Carbohydrate Research 344 (2009) 1487–1493

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

Synthesis of Lewis X epitopes on plant N-glycans

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ARTICLE INFO

Article history:
Received 25 February 2009
Received in revised form 26 April 2009
Accepted 6 May 2009
Available online 9 May 2009

Dedicated to Professor Hans Kamerling on the occasion of his 65th birthday

Keywords:
Tobacco
Transgenic
β-(1→4)-Galactosyltransferase
α-(1→3)-Fucosyltransferase
N-glycan
Lewis X

ABSTRACT

Glycoproteins from tobacco line xFxG1, in which expression of a hybrid β-(1→4)-galactosyltransferase (GalT) and a hybrid α-(1→3)-fucosyltransferase IXa (FUT9a) is combined, contained an abundance of hybrid N-glycans with Lewis X (Le^X) epitopes. A comparison with N-glycan profiles from plants expressing only the hybrid β-(1→4)-galactosyltransferase suggested that the fucosylation of the LacNAc residues in line xFxG1 protected galactosylated N-glycans from endogenous plant β-galactosidase activity.

1. Introduction

N-Glycosylation in plants and animals is similar up to the Golgi complex, where this process starts to diverge between and within both types of organisms depending on tissue type and developmental stage. The differences are mainly due to differences in the repertoire of glycosyltransferases encoded by these organisms and to some extent to differential compartmentalization.[1,2] A whole range of *N*-acetylglucosaminyltransferases and galactosyltransferases such as β-(1→4)-galactosyltransferase (GalT) do not occur in plants, although GalT can be expressed ectopically in tobacco using genetic modification as was shown in our laboratory.[3,4] Conversely, β-(1→2)-xylosyltransferase (XylT) appears to be ubiquitous in plants and seems to be absent from most animals except snails[5] and trematodes.[6]

There is at least one modification at the N-glycan antennae that is shared by plants and animals, that is, the Le^a epitope that is the result of the consecutive linkage of β-(1→3)-galactose and α-(1→4)-fucose to the terminal GlcNAc.[7] A plant homologue of the fucosyltransferase responsible for this addition has been cloned in our laboratory.[8] In our efforts to enable production of 'humanized' antibodies in plants, the human β-(1→4)-galactosyltransferase 1 (GalT)[3], and later a hybrid GalT comprising the N-terminal domain of the *Arabidopsis* XylT and the catalytic domain of GalT, were introduced in tobacco[4] and shown to induce synthesis of N-glycans with LacNAc structures that are typical of animal N-glycans. In tobacco plants expressing the hybrid GalT, we found a remarkable accumulation of the hybrid N-glycan $GlcNAcMan_5GlcNAc_2$, which was very likely to be due to de-galactosylation of the main galactosylated N-linked oligosaccharide $GalGlcNAcMan_5GlcNAc_2$. Under normal circumstances $GlcNAcMan_5GlcNAc_2$ is a transient intermediate in the synthesis of complex N-glycans and does not accumulate to a detectable level.

Plant β-galactosidases that might account for this de-galactosylation of β-(1→4)-galactosylated N-glycans, such as jack bean β-galactosidase, have been described decades ago.[9] In general, α-(1→3)-fucosylation of a Galβ-(1→4)-GlcNAc (LacNAc) structure renders the galactose residue insensitive to a range of β-galactosidases[10], a fact that has turned out to be very useful for sequential glycosidase sequencing of oligosaccharides.[11] Hence, it was deemed useful to try to modify the ectopic LacNAc structures in plants expressing the hybrid GalT by expression of a suitable α-(1→3)-fucosyltransferase from an animal source and thus create an Le^X epitope.

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doi:10.1016/j.carres.2009.05.003

The mouse FUT9 gene was the first of its kind to be cloned[12] and was subsequently shown to be highly homologous to its human counterpart.[13] It proved to be a distant member of a family of at least six mammalian α-(1→3)-fucosyltransferases, five of which were capable of fucosylating the nonreducing ends of glycans with type 2 chains yielding Le^X epitopes.[14]

In this paper we describe the results of MALDI-TOFMS analysis of N-glycans from tobacco plants transformed with a construct comprising two genes, the hybrid GalT used in earlier experiments and a hybrid FUT9. Both genes featured the N-terminal domain of *Arabidopsis* XylT. The stem region and catalytic domain of the hybrid FUT9 were derived from a pufferfish *Tetraodon nigroviridis* FUT9 homologue displaying 57% homology at the amino acid level with the corresponding segment of the mouse and human FUT9. One transgenic tobacco line was identified that synthesizes N-glycans with Le^X epitopes. Five other lines were found that lack galactosylation of N-glycans, but contain typical wild-type plant N-glycans with one or two additional fucose residues.

2. Results

2.1. Construction of plant transformation vectors

The gene fragment encoding the catalytic domain comprising residues 38–359 of a *T. nigroviridis* FUT9a homologue was obtained by PCR using genomic DNA and primers based on accession AJ783833. It was fused N-terminally with the sequence encoding the first 53 aa of the *Arabidopsis thaliana* XylT gene. Sequencing of a number of independent clones containing the truncated FUT9 gene revealed two non-silent mutations in all of them that lead to aa substitutions at positions 65 (I to V) and 176 (A to T). Neither of these mutations concerned residues that were highly conserved compared to other animal homologues. The hybrid gene was placed under control of the Cassava Vein Mosaic Virus (CVMV) promoter,[15] and the resulting expression cassette was combined with the one comprising the hybrid GalT under control of the CaMV 35S promoter.[4] Together they were transferred to a plant transformation vector conferring hygromycin resistance, which was designated xFxG.

2.2. N-Glycan profiles from a transgenic population

Eight plants were analyzed for N-glycan composition using MALDI-TOFMS. Seven transgenic lines contained the three abundant complex N-glycans at *m/z* 1211.4, 1414.5, 1617.5 that typically represent more than 70% of total N-glycans in wild-type plants. However, five of these displayed N-glycan profiles that at first sight appeared to be wild-type as well, but which proved to be supplemented with additional minor oligosaccharides. For example in Figure 1, a comparison of the N-glycan profiles from wild-type and xFxG5 plants showed that line xFxG5 contained unusual N-glycans at *m/z* 1560.5, 1763.7 and 1909.7, which seemed to represent mono- or difucosylated versions of the typical wild-type products at *m/z* 1414.5 and 1617.5.

Only transgenic line xFxG1 displayed a completely different N-glycan profile that contained oligosaccharides indicative of the presence of Le^X epitopes. A comparison of its N-glycans with that from line xylGalT12,[4] which contained only the xylGalT gene, revealed considerable increases of peaks at *m/z* 1444.5 and 1768.6 that suggested fucose addition to hybrid galactosylated N-glycans typical of xylGalT lines, that is, oligosaccharides at *m/z* 1298.4 and 1622.6 (cf. Fig. 2A and B). For example, efficient fucosylation of the most abundant galactosylated N-glycan in xylGalT12 shown in Fig. 2A at *m/z* 1622.6 ($GalGlcNAcMan_5GlcNAc_2$) would explain the occurrence of the most abundant hybrid shown in Fig. 2B at *m/z* 1768.6. The fact that PNGase F-mediated release of the N-glycans from glycoproteins of line xFxG1 gave an N-glycan profile that was virtually indistinguishable from the one obtained following PNGase A-mediated release, showed unequivocally that most of the N-glycans in this line including the major peak at *m/z* 1768.6 in line xFxG1 lacked the core-bound α-(1→3)-fucose (cf. Fig. 2B and C). At 20% of total peak area, the level of high-mannose in this line is somewhat lower than what is usually encountered in plants expressing only xylGalT. Unexpectedly, not one of the anti-Le^X monoclonals 128-4F9-A, 291-4D10-A, 291-2G3-A[16] and anti-L5[17] revealed differences between xFxG1 and wild-type plants in Western blot analyses of leaf proteins separated by denaturing SDS–PAGE (data not shown).

Further corroboration of the presence of Le^X epitopes in line xFxG1 was sought by digesting purified N-glycans from this line with either β-(1→4)-galactosidase, α-(1→3,4)-fucosidase or a combination of these enzymes followed by MALDI-TOFMS analysis. MALDI-TOFMS analysis of β-(1→4)-galactosidase-treated N-glycans from line xFxG1 showed that the products at *m/z* 1444.5, 1606.6 and 1768.6 were not cleaved (cf. Fig. 3A and B), though the disappearance of the product at *m/z* 1622.6 proved that the galactosidase had been active. Concurrently, β-(1→4)-galactosidase digestion greatly increased the peak at *m/z* 1460.5 to about the same extent as the peak at *m/z* 1622.6 was reduced, which strongly suggested a product–precursor relation between these two peaks (Table 1). It is likely that most of the N-glycan at *m/z* 1460.5 consists of $GlcNAcMan_5GlcNAc_2$ and only a minor fraction, detectable at *m/z* 1298.4 after β-galactosidase digestion, is made up of the galactosylated $GalGlcNAcMan_4GlcNAc_2$. The N-glycan at *m/z* 1298.4 in the untreated sample consists mainly of $GalGlcNAcMan_3GlcAc_2$, and it can be detected as a product at *m/z* 1136.4 after digestion with β-galactosidase. Digestion of the N-glycans with α-(1→3,4)-fucosidase revealed not only that the oligosaccharides at *m/z* 1444.5, 1606.6 and 1768.6 shown in Figure 3A and B contained fucose residues at their nonreducing ends (Fig. 3C), but their almost complete cleavage also confirmed the conclusions from PNGase F-mediated release of N-glycans (Fig. 2C): all three peaks were nearly lacking N-glycans with a core-bound fucose. Subsequent digestion of the de-fucosylated products with β-(1→4)-galactosidase demonstrated the presence of β-(1→4)-linked galactose residues on the antennae of products at *m/z* 1298.4 and 1622.6 (Fig. 3C), which resulted in the complete disappearance of the ion at *m/z* 1622.6 (Fig. 3D). The presence of the galactose residue on the N-glycan at *m/z* 1298.4 shown in Figure 3C can be inferred from the emergence of a peak at *m/z* 1136.4 that represents $GlcNAcMan_3GlcNAc_2$, (Fig. 3D) upon digestion with β-galactosidase. To sum up, the observation that β-(1→4)-galactosidase could only release the galactose from the hybrid N-glycans at *m/z* 1444.4, 1606.5 and 1768.6 shown in Figure 3A after removal of the fucose residue strongly suggests that they carried Le^X epitopes.

Independent confirmation of N-glycans with Le^X epitopes in line xFxG1 was obtained by Q-TOFMS/MS analysis of its most abundant N-glycan at *m/z* 1768.8 in the MALDI spectrum of Figure 2B. In the presence of LiOAc and formic acid, this N-glycan from line xFxG1 could be detected in the Q-TOF instrument as a doubly charged lithium adduct at *m/z* 879.88.[18] Collision-induced fragmentation of the ion at a collision cell voltage of up to 35 V produced a complex spectrum that revealed a prominent doubly charged $[M+2Li^+]$ ion peak at *m/z* 806.84, which resulted from loss of the fucose residue (Fig. 4). More importantly, the singly charged $[M+Li^+]$ ion at *m/z* 518.25, almost certainly containing a GlcNAc, a fucose and a hexose, implies that the fucose is indeed very likely to be part of a Le^X structure at the nonreducing end with the hexose representing the terminal β-(1→4)-galactose or the α-(1→3)-mannose of the trimannosyl core.

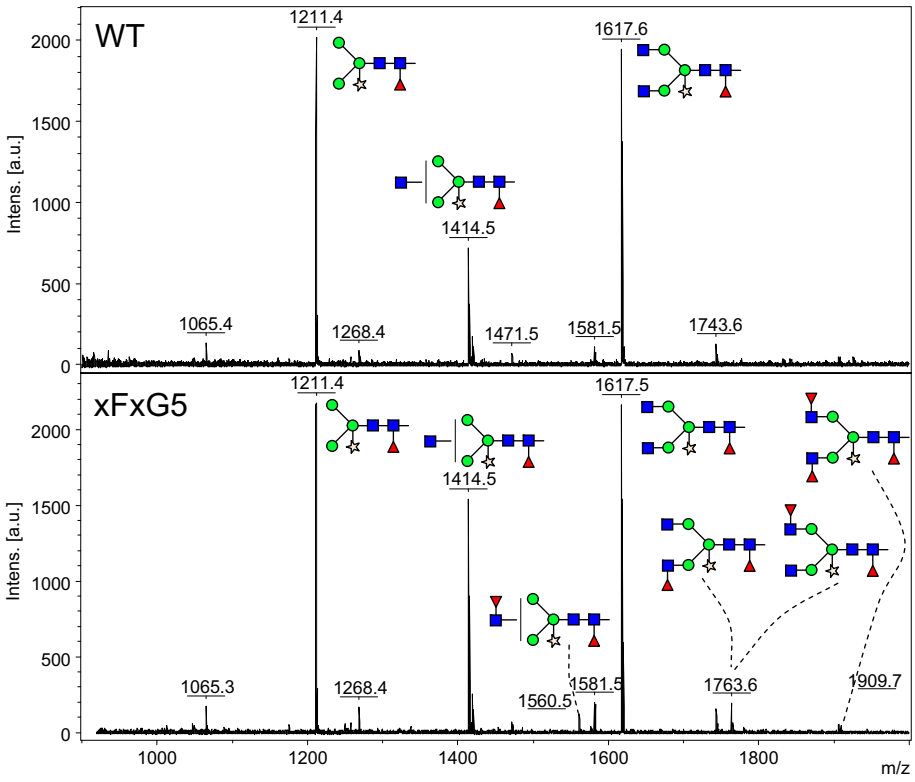

Figure 1. MALDI-TOFMS spectra of N-glycans [M+Na$^+$] from wild-type (upper panel) and xFxG5 plants (lower panel). The diagrams represent the proposed structures of the major peaks and those representing di- and trifucosylated products have been tentatively assigned. The diagrams of the analyzed oligosaccharides in Figures 1–4 use the following key: green circle, mannose; blue square, *N*-acetylglucosamine; red triangle, fucose; orange star: xylose; yellow circle, galactose.

3. Discussion

The enzymatic characteristics of the pufferfish FUT9 homologue employed in this study had not been verified experimentally, but the segment we used was 57% homologous at the amino acid level to both the mouse and human FUT9 proteins. As the human enzyme was shown to preferentially fucosylate terminal LacNAc structures,[14] unlike the other human FUT genes capable of fucosylating lactosamine, we considered it potentially useful for synthesizing Le^X on plant N-glycans. The choice of α-(1→3)-fucosyltransferase was narrowed down further by reports showing that human FUT9 displayed a broad spectrum of acceptor specificities,[19] including N-glycans, and that its mouse homologue was likely to be involved in synthesizing Le^X structures in the brain.[20] Later on, using Fut9$^{-/-}$ mice, further evidence was provided that FUT9 was responsible for fucosylating N-linked LacNAc in brain and kidney.[21,22] Finally, fucosyltransferase assays of HeLa cells that overexpressed the human FUT9 clearly showed that it fucosylated N-linked glycans of asialoEPO.[23]

The choice of the preferred FUT9 gene source was mainly decided by considerations regarding sequence characteristics that would lead to optimal expression in plants. In this respect, the *T. nigroviridis* FUT9a represented one of the three FUT9 genes that had been described for this pufferfish species. Its coding region was G + C rich, like the other two pufferfish FUT9 genes, with a G + C content of 48%. This was deemed superior for expression in plants to that of the human gene with its G + C content of 41% and a long stretch of more than 100 bp with a G + C level below 30%. In general, genes with high A + T content tend to be expressed poorly in plants.[24,25] The use of a hybrid FUT9 was invoked by the use of the hybrid GalT, which was chosen not only for its ability to induce high-level galactosylation, but also because it is associated with the occurrence of high levels of the N-glycan GlcNAcMan$_5$GlcNAc$_2$, a hybrid N-glycan that presumably arises due to de-galactosylation of the primary galactosylated N-glycan.[4] But, as the xylGalT hybrid enzyme tends to be localized in the medial Golgi region, the FUT9 catalytic domain was fused with the same CTS-region as the GalT catalytic domain to ensure that the FUT9 activity would also be present in this compartment.

Five out of eight transgenics that were examined contained unusual N-glycans in which typical wild-type plant N-glycans appeared to be fucosylated twice, presumably once at the usual α-(1→3)-position on the core and again somewhere else in the molecule. Exoglycosidase treatment with the *Xanthomonas manihotis* α-(1→3,4)-fucosidase did not lead to loss of fucose, but that does not imply that the second fucose is not located at the antennae. It could be that the enzyme requires a β-linked galactose on the GlcNAc residue substituted with a fucose residue. Perhaps, the presence of high levels of FUT9 in the absence of GalT activity allows some inadvertent transfer of fucose to an acceptor that would not otherwise act as such. Weak α-(1→3)-fucosyltransferase activity onto acceptors such as chitobiose and asialo-agalacto glycopeptides, which are found to be associated with recombinant forms of human FUT5 and FUT6,[26,27] suggests that possibly other representatives of this class of enzymes, like FUT9, may spuriously fucosylate the terminal GlcNAc on the nonreducing end of chitobiose or an N-glycan instead of the GlcNAc of a LacNAc.

Even though only one out of eight plants transformed with the xFxG construct appears to synthesize N-glycans with Le^X epitopes, the exoglycosidase digestions in conjunction with MALDI-TOFMS analyses of the products demonstrate that line xFxG1 contains high levels of N-glycans with the new structure. For example, the inability of the streptococcal β-(1→4)-galactosidase to remove the galactose from the oligosaccharide at *m/z* 1768.6, combined with the fact that it could be removed once this oligosaccharide had first been treated with the α-(1→3,4)-fucosidase, strongly suggests that

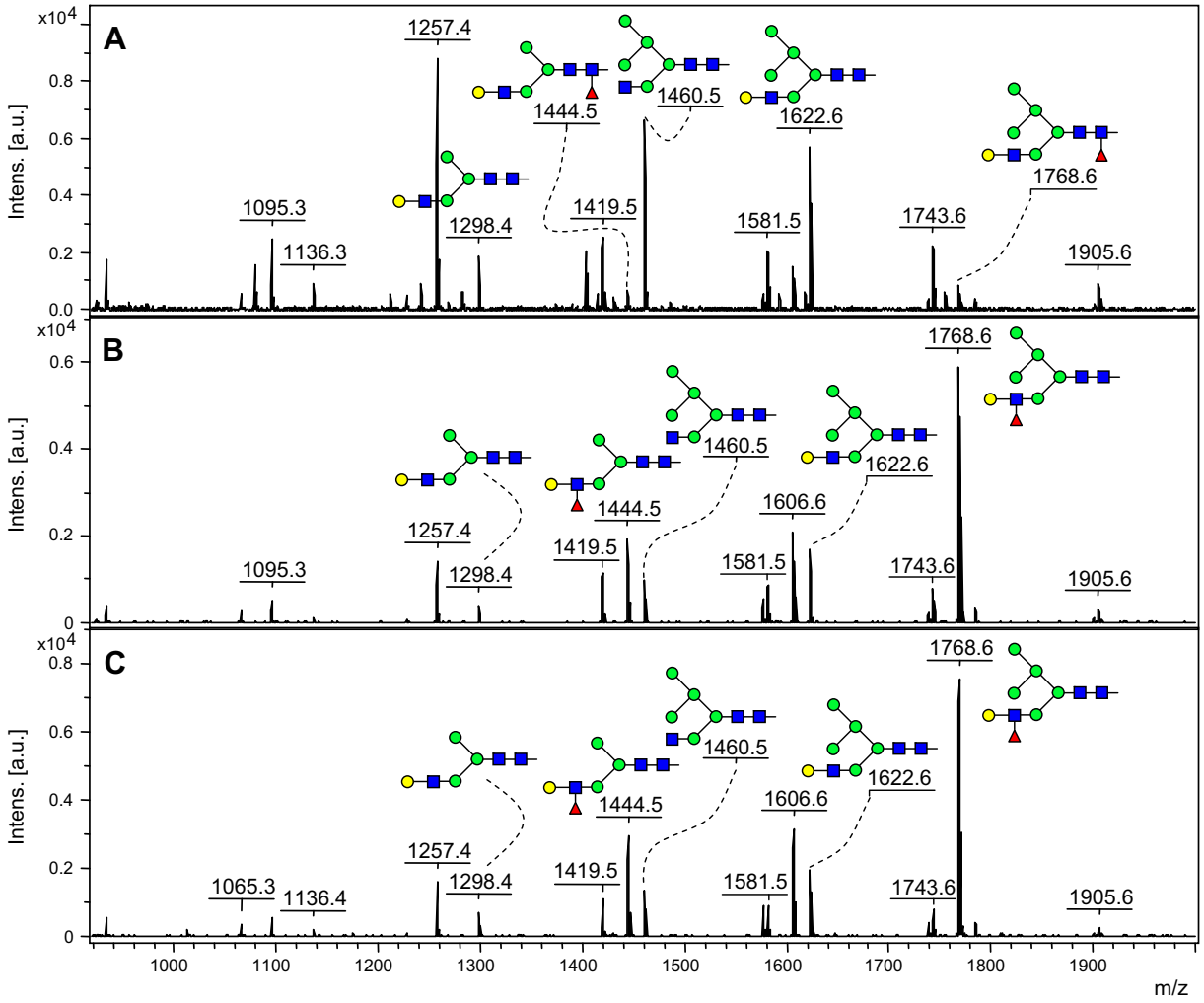

Figure 2. MALDI-TOFMS spectra of N-glycans [M+Na$^+$] from xylGalT12[4] (panel A) and xFxG1 plants (panels B and C). The samples in panels B and C were obtained following N-glycan release by PNGase A and F, respectively. The diagrams represent the proposed structures of the primary components of peaks (for details concerning the used symbols see Fig. 1).

the fucose had been protecting the β-(1→4)-galactose, as it would have in an Le^X structure. The presence of Le^X-containing N-glycans was substantiated even further using Q-TOFMS/MS analysis of the most abundant oligosaccharide at *m/z* 1768.6 in the MALDI spectrum. Despite the labile nature of the fucose residue, we could detect production of a small but detectable amount of an ion at *m/z* 518.25 that consisted of a fucose unit linked to a GlcNAc and a hexose residue, which provided unequivocal evidence for the occurrence of the Le^X epitope in the parent ion. This ion is likely to represent the cleavage product with the terminal β-(1→4)-galactose which requires only one cleavage reaction, and not the alternative product of two cleavages with the α-(1→3)-mannose of the trimannosyl core. The inability to detect the Le^X epitope on plant glycoproteins using immunoblotting with four different monoclonal antibodies could be due to the fact that it is incorporated in hybrid N-glycans, which might impede the binding of these monoclonals, as this molecular context is likely to be quite unlike the epitopes against which the monoclonals were elicited.

The conversion of LacNAc to fucosylated LacNAc appears to be incomplete as exemplified by the N-glycans GalGlcNAcMan$_5$GlcNAc$_2$ at *m/z* 1622.6, and its likely degradation product GlcNAcMan$_5$GlcNAc$_2$ at *m/z* 1460.5. The fact that the latter peak is mainly made up of this degradation product, and not the galactosylated N-glycan GalGlcNAcMan$_4$GlcNAc$_2$, can be deduced from the observation that β-galactosidase treatment leads to an increase in peak intensity at *m/z* 1460.5 that is proportional to the reduction of peak intensity at *m/z* 1622.6. In view of the fact that a plant-specific fucosidase has been identified[28] that could cleave the fucose from Le^X epitopes, we cannot rule out that the GalGlcNAcMan$_5$GlcNAc$_2$ peak at *m/z* 1622.6 is due to the fucosidase-mediated cleavage of the GalFucGlcNAcMan$_5$GlcNAc$_2$ peak at *m/z* 1768.6 instead of incomplete conversion of LacNAc termini.

Virtually no differences were found between the xFxG1 N-glycan profiles following release by either PNGase A or F suggesting the absence of an N-glycan with core α-(1→3)-linked fucose in this line. Similarly, and in agreement with earlier observations on plants expressing only the xylGalT gene,[4] the level of core-bound xylose is also low. In the xFxG1 line more than 70% of the N-glycans are galactosylated, of which more than 85% occur in the form of an Le^X epitope. Hence, it would seem that, like all other β-galactosidases characterized so far, none of the β-galactosidase activities encountered in plant leaves significantly cleave the galactose in an Le^X structure.[29,30]

The Le^X epitope is one of a group of fucosylated oligosaccharides that are bound by the human dendritic cell receptor DC-SIGN[31,32] that can also be used for efficient delivery of antigens through coupling to anti-DC-SIGN antibodies.[33] As Le^X-containing antigens have been shown to induce antigen-specific immune responses,

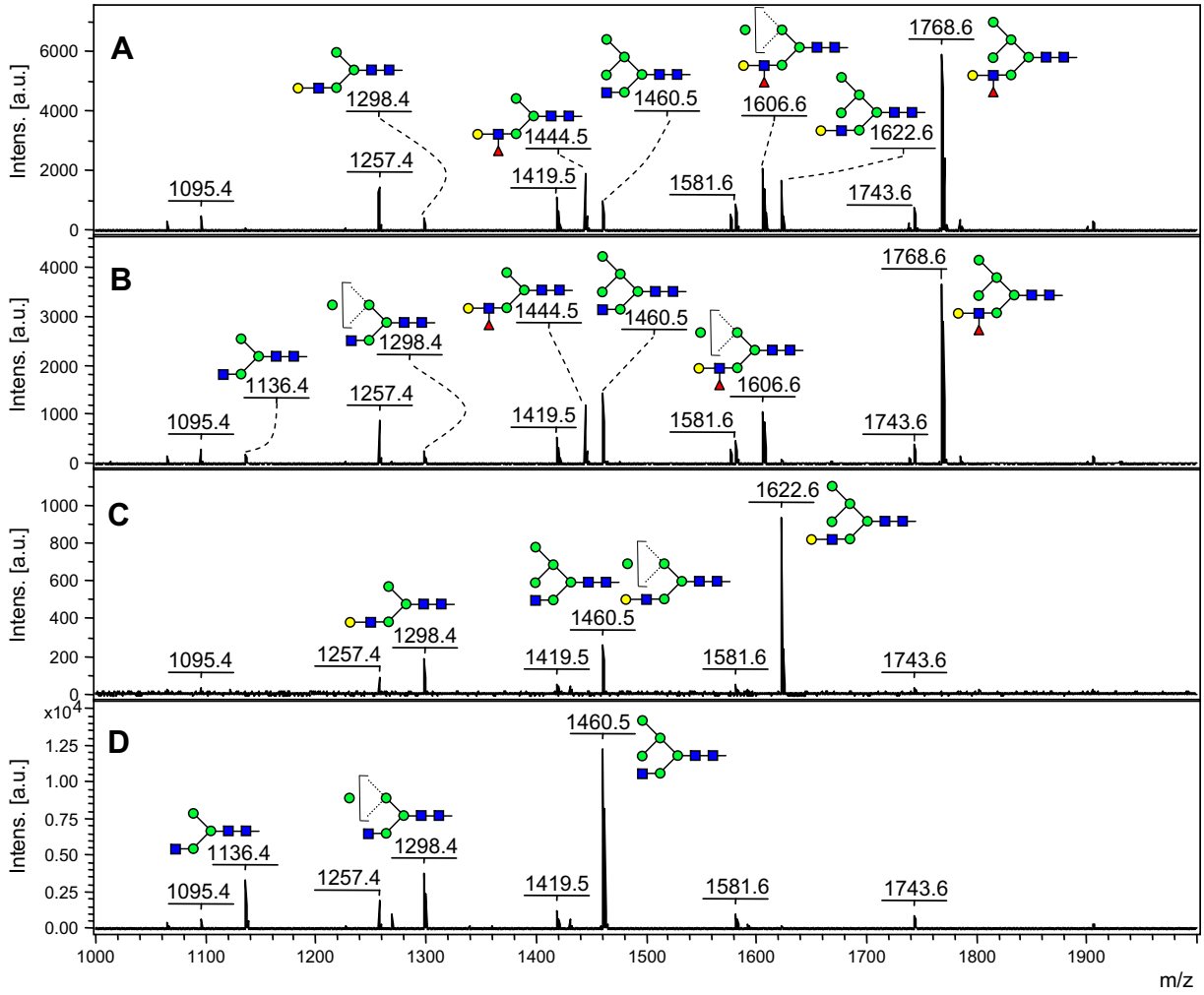

Figure 3. MALDI-TOFMS spectra of N-glycans [M+Na$^+$] from xFxG1 treated with β-(1→4)-galactosidase (panel B), α-(1→3,4)-fucosidase (panel C), sequentially with α-(1→3,4)-fucosidase and β-(1→4)-galactosidase (panel D) and untreated control (panel A). The diagrams represent the proposed structures of the primary components of peaks (for details concerning the symbols used see Fig. 1).

Table 1
MALDI-TOFMS analysis of N-glycans [M+Na$^+$] from xFxG1 leaf glycoproteins treated with β-(1→4)-galactosidase or left untreated[a]

Observed *m/z*	Proposed structure[b]	− β-Galactosidase	+ β-Galactosidase
933.3	Man3GlcNAc2	1.3	1.6
1095.4	Man4GlcNAc2	1.6	1.6
1136.4	GlcNAcMan3GlcNAc2	–	1.3
1257.4	Man5GlcNAc2	5.4	5.8
1298.5	GalGlcNAcMan3GlcNAc2	1.5	1.8
1419.5	Man6GlcNAc2	4.6	4.1
1444.5	GalGlcNAcFucMan3GlcNAc2	8.3	8.6
1460.5	GlcNAcMan5GlcNAc2	4.2	11.1
1576.6	GalGlcNAcFucXylMan3GlcNAc2	2.4	2.0
1581.5	Man7GlcNAc2	3.7	3.7
1606.6	GalGlcNAcFucMan4GlcNAc2	10.1	10.0
1622.6	GalGlcNAcMan5GlcNAc2	8.4	–
1738.6	GalGlcNAcFucXylMan4GlcNAc2	1.2	–
1743.6	Man8GlcNAc2	3.6	3.0
1768.6	GalGlcNAcFucMan5GlcNAc2	37.4	38.6
1784.6	GlcNAcMan7GlcNAc2	1.6	1.6
1905.6	Man9GlcNAc2	1.6	1.3
Total		96.9	95.9

[a] Values indicate percentage of total peak area.
[b] Proposed structure indicates the structure of the major component. N-Glycans representing <1% of total peak area have been omitted.

[34,35] possibly also as a result of DC-SIGN-mediated uptake, plants might be used to produce vaccines with enhanced immunogenicity resulting from N-linked Le^X epitopes. Furthermore, in view of the protective effect against β-galactosidases conferred by α-(1→3)-fucosylation of the LacNAc, it is conceivable that transgenic plants that synthesize the Le^X structure could also be used to produce monoclonal antibodies with much higher levels of galactosylated N-glycans than plants that synthesize only the LacNAc structure.

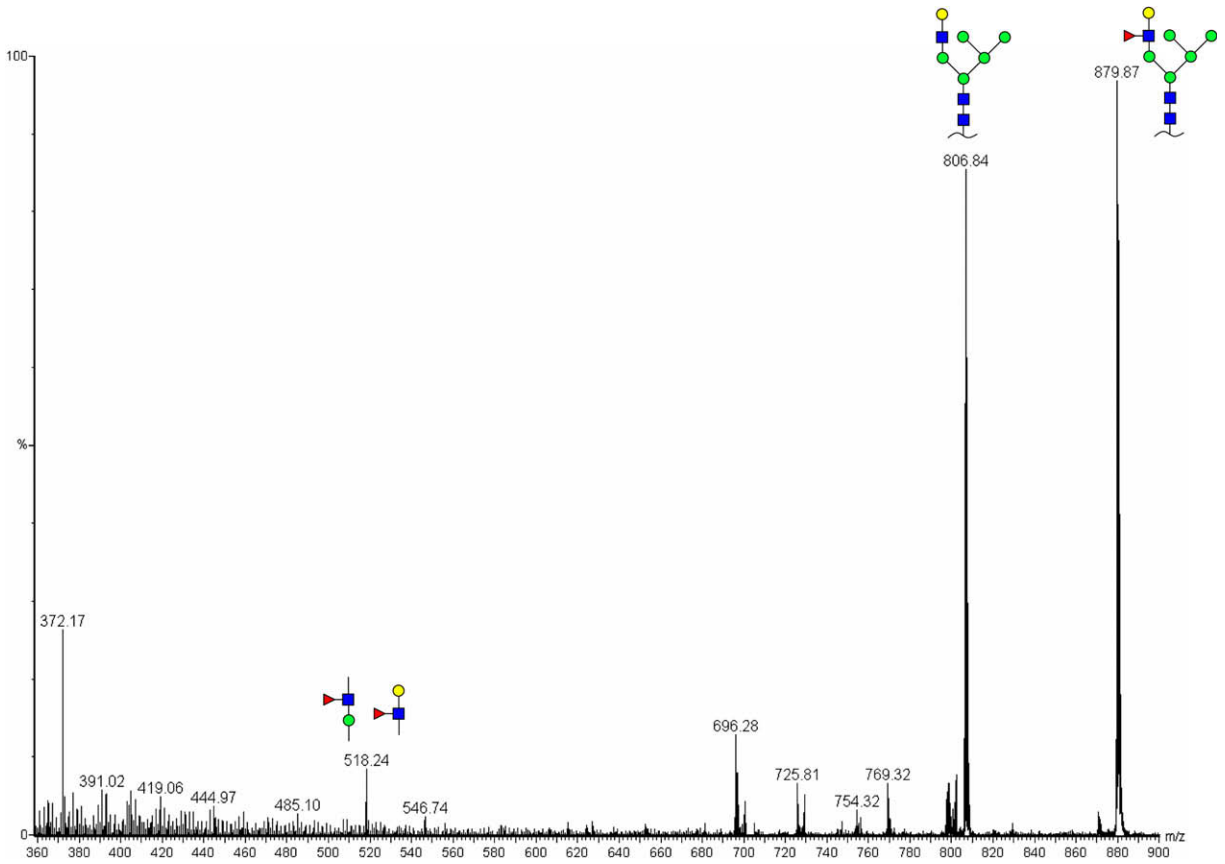

Figure 4. Electrospray-ionization tandem mass spectrum [M+Li$^+$] ions of N-linked glycan GalGlcNAcFucMan$_5$GlcNAc$_2$. The diagrams denote proposed structures of ions mentioned in the text (for details concerning the symbols used see Fig. 1).

Yet, this would require a (postharvest) processing step that can efficiently remove the terminal α-(1→3)-fucose, for example, through the use of a specific plant fucosidase.[28] The feasibility of employing plants with Le^X-containing N-glycans as production platforms for biopharmaceuticals would be boosted considerably if the negative effects of GalT and FUT9a co-expression on plant development (data not shown) could be suppressed, for example, through the use of tissue-specific promoters for at least the GalT gene.

4. Experimental

4.1. Construction of plant transformation vectors

A DNA fragment comprising the stem region and catalytic domain of *T. nigroviridis* FUT9a (accession no. AJ783833; Martinez-Duncker, I., Oriol, R. and Mollicone, R., unpublished) was amplified from genomic DNA using the following primers: FUTup, TGACCATG GCGTCTCACATGACTGAATTCTCCTCCGGACCAGTGGAGACAGGACTG A; FUTdw, GTGACGGATCCAATCAACCCCAGTACCACTTGTTAAG. The *Nco* I/*Bam* HI digested fragment was cloned into a likewise digested pMTL23[36] derivative lacking the *Eco* 31I site, giving clone pMTLtr-FUT9. A cDNA fragment covering the N-terminal part of the *A. thaliana* xylosyltransferase (accession no. AJ277603) was obtained by amplification with the following primers: AtxylBpi, GTGACGAAGA CAACATGAGTAAACGGAATCCGAAGATTC; Atxyl137c, GTGACGAATT CCGA-TTGGTTATTCACTGAAACGT. This fragment was digested with *Bpi* I and *Eco* RI and cloned into *Esp* 3I/*Eco* RI-digested pMTLtr-FUT9, yielding clone pMTLxylFUT9. The promoter–terminator cassette for cloning of the hybrid gene was made by removing the enhanced 35S promoter from pUCAP35S[37] by *Asc* I/*Nco* I digestion and replacing it with a likewise digested PCR fragment covering the cassava vein mosaic virus (CVMV) promoter[15] that had been obtained following amplification with primers: CVMV-Asc, GTCAG GCGCGCCGCGATCGCCAGAAGGTAATTATCCAAG; CVMV-Nco, GTGA CCATGGAACAAACTTACAAATTTCTCTGAAG of a plasmid with this promoter. The *Esp* 3I/*Bam* HI fragment containing the hybrid gene was cloned into *Nco* I/*Bgl* II digested pUCAP-CVMV. This clone was used to insert the xylGalT expression cassette constructed earlier.[4] The *Asc*I/*Pac*I fragment comprising the two expression cassettes in the same transcriptional orientation was cloned into a modified version of binary vector pMOG22[38] in which the *Eco*RI and *Hin*dIII sites of the multiple cloning site had been replaced by *Pac*I and *Asc*I, respectively. PCR amplifications were carried out using Accu-Prime *Pfx* DNA polymerase (Invitrogen), and the sequences of all PCR fragments were verified using DYEnamic™ ET Terminator Cycle Sequencing Kits (GE Healthcare) on a ABI Prism 3730XL DNA analyzer (Applied Biosystems).

4.2. Plant transformation and growth

The plant transformation vector comprising the two genes was used to transform *Nicotiana tabacum* (cv Samsun NN) as described previously.[3] Transgenic seedlings and plants were grown in a greenhouse at 21 °C.

4.3. N-Glycan analysis

N-Glycans were purified as described using PNGase A for release from the peptides unless specifically mentioned otherwise using PNGase F.[4] For galactosidase treatments, N-glycans were incubated overnight with 1.5 mU *Streptococcus pneumoniae* β-

(1→4)-galactosidase (Calbiochem) in 50 mM sodium phosphate buffer, pH 6.0, and for fucosidase treatments N-glycans were incubated overnight with 0.5 mU *X. manihotis* α-(1→3,4)-fucosidase (Sigma–Aldrich) in 50 mM sodium phosphate, pH 5.0. N-Glycans were purified away from salts and enzymes using an Ultra-Clean Carbograph column as described above, dissolved in 5 mM NaOAc and mixed with an equal volume of 10% DHB and 3% *N*,*N*-dimethylaniline in 50% acetonitrile.[39] Aliquots (1 μL) were spotted onto a stainless steel sample plate and dried under a stream of air at room temperature. Positive-ion MALDI-TOFMSs of $[M+Na]^+$ adducts were recorded on a Bruker Ultraflex fitted with delayed extraction and a nitrogen laser (337 nm). A maltodextrin series was used as an external molecular weight standard. Spectra were generated from the sum of 200–300 laser pulses.

4.4. Electrospray-ionization mass spectrometry

Electrospray-ionization mass spectrometry was performed with a Q-TOF mass spectrometer (Micromass). The N-glycans dissolved in MeOH–salt solution (1:1, v/v) were applied to the Q-TOF Z-spray source in 10-μL aliquots through nanoflow probe tips (Type F 6028634, Micromass). In the MS mode, the collision energy (CE) was maintained at 5.0 kV and the analysis time at 5 min. In the MS/MS mode, CE was maintained at 5.0 kV for the first 30 s to obtain parent peak information and then increased to 35 kV to acquire a balanced fragmentation pattern for the remainder of the analysis time for a total of 15 min. Interpretation of mass spectra was facilitated using the software tool GlycoWorkbench.1.1.3480.[40]

Acknowledgements

We thank Dr. A. Roesner and Professor T. Burmester (Johannes Gutenberg-University, Mainz, Germany) for providing us with *Tetraodon nigroviridis* genomic DNA and Dr. C.H. Hokke (University of Leiden, The Netherlands) and Dr. A. Streit (King's College. London, United Kingdom) for providing monoclonal antibodies 128-4F9-A, 291-4D10-A, 291-2G3-A and anti-L5, respectively.

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