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Generation of *Arabidopsis thaliana* plants with complex *N*-glycans lacking β1,2-linked xylose and core α1,3-linked fucose

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Abstract The plant glycosyltransferases, β1,2-xylosyltransferase (XylT) and core α1,3-fucosyltransferase (FucT), are responsible for the transfer of β1,2-linked xylose and core α1,3-linked fucose residues to glycoprotein *N*-glycans. These glycan epitopes are not present in humans and thus may cause immunological responses, which represent a limitation for the therapeutic use of recombinant mammalian glycoproteins produced in transgenic plants. Here we report the genetic modification of the *N*-glycosylation pathway in *Arabidopsis thaliana* plants. Knockout plants were generated with complete deficiency of XylT and FucT. These plants lack antigenic protein-bound *N*-glycans and instead synthesise predominantly structures with two terminal β*N*-acetylglucosamine residues ($GlcNAc_2Man_3GlcNAc_2$).
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1. Introduction

N-Glycosylation is one of the major posttranslational modifications of proteins. Although the first steps in the *N*-glycosylation pathway leading to the formation of oligomannosidic structures are conserved in plants and animals, the final steps in the formation of complex *N*-glycans differ [1,2]. In particular, plant *N*-linked glycans contain β1,2-xylose and core α1,3-fucose, which are not present in mammals and therefore constitute epitopes for carbohydrate reactive antibodies. Moreover, the antigenicity of complex plant *N*-glycans is well documented [3–7]. Complex plant *N*-glycans containing β1,2-xylose and core α1,3-fucose are regarded as the major class of the so-called 'carbohydrate cross-reactive determinants' reactive with IgE antibodies in the sera of many allergic patients [7–10].

Plants are attractive hosts for the production of recombinant proteins of pharmaceutical interest as they are inexpensive and versatile systems, amenable to rapid and economical scale-up [11]. However, the inability to perform authentic *N*-glycosylation is a major limitation of plants as expression systems. Different strategies have already been applied in plants to reduce β1,2-xylose and core α1,3-fucose residues. These include the overexpression of β1,4-galactosyltransferases [12,13], which compete for the same acceptor substrate as XylT and FucT, and the retention of glycoproteins within the endoplasmic reticulum [14]. However, the complete elimination of β1,2-xylose and core α1,3-fucose has not been achieved. Here we report the generation of knockout plants, which completely lack XylT and FucT activity. The plants accumulate high amounts of humanised *N*-glycan structures that do not contain any β1,2-xylose and core α1,3-fucose residues.

2. Materials and methods

2.1. Plant material

Arabidopsis thaliana wild-type plants ecotype Columbia and T-DNA insertion lines were grown in soil at 22°C with a photocycle of 16 h light/8 h dark. Homozygous mutants were crossed and allowed to self-pollinate in the F1 generation. Double and triple mutants were analysed in the F2 generation.

2.2. Identification of T-DNA mutants

The T-DNA mutant collection of the Arabidopsis Knockout Facility at the University of Wisconsin were screened as recommended (http://www.biotech.wisc.edu/Arabidopsis/) [15]. The facility contains two populations (ecotype Wassilewskija) of 60 480 and 72 960 lines respectively. Both populations were screened with the FucTB forward primer 5′-TTTAAAACCTCTAGACCATCAACCCAACT-3′ and reverse primer 5′-AACATTTATGCATCCAGCTATCAAGAACA-3′. The reverse primer in combination with the left border T-DNA primer JL202 (5′-CATTTTATAATAACGCTGCGGACATCTAC-3′) were used to amplify the insertion. For FucTA and XylT plants insertion mutant information was obtained from the SIGnAL database (http://signal.salk.edu) [16]. We thank the Salk Institute Genomic Analysis Laboratory for providing the sequence-indexed *A. thaliana* T-DNA insertion mutants. Seeds for the corresponding lines were purchased from the Nottingham Arabidopsis Stock Centre, Nottingham, UK (http://nasc.nott.ac.uk/).

2.3. Polymerase chain reaction (PCR) screening of mutant plants

Genomic DNA was extracted from young *A. thaliana* seedlings by macerating a young leaf with grinding balls in a mixer mill. 700 μl of extraction buffer (200 mM Tris–HCl, pH 8.0, 250 mM NaCl, 25 mM ethylenediamine tetraacetic acid (EDTA) and 0.5% sodium dodecyl sulphate (SDS)) were added and the suspension was mixed thoroughly. The extracts were centrifuged for 1 min at 16 000×*g* in a microcentrifuge and the supernatant was mixed with an equal volume of isopropanol and immediately centrifuged for 5 min at 16 000×*g*. The dried pellet was resuspended in 100 μl of autoclaved deionised water and 1 μl was used for PCR amplification. Each reaction contained 1×PCR buffer (Promega), 0.1 mM deoxyribonucleoside triphosphate (dNTP), 0.24 μM oligonucleotides and 2.5 units Taq polymerase (Promega). PCR products, which were to be subjected to DNA sequencing, were amplified using Pfu polymerase (Promega). DNA sequencing was performed in a thermocycler using the BigDye

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terminator v1.1 cycle sequencing kit and an ABI Prism 3100 genetic analyser (Applied Biosystems).

2.4. Reverse transcription (RT)-PCR

Leaves were ground in liquid nitrogen and RNA was extracted following the recommendations of the Trizol protocol. RT reactions were carried out from 500 ng of total RNA using either oligo(dT) primers or gene-specific primers and AMV reverse transcriptase (Promega) in a final volume of 20 µl. 1 µl was used for PCR amplification. Control reactions were performed without reverse transcriptase.

2.5. Immunoblot and dot blot analysis

Plant material was ground in liquid nitrogen, resuspended in 10 µl phosphate-buffered saline buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) per mg of plant material and cleared by centrifugation (two times 3 min at $16000\times g$). An aliquot of the supernatant was immediately mixed with SDS–polyacrylamide gel electrophoresis (PAGE) loading buffer, denatured at 95°C for 5 min and subjected to 12.5% SDS–PAGE under reducing conditions. Separated proteins were either stained according to the Bio-Rad silver staining protocol or blotted onto Hybond enhanced chemiluminescence (ECL) nitrocellulose membranes (Amersham Pharmacia Biotech). The blot was blocked in 5% (w/v) non-fat dry milk in Tris-buffered saline (TBS, 20 mM Tris–HCl, pH 7.6, 137 mM NaCl) for 1 h and incubated in a 1:5000 dilution of the rabbit anti-horseradish peroxidase antibody [4] in TBS supplemented with 0.1% (v/v) Tween 20. The detection was performed after incubation in a 1:10000 dilution of a horseradish peroxidase-conjugated goat anti-rabbit antibody (Sigma) in TBS-Tween with Supersignal West Pico chemiluminescent substrate (Pierce). The protein content was determined using the BCA protein assay protocol (Pierce) and bovine serum albumin as a standard. For the dot blot analysis plant material was resuspended in 50 µl phosphate-buffered saline per mg plant material and 1 µl of a 1:5 dilution was directly spotted onto Hybond ECL nitrocellulose membranes. The blot was developed as described for the immunoblot.

2.6. Preparation of N-linked glycans and matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) mass spectrometry

500 mg of fresh leaves from 8-week-old plants were ground and suspended in 2.5 ml of 5% (v/v) formic acid and 0.1 mg/ml pepsin. The slurry was incubated at 37°C for 20 h with occasional stirring. Insoluble material was then removed by centrifugation. From the supernatant glycopeptides were enriched by cation exchange and gel filtration as described previously [17]. Subsequently *N*-glycans were released from glycopeptides with peptide *N*-glycosidase A (Roche) and purified by cation exchange chromatography, gel filtration and passage through a reversed phase matrix. MALDI-TOF mass spectra were acquired on a DYNAMO (Thermo Bioanalysis) linear TOF mass spectrometer capable of dynamic extraction using 2,5-dihydroxybenzoic acid as the matrix.

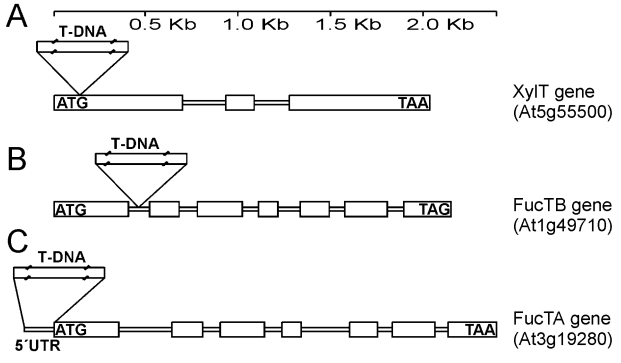

Fig. 1. Schematic representation of the gene structure and the T-DNA insertions. The size of the T-DNA insertion is not drawn to scale. The gene locus identifier is shown in brackets. A: XylT. B: FucTB. C: FucTA.

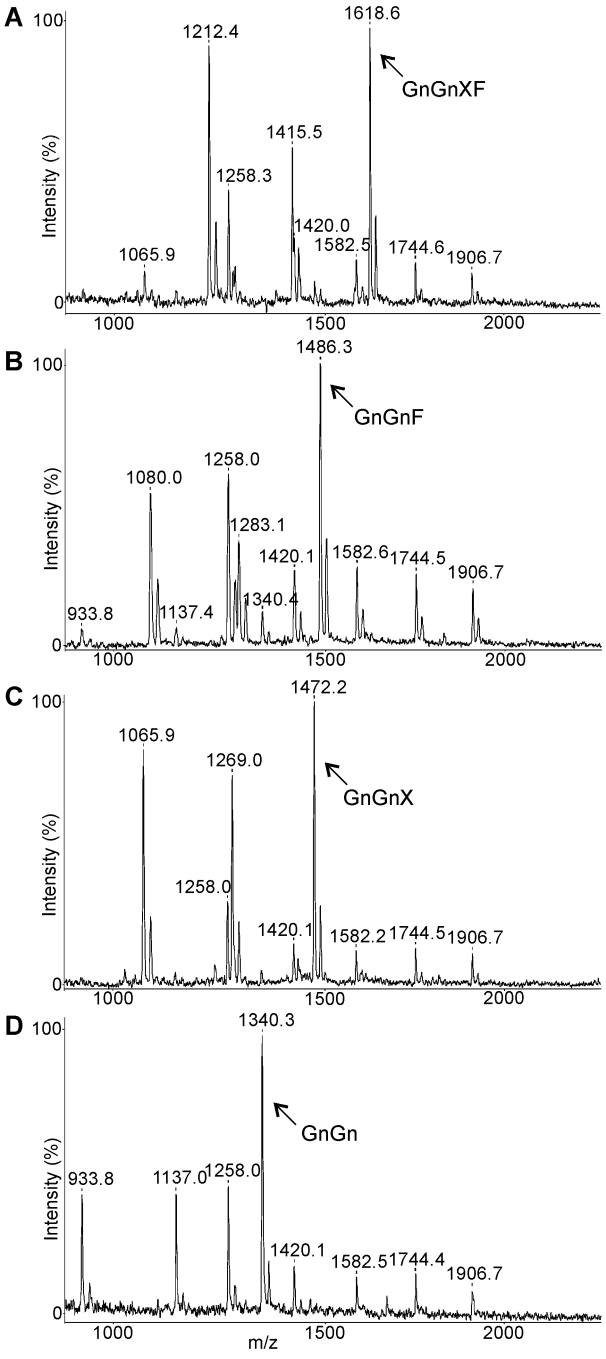

Fig. 2. MALDI-TOF mass spectra of oligosaccharides from endogenous proteins. A: Wild-type *A. thaliana*. B: XylT knockout (xylt). C: FucTA/FucTB double knockout (fuct). D: FucTA/FucTB/XylT triple knockout plants (xylt/fuct). $GlcNAc_2Man_3XylFucGlcNAc_2$ (GnGnXF); $GlcNAc_2Man_3FucGlcNAc_2$ (GnGnF); $GlcNAc_2Man_3XylGlcNAc_2$ (GnGnX); $GlcNAc_2Man_3GlcNAc_2$ (GnGn). The labelled peaks represent $(M+Na)^+$ ions. Other peaks are potassium adducts of the same glycans.

3. Results and discussion

3.1. Generation of β1,2-xylose-deficient plants

The enzyme that catalyses the transfer of xylose in β1,2-linkage to the *N*-linked oligosaccharides of glycoproteins is XylT and has been characterised in our laboratory recently [18,19]. In the present work we attempted to generate β1,2-

xylose-deficient *A. thaliana* lines by inactivation of XylT. According to information from the sequencing of the *A. thaliana* genome XylT should be present as a single-copy gene. We screened collections of insertion mutation lines for the presence of putative XylT knockouts. These collections (Arabidopsis Knockout Facility, Salk Institute Genome Analysis Laboratory (SIGnAL)) [15,16] contain stable *Agrobacterium tumefaciens* transferred DNA (T-DNA) insertions randomly distributed over the whole genome. While the 133 440 Arabidopsis Knockout Facility lines did not contain an insertion within the XylT gene, one line was identified in the SIGnAL collection (Salk_42226) that contained a T-DNA insertion in exon 1 (Fig. 1A). Subsequently homozygous plants were generated and analysed. The insertion resulted in the formation of a hybrid T-DNA/XylT mRNA as confirmed by RT-PCR and sequence analysis.

To monitor changes in the *N*-glycosylation pattern due to the inactivation of the XylT gene endogenous glycoproteins from the mutant line were subjected to total *N*-glycan analysis by MALDI-TOF mass spectrometry. Absence of xylose residues in the *N*-glycans can be monitored by a reduction of the mass of the respective peaks (132 mass units for xylose). The mass spectra of wild-type *A. thaliana* plants contained three major peaks. These three peaks were assigned to the complex-type *N*-glycans $Man_3XylFucGlcNAc_2$ (*m/z* 1212), $GlcNAcMan_3XylFucGlcNAc_2$ (*m/z* 1415) and $GlcNAc_2Man_3XylFucGlcNAc_2$ (*m/z* 1618), all of which contained β1,2-xylose and α1,3-fucose residues (Fig. 2A). The amount of all complex-type *N*-glycans that lacked xylose and fucose residues was below 2% (Table 1). Compared to the mass spectra derived from wild-type plants the spectra of the XylT insertion line lacked the three major complex-type *N*-glycan peaks carrying β1,2-xylose. Instead $Man_3FucGlcNAc_2$ (*m/z* 1080), $GlcNAcMan_3FucGlcNAc_2$ (*m/z* 1283) and $GlcNAc_2Man_3FucGlcNAc_2$ (*m/z* 1486) were most abundant (Fig. 2B and Table 1). This result clearly demonstrates the inactivation of XylT by the T-DNA insertion and confirmed that *A. thaliana* contains only one functional XylT gene responsible for the attachment of β1,2-xylose to *N*-glycans.

3.2. Generation of core α1,3-fucose-deficient plants

The attachment of α1,3-linked fucose to the asparagine-linked GlcNAc is catalysed by FucT, which has been characterised in our laboratories [20,21]. These findings demonstrated the presence of two genes for FucT (FucTA and FucTB) in *A. thaliana*, whereby in vitro enzyme activity was detected only for FucTA [21,22].

As described for XylT, T-DNA collections were screened for insertions within the two FucT genes. In the Arabidopsis Knockout Facility collection one line was identified with an insertion in the first intron of the FucTB gene (Fig. 1B) and a T-DNA insertion within the start codon of FucTA (Fig. 1C) was detected in the SIGnAL collection (Salk_87481). Sequence and RT-PCR analysis of the subsequently generated homozygous lines revealed T-DNA/FucT mRNA hybrids.

The two mutant FucT lines were subjected to total *N*-glycan analysis by MALDI-TOF mass spectrometry. Absence of fucose residues on the *N*-glycans can be monitored by a reduction of the mass by 146 mass units. In contrast to the mass spectra derived from XylT-deficient plants, the spectra of the FucTA and FucTB T-DNA insertion lines were not significantly different to wild-type (data not shown). Thus, both FucTA and FucTB seem to be active in vivo. Therefore, to generate plants deficient in FucT activity double knockouts that lack both FucTA and FucTB were generated. The two FucT insertion lines were crossed, double T-DNA insertion lines were isolated and the *N*-glycans from endogenous proteins were analysed by MALDI-TOF mass spectrometry. As expected the three major peaks of the complex-type *N*-glycans differed between wild-type and the double insertion line (Fig. 2C and Table 1). $Man_3XylGlcNAc_2$ (*m/z* 1066), $GlcNAcMan_3XylGlcNAc_2$ (*m/z* 1269) and $GlcNAc_2Man_3XylGlcNAc_2$

Table 1
Relative amounts of *N*-glycans detected in *A. thaliana* wild-type and knockout lines

m/z $(M+Na)^+$	Structures	wt (%)	xylt (%)	fuct (%)	xylt/fuct (%)
	truncated and complex-type structures				
933.8	$Man_3GlcNAc_2$	–	1.7	–	13.3
1065.9	$Man_3XylGlcNAc_2$	1.6	–	25.2	–
1080.0	$Man_3FucGlcNAc_2$	–	18.2	–	–
1137.0	$GlcNAcMan_3GlcNAc_2$	1.4	1.5	–	13.7
1212.1	$Man_3XylFucGlcNAc_2$	26.4	–	–	–
1228.1	$Man_4XylGlcNAc_2$	–	–	2.0	–
1269.1	$GlcNAcMan_3XylGlcNAc_2$	1.5	–	23.1	–
1283.2	$GlcNAcMan_3FucGlcNAc_2$	–	10.8	–	–
1340.2	**$GlcNAc_2Man3GlcNAc_2$**	**-**	**2.7**	**1.2**	**42.3**
1415.5	$GlcNAcMan_3XylFucGlcNAc_2$	15.0	–	–	–
1431.3	$GlcNAcMan_4XylGlcNAc_2$	–	–	1.3	–
1472.1	**$GlcNAc_2Man_3XylGlcNAc_2$**	**1.4**	**–**	**26.8**	**–**
1486.4	**$GlcNAc_2Man_3FucGlcNAc_2$**	**–**	**25.5**	**–**	**–**
1618.5	**$GlcNAc_2Man_3XylFucGlcNAc_2$**	**26.6**	**–**	**–**	**–**
	total	73.9	60.4	79.6	69.3
	oligomannosidic structures				
1258.1	$Man_5GlcNAc_2$	10.8	17.5	7.8	15.4
1420.2	$Man_6GlcNAc_2$	6.1	6.4	3.8	5.3
1582.4	$Man_7GlcNAc_2$	2.7	5.9	3.0	3.5
1744.5	$Man_8GlcNAc_2$	3.5	5.6	2.8	4.5
1906.7	$Man_9GlcNAc_2$	3.0	4.2	3.0	2.0
	total	26.1	39.6	20.4	30.7

Percentages were calculated based on peak areas from MALDI-TOF mass spectra. wt, *A. thaliana* wild-type line; xylt, β1,2-xylosyltransferase knockout line; fuct, core α1,3-fucosyltransferase double knockout line; xylt/fuct, triple knockout line. The amounts of the *N*-glycans with two terminal *N*-acetylglucosamine residues are shown in bold.

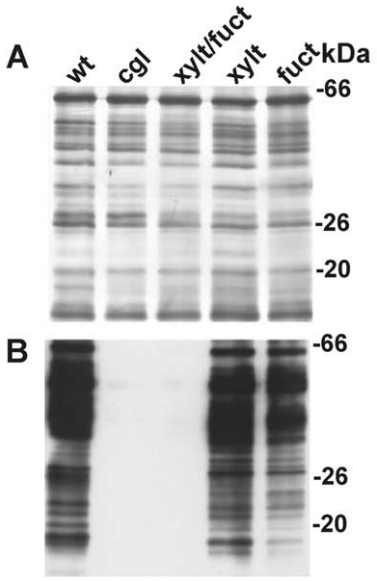

Fig. 3. Detection of immunogenic epitopes in endogenous proteins from different *A. thaliana* lines. A: SDS–PAGE and silver staining. B: Immunoblot probed with antibodies specific for β1,2-xylose- and α1,3-fucose-containing *N*-glycans. 3 μg of endogenous proteins were loaded per lane. *A. thaliana* wild-type (wt); *A. thaliana* mutant (cgl), which does not produce complex *N*-glycans [23]; FucTA/FucTB/XylT triple knockout plants (xylt/fuct); XylT knockout (xylt) and FucTA/FucTB double knockout (fuct).

(*m*/*z* 1472) oligosaccharides were the main structures present, demonstrating the complete elimination of complex-type *N*-glycans with core α1,3-fucose residues. Our results prove that inactivation of both FucTA and FucTB is necessary to abolish core α1,3-fucosylation in *A. thaliana*. Hence, the *A. thaliana* genome contains two functionally active core α1,3-fucosyltransferases, which seem fully capable to complement each other.

3.3. Generation of β1,2-xylose- and core α1,3-fucose-deficient plants

To obtain plants lacking both β1,2-xylose and core α1,3-fucose the XylT knockout line was crossed with the FucTA/FucTB double knockout. The resulting progeny was screened for the presence of triple knockout plants using dot blots and anti-horseradish peroxidase antibodies, which recognise β1,2-xylose- and core α1,3-fucose-containing epitopes [4]. Out of 381 seedlings analysed four extracts of endogenous proteins did not show any detectable staining, which indicated the successful generation of homozygous triple knockout plants. The presence of all three T-DNA insertions was confirmed by PCR. Total *N*-glycan profiling revealed the complete absence of β1,2-xylose- and core α1,3-fucose-containing oligosaccharides (Fig. 2D). The main peak (42% of all *N*-glycans) was $GlcNAc_2Man_3GlcNAc_2$ (*m*/*z* 1340) accompanied by $GlcNAcMan_3GlcNAc_2$ (*m*/*z* 1137) and $Man_3GlcNAc_2$ (*m*/*z* 934). To confirm the complete absence of β1,2-xylose and core α1,3-fucose on endogenous glycoproteins of the triple knockout plants immunoblot analysis using the polyclonal anti-horseradish peroxidase antibodies was carried out. Wild-type, XylT and FucTA/FucTB knockout plants displayed an intense staining of multiple bands. In contrast, no staining was detected in the triple insertion line clearly indicating the absence of any antigenic *N*-glycans on endogenous glycoproteins (Fig. 3). Absence of staining was also observed for the *A. thaliana* cgl mutant, which accumulates high-mannose instead of complex-type *N*-glycans as a consequence of the inactivity of *N*-acetylglucosaminyltransferase I [23]. The strong reactivity of endogenous proteins from XylT and FucTA/FucTB knockout plants, with the anti-horseradish peroxidase antibodies, is consistent with the observation that both β1,2-linked xylose and core α1,3-linked fucose, independently of each other constitute antigenic epitopes [7]. This result is in agreement with previous results, where the expression of XylT in insect cells led to the production of antigenic glycan epitopes on insect proteins [18]. Therefore, strategies that lead to the deficiency of only one of the two antigenic sugar residues as discussed by Ko et al. [14] will not be sufficient for the production of recombinant glycoproteins devoid of immunoreactive *N*-glycans in plants.

FucTA/FucTB/XylT triple knockout lines were viable and revealed no obvious morphological phenotype under standard growth conditions. Hence, we have generated, for the first time, higher plants that lack potentially allergenic and immunogenic β1,2-xylose and core α1,3-fucose residues without any deleterious impact on plant growth and development. Importantly, the FucTA/FucTB/XylT triple knockout lines display less *N*-glycan heterogeneity than wild-type plants. Furthermore, the surprisingly high proportion of complex *N*-glycans carrying terminal β*N*-acetylglucosamine residues on both the α1,3- and α1,6-linked mannoses is the optimal prerequisite for the further restoration of human-type *N*-glycosylation in plants with the addition of β1,4-galactose residues [12,13] and engineering of the sialic acid pathway [24] as the final aims.

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