently, Kaneko et al. [31] have shown that the sialylated Fc of mAb conferred anti-inflammatory properties. As described here, for the galactosylation of plant glycans, some technologies have been developed. The extension of the β(1,4)-linked Gal residue on complex-type glycans enables the production of antibodies in plants with human-compatible glycans, enabling to the use of plant cells as efficient bioreactors.

Plant systems for the production of human pharmaceutical proteins have some advantages, such as a low production cost, easy scale-up, and the lack of animal pathogens. Suspension cultured plant cells also have potential for the production as alternative hosts [3,32]. One of the advantages of using suspension cultured cells is the ease of controlling their culture conditions to achieve a uniform environment. Because the transgenic tobacco BY2 cell line expressing human β(1,4)-GalT has the potential to produce (asialo)galactosylated mAbs and reduce plant-specific glycosylated mAbs, such as β(1,2)-xylosylated and α(1,3)-fucosylated mAbs, this GT6 cell line could be used as a high potential bioreactor for the production of pharmaceutical proteins.

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