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COMMUNICATION

Generation of glyco-engineered BY2 cell lines with decreased expression of plant-specific glycoepitopes

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ABSTRACT

Plants are known to be efficient hosts for the production of mammalian therapeutic proteins. However, plants produce complex N-glycans bearing β1,2-xylose and core α1,3-fucose residues, which are absent in mammals. The immunogenicity and allergenicity of plant-specific N-glycans is a key concern in mammalian therapy. In this study, we amplified the sequences of 2 plant-specific glycosyltransferases from *Nicotiana tabacum* L. cv Bright Yellow 2 (BY2), which is a well-established cell line widely used for the expression of therapeutic proteins. The expression of the endogenous xylosyltranferase (XylT) and fucosyltransferase (FucT) was down-regulated by using RNA interference (RNAi) strategy. The xylosylated and core fucosylated N-glycans were significantly, but not completely, reduced in the glyco-engineered lines. However, these RNAi-treated cell lines were stable and viable and did not exhibit any obvious phenotype. Therefore, this study may provide an effective and promising strategy to produce recombinant glycoproteins in BY2 cells with humanized N-glycoforms to avoid potential immunogenicity.

KEYWORDS BY2 cells, N-glycosylation, glycosyltransferase, RNA interference

INTRODUCTION

Plants have emerged as economical and convenient factories for large-scale production of recombinant therapeutic proteins (Twyman et al., 2003). Most pharmaceutical proteins are glycoproteins; N-glycosylation of these proteins is essential for their solubility, stability, bioactivity, proper folding, and pharmacokinetics (Matsumoto et al., 1995; Dirnberger et al., 2001). Plants can perform N-glycosylation similar to mammals, which gives these plants a key advantage over other heterologous expression systems (Catherine Rayon, 1998; Lerouge et al., 1998). However, the process of glycosylation in plants and mammals varied greatly in the late Golgi apparatus (Gomord and Faye, 2004). This difference is mainly because of the residues in the core structure of plant complex N-glycans substituted by β1,2-xylose (Xyl) and α1,3-fucose (Fuc), unlike α1,6-Fuc in mammalian N-glycans. Moreover, plant N-glycans are generally much smaller and lack the characteristic β1,4-galactose and sialic acid residues which were found in their mammalian counterparts. Consequently, the expression of mammalian glycoproteins in plants results in chimeric glycoprotein that have an N-glycan profile mainly of complex N-glycans containing β1,2-Xyl and core α1,3-Fuc residues (Cabanes-Macheteau et al., 1999).

Although it has been previously reported that the absence of core α 1,6-Fuc may serve as an advantage for increasing Fc receptor (FcR) binding and antibody-dependent cellular cytotoxic (ADCC) function of antibodies, the unwanted immunogenicity and allergenicity of the non-mammalian sugars β1,2-Xyl and core α1,3-Fuc cannot be excluded (van

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Ree et al., 2000; Niwa et al., 2004). These plant-specific N-glycans contain IgE binding carbohydrate determinants (Gomord et al., 2005). Furthermore, the production of β1,2-Xyl- and/or core-α1,3-Fuc-specific antibodies in goats, rabbits, and rats can be elicited by immunization with plant glycoproteins (Bardor et al., 2003). Hence, the immunogenicity of plant glycoepitopes remains a major concern in human therapy.

It has been documented that RNA interference (RNAi) is a valuable tool to eliminate the expression of core α1,3-fucosyltransferase (FucT) and β1,2-xylosyltransferase (XylT), which are enzymes responsible for the transfer of β1,2-Xyl and core α1,3-Fuc. This promising strategy effectively reduced the core Xyl and Fuc levels in *Lemna minor* (Cox et al., 2006). The glycoproteins synthesized in these RNAi-targeted lines carried predominantly humanized structures (GnGn) devoid of plant-specific glycoepitopes.

The tobacco BY2 cell line has often been chosen as a well-characterized host for the production of exogenous proteins because of rapid growth rate, product purification and no contamination to environments (Fischer et al., 1999; Doran, 2000). In this study, tobacco BY2 cells (N. tabacum L. cv Bright Yellow 2) were engineered to achieve targeted down-regulation of the expression of endogenous FucT and XylT genes by using RNAi strategies. These glyco-optimized cell lines contained complex N-glycan structures, but they strongly inhibited the incorporation of β1,2-Xyl and core α1,3-Fuc, which are responsible for the immunogenicity of plant N-glycans, as determined by immunoblotting and matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS).

RESULTS

Cloning of β1,2-XylT and α1,3-FucT cDNA fragments from tobacco BY2 cells

To generate the glyco-engineered BY2 cell lines, we planned to use the gene knockout strategy for preventing the transference of plant-specific glycoepitopes. To achieve this, cDNAs encoding β1,2-XylT and α1,3-FucT were isolated from tobacco BY2 cells and used as probes. First, cDNA sequences of XylT (EC 2.4.2.38) and FucT (EC 2.4.1.214) of several plant species were collected (http://www.cazy.org/), including *Arabidopsis thaliana*, *N. tabacum*, *Oryza sativa*, and *Medicago truncatula*; the conserved region was identified by aligning the sequences and degenerate primers were generated. Two DNA fragments, namely, NtXT (233 bp) and NtFT (933 bp) encoding β1,2-XylT and core α1,3-FucT, respectively, were amplified from tobacco BY2 cells using degenerate primers designed according to the homologous region sequences of these species. As predicted by NetPlantGene analysis, NtXT was a partial coding domain of β1,2-XylT gene without an intron. Sequence similarity search with XylT cDNA of other species was performed (http://bioinfo.genopole-toulouse.prd.fr/multalin/). The NtXT amino acid sequence exhibited 98.3% and 67.0% similarity to α1,2-XylT from *A. thaliana* (At5g55500) and *O. sativa* (Os08 g0503800), respectively.

NetPlantGene analysis and sequence alignment with other plant FucTs revealed that NtFT contains an intron sequence from 194 to 854 bp. After splicing the intron domain, we performed sequence similarity search with FucT cDNA of other species (http://bioinfo.genopole-toulouse.prd.fr/multalin/). The NtFT amino acid sequence showed 75.3% and 77.1% identity with α1,3-FucT from *A. thaliana* (At3g19280, AtFucTA) and *O. sativa* (AP004457), respectively.

Generation of glyco-engineered lines of tobacco BY2 cells

To use the potential of tobacco BY2 cells for the production of recombinant pharmaceutical glycoproteins, it is highly desirable to prevent the incorporation of α1,3-Fuc- and β1,2-Xyl-containing glycoepitopes to the proteins. This step is essential to prevent the immunogenicity of plant-specific N-glycans in human therapy. To obtain a targeted down-regulation of endogenous XylT and FucT gene expression, the amplified cDNA sequences of NtXT and NtFT were used to generate a binary RNAi construct. This construct, which was named FXR, contained the amplified 233 bp fragment of XT and 192 bp fragment of FT cDNAs in the sense and antisense orientations, separated by an intron. The sense–intron–antisense hairpin RNA (ihpRNA) was supposed to target both the endogenous core α1,3-FucT and β1,2-XylT of BY2 cells (Fig. 1C). BY2 transgenic lines were generated by *Agrobacterium*-mediated transformation, and the first screening based on kanamycin resistance was performed on a selection plate. Interestingly, all FXR lines were viable and exhibited no obvious morphological phenotype under standard growth conditions.

Western blot analysis of glycoprotein xylosylation and fucosylation in FXR lines

Independent transgenic XylT/FucT-RNAi lines (named as FXR1–12) were further screened by Western blot analysis to detect the presence or absence of the respective products: N-glycans with Xyl and core α1,3-Fuc residues. Protein extracts from FXR lines were subjected to Western blot analyses using anti-horseradish peroxidase (anti-HRP) antibodies that recognize β1,2-Xyl- and core α1,3-Fuc-containing structures. As shown in Fig. 2A, varying staining intensities were observed, indicating the presence of different amounts of Xyl and Fuc residues. Most of these knock-down transformants exhibited a large decrease in β1,2-xylosylation and α1,3-fucosylation. One XylT/FucT-RNAi line (line 7; which was named FXR7), which exhibited very weak intensity after

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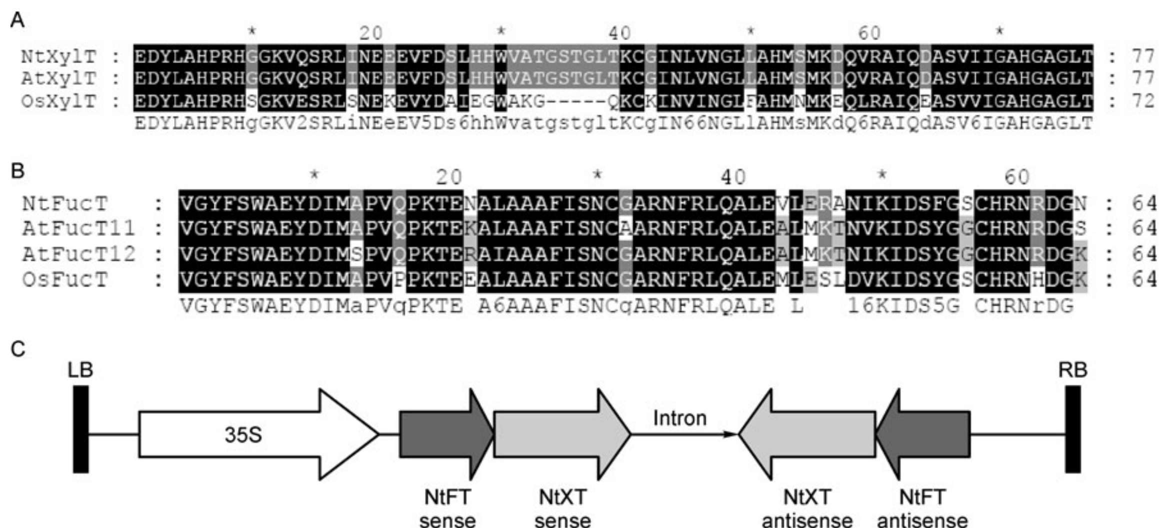

Figure 1. Mutiple sequence alignment and diagram of RNAi constructs. (A) Alignment of conserved motifs from β1,2-XylT protein sequences. (B) Alignment of conserved motifs from α1,3-FucT protein sequences. The aligned amino acid sequences are from the plants *Nicotiana tabacum* L. cv Bright Yellow 2 (Nt), *Arabidopsis thaliana* (At) and *Oryza sativa* (Os). Black blocks represent amino acid identity between all sequences and gray blocks between two or three sequences. Broken lines correspond to the gaps required for the alignment of these sequences. (C) The construction diagram of FXR. The amplified DNAs encoding hairpin RNA (ihpRNA) designed to target silencing of endogenous tobacco BY2 genes encoding α1,3-fucosyltransferase and β1,2-xylosyltransferase. RB and LB, right and left borders; 35S, cauliflower mosaic virus 35S promoters.

staining with anti-HRP antibody, was identified and used for further analysis. It has been reported that cell growth influences the N-glycosylation profile of endogenous proteins in tobacco BY2 cells (Elbers et al., 2001). Moreover, the levels of β1,2-Xyl and α1,3-Fuc residues were elevated markedly in the late growth phase (from 5th to 7th day after subculture) (Yin et al., 2009). Therefore, we determined the growth pattern of wild-type and FXR7 lines, and no obvious differences were observed between these 2 lines. Subsequently, the proteins from wild-type and FXR7 lines from the late growth phase were extracted and subjected to Western blot analyses using Xyl- and Fuc-specific antibodies, respectively. As compared to the wild-type cell line, the FXR7 line exhibited a marked decrease in β1,2-Xyl- and core α1,3-Fuc-containing structures in the entire late growth phase (Fig. 2B). This observation indicated that rather than cell growth, ihpRNA effectively eliminated the mRNA and significantly reduced the levels of β1,2-Xyl and core α1,3-Fuc residues, thereby reducing the amount of endogenous glycosyltransferases.

Structural analysis of N-glycans on total endogenous proteins from tobacco BY2 cell wild-type and RNAi lines

To investigate the precise N-glycoform profile of endogenous proteins, we subjected the wild-type and FXR7 lines of tobacco BY2 cells to MALDI-TOF MS. The total N-glycans were isolated from tobacco BY2 cells, as reported by Bakker et al. (2006). Briefly, the resulting N-glycan mixtures were analyzed by MALDI-TOF MS after digestion of total proteins by pepsin and peptide-N-glycosidase A. The mass spectrum showed a series of high-mannose-type N-glycan ions ranging from Man5 to Man9 and complex-type N-glycans from a (MMXF) to e (GnGnXF) (Fig. 3). As expected, the mass spectrum revealed that the N-glycan profile of wild-type tobacco cells had mainly complex-type N-glycans bearing Xyl and α1,3-Fuc residues, including MMXF (a), GnMXF (c), Gn2MX (d), and GnGnXF (e). In contrast, the composition of N-glycans in FXR7 lines revealed a remarkable decrease in plant-specific N-glycans. Although a small but significant amount of GnGnXF was still detected in FXR7, a major peak was identified as GnGn, and the complex-type N-glycan without Xyl and Fuc residues was considerably increased and accounted for over 10% of the total oligosaccharides. It was worth noting that no N-glycan with a GnGn (b) structure was detected in wild-type cells. Further, in the XylT/FucT RNAi lines, some of complex-type N-glycans did not contain the plant-specific residues and exhibited a sharp reduction in xylosylated and core fucosylated N-glycans (GnGnXF) because of incomplete downregulation of XylT and FucT activities. This result showed that the expression of an RNAi construct targeting the XylT and FucT in tobacco BY2 cells effectively prevented the accumulation of the plant-specific glycoepitope on complex N-glycan structure.

DISCUSSION

In this study, we investigated the feasibility of glyco-engineering in tobacco BY2 cells. Partial cDNA sequences

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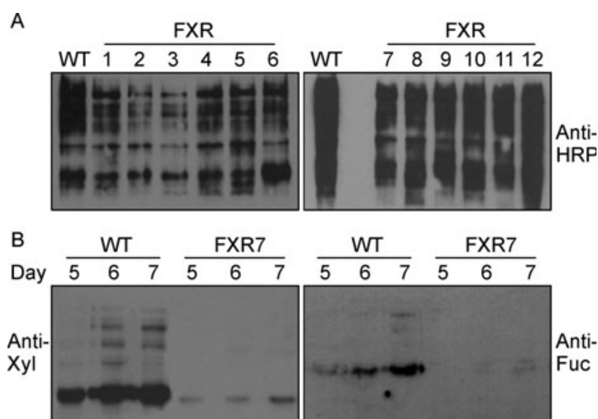

Figure 2. Analysis of the protein xylosylation and fucosylation in FXR lines. (A) Western blot analysis of total protein extracts from transgenic RNAi lines (FXR1–12). The presence/absence of fucose and xylose residues was determined with anti-HRP antiserum. Numbers indicated the different transformed lines. (B) Western blot analysis of total protein extracts from WT and FXR7 lines from late growth phase using anti-xylose (left panel) and anti-fucose (right panel).

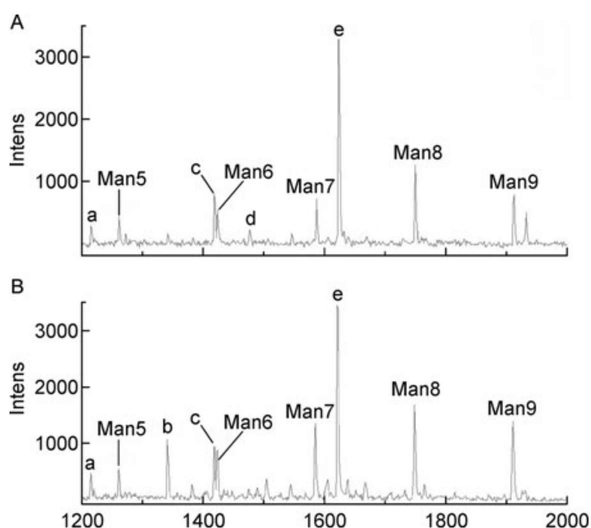

Figure 3. MALDI-TOF mass spectrum of N-glycans isolated from endogenous proteins of WT (A) and FXR7 (B) cells.

of β1,2-XylT and α1,3-FucT were cloned from tobacco BY2 cells. The sequences of these 2 proteins were used for generating RNAi construct for targeted down-regulation of endogenous XylT and FucT genes. The generation of glyco-engineered transformants with a low level of β1,2-Xyl and α1,3-Fuc residues was confirmed by Western blotting and MALDI-TOF MS.

Interestingly, XylT/FucT k.o. plants of *A. thaliana* were viable and exhibited no obvious phenotype under standard growth conditions (Strasser et al., 2004). In addition, the transgenic plants (*L. minor*, *N. benthamiana*, and *M. sativa)*, in which Xyl and Fuc residues were eliminated by RNAi, did not exhibit any obvious morphological phenotype (Cox et al., 2006; Sourrouille et al., 2008; Strasser et al., 2008). In our study, we also found that the deficiency of XylT and FucT did not affect the morphology, growth rate, or growth status of tobacco BY2 cells.

In previous studies, several strategies have been adopted to minimize the structural differences observed in complex N-glycans of plants and mammals (Palacpac et al., 1999; Triguero et al., 2005). Cox et al. reported that RNAi was a valuable tool to eliminate the expression of core α1,3-FucT and β1,2-XylT in the aquatic plant species *L. minor* (Cox et al., 2006). Surprisingly, this strategy was effectively applied to the production of a chimeric monoclonal antibody (mAb) that predominantly contained a single N-glycan species (GnGn) without any detectable plant-specific N-glycans. More importantly, the glycan-optimized mAb exhibited high effector activities as compared to its Chinese hamster ovary (CHO)-derived homologs. Recently, several studies also showed that RNAi effectively prevented the function of β1,2-XylT and core α1,3-FucT in the whole plant of *N. benthamiana* and alfalfa, respectively (Sourrouille et al., 2008; Strasser et al., 2008). As compared to the whole plant, tobacco BY2 cells possess the merits of precise control over growth conditions, easy downstream processing, and no pollen contamination (Hellwig et al., 2004). However, little information is available about the 2 plant-specific glycosyltransferases in BY2 cells. According to the sequences of the conserved domain in several plant species, we designed degenerate primers to amplify the partial cDNA of endogenous β1,2-XylT and core α1,3-FucT. The nucleotide sequences of these 2 amplified cDNAs exhibited high identity with XylT and FucT from *A. thaliana* and other plant species; furthermore, high sequence homologies were observed in the amino acid sequences of these glycosyltransferases. Previous studies have shown that β1,2-XylT and core α1,3-FucT are type II membrane glycoproteins located in the membrane of Golgi apparatus (Wilson et al., 2001; Pagny et al., 2003). Similar to other glycosyltransferases, β1,2-XylT and core α1,3-FucT contain a short N-terminal cytosolic tail, a transmembrane domain, and a large luminal catalytic domain. Sequence analysis of the new amplified cDNAs in BY2 cells revealed that these structures were located in the conserved catalytic domain at the C-terminal end.

Western blot analysis revealed that the transgenic FXR lines exhibited a significant decrease in β1,2-Xyl and core α1,3-Fuc, although the N-glycans of wild-type cells mainly contained these glycan residues. It has been reported that the developmental stage may influence the glycosylation profile of endogenous proteins in tobacco leaves and BY2 cells (Elbers et al., 2001; Yin et al., 2009). To exclude the effect of

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Peak	Abbreviation	Structure
	Man5-Man9	(Manα1-2)Manα1 6 Manα1 3 6 (Manα1-2)Manα1 Manβ1-4GlcNAcβ1-4GlcNAc 3 (Manα1-2Manα1-2)Manα1
a	MMXF	Manα1 6 Manβ1-4GlcNAcβ1-4GlcNAc 3 2 3 Manα1 Xylβ1 Fucα1
b	GnGn	GlcNAcβ1-2Manα1 6 Manβ1-4GlcNAcβ1-4GlcNAc 3 GlcNAcβ1-2Manα1
c	GnMXF	GlcNAcβ1-2Manα1 3/6 Manβ1-4GlcNAcβ1-4GlcNAc 3/6 2 3 Manα1 Xylβ1 Fucα1
d	GnGnX	GlcNAcβ1-2Manα1 6 Manβ1-4GlcNAcβ1-4GlcNAc 3 2 GlcNAcβ1-2Manα1 Xylβ1
e	GnGnXF	GlcNAcβ1-2Manα1 6 Manβ1-4GlcNAcβ1-4GlcNAc 3 2 3 GlcNAcβ1-2Manα1 Xylβ1 Fucα1

Figure 4. Structures of high-mannose-type (Man-5 to Man-9) and complex-type (a to e) N-linked oligosaccharides extracted from wild-type BY2 cells.

cell growth on protein N-glycosylation in this cell line, we determined its typical growth pattern. Interestingly, there was no influence of the status of cell growth on N-glycosylation. To further evaluate the RNAi efficiency, we analyzed protein extracts from wild-type and FXR cells in the late growth phase (5th to 7th day) by Western blotting. The 2 plant-specific N-glycans were hardly detected during the entire growth cycle. Complete elimination of plant-specific oligosaccharides could not be achieved in the FXR7 line; however, the main epitope structure (GnGnXF) was present in only 21.4% of the total N-glycans as compared to 45% of this structure in wild-type cells. Furthermore, an elevated level of GnGn structures devoid of plant-specific carbohydrate was detected by MALDI-TOF MS. The results of mass spectroscopy suggested that over 10% of total N-glycans detected in the line FXR7 had the GnGn structure that was not detected in the wild-type cells. This result suggests an obvious reduction of Xyl and Fuc residues in the RNAi lines despite incomplete downregulation of XylT and FucT activity. One explanation for this finding could be the presence of several copies of glycosyltransferases genes in tobacco BY2 cells. This observation has been described for other plant species. For example, 2 copies of α1,3-FucT (FucT A and FucT B) have been detected in *Arabidopsis*. Although there was no significant difference between FucT T-DNA insertion lines and wild-type line, the double-insertion line deficient in both FucT A and FucT B showed complete elimination of complex-type N-glycans with core α1,3-Fuc residues. It appears that both of FucT A and FucT B were active *in vivo*. In addition, there were no obvious changes in the other 2 minor peaks

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that were identified as MMXF and GnMXF, probably because of incomplete elimination of the 2 plant glycosyltransferases and the low level of MMXF and GnMXF.

Therefore, in this study, new partial cDNAs of XylT and FucT were amplified and the inhibition of the endogenous α1,3-FucT and β-1,2-XylT activities in glycol-optimized lines was accomplished by co-expression with a single RNAi transcript. The absence of plant-specific N-glycans on the FXR transgenic BY2 line was confirmed by affinoblotting and MALDI-TOF MS. This strategy will pave the way to produce recombinant proteins devoid of plant glycol-epitopes in plant cells.

MATERIALS AND METHODS

Isolation of cDNAs encoding glycosyltransferases from tobacco BY2 cells

The core α1,3-FucT fragment was isolated from the cDNA of tobacco BY2 cells using the forward primer Fuc-Fw (5′-GTTGGVTAYTTTTCRTGGG-3′) and reverse primer Fuc-Rev (5′-TCTGTDACATAATCYTCCTC-3′). Further, β1,2-XylT sequence was amplified by polymerase chain reaction (PCR) using the primers Xyl-Fw (5′-GARGAYTACTTRGCCCATCC-3′) and Xyl-Rev (5′-TGAG-TYAGWCCWGCYCCATG-3′). These 2 pairs of degenerate primers were designed based on the results of sequence homology analysis with annotated glycosyltransferases sequences and with known proteins from the database. The partial cDNA products were subjected to gel electrophoresis, purified, and then ligated into the pMD18-T vector (Takara) for sequencing.

Construction of RNAi vectors for glycosyltransferases

The PCR product of FucT (191 bp) was amplified using primers FucTS-Fw (CTCGAGACTAGTgttgggtattttcgtggg) and FucTS-Res (AAGCTTgggcatttccatcccggtt). Then, FucT was ligated into the *Xho*I/*Hin*d III sites of the cloning vector psk-int to create psk-int-FI. The antisense fragment obtained by PCR using the primers FucTA-Fw (5′-TCTAGAACTAGTgttgggtattttcgtggg-3′) and FucTA-Res (5′-CCCGGGcatttccatcccggttac-3′) was cloned into the *Xba*I/*Sma*I site of the vector psk-int-FI to create psk-int-FR. The XylT fragment (233 bp) was then ligated into the *Hin*d III sites of psk-int-FR, while the antisense XylT was cloned into the *Sma*I site. Consequently, the “sense–intron–antisense” cassette was digested at the *Spe*I site, and the product was cloned into the binary expression vector pCanG to create pCanG-FXR. The plasmid pCanG-FXR was transferred into the *Agrobacterium tumefaciens* strain LBA4404 by electroporation.

Cultivation of tobacco BY2 cells and transformation

The tobacco BY2 cells (*N. tabacum* L. cv Bright Yellow 2) were subcultured on every 7th day in Murashige and Skoog medium (Swiatek et al., 2002) (Sigma) of pH 5.8, which contained 3% (*w/v*) sucrose, 0.2 g/L KH_2PO_4, 10 mg/L myo-inositol, 1 mg/L thiamin hydrochloride, and 0.2 mg/L 2,4-D. The cell line was regularly subcultured by transferring 1 mL of culture into 20 mL of fresh medium in a 100-mL flask. The cultures were incubated in the dark at 26°C on a gyrating shaker at 120 rotation/min.

The tobacco BY2 cells were transformed by the *Agrobacterium*-mediated method (Rempel and Nelson, 1995). Transgenic lines were selected and maintained in the abovementioned antibiotics-containing medium (250 mg/L carbenicillin sodium salt and 100 mg/L kanamycin).

Glycoprotein analysis

For N-glycans analysis, the total protein extracts from BY2 cells were prepared as described in the literature (Bakker et al., 2006). Tobacco cells were collected by centrifuge and then grinded in liquid nitrogen. The ground material was extracted in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer containing 20 mM Tris-HCl (pH 6.8), 6% (*v/v*) glycerol, 0.4% (*v/v*) SDS, 20 mM DTT, and 2.5 µg/mL bromophenol blue. The insoluble material was pelleted after incubation at 100°C for 5 min. The supernatants (12.5 µL of each sample) were collected, subjected to 12% SDS-PAGE, and blotted to nitrocellulose membranes. Affinoblotting of β1,2-xylosylated and/or α1,3-fucosylated proteins was performed with rabbit anti-HRP (Dingguo, Beijing), anti-β1,2-Xyl, and anti-α1,3-Fuc antibodies.

N-glycan purification

The cultured BY2 cells were centrifuged, and total proteins were isolated from BY2 cells and N-glycans were prepared as described (Yin et al., 2009).

N-glycan analysis

The lyophilized N-glycans were dissolved in 5 µL of water per sample; 1 µL of this solution was spotted on a stainless steel sample plate to which 1 µL of S-DHB matrix (Sigma) was added, and this mixture was allowed to dry at room temperature. Positive-ion MALDI-TOF spectra were recorded on an Autoflex mass spectrometer (Bruker) fitted with delayed extraction and a nitrogen laser (337 nm). The spectra were generated from the sum of 200–300 laser pulses.

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ABBREVIATIONS

Fuc, fucose; FucT, fucosyltransferase; GlcNAc, N-acetyl-glucosamine; HRP, horseradish peroxidase; ihpRNA, sense–intron–antisense hairpin RNA; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight mass spectrometry; Man, mannose; RNAi, RNA interference; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Xyl, xylose; XylT, xylosyltransferase

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