Galactose-extended glycans of antibodies produced by transgenic plants

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Plant-specific *N*-glycosylation can represent an important limitation for the use of recombinant glycoproteins of mammalian origin produced by transgenic plants. Comparison of plant and mammalian *N*-glycan biosynthesis indicates that β1,4-galactosyltransferase is the most important enzyme that is missing for conversion of typical plant *N*-glycans into mammalian-like *N*-glycans. Here, the stable expression of human β1,4-galactosyltransferase in tobacco plants is described. Proteins isolated from transgenic tobacco plants expressing the mammalian enzyme bear *N*-glycans, of which about 15% exhibit terminal β1,4-galactose residues in addition to the specific plant *N*-glycan epitopes. The results indicate that the human enzyme is fully functional and localizes correctly in the Golgi apparatus. Despite the fact that through the modified glycosylation machinery numerous proteins have acquired unusual *N*-glycans with terminal β1,4-galactose residues, no obvious changes in the physiology of the transgenic plants are observed, and the feature is inheritable. The crossing of a tobacco plant expressing human β1,4-galactosyltransferase with a plant expressing the heavy and light chains of a mouse antibody results in the expression of a plantibody that exhibits partially galactosylated *N*-glycans (30%), which is approximately as abundant as when the same antibody is produced by hybridoma cells. These results are a major step in the *in planta* engineering of the *N*-glycosylation of recombinant antibodies.

Plants are potential cost-efficient and contamination-safe factories for the production of recombinant mammalian proteins (1, 2). A major limitation shared with other heterologous expression systems like bacteria, yeast, and insect cells is their different glycosylation profile compared with mammals (3, 4). In contrast to bacteria, having no *N*-linked glycans, and yeast, having polymannose glycans, plants are able to produce proteins with complex *N*-linked glycans having a core substituted by two *N*-acetylglucosamine (GlcNAc) residues as observed in mammals. In plants, however, this core is substituted by β1,2-linked xylose (Xyl) and α1,3-linked core fucose (Fuc) instead of α1,6-linked core Fuc in mammals. β1,2-Xyl and α1,3-Fuc epitopes are known to be highly immunogenic and might play a role in allergenicity (5). Furthermore, plant glycoproteins lack the characteristic galactose (Gal)- and sialic acid-containing complex *N*-glycans (*N*-acetylneuraminic-α2–6/3Galβ1–4) found in mammals. Consequently, expression of mammalian glycoproteins in plants results in the production of glycoproteins whose glycans reflect the differences in the *N*-glycan processing between plants and mammals (6).

Antibodies have conserved *N*-linked glycosylation at Asn-297 of the Fc region of each of the two heavy chains. These glycans are buried between the two heavy chains and exhibit reduced mobility through interactions with the protein backbone (7). In particular, the terminal β1,4-galactose residues are in close contact with the protein backbone. Therefore, it has been suggested that the Gal residues are of broad significance for antibody performance, because not only are they involved in immunological effector functions as complement interactions, but they also contribute to correct antibody folding (8–10). Thus, Gal may be critical for pharmacokinetic activity of certain therapeutic antibodies. In contrast, glycans on antibodies generally are of low sialic acid content (11), and it has been suggested that sialic acid contributes little or not at all to the functionality of antibodies (10).

Antibodies have been identified as very promising candidates for commercial production by transgenic plants. Clinical trials of therapeutical plantibodies have already been reported (12). Because the terminal β1,4-galactose residues contribute to many aspects of antibody quality, their presence on plantibodies is relevant for many applications. Consequently, engineering the plant glycosylation machinery with the capacity to produce galactosylated glycans is of major importance, and it is our prime target in the humanization of glycans on antibodies produced by plants.

The missing enzyme in plants to achieve this is β1,4-galactosyltransferase (GalT). This enzyme transfers Gal from UDP-Gal in β1,4-linkage to GlcNAc residues in *N*-linked glycans. cDNAs encoding this enzyme have been cloned from several mammalian species (13, 14), and the enzyme has been introduced successfully in insect cell cultures (15, 16) or into a tobacco cell suspension culture (17) to extend the *N*-glycosylation pathway. Although changes in *N*-glycan structure usually have little effect at the cellular level, it is not obvious that GalT can be expressed in whole plants because severe effects at the organism level have been reported (18–20). In this manuscript, we show that human GalT can be expressed successfully in tobacco plants and is able to partially humanize *N*-linked glycans of endogenous glycoproteins as well as of transgenically expressed mammalian antibodies. Moreover, this feature is inheritable, and there is no visible phenotypical difference between plants expressing the human GalT and wild-type tobacco plants.

Materials and Methods

Plasmids and Plant Transformation. A plant transformation vector containing human GalT was constructed with a 1.4-kb *Bam*HI/*Xba*I fragment of pcDNAI-GalT (21, 22) that was ligated in the corresponding sites of pUC19. Subsequently, this fragment was reisolated by using the surrounding *Kpn*I and *Hin*cII sites and was cloned into the *Kpn*I and *Sma*I site of pUCAP35S (ref. 23;

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Abbreviations: GlcNAc, *N*-acetylglucosamine; Fuc, fucose; Gal, galactose; Man, mannose; Xyl, xylose; GalT, β1,4-galactosyltransferase; RCA, ricinus communis agglutinin; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.

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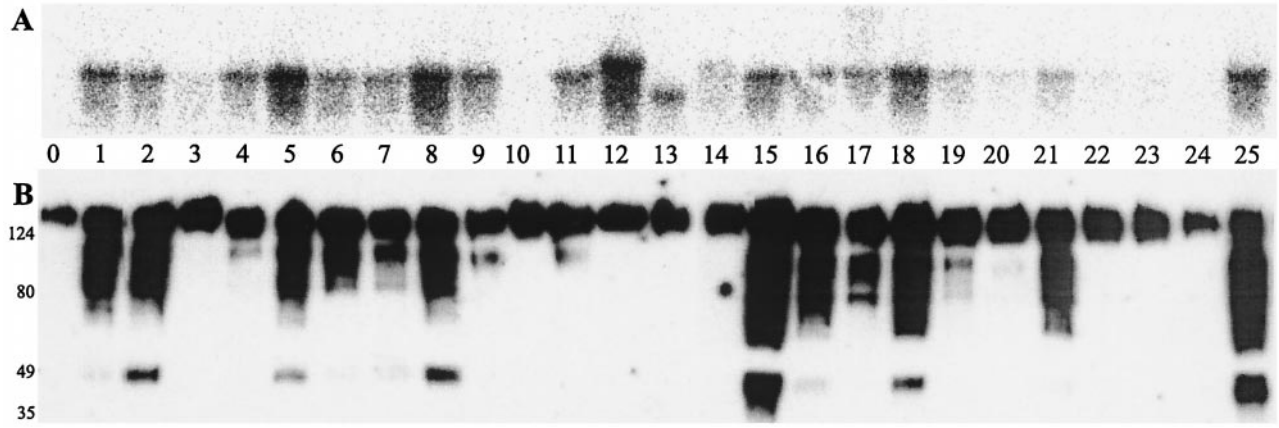

Fig. 1. Correlation of mRNA expression of GalT and RCA binding. (*A*) Northern blot showing the mRNA expression of GalT in 25 GalT plants and an untransformed control plant (lane 0). (*B*) Western blot of the same plants showing binding of the Gal-specific lectin RCA to isolated proteins.

named pUCAP35S-HgalT). By using the *Asc*I and *Pac*I sites, the CaMV35S promotor-cDNA-Nos terminator cassette of pUCAP35S-HgalT was cloned in the binary vector pBINPLUS (23) resulting in the vector pBINPLUS-HgalT. *Nicotiana tabacum* (variety Samsun NN) was transformed by using an *Agrobacterium tumefaciens*-mediated transfer method (24). Modifications to the published protocol are: after incubation with *A. tumefaciens*, leaf discs were incubated for 3 days in medium containing 1 mg/ml of α-naphtalene acetic acid and 0.2 mg/ml 6-benzylaminopurine and the use of 0.25 mg/ml cefotaxime and vancomycine to inhibit bacterial growth in the callus and shoot-inducing medium. Twenty-five rooted shoots were transferred from *in vitro* medium to soil and, after several weeks, the leaf material of these plants was analyzed.

Northern Blotting. The GalT RNA level in the transgenic plants was analyzed by Northern blotting (25). RNA was isolated as described (24) from the first fully grown leaves of transgenic and control plants that were in the same developmental stage. Of the total RNA, 10 μg was used per sample. The blot was probed with a [^{32}P]dATP-labeled *Sst*I/*Xho*I fragment containing the whole GalT cDNA isolated from pBINPLUS-HgalT.

Glycoprotein Analysis. Total protein extracts of tobacco were prepared by grinding leaves in liquid nitrogen. Ground material was diluted 10 times in SDS/PAGE loading buffer [20 mM of Tris·HCl (pH 6.8)/6% (vol/vol) glycerol/0.4% (vol/vol) SDS/20 mM DTT/2.5 μg/ml bromophenol blue]. After incubation at 100°C for 5 min, insoluble material was pelleted. Supernatants (12.5 μl/sample) were run on 10% (vol/vol) SDS/PAGE and blotted to nitrocellulose. Affinoblotting with peroxidase-conjugated ricinus communis agglutinin $(RCA)_{120}$ (Sigma) was carried out according to the manufacturer's protocol and was revealed with Lumi-Light Western blotting substrate (Roche Diagnostics). Immunodetections with anti-β1,2-Xyl, anti-α1,3-Fuc, and anti-Lewis^a antibodies were carried out according to the literature (26, 27). Western blots with purified antibody (see below) were performed for total protein by using 0.17 μg per lane.

Plant Crossings. Mgr-48 (28) is a mouse monoclonal IgG1 that has been expressed in *N. tabacum* plants (unpublished work). The construct used for transformation was identical to monoclonal antibody 21C5 expressed in tobacco (23). Flowers of three selected tobacco plants with a high expression of GalT (nos. GalT6, GalT8, and GalT15 from Fig. 1) were pollinated with a tobacco plant expressing monoclonal antibody Mgr-48. Of the F1 generation, 12 plants of each crossing were analyzed for the expression of antibody by Western blots probed horseradish peroxidase-conjugated sheep-anti-mouse IgG, and GalT expression by affinodetection with RCA as described above. Of crossings with GalT6 $\times$ Mgr-48 and GalT-15 $\times$ Mgr-48, no plants were found with both Mgr-48 and GalT expression. Several were found in crossing GalT-8 $\times$ Mgr-48. Two plants were selected (GalT-8 $\times$ Mgr-48–11 and GalT-8 $\times$ Mgr48–12) to isolate IgG (compare lanes 3 and 4 in Fig. 3). MS data were obtained from plant GalT-8 $\times$ Mgr-48–11.

Isolation of *N*-Linked Glycans from Tobacco Leaves. Protein isolation from tobacco leaves of wild type and plants transformed with human GalT (GalT-8, lane 8, Fig. 1) and *N*-glycan preparation were performed as described (29). The *N*-glycans were desalted on a nonporous, graphitized carbon-black column (Carbograph Ultra Clean Tubes, Alltech Associates) before mass spectrometry analysis as described (30).

Purification of the Recombinant Mgr-48 Antibody Expressed in Tobacco Plants. Frozen leaves were ground in a stainless steel blender that was precooled with liquid nitrogen. This powder (200 g) was dissolved in 600 ml of 150 mM phosphate buffer (pH 7) containing 5 mM EDTA, 10 g polyvinylpyrrolidone, 20 mM sodium bisulfite, and 0.5 mM PMSF, and stirred at 4°C for 15 min. After centrifugation for 10 min at 10,000 $\times$ *g*, the supernatant was filtered, and the pellet was washed with 150 mM phosphate buffer (pH 7) containing 5 mM EDTA. The proteins were precipitated with ammonium sulfate (20% saturation). The sample was centrifuged for 6 min at 4,200 $\times$ *g* and the proteins of the supernatant were then submitted to a second precipitation with ammonium sulfate (60% saturation) overnight at 4°C. The second precipitate was dissolved in 50 mM phosphate buffer (pH 7), 100 mM NaCl, centrifuged, and filtered. The Mgr-48 plantibodies were purified from protein preparations by using affinity chromatography on a Hi-Trap Protein G column (Amersham Pharmacia) by using a 0.1 M glycine-HCl buffer (pH 2.7) for elution and cation chromatoghraphy on a Mono S column (Amersham Pharmacia) equilibrated with a 50 mM Mes buffer (pH 6). Elution was performed with a linear 0–0.3 M NaCl gradient in 50 mM Mes (pH 6).

Preparation of *N*-glycans Isolated from Mgr-48 Antibodies. *N*-glycans from hybridoma Mgr-48 antibody were isolated as described (6). Glycans *N*-linked to tobacco Mgr-48 antibodies were isolated from 1 mg of purified antibodies from Mgr-48 plants and the

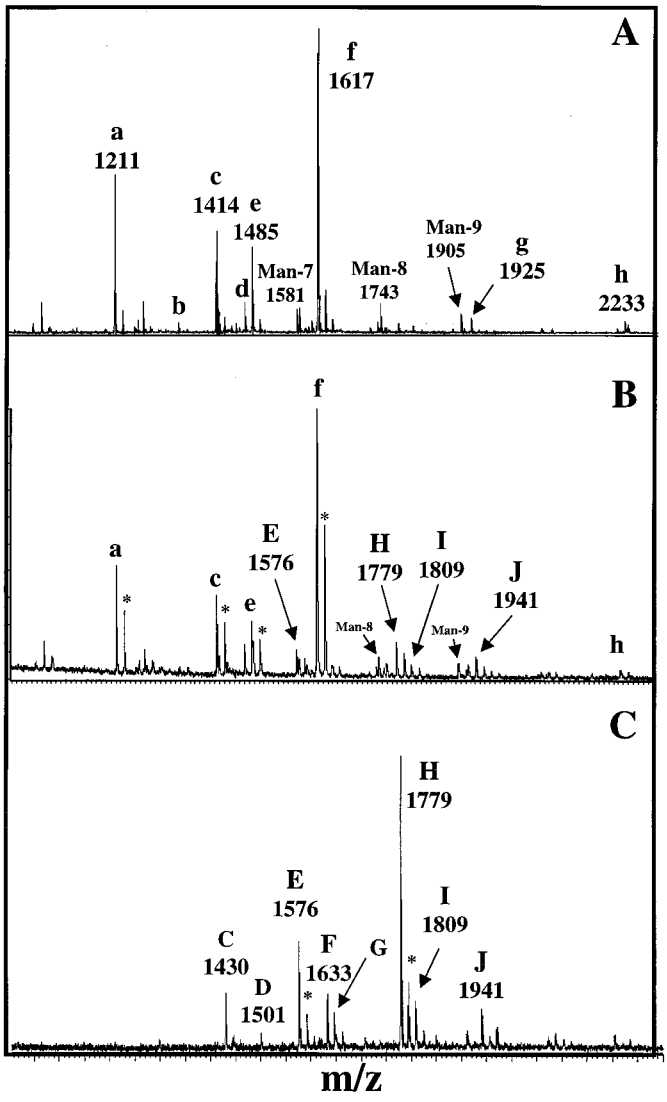

Fig. 2. MALDI-TOF mass spectra of (*A*) *N*-linked glycans isolated from control tobacco leaves, (*B*) *N*-linked glycans isolated from leaves of tobacco plants transformed with human GalT, and (*C*) *N*-linked glycans affinity purified on RCA. Asterisk, $(M + K)^+$ adducts. See Tables 1 and 2 for structures.

crossing of GalT-8 × Mgr-48–11 as described above for the isolation of *N*-glycans from tobacco protein extracts.

Affinity Purification on RCA. *N*-glycan mixture was applied to a 1-ml column of RCA_{120}-Sepharose and eluted in 10 mM Tris·HCl (pH 7.4). Fractions (1 ml) were collected. The first two fractions contained nonretained material. Fractions 3–7 contained retarded glycans. The column was then washed with 0.1 M lactose, and this fraction was combined with the retarded fraction and desalted on a Carbograph column as described previously for total *N*-glycans.

Matrix-Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometry. MALDI-TOF mass spectra were measured on a Micromass (Manchester, U. K.) Tof spec E MALDI-TOF mass spectrometer. Mass spectra were performed in positive ion mode and in the reflector mode by using 2,5-dihydroxybenzoïc acid as the matrix (29). Assignments to both $(M + Na)^+$ and $(M + K)^+$ adducts were confirmed by running an additional spectrum with CsCl. In these conditions, both $(M + Na)^+$ and $(M + K)^+$ adducts were converted into a single $(M + Cs)^+$ ion.

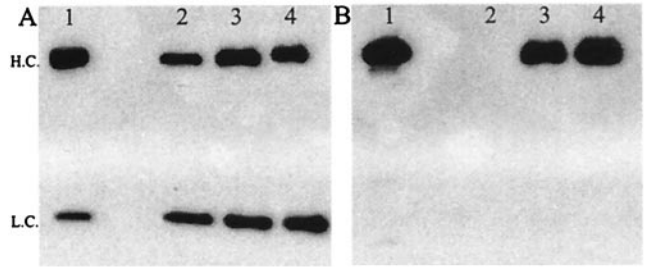

Fig. 3. Western blot showing sheep-anti-mouse-IgG [polyclonal, directed against both heavy and light chains] (*A*) and RCA binding (*B*) to purified antibody produced in hybridoma culture (lane 1), tobacco plants (lane 2), and two tobacco plants (GalT-8 × Mgr-48–11 and -12) coexpressing GalT (lanes 3 and 4). An amount of 0.17 μg hybridoma antibody was used per lane, and equal amounts of the other antibodies as judged from the intensity of the heavy chain bands on Coomassie-stained gels (not shown). H.C., heavy chain; L.C., light chain.

Enzyme Sequencing. Jack Bean β-*N*-acetylglucosaminidase (25 milliunits), 50 milliunits of bovine testis β-galactosidase, or 200 milliunits of Jack Bean α-mannosidase (Sigma) were desalted by ultrafiltration and incubated overnight with 50 pmols of the glycan mixture. Then the digests were directly analyzed by using MALDI-TOF MS (31).

Results

Human GalT Modifies Endogenous Protein *N*-Glycans in *N. tabacum*. Human GalT (13) was introduced in tobacco plants by agrobacterium-mediated leaf-disk transformation of plasmid pBIN-PLUS-HgalT containing a cDNA that included the complete coding sequence. Twenty-five plants selected for kanamycin resistance were analyzed for mRNA levels by Northern hybridization (Fig. 1*A*). The same plants were analyzed by affinity blotting with RCA_{120}, a lectin from *Ricinus communis* that binds to the Galβ1–4GlcNAc sequence, but also in a minor extent to other terminal β-linked Gal residues. Fig. 1*B*, lane 0, shows that RCA binds to high molecular-weight proteins from nontransgenic control tobacco plants, which probably are arabinogalactan proteins, because RCA is known to bind to such plant proteoglycans (32). In a number of plants transformed with human GalT, RCA binds in addition to a smear of other proteins. Apparently, through the GalT activity, many proteins have been modified into glycoforms that bind to RCA. As illustrated in Fig. 1, there is a good correlation between the GalT RNA expression level and the increase of RCA reactivity with glycoproteins in transgenic plants, except for plant nos. 12 and 13, in which the mRNA size is altered.

Because it has been reported previously that galactosylated *N*-glycans are poor acceptors for plant β1,2-xylosyl- and α1,3-fucosyltransferase (33), the influence of expression of GalT on the occurrence of the Xyl- and Fuc-containing glycan epitopes was investigated by immunoblotting with specific antibodies. Immunodetection with antibodies that react with β1,2-Xyl or α1,3-Fuc epitopes, specific of plant *N*-glycans, showed no clear difference in binding to isolated protein from both control and transgenic plants (not shown). Furthermore, both protein extracts were similarly detected with anti-Lewis[a] antibodies (26).

Effects of Human GalT on *N*-Linked Glycan Structures in Tobacco Plants. To investigate in further detail the effect of human GalT expression on the structure of glycans *N*-linked to glycoproteins, we have compared the structures of *N*-glycans isolated either from leaves of wild-type control plants or from plants expressing

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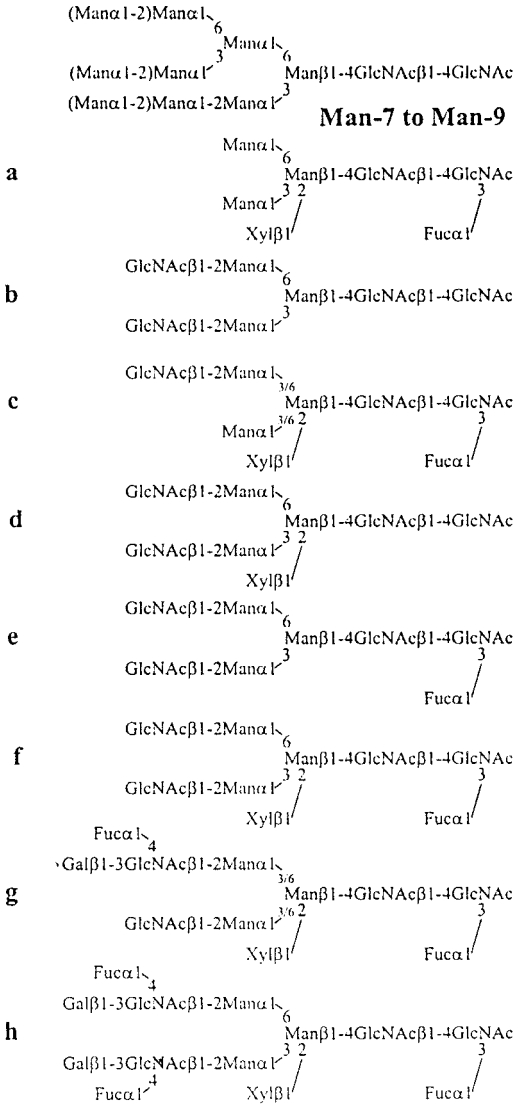
Table 1. Structure of *N*-glycans isolated from control plants



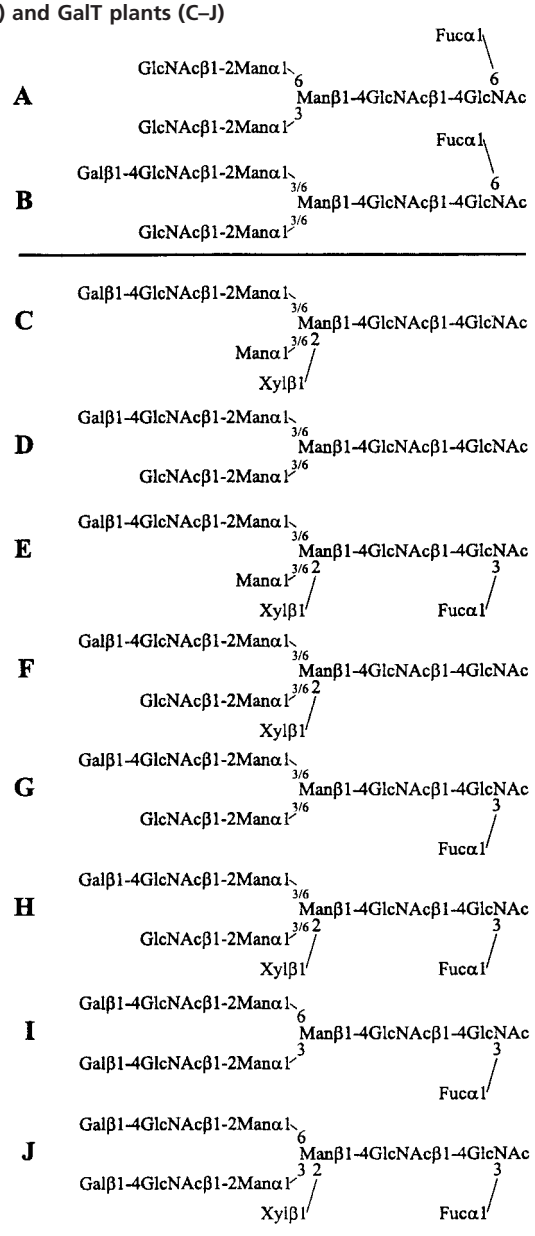
human GalT (GalT plants). A pool of *N*-glycans was first isolated from control leaves as reported in the literature (29) after digestion with peptide-*N*-glycosydase-A of glycopeptides generated by pepsin treatment of a leaf protein extract. The resulting oligosaccharide mixture was analyzed by using MALDI-TOF MS, which allows for the detection of the different molecular species contained in the pool of the *N*-glycans. Moreover, data on the ratio between the different *N*-glycans can be deduced from their relative corresponding $(M + Na)^+$ intensities. The MALDI-TOF MS (Fig. 2*A*) shows a mixture of ions that were assigned to $(M + Na)^+$ adducts of high-mannose (Man)-type *N*-glycans ranging from Man-7 to Man-9 and of mature *N*-glycans from the truncated structure **a**, $Man_3XylFucGlcNAc_2$ to glycans **h**, harboring two Lewis[a] epitopes (refs. 26 and 34; Table 1). These results are in line with the immunoblotting data. To confirm these assignments, the glycan mixture was treated successively with β-*N*-acetylglucosaminidase and α-mannosidase, and the resulting digests were reanalyzed by MALDI-TOF MS (31). Major complex *N*-glycans (**a**–**f**) were found to be converted into $ManXylGlcNAc_2$ [$(M + Na)^+$; $m/z = 741$], $ManFucGlcNAc_2$ [$(M + Na)^+$; $m/z = 755$], or $ManXylFucGlcNAc_2$ [$(M + Na)^+$; $m/z = 887$]. Furthermore, high-Man-type *N*-glycans, $Man_7GlcNAc_2$ and $Man_9GlcNAc_2$, were converted into $ManGlcNAc_2$ ($m/z = 609$) (not shown).

N-glycans were then isolated as described previously from plants transformed with the cDNA of human GalT. In addition to the *N*-glycans characterized in the control plants, the MALDI-TOF MS of the glycan mixture showed numerous ions assigned to *N*-linked glycans resulting from the action of the human enzyme (Fig. 2*B*). These additional oligosaccharides, representing about 15% of the population, were isolated by affinity chromatography on an RCA-Sepharose column. The MALDI-TOF MS of the RCA-purified fraction (Fig. 2*C*) shows $(M + Na)^+$ ions that can be assigned to glycans **C**–**J** (Table 2) on the basis of their molecular weight and the fact that they contain the *N*-acetyl lactosamine sequence necessary for RCA binding. The structure of these ions was confirmed by enzyme sequencing combined with MALDI-TOF MS. For instance, glycans **H** or **J** were converted to **f** by treatment with β-galactosidase and further to $ManXylFucGlcNAc_2$ by additional treatments with β-*N*-acetylglucosaminidase and α-mannosidase.

Plantibody Produced in GalT-Plants Is Gal Terminated. The effect of expression of GalT on a plantibody (Mgr-48) was investigated. Plantibodies were isolated by affinity chromatography on protein G from tobacco plants expressing Mgr-48 and from two selected plants (GalT-8 × Mgr-48–11 and -12) resulting from a crossing between GalT plants and tobacco expressing Mgr-48 (28). Isolated Mgr-48 from hybridoma cells, tobacco, and the crossings of GalT-8 × Mgr-48 were analyzed on blot with sheep-anti-mouse-IgG and RCA (Fig. 3). Sheep-anti-mouse-IgG bound to both the heavy and light chains of all isolated antibodies. In contrast, RCA bound to hybridoma and to antibody produced in GalT plants but not to the antibody produced in plants expressing only Mgr-48. Furthermore, both anti-α1,3-Fuc and anti-β1,2-Xyl antibodies bound to Mgr-48 isolated from tobacco and GalT plants (not shown). This result indicates that, as observed on endogenous plant glycoproteins, antibodies produced in plants that coexpress human GalT carry both plant and mammalian glycan epitopes. Furthermore, the proportion of glycans of this monoclonal antibody that are galactosylated (as judged from the RCA binding) is similar in plants and in hybridoma cells.

N-glycans were isolated from the antibodies produced in these different expression systems by treatment with peptide-*N*-glycosydases and then characterized by MALDI-TOF MS and glycosidase sequencing as reported (6). Two major ions of *N*-glycans isolated from Mgr-48 produced in hybridoma cells were detected by MALDI-TOF MS (Fig. 4*A*). These ions were assigned to the fucosylated and partially galactosylated oligosaccharides **A** and **B** by reference to previous reports on mouse antibody *N*-glycosylation (6). Fig. 4*B* shows ions corresponding to *N*-glycans released from Mgr-48 antibody expressed in tobacco plants. A major population of complex *N*-glycans **a**–**f** is detected in addition to a minor population of high-Man-type *N*-glycans Man-7–Man-9. This heterogeneity already was reported in a previous work on plantibody *N*-glycosylation (6). The proposed structures for oligosaccharides *N*-linked to tobacco Mgr-48 antibody were confirmed by enzyme sequencing coupled with MALDI-TOF MS analysis as described previously. Fig. 4*C* shows the MALDI-TOF MS of *N*-glycans isolated from Mgr-48 antibody produced in tobacco plants expressing human GalT. As observed for the endogenous glycoproteins of GalT plants, additional ions representing about 30% (compared **H** + **J** to **f**), assigned to glycans **E**, **G**, **H**, **I**, and **J**, were detected and affinity purified on RCA. The structures of these oligosaccharides, of the IgG glycan population, were confirmed by enzyme sequencing analysis.

Table 2. Structure of *N*-glycans isolated from hybridoma IgG (A and B) and GalT plants (C–J)



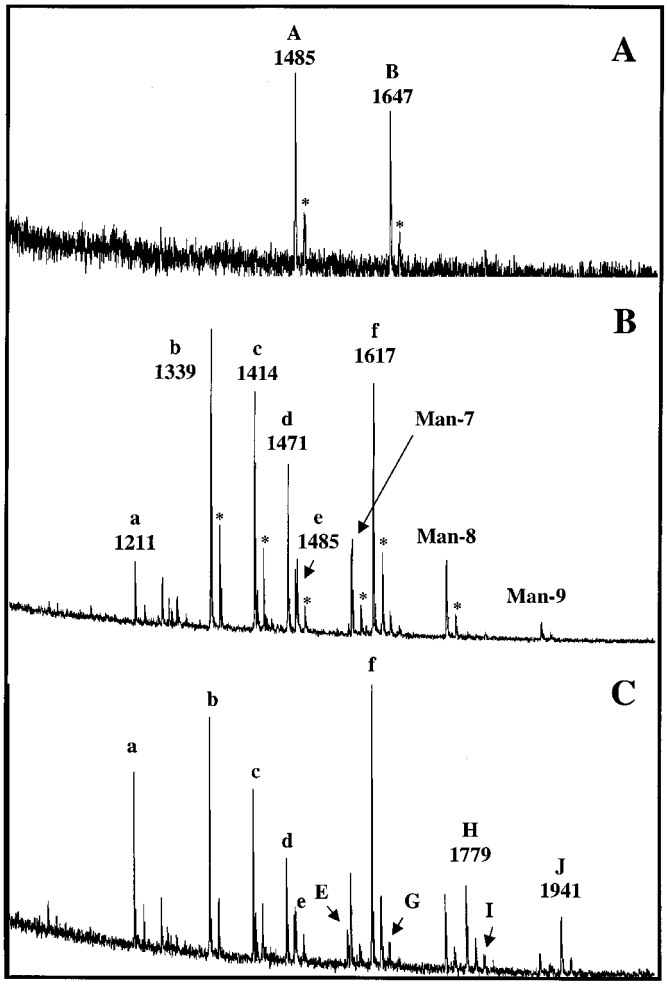


Fig. 4. MALDI-TOF mass spectra of *N*-linked glycans isolated from (*A*) hybridoma Mgr-48 antibody, (*B*) tobacco Mgr-48 antibody, and (*C*) Mgr-48 antibody expressed in tobacco plants transformed with human GalT. Asterisk, $(M + K)^+$ adducts. See Tables 1 and 2 for structures.

Discussion

There can be considerable variation in the structure of the *N*-linked glycans located in the Fc region of antibody heavy chains, but, in general, sialic acid content is low, and *N*-glycans are of the biantennary type with or without a core α1,6-linked Fuc (11, 35). For instance, *N*-glycans isolated from Mgr-48 IgG used in this study harbor a core α1,6-linked Fuc and are β1,4-galactosylated for about 45% when produced in hybridoma cells (Fig. 4*A*). The variation in *N*-glycan structures isolated from plantibody produced in GalT plants is much greater, but this diversity mostly arises from the variability in Xyl and α1,3-Fuc contents. With respect to galactosylation, the profile of *N*-glycans of immunoglobulins isolated from GalT plants is comparable to that isolated from hybridoma cells and as they naturally occur in blood.

From our results, it can be concluded that expression of human GalT has no detectable effect on the occurrence of xylosylation and fucosylation on *N*-glycans in plants. In contrast, Palacpac *et al.* (17) found that after expression of human GalT in BY2 tobacco suspension cells, Xyl and Fuc residues are completely absent in β1,4-Gal-containing *N*-glycans (47.3% of the population). The major modified product formed in transformed BY2 tobacco cell is a hybrid structure having a single Galβ1–4GlcNAc, which is a result of the action of human GalT on $GlcNAcMan_5GlcNAc_2$ (17). This $GalGlcNAcMan_5GlcNAc_2$ structure normally is not found in mammals. In mammals, Gal is transferred to $GlcNAc_2Man_3GlcNAc_2$, a structure formed after the action of α-mannosidase-II and GlcNActransferase-II. This observation indicates that in BY2 cells, GalT is acting too early in the Golgi apparatus, resulting in the formation of abnormal structures on which not only can the β1,2-xylosyltransferase and α1,3-fucosyltransferase not act, but this transfer also prevents trimming of the α1,6-Man arm by α-mannosidase-II. A possible explanation for this early action of human GalT in the Golgi apparatus can be related to the difference in the glycosyltranferase targeting or Golgi organization between cell culture and plants. The transformed tobacco plants described in this paper have a more mammalian-like galactosylation profile, because biantennary bigalactosylated structures are synthesized. This result indicates that in our tobacco plants, GalT is probably properly located in the median and in the *trans*-Golgi

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compartments and acts with the endogenous β1,2-xylosyl- and α1,3-fucosyltransferase on a common $GlcNAc_2Man_3GlcNAc_2$ substrate. Further studies are now being carried out in our laboratories to optimize the galactosylation levels in GalT transformants and to investigate the effects of a modified GalT targeting within the plant Golgi apparatus by changing its natural Golgi retention signal for a plant glycosyltransferase retention signal.

Probably all tobacco glycoproteins have an extended *N*-glycosylation resulting from the expression of GalT. However, no obvious phenotype is observed, and no problems occur in the reproduction of the plants. It also has been shown before that reducing the complex *N*-glycan maturation potential in plants has no severe effect on the phenotype as demonstrated by the *Arabidopsis thaliana cgl* mutant. Because of the absence of *N*-acetylglucosaminyltransferase I activity in this mutant, these plants completely lack Xyl- and Fuc-containing complex *N*-glycans (36). In contrast, changes in animal glycosylation usually have a quite severe effect. This ability of plants to be unaffected by drastic changes in their *N*-glycan processing can be an advantage in using transgenic plants for production of glycan-engineered therapeutic glycoproteins.

Independent of the Gal addition, glycosylation of antibodies is quite different from the total glycoprotein population (compare Figs. 2*A* and 4*B*). Mgr-48 antibodies contain more GlcNAc and fewer Xyl and Fuc residues. These differences might reflect both localization and protein specificity. Secreted proteins, like the expressed antibody, contain more GlcNAc, whereas vacuolar proteins, which are included in the total protein population, contain less GlcNAc (4). The reduction of Xyl and Fuc content might be because of the buried nature of the *N*-glycan within the antibody, making it inaccessible for glycosyltransferases, which can also explain the low sialic acid content of natural antibodies (11, 35).

In conclusion, expression of GalT in tobacco plants results in galactosylation of plantibodies similar to that observed in mammals. Human GalT, therefore, localizes correctly in the plant Golgi apparatus. Because Gal is important for the function of antibodies, this is a major improvement in the *in planta* engineering of antibodies and an important step toward obtaining recombinant therapeutic glycoproteins with fully humanized *N*-glycans. To obtain these fully humanized glycoproteins, further modification is required to prevent Xyl and Fuc addition to *N*-linked glycans. For proteins that do not require sialic acid to be functional, inactivation of the β1,2-xylosyl- and α1,3-fucosyltransferase and expression of GalT will be sufficient to produce human-compatible proteins.

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