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# *In vivo* activity of recombinant human Lewis fucosyltransferase III in leaves of *Nicotiana tabacum* L.

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## Abstract

Fucosylation in plants occurs in glycoproteins and polysaccharides but the function of fucosylation is largely unknown. We aimed to analyze the effects of over-expression of human fucosyltransferase III (hFucT III) on *in vivo* *N*-glycan accumulation in tobacco plant leaves and focused on comparing the amount of Lewis a (Le[a])-epitope accumulation in transgenic and in wild-type plants. Fucosyltransferase assays, Western blot and mass spectrometry were used to identify, quantify and analyse Le[a] *N*-glycans. We found that constitutive overexpression of hFucT III activity had no effect on Le[a] complex type *N*-glycans accumulation. Our results suggest that tobacco recombinant hFucT III acts more as a hydrolase than as a transferase.

*Additional key words*: fucose, *N*-glycans, polysaccharides.

## Introduction

Fucosylation in plants occurs in glycoproteins and in some polysaccharides (Fig. 1). Rhamnogalacturonans (RGI and II) are heteropolymers possessing repeat units of α(1,2)Rha (Fig. 1*A*). Between 20 and 80 % of the rhamnose residues of RG-I are substituted by neutral sugars such as fucose (Fuc) (O'Neill *et al.* 1990). Fuc can also be found in RG-II with α2-linkage as methylated or unmethylated sugar (Ridley *et al.* 2001). Xyloglucan (XG), the major compound of hemicellulose in dicotyledonous and non-graminaceous monocotyledonous plants, has a cellulose backbone (Fig. 1*B*). Some of the β(1,4)-linked glucose are substituted by a xylose, itself carrying in some cases a galactose that can be sustituted by a fucose residue (Levy *et al*. 1997). Arabino-galactan proteins are *O*-glycosylated proteoglycans constituted by arabinosyl and galactosyl residues as an oligosaccharide backbone fraction (Fig. 1*C*). Fucose can be linked in two positions: with *O*-2 linkage to the arabino-galactan residue of the trisaccharides Araα(1,3)Galβ(1,6)Gal and with α4-linkage as described by Van Hengel and Roberts (2002).

Plant *N*-glycans are fucosylated in α(1,3) to the proximal *N*-acetylglucosamine (GlcNAc) of the chitobiose. In some cases Fuc residue is linked to the *O*-4 GlcNAc at the non-reducing end of the lacto-*N*-biose. Fucosylated *N*-glycans were first described in humans (Yamashita *et al.* 1977) and more recently in some primates (Johnson and Watkins 1992, Dupuy *et al.* 2002). Complex *N*-glycans harboring the Lewis a (Le[a]) motif are also found in plants (Fitchette-Laine *et al*. 1997, Lhernould *et al.* 1997, Mélo *et al.* 1997, Wilson *et al.* 2001, Léonard *et al.* 2002). The α(1,4)-fucosyltransferase activity in plants was first described by Crawley *et al.* (1989). The corresponding genes have been more recently cloned in *Beta vulgaris* (Bakker *et al.* 2001), in *Lycopersicon esculentum* (Wilson 2001) and in *Arabidopsis thaliana* (Léonard *et al.* 2002). The function of α4-fucosylation is largely unknown, although the over-expression of the human α3/4-fucosyltransferase (*hFUT3*) has been investigated in tobacco plants (Joly *et al.* 2002a,b) where high amounts of hFucT III were found in the over-expressors accompanied by a strong increase in α4-fucosyl-transferase activity in the plants' sexual organs. The over-expressed α4-fucosyltransferase

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*Abbreviations*: AG - arabinogalactan; GlcNAc - *N*-acetylglucosamine; Fuc - fucose; FucT - fucosyltransferase; hFucT III - human FUCT III (AC:P21217); Le[a] - Lewis a; TLC - thin layer chromatography; MALDI-TOF MS - matrix-assisted laser desorption ionisation time-of-flight spectrometry; RG - rhamnogalacturonan; Tt - transgenic type tobacco; Wt - wild-type tobacco; XG - xyloglucan.
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activity in tobacco was positively correlated with a more significant immuno-staining of $Le^a$ motifs in transgenic than in control pollen grains. Pollen grain development was shown to be altered in transgenic plants, which had disorganized cell wall surfaces in hFucT III over-expressor pollen grains. The alteration was suggested to reduce seed production in hFucT III over-expressing plants.

In our work, we aimed to analyze the effects of an over-expression of human FucT III (hFucT III) on *in vivo* *N*-glycan accumulation in mature leaves of tobacco plants. Our particular focus was a comparison of the amount of $Le^a$-epitope accumulation in transgenic (Tt) and in wild-type (Wt) plants. We showed that constitutive over-expressed hFucT III activity had no effect on $Le^a$ complex type *N*-glycan accumulation. More remarkably, we demonstrated that recombinant hFucT III has a GDP-Fuc hydrolytic activity.

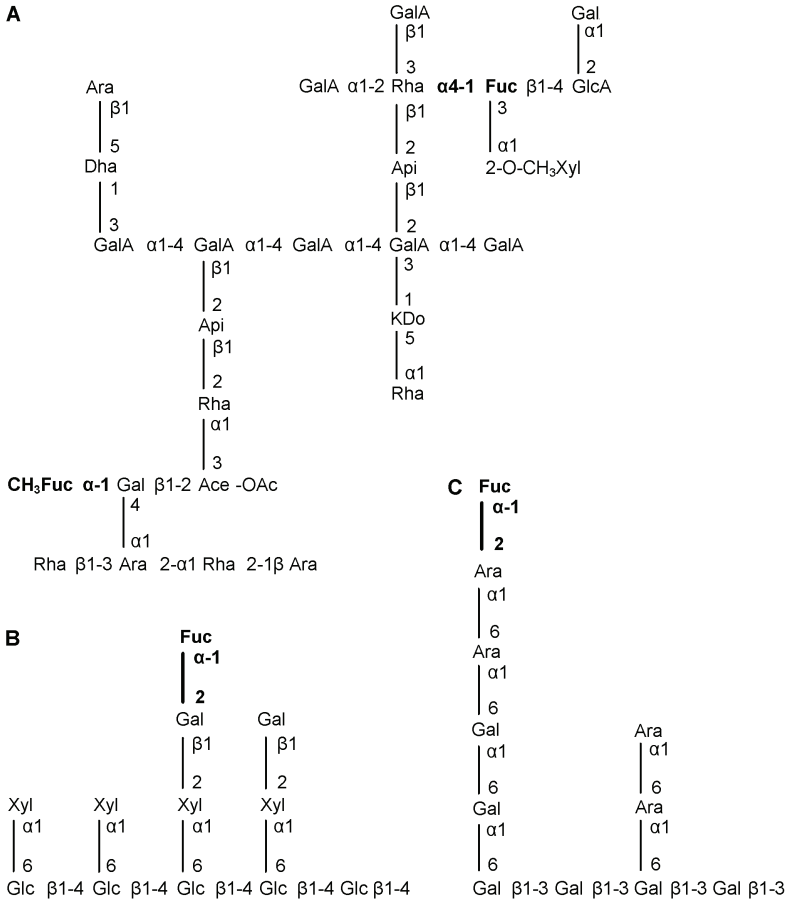


Fig. 1. Plant oligosaccharide structures harboring Fuc linkage: *A* - rhamnogalacturonan, *B* - xyloglucan, *C* - arabinogalactan.

## Materials and methods

**Plants:** Seeds of transgenic (Tt) and wild-type (Wt) tobacco plants (*Nicotiana tabacum* L. var. *Xanthi*) were grown in a greenhouse at a temperature of 25 °C and an irradiance of 250 μmol(photon) $m^{-2}$ $s^{-1}$ with a 16-h photoperiod for 3 months before leaf harvesting. Transgenic plants were obtained after *Agrobacterium* transformation of Wt with the human *FUT3* gene (accession: NM_000149) as described by Joly *et al.* (2002b).

**Enzyme assays:** Fucosyltransferase assays were conducted in a 60-$mm^3$ reaction volume containing 20 mM sodium cacodylate (pH 7), 5 mM ATP, 20 mM $MnCl_2$, 10 mM α-L-fucose, 3 μM GDP-[$^{14}$C]-fucose (115 GBq $mmol^{-1}$; *Amersham Pharmacia Biotech,* Quebec, Canada), and 25, 50 or 75 μg crude protein extracts from Wt and Tt leaves or supernatant of transfected COS-7 cells. The proteins used to measure α(1,4)-Fuc-T activity were extracted into cacodylate buffer [200 mM sodium cacodylate, pH 7.0, containing 1 % (m/v) Triton X-100]. The homogenate was centrifuged at 14 000 *g* for 30 min and the crude proteins collected from the supernatant. The mixture was incubated 2 h at 25 °C with 0.1 mM acceptor substrate. The reaction was stopped by addition of 3 $cm^3$ of cold water.

For hydrophobic substrates, the radio-labeled reaction product was collected and counted as described by

Léonard *et al.* (2002). For hydrophilic substrates, the reaction mixture was then applied to a *Dowex* (anion exchange column, *Sigma-Aldrich*, St. Louis, USA) 1 × 8 column (1 $cm^3$; 0.5 g $cm^{-3}$). The radio-labeled reaction product was eluted off successively with 6 $cm^3$ of water, with 3 $cm^3$ of 100 mM of sodium acetate buffer followed by sodium acetate buffer 200 mM and counted.

**Western blot experiments:** Protein extracts for immuno-chemical analysis were separated by SDS-PAGE using a 10 - 14 % acrylamide Tris-tricine gel (Schagger and Von Jagow 1987). Proteins were visualized after Coomassie blue staining or transferred onto nitrocellulose membranes (*Schleicher and Schuell*, Dassel, Germany). Immuno-detections with anti-$Le^a$, anti-Fucα3 antibodies were performed according to Léonard *et al.* (2002). Anti-Fucα3 was prepared from a mixture of a polyclonal anti-horseradish peroxidase antibodies according to Wilson *et al.* (1998).

**Protein extraction for *N*-glycans purification:** Harvested Wt and Tt tobacco leaves were lyophilized and disrupted with liquid nitrogen. The powder obtained was suspended in 50 mM Hepes-NaOH buffer, pH 7.5, containing, 2 mM $Na_2S_2O_4$, 0.1 % sodium dodecyl-sulphate (m/v) and 0.1 % polyvinylpyrrolidone (m/v). The suspension was centrifuged twice (4 600 *g*, 4 °C, 15 min), and supernatants filtered (0.48 µm). Soluble polymers were then precipitated in 12.5 % trichloracetic acid (m/v) for 18 h at 4 °C. The pellet was washed twice in 90 % acetone before being resuspended in 0.01 M HCl. Protein content was determined by the Lowry method using bovine serum albumin as a standard (Lowry *et al.* 1951).

Before the *N*-glycans were harvested, proteins were partially digested by 2.35 U $g^{-1}$(d.m.) pepsin (*Sigma*) in 0.01 M HCl at 37 °C. Proteolysis was performed for 24 h and repeated once. The reaction was neutralized by 1 M NaOH and stopped by heating at 100 °C for 5 min. Digestion mixtures were then centrifuged (600 *g*, 15 min, 4 °C) and the supernatants lyophilized. These digested extracts were also used for dot-blot experiments and activity assays.

The samples were resuspended in 0.1 M $CH_3COOH$ before being loaded on a *Biogel P4* (*BioRad*, Hercules, USA) column (45 × 3 cm) equilibrated in 0.1 M $CH_3COOH$ at a flow rate of 10 $cm^3$ $h^{-1}$. The presence of oligosaccharides in the recovered fractions was revealed by using thin layer chromatography (TLC). The TLC plates were dried and oligosaccharides were revealed with orcinol-$H_2SO_4$ reagent (0.1 % orcinol in 20 % $H_2SO_4$) at 110 °C. Recovered fractions were lyophilized before being resuspended in distilled water. Fractions containing mannose and GlcNAc were pooled and loaded onto C-18 cartridges (*Supelco,* Bellefonte, USA; *LC-18* packing).

The polysaccharidic fractions were eluted with distilled water whereas hydrophobic fractions were eluted with acetonitrile:$H_2O$ (80:20 v/v) and methanol. Methanol and acetonitrile were eliminated from the extract by rotary evaporation. F1 and F2 fractions were lyophilized and resuspended in 0.1 M sodium acetate buffer, pH 5. *N*-glycans from glycopeptides were harvested with 50 U $cm^{-3}$ almond peptide *N*-glycosidase or PNGase A (*Roche*, Basel, Switzerland) for 18 h at 37 °C. *N*-glycans were removed from hydrophilic peptides on a *Dowex AG50W-X 2* (*BioRad*) column (8 × 1.5 cm), and equilibrated in 2 % acetic acid (v/v), whereas those from hydrophobic peptides were purified on a C-18 cartridge (*Supelco LC-18* packing) and equilibrated in 50 mM $CH_3COONa$ buffer, pH 5. *N*-glycans were then desalted on envicarb cartridges (*Supelclean ENVI-Carb*, *Supelco*) equilibrated in $H_2O$. Glycan elution was performed with 25 % acetonitrile (v/v) and 0.05% TFA (v/v) and lyophilized.

**Saccharide composition:** Monosaccharides were determined by using the Kamerling *et al.* procedure (1975), as modified by Montreuil *et al.* (1986). TMS derivatives of *O*-methyl-glycosides from glycopeptides were separated on a 50-m capillary column (*CP-SIL-5CB Chrompack*, *ChromTech*, Apple valley, USA) and detected by flame ionization. Matrix-assisted laser desorption ionisation time-of-flight spectrometry (*MALDI-TOF MS*) and HPLC analysis were performed as described by Wilson *et al.* (2001).

## Results and discussion

**Endogenous and tobacco recombinant hFucT III α4-fucosyltransferase activity:** L-Fuc transfer to Galβ1,3GlcNAcβ-O-$(CH_2)_7CH_3$ acceptor (type I) was monitored by the quantification of radio-labeled products of the enzyme. The *in vitro* transfer of Fuc was found to be strongly increased in Tt [1460 ± 12 pmol $mg^{-1}$(protein) $min^{-1}$] in comparison to Wt plants [22 ± 3 pmol $mg^{-1}$ (protein) $min^{-1}$], which is in agreement with the findings of Joly *et al.* (2002b). The α(1,4)-FucT activity in 3-month-old leaves was 70 times higher in Tt than Wt plants. At the same developmental stage, the recombinant hFucT III could be easily detected by SDS-PAGE and revealed two bands in Tt plants (Fig. 2) corresponding to two states of glycosylation. The specificity of the polyclonal anti-hFucT III antibody was sufficient to reveal the presence of hFucT III (Fig. 2) but not the presence of endogenous tobacco enzymes (Fig. 2).

***In situ* detection of *N*-glycans from Wt and Tt tobacco over-expressing hFucT III:** Two approaches were used to identify $Le^a$ *N*-glycans in tobacco leaves: *1*) The immuno-detection of $Le^a$ epitopes in a complex mixture

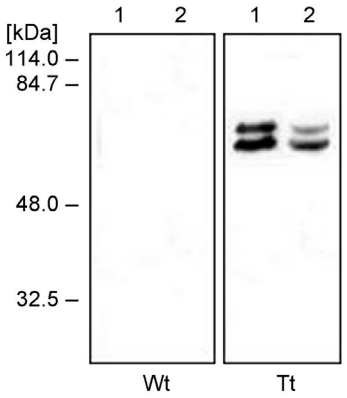


Fig. 2. Western blot analysis of tobacco proteins. Wild-type (Wt) and transgenic (Tt) tobacco leaf extract. *Lanes* 1 and 2 immuno-detection with the polyclonal anti-hFUT3 antibody.

of glycopeptides and polysaccharides, and *2*) a quantitative HPLC of pyridylaminated *N*-glycans. $Le^a$-epitopes were found from a plant extracts digested by pepsin in Wt as well as in Tt tobacco leaves (Fig. 3*B*). Although *N*-glycans could not be quantified in non-diluted extracts, we demonstrated that 3-fold dilution reduced (Fig. 3*B*) and 6-fold dilution washed out (Fig. 3*B*) the signal in Tt but not in Wt samples. Immuno-detection data seemed to indicate that $Le^a$ glyco-epitopes accumulated less in recombinant hFucT III than in Wt plants.

The purified and pyridylaminated *N*-glycans obtained from the complex mixture of glycopeptides of Wt and Tt tobacco leaves were then submitted to HPLC (Table 1) and mass spectrometry analyses (Fig. 4). All *N*-glycans with high mannose types, from $Man_5GlcNAc_2$ to $Man_9GlcNAc_2$, were found in Wt as well as in Tt tobacco leaves (Fig. 4, Table 1) but represented less than 15 % of the total *N*-glycans. Similar results have been published for suspension culture of tobacco BY2 cells (Fitchette *et al.* 1999) and tobacco plants (Elbers *et al.* 2001). Even though the proportion of complex type *N*-glycans compared with oligomannosidic types was slightly higher (92 %) in transgenic plants, they represented only a little more than 55 % in Wt. Transgenic plants that over-expressed hFucT III showed a qualitative and quantitative reduction in *N*-glycans, suggesting that hFucT III affects *N*-glycan metabolism. All these data indicate that if the recombinant hFucT III is well expressed *in planta*, its $Le^a$ activity on *N*-glycans cannot be demonstrated. hFucT III has been described as having an α4 as well as equally an α3 fucosyltransferase activity (De Vries *et al.* 1995, Costache *et al.* 1997). The question is to determine whether the tobacco recombinant hFucT III expresses more α3 than α4 activity *in planta*. Plant α3 as well as α4 fucosylation occurs in *N*-glycans (Fig. 3*A*), but α3 is restricted to the chitobiose core *N*-glycans and not to the lacto-*N*-biose as in mammalian cells (Costa *et al.* 2002). As demonstrated for the $Le^a$ epitope, core α3 *N*-glycan levels are lower in Tt than in Wt plants (Fig. 3*C*). HPLC of pyridylaminated *N*-glycans (Table 1) as well as mass spectrometry analyzes, confirmed the immuno-detection of core α3 *N*-glycans. The α3-Fuc linkage is approximately 55 % less in Tt than in Wt plants. This suggests that recombinant hFucT III possesses neither α4 nor α3 fuco-syltransferase activities on *N*-glycans.

To investigate the potential function of recombinant hFucT III on 3-month-old tobacco leaves, we analyzed its ability to fucosylate *in vitro* plant compounds [RG, XG, arabinogalactan (AG)] other than *N*-glycans. To decrease artifacts induced by endogenous tobacco substrates, the recombinant hFucT III was expressed in COS7 cells as described by Dupuy *et al.* (1999). The COS7 recombinant hFucT III was assayed for fucosyltransferase activity on type I as a positive control and on commercial (XG from

Table 1. Pyridylaminated 3-month-old tobacco *N*-glycans separated by HPLC on C18 column. The Wt and Tt samples were prepared according to their dried mass, and values collected here correspond to the mean surface area of each peak [arbitrary units]. Nomenclature is as in Strasser *et al.* (2007).

| | Wt | Tt |
|---|---|---|
| Oligomannosidic type *N*-glycans | | |
| $M_9Gn_2$ | 8.6 | 2.1 |
| $M_8Gn_2$ | 5.0 | 0.8 |
| $M_7Gn_2$ | 2.6 | 0.8 |
| $M_6Gn_2$ | 5.5 | 1.7 |
| $M_5Gn_2$ | 4.3 | 3.1 |
| Total | 26.0 | 8.5 |
| Complex type *N*-glycans | | |
| $M_3XGn_2$ | 8.8 | 5.6 |
| $M_3XFGn_2$ | 67.4 | 36.7 |
| $GnM_3XFGn_2$ | 19.2 | 12.4 |
| $Gn_2M_3XFGn_2$ | 63.8 | 35.2 |
| $(G/F)Gn_2M_3XFGn_2$ | 0.4 | 0.6 |
| $GFGn_2M_3XFGn_2$ | trace | trace |
| $(GF_2/G_2F)Gn_2M_3XFGn_2$ | trace | trace |
| $G_2F_2Gn_2M_3XFGn_2$ | 1.4 | trace |
| Total | 161.0 | 90.5 |

Table 2. Saccharide content [μg] of tobacco leaves. Before GC separation, a trimethylsilylation of sugars was performed as described in the Materials and methods. Sugar are extracted starting from 30 g of fresh leaves.

| Sugar composition | Wt | Tt |
|---|---|---|
| Rha | 172.5 | 84.6 |
| Fuc | 61.7 | 45.5 |
| Ara | 216.3 | 126.8 |
| Xyl | 55.6 | 68.4 |
| Man | 104.0 | 59.8 |
| Glc | 216.1 | 54.0 |
| Gal | 327.6 | 198.2 |
| GalA | 39.7 | 44.2 |
| GlcA | 145.3 | 69.9 |
| Total | 1338.8 | 751.4 |

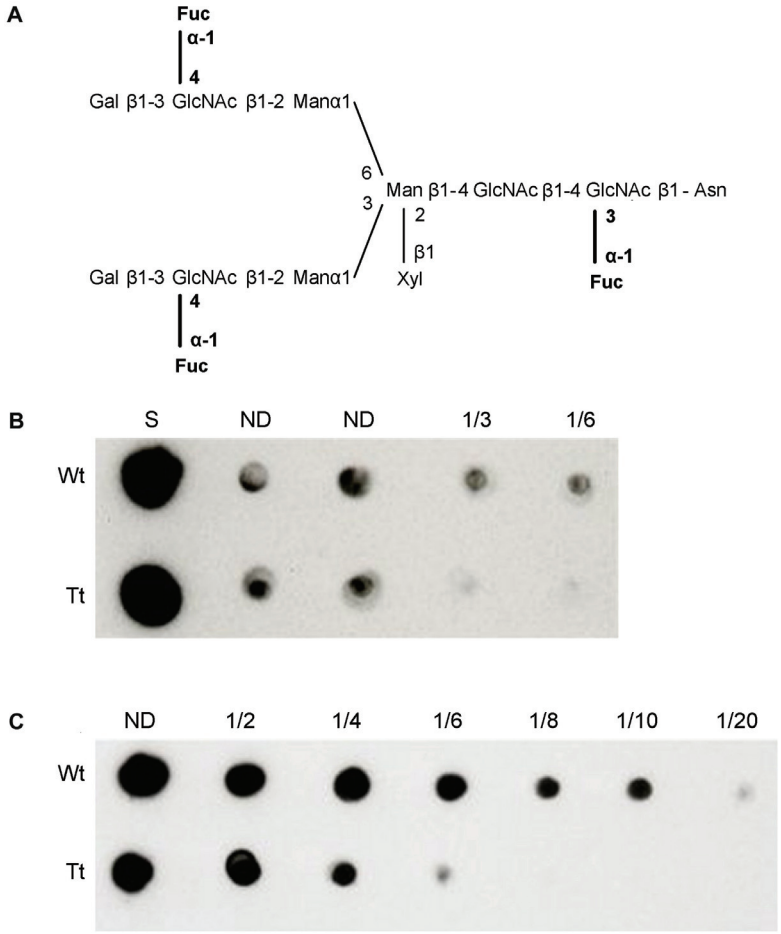


Fig. 3. *A* - Plant *N*-glycan structure. *B*, *C* - Dot-blot analysis of pepsin-digested plant extracts. Immuno-detection with antibody raised against Lewis a (*B*) or core-α(1,3)fucose (C). Tt - transgenic tobacco, Wt - wild-type tobacco, S - saliva (positive control), ND - non-diluted extract.

tamarind, AG from arabic gum) and plant-purified polysaccharidic substrates (arabinogalactan proteins from tobacco, RG from pine). The COS7 recombinant hFucT III showed an activity on type I (34 174 DPM corresponding to 100 % of activity) and no incorporation of radiolabeled L-Fuc onto any polysaccharide acceptors. Dot-blot analysis using antibody directed against arabinogalactan (JIM 13) was tested on this fraction (data not shown), but did not show any increase in the amount arabinogalactan of transgenic plants.

The amount of Fuc residue in polysaccharide-rich fractions was analyzed and Tt found to have approximately 40 % less polysaccharide content than Wt (Table 2). The relative Fuc content between Wt and Tt tobacco leaves was approximately similar (~5 % of the sugar content of the corresponding fraction). The lack of activity measured in COS7 recombinant hFucT III was not associated to any relevant differences in the relative amount of Fuc in the polysaccharide fraction, which confirms that hFucT III is not able to fucosylate such acceptors.

**GDP-Fuc hydrolysis by the recombinant hFUCT III:** Until now we have demonstrated that tobacco recombinant hFucT III, which is well expressed *in planta,* is not suitable to fucosylate any plant endogenous acceptors. We hypothesize that plant substrates are poor acceptors for hFucT III. So the enzyme binds GDP-Fuc to its catalytic domain, hydrolyses Fuc and fails to effectively transfer Fuc from GDP to the final acceptor. Firstly, a basic auto-hydrolysis of GDP-Fuc was demonstrated when activity was monitored in a free enzyme reaction medium (Fig. 5). Secondly, kinetic analysis of the recombinant hFucT III activity in a mix lacking the final acceptor (type I or plant polysaccharide) showed strong GDP-Fuc hydrolysis (Fig. 5). Further, the addition of type I acceptor to the reactional mix was positively correlated with a strong increase in Fuc transfer from GDP-Fuc to the type I acceptor. In addition, the replacement of type I by plant polysaccharides as the final acceptor was not associated with fucosyltransferase activity but simply with a GDP-Fuc hydrolysis (Fig. 5). Finally, similar results were obtained in a new set of

experiments where only the amount of recombinant hFucT III was changed (from 25 to 75 μg). All these data suggest that hFucT III acts more as a hydrolase in the absence or in the presence of a poor final acceptor. We suggest that this state can reduce the amount of GDP-Fuc available for the activity of the other plant fucosyl-transferases. This in turn reduces the possibility of all potentially fucosylated molecules reaching maturation.

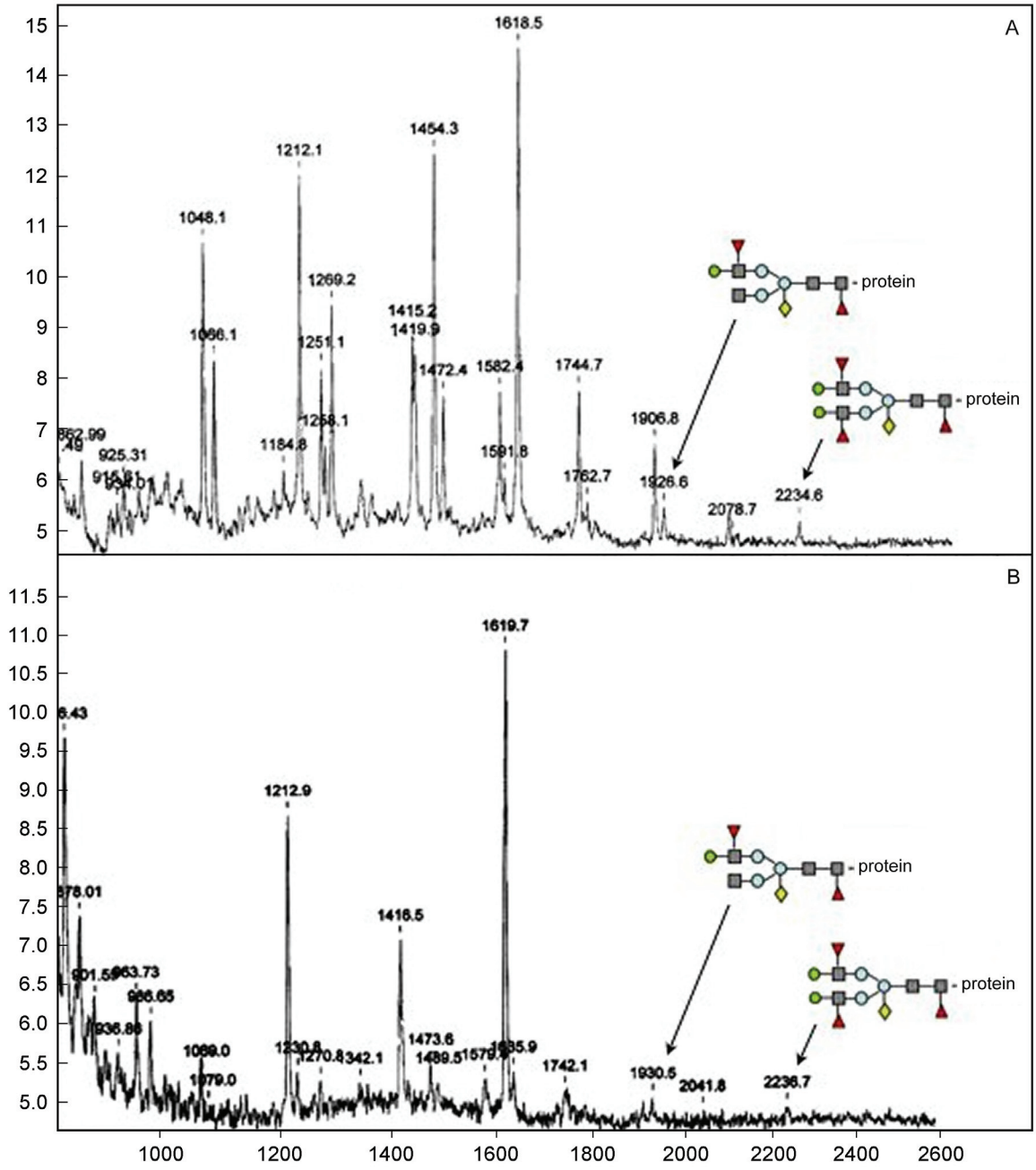


Fig. 4. MALDI-TOF MS analysis of N-linked glycans. *A* - wild-type tobacco, *B* - 3-month-old transgenic tobacco leaves.

**Conclusion:** Although over-expressed hFucT III was clearly demonstrated *in vitro*, no relevant difference could be observed in the $Le^a$ amount between leaves of Wt and Tt 3-month-old tobacco plants. However, even though recombinant tobacco hFucT III does not affect plant $Le^a$ production, it does quantitatively and qualitatively reduce *N*-glycans. Moreover, the composition of the poly-saccharides reveals that the hFuct III expressed in tobacco strongly disturbs polysaccharide metabolism. In fact, the contents of individual saccharides were changed more in Tt than the total amount of the polysaccharides. The contents of xylose, arabinogalactan and mannose

increased Tt. In addition, arabinogalactan and xylan were more accumulated in Tt than in Wt, which probably partially explains the observed phenotype (Lejoly *et al*. 2002b). More than this indirect effect of tobacco hFucT III, we suggest that the enzyme maintains its hydrolytic activity without the possibility to transfer released Fuc from GDP to the final acceptor. *In planta*, tobacco recombinant hFucT III probably acts more as a hydrolase than as a transferase.

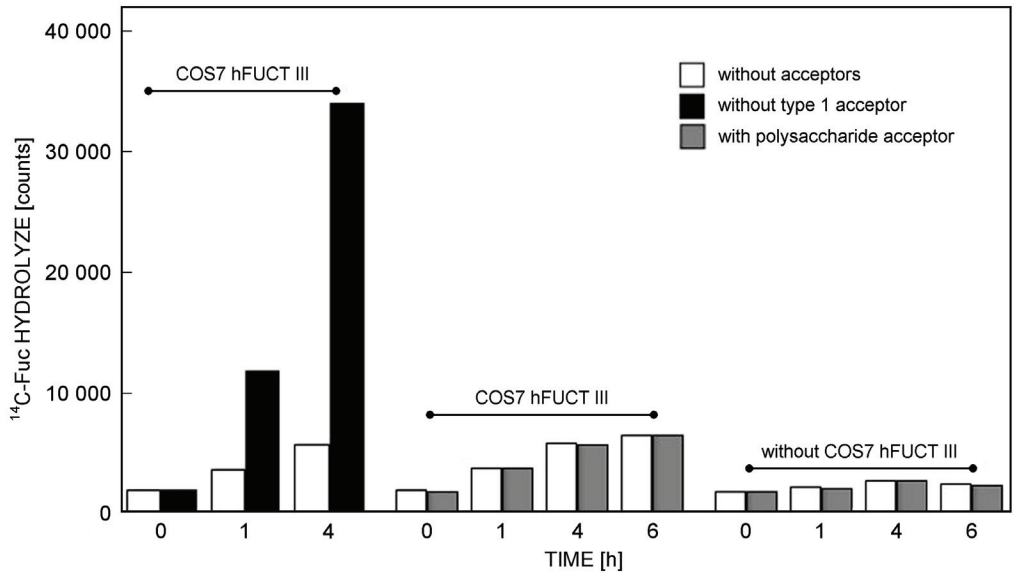


Fig. 5. Fucosyltransferase activity of hFucT III transfected in COS7 cells on type 1 and polysaccharides acceptors (xyloglucan from tamarind, arabinogalactan from arabic gum and rhamnogalacturonan from pine). Incubation was done at 25 °C as described in the Materials and methods. Results presented here are made with 25 μg proteins. GDP-Fuc was separated from acceptor on C18 cartridge for type 1 and on *Dowex* 1*8 (0.5 g cm$^{-3}$). Elution was performed with $H_2O$ and 200 mM $CH_3COONa$, pH 5.5.

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