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Stable expression of human β1,4-galactosyltransferase in plant cells modifies N-linked glycosylation patterns

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ABSTRACT **β1,4-Galactosyltransferase (UDP galactose: β-*N*-acetylglucosaminide: β1,4-galactosyltransferase; EC 2.4.1.22) catalyzes the transfer of galactose from UDP-Gal to *N*-acetylglucosamine in the penultimate stages of the terminal glycosylation of N-linked complex oligosaccharides in mammalian cells. Tobacco BY2 cells lack this Golgi enzyme. To determine to what extent the production of a mammalian glycosyltransferase can alter the glycosylation pathway of plant cells, tobacco BY2 suspension-cultured cells were stably transformed with the full-length human galactosyltransferase gene placed under the control of the cauliflower mosaic virus 35S promoter. The expression was confirmed by assaying enzymatic activity as well as by Southern and Western blotting. The transformant with the highest level of enzymatic activity has glycans with galactose residues at the terminal nonreducing ends, indicating the successful modification of the plant cell N-glycosylation pathway. Analysis of the oligosaccharide structures shows that the galactosylated *N*-glycans account for 47.3% of the total sugar chains. In addition, the absence of the dominant xylosidated- and fucosylated-type sugar chains confirms that the transformed cells can be used to produce glycoproteins without the highly immunogenic glycans typically found in plants. These results demonstrate the synthesis in plants of N-linked glycans with modified and defined sugar chain structures similar to mammalian glycoproteins.**

Transgenic plants are one of the promising systems for the production of human therapeutic proteins because of ease of genetic manipulation, lack of potential contamination with human pathogens, low-cost biomass production, and the inherent capacity to carry out most posttranslational modifications similar to mammalian cells (1–3). Among the posttranslational modifications, glycosylation has been shown to play critical roles for various physiological activities of mammalian glycoproteins (2, 4–6). Modifying the glycosylation activities is important not only for medical and biotechnological purposes (7) but also as a tool to investigate the role of plant glycan structures and the N-link glycosylation pathway in plants (8).

The asparagine-linked (N-linked) glycosylation mechanism in mammalian and plant systems is conserved evolutionarily; however, the fine details in the oligosaccharide trimming and further modification of glycans in the Golgi differ (5, 8–10). Thus, high-mannose-type *N*-glycans in plants have structures identical to those found in other eukaryotic cells; however, plant complex N-linked glycans differ substantially (8). In the mammalian system, the $Man_3GlcNAc_2$ (M3) core structure is extended further to contain penultimate galactose and terminal sialic acid residues (5, 11). In contrast, typically processed *N*-glycans in plants are mostly of a $Man_3GlcNAc_2$ structure with or without β1,2-xylose and/or α1,3-fucose residues (8–10). Larger, complex-type plant *N*-glycans are rare and recently have been identified as additional α1,4-fucose and β1,3-galactose residues, giving rise to mammalian Lewis a (Le^a) structures (9). The presence of xylose and/or fucose residues makes plant recombinant therapeutics less desirable (8–10). Complementing the *N*-glycan machinery with heterologous glycosyltransferases may help achieve the production of glycoproteins with human-compatible-type oligosaccharide structures. In plants, transformation of mutant *Arabidopsis cgl* cells with the cDNA encoding human *N*-acetylglucosaminyl transferase I (GnT-I) resulted in the conversion of high-mannose glycans into complex glycans that may be fucose-rich and xylose-poor, implying that the human enzyme can be integrated functionally in the normal pathway for biosynthesis of complex glycans in *Arabidopsis* (8, 12).

We expressed the human β1,4-galactosyltransferase gene (UDP galactose: β-*N*-acetylglucosaminide: β1,4 galactosyltransferase; EC 2.4.1.22), placed under the control of the cauliflower mosaic virus 35S (CaMV35S) promoter, in *Nicotiana tabacum* L. *cv*. Bright Yellow 2 (BY2) cells as a first step to evaluate the possibility of enlarging the spectrum of glycosyltransferases in plant suspension-cultured cells, which potentially could lead to transgenic plants. We chose the mammalian glycosyltransferase for two reasons: β1,4-galactosyltransferase is the first glycosyltransferase in mammalian cells that initiates the further branching of complex N-linked glycans after the action of GnT-I and -II (9, 11, 13); and though the glycosyltransferase is ubiquitous in the vertebrate kingdom (13), its presence has not yet been conclusively proven in plants (9, 10). Moreover, we recently have shown by HPLC and ion-spray tandem (IS)-MS/MS analyses that tobacco BY2 suspension-cultured cells do not contain any galactosylated *N*-glycan (14), suggesting that the glycosyltransferase either may be absent or too low to be effective in these cells. The transformed cells expressed β1,4-galactosyltransferase activity and possess glycans that bind to *Ricinus communis* agglutinin (RCA_{120}; specific for β1,4-linked galactose). Galactosylated glycans did not react with an antibody specific for complex glycans containing β1,2-xylose residues, indicating that the sugar chains do not contain β1,2-xylose residues. Glycans with α1,3-fucose were not observed based on HPLC and IS-MS/MS determinations. Structural analysis of the oligosaccharide moieties from glycoproteins of transformed cells provides proof of

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Abbreviations: hGT, human β1,4-galactosyltransferase; BY2, *Nicotiana tabacum* L. *cv*. Bright Yellow 2; RCA_{120}, *Ricinus communis*$_{120}$ agglutinin; PA, pyridylamino; RP- and SF-HPLC, reversed-phase and size fractionation HPLC; IS-MS/MS, ion-spray tandem MS/MS; GnT-I and -II, *N*-acetylglucosaminyl transferase I and II; CaMV35S, cauliflower mosaic virus 35S.

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the changes in plant *N*-glycan structure and confirms that galactosylation influenced and improved the N-linked pathway in tobacco BY2 cells.

MATERIALS AND METHODS

Construction of the Plant Expression Vector. The gene for human β1,4-galactosyltransferase (hGT) was amplified by PCR using two sets of primers based on the cDNA sequence reported by Masri *et al.* (15). Primers 1 (5′-AAG*ACTAGT*-GGGCCCCATGCTGATTGA-3′, *Spe*I site in italics) and 2 (5′-GTA*GTCGAC*GTGTACCAAAACGCTAGCT-3′, *Sal*I site in italics) were used to amplify an 813-bp fragment from human placenta cDNA (CLONTECH) by using *Taq* polymerase (Takara Shuzo, Kyoto). For the N-terminal portion of the gene, primers 3 (5′-AAA*GAATTC*GCGATGCCAG-GCGCGTCCCT-3′, *Eco*RI site in italics) and 4 (5′-AAT*ACTAGT*AGCGGGGACTCCTCAGGGCA-3′, *Spe*I site in italics) were used to obtain a 376-bp fragment from human genomic DNA (CLONTECH). The PCR products were cloned in M13mp18, sequenced with an AutoRead Sequencing Kit (Pharmacia), and analyzed by using an ALF DNA sequencer (Pharmacia). The truncated coding sequences were juxtaposed to obtain the complete β1,4-galactosyltransferase gene (1.2 kbp). Sequence alignment of the entire gene shows 99% overall sequence identity to that reported by Watzele and Berger (ref. 16; GenBank accession no. X55415) and Uejima *et al.* (ref. 17; accession no. X13223).

The 1.2-kbp DNA fragment was subcloned into pBluescript KS(–) (Stratagene), designated as pSKhGT (18). Plasmid pSKhGT was digested with *Eco*RI and *Sal*I, and the resulting fragment was inserted between the CaMV35S promoter and nopaline synthase terminator (nos-t) in pBI221 (CLONTECH). The resulting 2.2-kbp fragment, CaMV35S/hGT/nos-t chimeric gene, was excised and ligated to the binary vector, pGA482 (19), to construct the hGT expression vector pGAhGT.

Plant Transformation. The pGAhGT plasmid was mobilized into *Agrobacterium tumefaciens* strain EHA101 by triparental mating (20). Tobacco BY2 suspension-cultured cells (*Nicotiana tabacum* L. *cv*. Bright Yellow 2) were transformed as described (21). Transformants were selected and maintained on modified Linsmaier and Skoog medium (14, 22) supplemented with 150 μg/ml of kanamycin and 250 μg/ml of carbenicillin. Kanamycin-resistant transformants were screened for hGT expression by Southern and Western blotting.

DNA Isolation and Southern Blot Analyses. Chromosomal DNA (40 μg) was isolated from tobacco cell transformants (23) and digested with *Eco*RI and *Hin*dIII. DNA fragments were electrophoresed, blotted, and hybridized with a [^{32}P] random-primed, 2.2-kbp *Eco*RI and *Hin*dIII fragment from pGAhGT (Takara random-priming labeling kit).

Preparation of Cell Extracts for Western Blotting, Enzyme Assay, and Affinity Chromatography. Tobacco suspension cultures (5–7 days old) were harvested, suspended in extraction buffer (25 mM Tris·HCl, pH 7.4/0.25 M sucrose/1 mM $MgCl_2$/50 mM KCl), and disrupted by brief sonication (200 W; Kaijo Denki, Japan) or homogenized. Cell extracts and microsome fractions were prepared as described (24).

Protein concentration was measured by the Coomassie blue dye-binding assay (Bio-Rad) with BSA (Sigma) as standard.

Immunoblotting and β1,4-Galactosyltransferase Activity. Cell homogenates were solubilized in Laemmli sample buffer (25) and subjected to 10% SDS/PAGE. The hGT protein was detected by Western blotting using a monoclonal anti-human galactosyltransferase antibody (mAb 8628; 1:5,000) kindly provided by H. Narimatsu (Soka University) (17, 26). Blots then were incubated with a horseradish peroxidase-conjugated goat anti-mouse IgG (1:1,000 in 5% skim milk; EY Laboratories) and washed, and the horseradish peroxidase color reaction was carried out by using the POD immunostain kit (Wako Chemicals, Osaka).

Immunoblot analysis for plant-specific complex glycans was carried out by using a polyclonal antiserum raised against carrot cell-wall β-fructosidase (27).

β1,4-Galactosyltransferase activity was assayed by using UDP-Gal and pyridylamino-labeled $GlcNAc_2Man_3GlcNAc_2$ ($GlcNAc_2Man_3GlcNAc_2$-PA) as substrate (28). The enzyme reaction contained 1–120 μg protein, 25 mM sodium cacodylate (pH 7.4), 10 mM $MnCl_2$, 200 μM UDP-Gal, and 100 nM $GlcNAc_2Man_3GlcNAc_2$-PA. Reaction products were analyzed by HPLC using a PALPAK Type R and a Type N column (Takara Shuzo) according to the manufacturer's recommendation. PA-labeled standards $GlcNAc_2Man_3GlcNAc_2$-PA and $Gal_2GlcNAc_2Man_3GlcNAc_2$-PA and two isomers of $GalGlcNAc_2Man_3GlcNAc_2$-PA were from Takara Shuzo and Honen Co. (Tokyo), respectively.

Affinity Chromatography on RCA_{120}. Crude cell extracts and microsome fractions of transformed cells with highest enzymatic activity were loaded onto an RCA_{120}-agarose column (Wako). The column was washed with 15 volumes of 10 mM ammonium acetate, pH 6.0. Bound proteins were eluted with 0.2 M lactose and fractionated on SDS/PAGE before silver (Wako Silver Staining Kit) or lectin staining. For lectin staining, membrane blots were washed in TTBS buffer (10 mM Tris·HCl, pH 7.4/0.15 M NaCl/0.05% Tween 20) and incubated with horseradish peroxidase-labeled RCA_{120} (Honen), and galactosylated glycoproteins were visualized by using the POD immunostain kit (Wako).

Preparation, Derivatization, and Characterization of N-Linked Glycans. Structures of N-linked glycans in transformed cells were analyzed by a combination of reversed-phase and size-fractionation HPLC (RP- and SF-HPLC, respectively), two-dimensional sugar chain mapping, exoglycosidase digestions, and IS-MS/MS as described earlier (14). Briefly, cell extracts were defatted with acetone and sugar chains were released by hydrazinolysis (100°C, 12 hr). The hydrazinolysate was N-acetylated, desalted by Dowex 50X2 and Dowex 1X2, and fractionated on a Sephadex G-25 gel-filtration column (1.8 × 180 cm) in 0.1 N ammonia. Pyridylamination of the oligosaccharides obtained was as described (29). PA-sugar chains were fractionated on a Jasco 880-PU HPLC apparatus equipped with a Jasco 821-FP Intelligent Spectrofluorometer by using a Cosmosil 5C18-P column (6 × 250 mm; Nacalai Tesque, Kyoto) or an Asahipak NH2P-50 column (4.6 × 250 mm; Showa Denko, Tokyo). Elution positions of sugar chains were compared with authentic sugar chains as prepared previously (30) or purchased (Wako).

Glycosidase digestions using β-*N*-acetylglucosaminidase (*Diplococcus pneumoniae*; Boehringer Mannheim) or α-mannosidase (jackbean; Sigma) were done by using about 1 nmol of PA-sugar chains (30). For β-galactosidase (*D. pneumoniae*; Boehringer Mannheim) and α1,2-mannosidase (*Aspergillus saitoi*; a kind gift from T. Yoshida, Tohoku University) digestions, 1 nmol of PA-sugar chain in 50 mM sodium acetate buffer (pH 5.5) was incubated with either 200 milliunits β-galactosidase or 60 μg α1,2-mannosidase at 37°C. The reactions were stopped by boiling, and an aliquot of the digests was analyzed by SF-HPLC. The molecular masses of the resulting digests were analyzed by IS-MS/MS and/or compared with authentic sugar chains (14, 30).

IS-MS/MS experiments were performed by using a Perkin–Elmer Sciex API-III, triple-quadrupole mass spectrometer (14). Scanning was done with a step size of 0.5 Da, and the collisionally activated dissociation daughter ion spectrum was recorded from m/z 200.

RESULTS

Transformation of Tobacco BY2 Cells with the hGT Gene. The constructed hGT gene encoding human β1,4-galactosyltransferase cDNA for expression in tobacco cells is depicted in Fig. 1*A*. The gene was placed under the control of the CaMV35S promoter. pGAhGT also carries a neomycin phosphotransferase expression cassette conferring kanamycin resistance in transformed cells. Southern hybridization of genomic DNA obtained from transformed cells confirmed the presence of a 2.2-kbp fragment derived from the T-DNA of pGAhGT (Fig. 1*B*). No hybridization signal was observed in nontransformed control cells, establishing that BY2 cells were transformed with the hGT gene. Extra hybridizing bands from cell line 6 and 8 may be due to some truncated T-DNA insertions because of *Agrobacterium*-mediated transformation.

Western immunoblotting of the proteins from transformed cells gave a positive signal at a molecular mass of about 50 kDa (Fig. 1*C*), which is slightly higher than that estimated from the amino acid sequence (which is about 40 kDa), but is similar to the galactosyltransferase purified from ascitic fluids or obtained from yeast-expressed bovine galactosyltransferase (26, 31). Strong immunoreactive bands observed in microsome fractions compared with cell lysates may suggest that hGT was preferentially localized intracellularly. No immunoreactive bands were detected from wild-type cells.

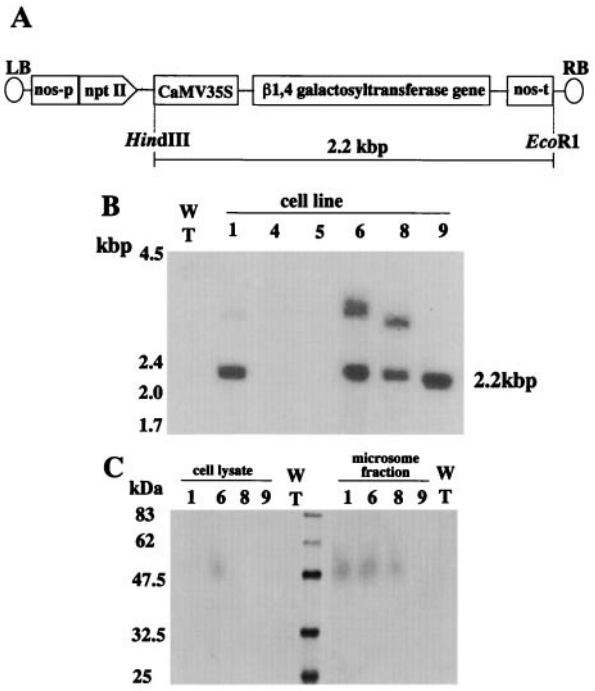

FIG. 1. Expression of human β1,4-galactosyltransferase (hGT) gene in tobacco BY2 cells. (*A*) The recombinant hGT plasmid for expression of human β1,4-galactosyltransferase in tobacco cells. The hGT coding region lies downstream of the CaMV35S promoter, followed by the nopaline synthase terminator (nos-t) from pBI221. The construct also has the neomycin phosphotransferase II (*npt*II) gene under the control of the nopaline synthase promoter (nos-p) from pGA482. RB/LB, right and left borders, respectively. (*B*) Southern blot analyses of genomic DNAs from transformed and wild-type BY2 tobacco cells. DNAs were digested with *Hin*dIII and *Eco*RI and probed with a ^{32}P-labeled hGT gene fragment as described in *Materials and Methods*. WT, wild type; 1, 4, 5, 6, 8, and 9, cell line number for transformed tobacco cells. The numbers to the left indicate the sizes (kbp) and positions of λ DNA fragments digested with *Hin*dIII. Size of the hybridizing fragment (2.2 kbp) is indicated. (*C*) Western blots of immunoreactive proteins from transgenic and nontransgenic tobacco cells. Proteins were denatured, resolved by SDS/PAGE, and electroblotted onto a nitrocellulose membrane. The blots were probed with anti-hGT antibody as described in *Materials and Methods*. Lanes contain proteins from cell extracts of cell lines 1, 6, 8, 9 and wild type and microsome fractions of cell lines 1, 6, 8, 9 and wild type. Molecular mass of marker proteins are indicated on the left.

Selection of Transformed Cells with Highest β1,4-Galactosyltransferase Activity. β1,4-Galactosyltransferase activity was assayed by using Triton X-100-solubilized proteins from the microsomal fraction. Based on HPLC analysis, crude enzyme preparations from transformed cells were able to transfer galactose from UDP-Gal to $GlcNAc_2Man_3GlcNAc_2$-PA (data not shown). No reaction product was obtained by using protein extracts of wild-type BY2 cells. Thus, the activity was obtained in transformed tobacco suspension cells alone. β1,4-Galactosyltransferase activity varied among the different cell lines (GT1, 1.2×10^2; GT6, 9.0×10^2; GT8, 1.0×10^1; wild type, $<2.1 \times 10^{-1}$ pmol/hr per mg protein). One line with the highest activity, designated GT6, was used for further study.

Effect of hGT Activity on the Glycan Structure in Plant Cells. RCA_{120} affinity chromatography was used to examine whether hGT transferred galactose residues to glycoproteins in transformed cells. Proteins from both cell lysates and microsome fractions of the GT6 line were bound to the RCA_{120} column and subsequently eluted by using a hapten sugar (Fig. 2*A*). Eluted proteins blotted onto nitrocellulose membranes were positive in subsequent RCA_{120} lectin-staining analysis (Fig. 2*B*). RCA_{120} binding was not observed in wild-type BY2, which further substantiates that GT6, unlike BY2, has glycoproteins with galactose at the nonreducing end of their glycan moieties.

Total protein extracts from BY2 and GT6 cells and eluted proteins of GT6 from RCA_{120} affinity chromatography were, likewise, probed with a polyclonal antiserum specific for plant complex glycans (Fig. 2*C*). The serum used primarily binds to the β1,2-xylose residues on plant glycoproteins (27). BY2 cells contained several glycoproteins that reacted with the antibody (Fig. 2*C*, lane 1). GT6 cells also contained glycoproteins that crossreacted, although the intensity was much less (lane 2). The glycoproteins eluted from RCA_{120} did not bind to the antibody (lane 3), suggesting that galactosylated glycoproteins may not contain any β1,2-xylose residues.

Analysis of Glycans in GT6 Cells. Structural elucidation of N-linked glycans in transformed cells further confirmed the presence of galactose residues and allowed analysis and comparison of glycan processing between wild-type and transformed cells. The PA-sugar chains obtained from GT6 were

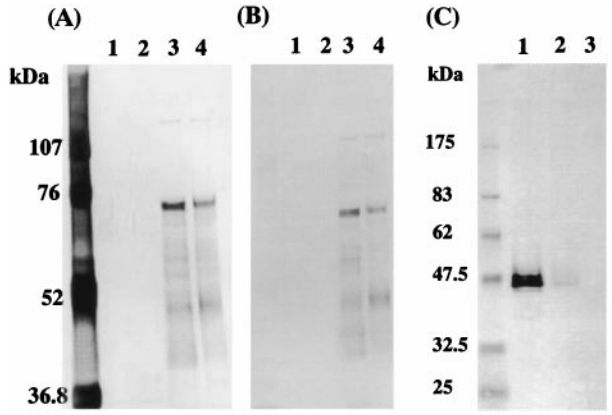

FIG. 2. Detection of galactosylated glycoproteins by using *R. communis* RCA_{120} affinity chromatography. Eluted fractions were subjected to SDS/PAGE, and the gel was visualized by silver staining (*A*) or blotted onto a nitrocellulose membrane and lectin (RCA_{120}) stained (*B*). Lanes 1 and 2, proteins from wild-type BY2 (WT); 3 and 4, proteins from transformed GT6. (*C*) Blots probed with a xylose-specific antiserum for plant complex glycans. Lanes 1 and 2, total protein extracts from BY2 and GT6; 3, glycoproteins from GT6 after RCA_{120} affinity chromatography. Molecular mass markers are in kDa.

purified and characterized by a combination of RP- and SF-HPLC (Fig. 3). Fig. 3*A* shows several peaks of PA derivatives as analyzed by RP-HPLC. Each collected fraction (I-XI) was rechromatographed in SF-HPLC (Fig. 3*B*).

No N-linked oligosaccharides were eluted from SF-HPLC in fraction I (between 10 and 25 min), suggesting that this fraction is a run-through fraction containing by-products during hydrazinolysis (Fig. 3*B*). MS/MS analyses of these peaks gave no fragment ions with an m/z of 300 (PA-GlcNAc). Similarly, fraction XI (50–60 min) did not contain any *N*-glycans because no peaks were observed in SF-HPLC. On the contrary, a total of 17 peaks were purified from analyses of fractions II–X by SF-HPLC (Fig. 3*B*). IS-MS/MS analyses, however, indicated that only seven of these are N-linked sugar chains. The elution position and molecular mass of oligosaccharides A, E, H, I, M, O, P, and Q (Fig. 3*B*) did not correspond to standard PA-sugar chains, and, although these fractions gave signals at m/z 300 and 503 ($GlcNAc_2$-PA), no signal at m/z 665 ($ManGlcNAc_2$; M1) was obtained (data not shown). Other peaks found (B, D, and N) were confirmed to be non-*N*-glycans because no fragment ions were observed at m/z 300. The characterization of the seven N-linked sugar chains is described below.

The elution position and molecular mass of peaks C (m/z 1,637.5; 9.3% as molar ratio), F (m/z 819.5 for $[M+2H]^{2+}$, 1,639.0 for $[M+H]^{+}$; 15.9%), and G (m/z 1,475.5; 19.5%) corresponded to high-mannose-type sugar chains $Man_7GlcNAc_2$ (isomers M7A and M7B) and $Man_6GlcNAc_2$ (M6B), respectively. Jackbean mannosidase digestion trimmed each *N*-glycan to $ManGlcNAc_2$ (M1) as analyzed by SF-HPLC (data not shown). By IS-MS analysis of the resulting digests, the ion at m/z 665.0 corresponded to the calculated mass of 664.66 for M1, confirming their structural identity to corresponding reference PA-oligosaccharides (Fig. 4).

Peak J (6.6%), with a molecular mass of 1,121.5, agreed well with the calculated mass for $Man_3Xyl_1GlcNAc_2$-PA (M3X; m/z 1,121.05). The location of the fragment ions at m/z 989.5, 827.5, 665.5, 503.3, and 300 was consistent with the serial loss of each monosaccharide from the parent molecule in the order of Xyl, Man, Man, Man, and GlcNAc. Digestion of the sugar chain with α-mannosidase (jackbean) cleaved the nonreducing terminal mannose residues, resulting in a change in elution position equivalent to $ManXylGlcNAc_2$-PA (data not shown). The deduced structure of the sugar chain is shown in Fig. 4.

IS-MS analysis of peak K showed that the fraction contained two *N*-glycans, one at a molecular mass of 1,314.0 and the other at 1,354.5. The fraction thus was reinjected in RP-HPLC, resulting in two peaks, K-1 (1.4%) and K-2 (11.8%). The sugar chain at m/z 1,314.0 (K-1) corresponded exactly to authentic $Man_5GlcNAc_2$ (M5) (Fig. 4), and subsequent α-mannosidase (jackbean) digestion removed four mannose residues, as indicated by the elution shift that matched the elution position of M1 (data not shown).

Galactosylated *N*-Glycans in GT6 Cells. The sugar chain at m/z 1,354.5 (peak K-2) is in good agreement with the expected mass (m/z 1,354.3) for $Gal_1GlcNAc_1Man_3GlcNAc_2$-PA (GalGNM3). Relevant signals observed could be reasonably assigned as fragment ions derived from the parent: m/z 1,193.5 ($GlcNAcMan_3GlcNAc_2$-PA), 989.5 ($Man_3GlcNAc_2$-PA), 827.5 ($Man_2GlcNAc_2$-PA), 665 ($ManGlcNAc_2$-PA), 503 ($GlcNAc_2$-PA), 336 (ManGlcNAc), 300 (GlcNAc-PA), and 204 (GlcNAc). Based on the deduced *N*-glycan structure, there are two possible isomers for GalGNM3, i.e., Galβ4GlcNAcβ2Manα6(Manα3)Manβ4GlcNAcβ4GlcNAc-PA and Manα6(Galβ4GlcNAcβ2Manα3)Manβ4GlcNAcβ4GlcNAc-PA. The elution position of the purified PA-sugar chain matched with that of the latter (Fig. 5).

Ensuing exoglycosidase digestions confirmed the structural identity as shown in Fig. 6*A*. A product derived from β-galactosidase (*D. pneumoniae*) digestion, whose main specificity is

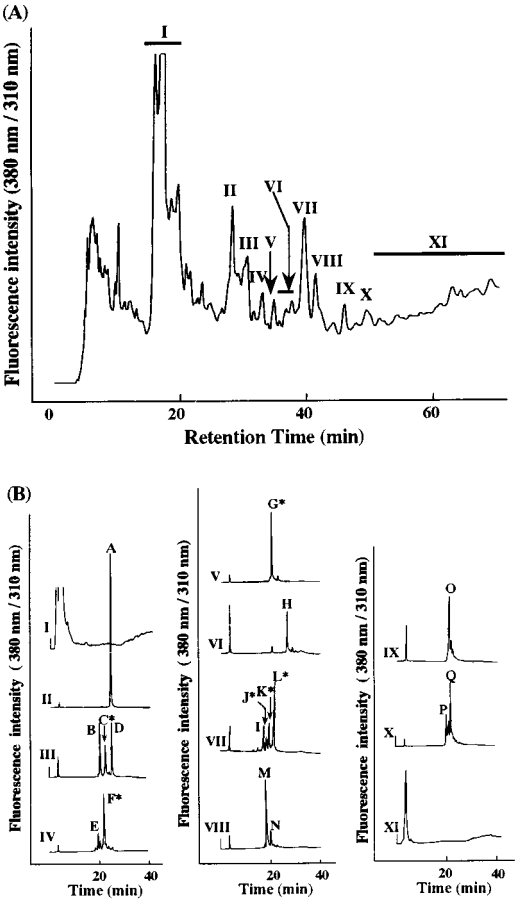

FIG. 3. PA derivatives from glycoproteins expressed in transformed cells. (*A*) RP-HPLC pattern of PA-sugar chains eluted by increasing the acetonitrile concentration in 0.02% trifluoroacetic acid linearly from 0 to 15% for 60 min at a flow rate of 1.2 ml/min. I–XI, individual fractions collected and purified in SF-HPLC. (*B*) SF-HPLC of PA-sugar chains in *A*. PA-sugar chains were eluted by increasing the water content in the water-acetonitrile mixture from 30 to 50% for 40 min at a flow rate of 0.8 ml/min. Excitation and emission wavelengths were 310 and 380 nm, respectively. Fractions that contain N-linked sugar chains are marked with *.

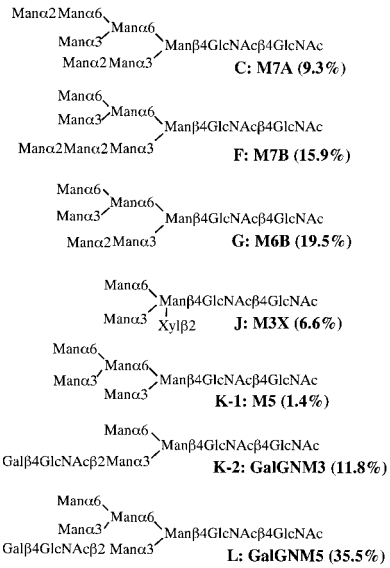

FIG. 4. Proposed structures of N-linked glycans obtained from transformed cells. Enclosed numbers in parentheses represent molar ratios.

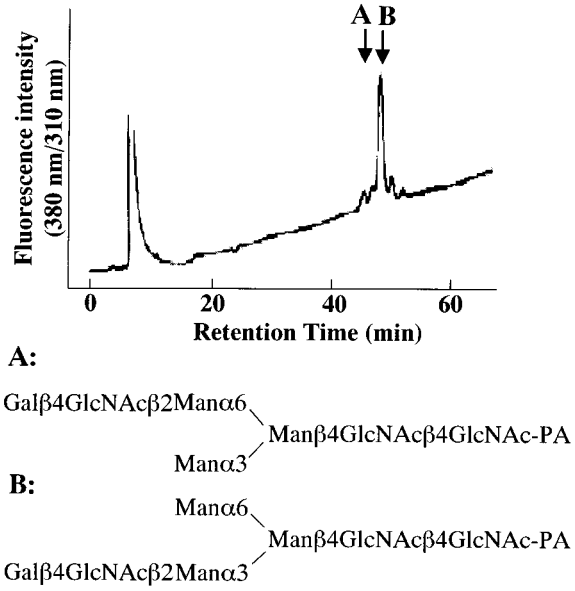

FIG. 5. Elution position of peak K-2 in RP-HPLC compared with two authentic sugar chains, A and B. Elution conditions are described as in Fig. 3*A*.

toward Galβ1,4GlcNAc linkage (32), has the same elution position with GlcNAcMan$_3$GlcNAc$_2$-PA (Fig. 6*A*, II). IS-MS/MS analysis gave a signal at m/z 1,192.0. These results suggest that one β1,4-linked galactose residue was removed from one nonreducing-end GlcNAc. Digestion of this product with *N*-acetyl-β-D-glucosaminidase (*D. pneumoniae*), which specifically hydrolyzes β1,2-GlcNAc linkages (33), resulted in a peak that coeluted with Man$_3$GlcNAc$_2$-PA (Fig. 6*A*, III), which was converted further to ManGlcNAc$_2$-PA by digestion with jackbean α-mannosidase (Fig. 6*A*, IV). The deduced structure of peak K-2 is shown in Fig. 4.

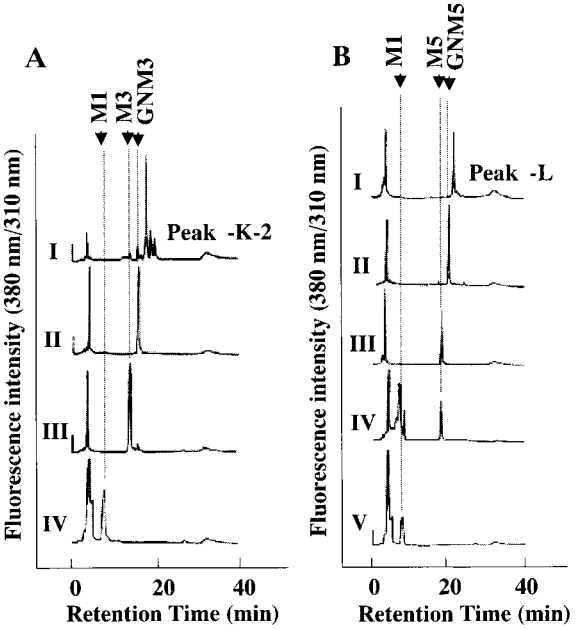

FIG. 6. SF-HPLC profiles of galactosylated PA-sugar chains obtained after exoglycosidase digestions. PA-sugar chains were eluted by increasing the water content in the water-acetonitrile mixture from 30 to 50% for 25 min at a flow rate of 0.8 ml/min. (*A*) PA-sugar chain K-2. I, elution position of native galactosylated PA-sugar chain; II, β-galactosidase digest of I; III, β-*N*-acetylglucosaminidase digest of II; IV, jackbean α-mannosidase digest of III. (*B*) PA-sugar chain L. I, elution position of native galactosylated PA-sugar chain; II, β-galactosidase digest of I; III, β-*N*-acetylglucosaminidase digest of II; IV, α1,2 mannosidase digest of III; V, jackbean α-mannosidase digest of III.

Peak L (35.5%) has a molecular mass of 840.0 for $[M+2H]^{2+}$ and 1,680.0 for $[M+H]^{+}$ and corresponds to the expected mass of Gal$_1$GlcNAc$_1$Man$_5$GlcNAc$_2$-PA (GalGNM5; m/z 1,678.55). The locations of the fragment ions at m/z 1,476, 1,313.5, 1,152, 989.5, 827.5, 665.5, 503, and 300 were consistent with the serial loss of each monosaccharide from the parent molecule. β-Galactosidase digestion suggested the presence of galactose β1,4-linked to one nonreducing-end GlcNAc residue (Fig. 6*B*, II). The removal of the galactose residue also was confirmed by IS-MS/MS analysis, with the resulting molecular mass of 759.0 for $[M+2H]^{2+}$ and 1,518.0 for $[M+H]^{+}$. From the parent signal at m/z 1,518.0 (GlcNAc$_1$Man$_5$GlcNAc$_2$-PA), the fragment ions at 1,314, 1,152, 990, 827.5, 665.5, 503.5, and 300 corresponded to Man$_5$GlcNAc$_2$-PA, Man$_4$GlcNAc$_2$-PA, Man$_3$GlcNAc$_2$-PA, Man$_2$GlcNAc$_2$-PA, Man$_1$GlcNAc$_2$-PA, GlcNAc$_2$-PA, and GlcNAc-PA, respectively. Digestion of GlcNAc$_1$Man$_5$GlcNAc$_2$-PA with *N*-acetyl-β-D-glucosaminidase yielded a product with an elution position similar to Man$_5$GlcNAc$_2$-PA (Fig. 6*B*, III). No elution shift was observed when the PA-sugar chain was treated further with α1,2-mannosidase (Fig. 6*B*, IV). Treatment with jackbean mannosidase (Fig. 6*B*, V), however, resulted in an elution position corresponding to M1, suggesting the removal of four nonreducing terminal mannose residues. Results indicate that the PA-sugar chain consists of five α-mannosyl residues and verifies that neither one of these mannose residues is α1,2 linked to the α1,3-mannose residue. Based on exoglycosidase digestions, two-dimensional sugar chain mapping, and IS-MS/MS, the structure of peak L was identified as GalGNM5 (Fig. 4).

DISCUSSION

We expressed the human β1,4-galactosyltransferase gene in tobacco BY2 cells to determine whether this mammalian glycosyltransferase could extend and modify the N-linked processing pathway in plants. Our results demonstrate that the hGT gene was expressed in transformed tobacco cells and the transformed cells exhibited β1,4-galactosyltransferase activity. High activity in the Triton X-100-solubilized 100,000 $\times$ *g* fraction compared with other fractions tested (data not shown) suggests that the catalytic form of the glycosyltransferase is oriented toward the luminal side of the Golgi, similar to the mammalian glycosyltransferase (13). The presence of galactosylated glycans was demonstrated by affinity chromatography on RCA$_{120}$. Transformed cells have glycoproteins that bound to RCA, suggesting that the human enzyme extended some complex-type sugar chains from nongalactose- into galactose-containing types. Galactosylated glycoproteins do not contain any β1,2-xylose residues, as implied from immunodetection with a xylose-specific antiserum. No α1,3-fucose residues were observed based on structural studies. Interestingly, there were no significant differences in the *in vitro* growth properties of wild-type BY2 or transformed GT6 cells. We have maintained GT6 cells continuously for more than 150 weekly transfers in the laboratory.

Structural studies of *N*-glycans highlight the diversity of oligosaccharide structures in transformed cells. Our recent studies (14) on *N*-glycans in tobacco BY2 cells indicate that (*i*) cells are deficient in galactosyltransferase activity, (*ii*) the pathway of asparagine-bound oligosaccharides leads to a majority of complex glycans with either xylose or xylose and fucose residues (92.5%), and (*iii*) *N*-glycan modification proceeds rapidly as inferred from the low amount of high-mannose-type glycans obtained (7.5%). In contrast, galactosylated glycans in transformed GT6 cells comprise 47.3% of the total sugar chains obtained. Together with the 6.6%

N-linked glycan containing a xylose residue (M3X), complex sugar chains account for only 53.9%. The lower conversion efficiency of oligomannose-type sugar chains to complex glycans also can be inferred from the amount of high-mannose-type sugars containing α1,2 linkages (44.7%). Clearly, the expression of hGT extends and modifies the N-linked glycosylation pattern in BY2.

Based on the structural diversity of N-linked sugar chains obtained, a possible processing scheme for the biosynthesis of complex oligosaccharides containing galactose can be deduced. The presence of GalGNM5, a hybrid *N*-glycan, in GT6 may infer that once $Man_5GlcNAc_2$ (M5) is formed, GnT-I rapidly transfers GlcNAc residues to M5. This agrees with the substrate specificity of GnT-I from plants (34, 35). The resulting hybrid glycan then may be acted on immediately by galactosyltransferase (Gal-T). The modification of M5 to GalGNM5 seems to occur quickly, because GalGNM5 occupies 35.5% of the total sugar chain obtained. Whether further trimming by mannosidase II (Man II) is possible after galactose addition (resulting to GalGNM3) needs to be investigated further because plant Man II substrate specificity studies did not include galactose-containing sugar chains (36). It was inferred, however, that the purified Man II from mungbean seedlings is, in most respects, similar to the mammalian Man II reported, and, thus, the activity can be reduced greatly with the presence of additional residues in the GlcNAcβ1,2Manα1,3 branch. Likewise, GalGNM5 acted as a poor substrate for jackbean mannosidase when assayed *in vitro* (data not shown).

Alternatively, Man II also may cleave GNM5 to GNM3, which, in turn, also might be a possible substrate for Gal-T, based on substrate specificity studies of mungbean Man II (36). The absence of *N*-glycans bearing GlcNAc residues at the α1,6-linked mannose may suggest that the sugar chains with GlcNAc residues at the α1,3-mannose branch are acted on rapidly by Gal-T and that these galactosylated glycans are poor substrates for further action of GnT-II (37, 38). In the same manner, galactosylated glycans cannot serve as acceptors for both fucosyl- and xylosyltransferases (39, 40).

The relative amounts of xylose-containing-type sugar chains are also indicative of the modification in the glycosylation activities in GT6. The significant reduction (92.5% in BY2 vs. 6.6% in GT6 cells) in plant-specific oligosaccharides bearing xylosyl and fucosyl residues not only may provide a system where questions on immunogenicity of various glycans can be tested but also suggest alternatives for the production of recombinant proteins with more mammalian-like *N*-glycans. More importantly, GT6 can be used to study a specific, transgene-encoded glycoprotein to demonstrate the cell lines' ability to modify, in a predictable manner, the glycosylation status of the foreign protein. GT6 cells also may be used to produce galactosylated and nongalactosylated versions of recombinant proteins, which will help us access the functional consequences of galactosylation, in plants as well as in the recombinant proteins, inasmuch as there are no specific inhibitors for this later step in the N-linked oligosaccharide-processing pathway.

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