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Unaltered complex N-glycan profiles in *Nicotiana benthamiana* despite drastic reduction of β1,2-*N*-acetylglucosaminyltransferase I activity

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UDP-GlcNAc:α3-D-mannoside β1,2-*N*-acetylglucosaminyltransferase I (GnTI; EC 2.4.1.101) is a Golgi-resident glycosyltransferase that is essential for the processing of oligomannose to hybrid and complex N-glycans in higher eukaryotes. The cDNA of *Nicotiana tabacum* GnTI has been cloned and characterised previously. To assess the influence of GnTI expression levels on the formation of complex N-glycans we used posttranscriptional gene silencing to knock down the expression of GnTI in the tobacco related species *Nicotiana benthamiana*. 143 independent transgenic plants containing GnTI constructs in either sense or antisense orientation were generated. 23 lines were selected for measurement of GnTI activity and 10 lines thereof showed a reduction of more than 85% in *in vitro* assays as compared to wildtype plants. GnTI reduction was stably inherited and did not interfere with the viability of the transformants. Noteworthy one line, 34S/2, exhibited a residual GnTI activity below the detection limit. β1,2-*N*-acetylglucosaminyltransferase II (GnTII), an enzyme which acts further downstream in the N-glycosylation pathway, as well as other control enzymes (α-mannosidase, β-*N*-acetylglucosaminidase) were not affected indicating the specific downregulation of GnTI. Remarkably, immunoblots and mass spectrometric N-glycan profiling revealed no significant changes of the total N-glycan pattern. Thus, even the undetectable residual GnTI activity was sufficient for the synthesis of complex N-glycans comparable to wildtype plants.
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Abbreviations: **CaMV, cauliflower mosaic virus; CTAB, cetyltrimethylammonium bromide; GnTI, β1,2-*N*-acetylglucosaminyltransferase I; GnTII, β1,2-*N*-acetylglucosaminyltransferase II; HPLC, high performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; MES, 2-(N-morpholino)ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; RT, reverse transcriptase; SDS, sodium dodecyl sulfate; TTR, transcription termination region.**

Introduction

N-Glycosylation is one of the most common posttranslational protein modifications in plants as well as in animals. The synthesis of N-glycans begins with an oligosaccharyltransferase, which transfers $Glc_3Man_9GlcNAc_2$ *en bloc* from dolichol-pyrophosphate-$Glc_3Man_9GlcNAc_2$ to an asparagine residue of the nascent polypeptide chain in the lumen of the endoplasmic reticulum. Oligosaccharide processing in the endoplasmic reticulum and Golgi apparatus lead to the formation of $Man_5GlcNAc_2$-Asn-Xaa, which is the entry point for the conversion of oligomannose to hybrid and complex N-glycans due to the action of UDP-GlcNAc:α3-D-mannoside β1,2-*N*-acetylglucosaminyltransferase I (GnTI; EC 2.4.1.101) [1]. In mammals as well as in plants GnTI is essential for the subsequent *in vivo* action of other enzymes in the processing pathway including UDP-GlcNAc:α6-D-mannoside β1,2-*N*-acetylglucosaminyltransferase II (GnTII). GnTI cDNA sequences from a variety of animal [2] as well as plant species [3–5] have been characterised. Like other Golgi-resident glycosyltransferases GnTI is a type II transmembrane protein. The large C-terminal catalytic domain of the enzyme is particularly

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well conserved through evolution, and its activity can be complemented between plants and mammals indicating the conserved status of the enzyme [4,6]. Shortly beyond GnTI activity the pathways for complex N-glycan biosynthesis in plants and animals begin to diverge. Whereas high-mannose N-glycans have the same structure in plant and in animal cells, complex N-glycans of plants lack sialic acid, are generally smaller and contain β1,2-xylose and/or core α1,3-fucose residues [7].

Although it has been demonstrated that animal GnTI mRNA is differentially expressed in various tissues [8–10] it is not clear yet, whether these changes in GnTI mRNA levels alter the *N*-glycosylation pattern of the tissues. For plant GnTI it has been reported that the mRNA expression is largely constitutive in different tissues of *Arabidopsis thaliana* plants [5]. To assess the influence of GnTI expression levels to subsequent formation of complex N-glycans we used posttranscriptional gene silencing (PTGS) to knockdown the expression of GnTI in the tobacco related species *N. benthamiana*. PTGS using sense or antisense constructs is a widely used method to specifically eliminate gene expression in plants [11]. We show, that transformed plants that exhibit specific reduction of GnTI activity below detection limit, do not exhibit significantly altered complex N-glycan structures as determined by immunological means and MALDI-TOF mass spectrometry.

Materials and methods

Plant material and cultivation

N. benthamiana plants were cultivated in a controlled growth chamber with 22°C day and night temperature, a 16 h photoperiod and 50% humidity.

Generation of transgenic plants

The binary expression vector pGA643 [12] was used to insert the cDNA of tobacco GnTI in sense or antisense orientation between the CaMV 35S promoter and the transcription termination region of the gene 7 of pTiA6 (7TTR), resulting in the two plasmids pAGT1s (-sense) and pAGT1as (-antisense). *Agrobacterium tumefaciens* strain UIA143 [13,14] was transformed either with vector pAGT1s or pAGT1as following standard protocols [15]. Leaf disc transformation from *N. benthamiana* with recombinant *Agrobacteria* cultures were done as described [16] and positive transformants were transferred to soil and grown to maturity.

Characterisation of transgenic plants on DNA and RNA level

Genomic DNA was isolated from putative transgenic plants using the CTAB method [17]. PCR amplification was done using a combination of pGA643 specific primers (JH020, 5′-GCTAGCTTAGCTCATCGC-3′, for antisense and JH006 5′-CCTTCGCAAGACCCTTCC-3′ for sense constructs) and one GnTI specific primer (tab9r: 5′-AGCCCGATTGCAAGCCATAAC-3′). RNA was isolated with Trizol (Life Technologies, Inc.). cDNA was synthesised from 1 μg of total RNA using the GnTI specific reverse primer tab2r (5′-CTGAGCGGTAAAGAGCATAAGGATC-3′) and AMV reverse transcriptase (Promega). PCR was done using primer tab4f (5′-CGGAGTGGCTGCTGTAGTTGTT-3′) and tab2r and the resulting PCR product was precipitated and subsequently digested with CfoI (Roche Diagnostics) in order to distinguish between endogenous (no CfoI site) and transgenic GnTI mRNA expression. For semiquantitative RT-PCR the RT reaction was carried out using oligo(dT) primers and the subsequent PCR was done using NbGnTI1 (5′-TTATGTATCTTCAATTCCGAGC-3′) and NbGnTI2 (5′-CTCAGATTTTTTTCCTGGTCTC-3′) or catalase specific primers NbCat1 (5′-CATTCGCGGTTTTGCTGTC-3′) and NbCat2 (5′-TGGTGGCGTGGCTATGATTTGTA-3′) to control for RNA quality and RT efficiency.

Preparation of microsomes from *N. benthamiana* leaves

450 mg of upper leaves from 5 to 7 week old *N. benthamiana* plants were harvested and cut into small pieces. 10 ml of Buffer M (25 mM Tris-HCl, pH 7.2, 250 mM sucrose, 1 mM EDTA, 0.5 mM dithioerythritol, 0.5 mM phenylmethylsulfonyl fluoride) was added and the leaves were disrupted using an Ultra-Turrax homogenisator. The suspension was centrifuged at 3000 × g for 15 min to remove nuclei, mitochondria and undisrupted cells. The supernatant was filtered and centrifuged for 1 h at 100000 × g to isolate the microsomal fraction. The microsomal pellet was dissolved in 46 μl 0.2 M MES, pH 6.3 containing 0.5% (v/v) Triton X-100. The suspension was centrifuged for 1 min at 2000 × g and the supernatant was used for enzyme activity measurements. The protein content was determined using the BCA Protein Assay protocol (Pierce) and bovine serum albumin as a standard.

Assay for endogenous GnTI activity

GnTI activity was measured using $Man_5GlcNAc_2$-pyridylamine (Man5-PA) as an acceptor and UDP-GlcNAc as a donor substrate [18]. AMP, GlcNAc and swainsonine were added to the reaction mixture to inhibit pyrophosphatase, hexosaminidase and Golgi-mannosidase II, respectively. The amount of product, $GlcNAcMan_5GlcNAc_2$-pyridylamine (Man5Gn-PA), was analysed by reversed-phase HPLC as described [3].

Reaction mixtures for the assay of endogenous GnTI contained the following components in a final volume of 20 μl: 100 mM MES buffer, pH 6.3, 20 mM $MnCl_2$, 0.25% (v/v) Triton X-100, 5 mM AMP, 100 ng swainsonine (Sigma), 100 mM GlcNAc, 2 mM UDP-GlcNAc and 0.125 mM Man5-PA. Upon addition of 10 μl of the microsomal fraction incubations were done at 37°C. After 4 h, 180 μl of water was added and enzymes were inactivated by incubation at 95°C for 5 min. Following centrifugation for 3 min at 16000 × g. 50 μl of the

reaction was injected and separated on a 5 μm Hypersil ODS-column (4 × 250 mm, ÖFZ Seibersdorf) with a flow rate of 1.5 ml/min. Elution was performed using 3.75% (v/v) methanol in 0.1 M ammonium acetate, pH 4.0. Fluorescence of the eluate was monitored using excitation and emission wavelengths of 320 and 400 nm, respectively. The product peak (Man5Gn-PA) eluted approximately 1 min earlier than the substrate Man5-PA. Enzyme activities were calculated from peak areas of the product. All assays were performed in duplicate.

Assay for endogenous GnTII

Reaction mixtures for the assay of endogenous GnTII contained in a final volume of 20 μl the following components: 100 mM MES buffer, pH 6.3, 20 mM $MnCl_2$, 0.25% (w/v) Triton X-100, 5 mM AMP, 100 mM GlcNAc, 2 mM UDP-GlcNAc and 0.1 mM $GlcNAcMan_3GlcNAc_2$-pyridylamine (MGn-PA) [18]. The reactions were started by the addition of 10 μl of the microsomal fraction and incubated at 37°C for 4 h. The reaction was stopped by the addition of 180 μl water, incubated for 5 min at 95°C and centrifuged for 3 min at 16000 × g. 50 μl of the sample were subjected to reversed-phase HPLC as described above except that the solvent contained 4.5% (v/v) methanol.

Assay for endogenous α-mannosidase and β-*N*-acetylglucosaminidase activity

α-Mannosidase and β-*N*-acetylglucosaminidase activities were determined photometrically. Enzyme incubations were performed for 4 h at 37°C. The reaction mixture contained in a total volume of 50 μl 1 to 4 μl of the microsomal fractions and 5 mM 4-nitrophenyl-α-D-mannopyranoside or 4-nitrophenyl *N*-acetyl-β-D-glucopyranoside (Sigma) in 0.1 M MES at pH 6.3 [19,20]. The reactions were terminated by the addition of 250 μl 0.4 M glycine/NaOH buffer, pH 10.4, and absorbence at 405 nm was measured. All assays were run with duplicate samples.

Immunoblot analysis

Plant material was ground in liquid nitrogen, resuspended in extraction buffer containing 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 1% (w/v) SDS and cleared by centrifugation (two times 3 min at 16000 × g). An aliquot of the supernatant and serial dilutions thereof were subjected to 12.5% SDS-PAGE under reducing conditions. Proteins after separation were either stained according to the BioRad silver staining protocol or blotted onto Hybond 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 [21] 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 anti-rabbit antibody (Sigma) in TBS-Tween with Supersignal West Pico Chemiluminescent substrate (Pierce) and X-ray films.

N-Glycan analyses from *N. benthamiana* leaves

2 g of fresh leaves from 5–7 weeks old *N. benthamiana* were homogenised in 10 ml of 5% (v/v) formic acid and digested with 0.1 mg/ml pepsin (Sigma) for 20 h at 37°C. Insoluble material was then removed by centrifugation. From the supernatant glycopeptides were enriched by cation exchange and gel filtration as described previously [22]. 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 time of flight mass spectrometer capable of dynamic extraction using 2,5-dihydroxybenzoic acid as the matrix.

Results

Generation of transgenic *N. benthamiana* lines

N. benthamiana leaf discs were incubated with *A. tumefaciens* carrying plasmids with GnTI cDNA from the closely related species *N. tabacum* in either sense or antisense orientation (Figure 1). Putative transformed plants were selected on kanamycin containing media and screened for the stable integration of the GnTI construct. Genomic DNA was isolated from young leaves and the presence of the foreign sequence was determined by PCR. From a total of 152 lines, 143 (94%) carried the GnTI sequence in either sense or antisense orientation. Subsequently, seeds were generated from the primary transformants (T0) whereby no seeds could be obtained from 31 (22%) of the T0 plant lines. For further analyses seeds (T1 generation) were selected on kanamycin containing medium to eliminate homozygous non-transformed siblings.

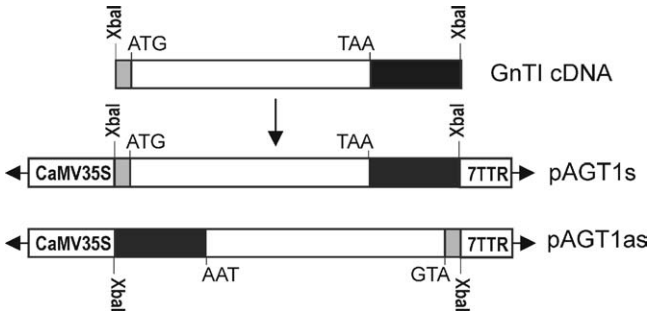

Figure 1. Schematic presentation of GnTI sense and antisense constructs. The complete cDNA fragment of tobacco GnTI (2017 bp) was inserted between the CaMV 35S promoter and the 7TTR sequence of the binary vector pGA643 resulting in plant transformation vectors pAGT1s (sense construct) and pAGT1as (antisense construct). White bars: coding region, grey bars: 5′-untranslated region, black bars: 3′-untranslated region of GnTI. ATG and TAA correspond to the start and stop codon of GnTI cDNA, respectively.

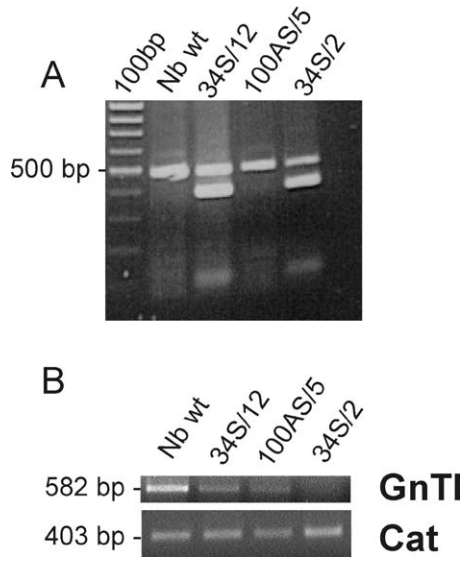

Figure 2. GnTI mRNA expression in transgenic *N. benthamiana* T2 lines. (A) CfoI digested RT-PCR products: RT reaction was performed with a gene-specific reverse primer (tab2r) and PCR was done with primers, that amplify both *N. benthamiana* and *N. tabacum* GnTI cDNA. CfoI digestion of PCR products from transgenic sense lines (34S/12 and 34S/2) resulted in the production of 377 and 93 bp fragments, whereas wildtype (Nb wt) and antisense lines (100AS/5) displayed only the uncut endogenous GnTI fragment (470 bp). (B) RT reaction was performed using oligo(dT) primer and PCR was done with primers that amplify only *N. benthamiana* GnTI. Catalase (Cat) mRNA expression was used as a control.

Molecular analysis of transgenic *N. benthamiana* plants

To determine whether transgenic GnTI cDNA derived from *N. tabacum* is expressed in transformed plants it was necessary to distinguish between endogenous and transgenic GnTI mRNA. Since the GnTI cDNA sequence from *N. benthamiana* was not known the corresponding cDNA was cloned from wildtype plants by RT-PCR using *N. tabacum* GnTI specific primers. The resulting cDNA sequence (GenBank No. AJ295993) contains 39 bp of 5′-untranslated region and an open reading frame of 1338 bp. The *N. tabacum* and *N. benthamiana* cDNA sequences show 97.8% identity. The two mRNA species can be distinguished upon CfoI restriction enzyme digestion of the corresponding RT-PCR product. The site is present only in the *N. tabacum* GnTI cDNA (Figure 2A). Expression of *N. tabacum* GnTI sense and antisense RNA could be detected in all transgenic *N. benthamiana* plants (Figure 2A, and data not shown). As expected, endogenous GnTI mRNA was monitored in all transgenic sense and antisense plants (Figure 2A). A significant specific reduction of endogenous mRNA expression in transgenic plants could be detected using semiquantitative RT-PCR (Figure 2B). This result indicated the activation of a gene silencing mechanism in sense and antisense lines.

Determination of GnTI activity in transgenic lines

Enzyme activity measurements were used to determine endogenous GnTI expression levels in transgenic lines. For this purpose microsomal fractions were isolated from upper leaves of 5 to 7 week old *N. benthamiana* plants. GnTI activity was measured *in vitro* using $Man_5GlcNAc_2$-pyridylamine (Man5-PA) as an acceptor and UDP-GlcNAc as a donor substrate. The amount of product, $GlcNAcMan_5GlcNAc_2$-pyridylamine (Man5Gn-PA), was analysed by reversed phase HPLC and was found to be proportional to incubation time until about 40% of substrate had been converted to product. As a control for equal enrichment of microsomal fractions α-mannosidase and β-hexosaminidase assays using chromogenic substrates were performed. While the activities of these control enzymes were the same in transgenic lines and wildtype plants, remarkable differences were detected regarding endogenous GnTI activity. In total 23 individual transgenic lines of the T1 generation were tested (Figure 3). Out of these lines 4 exhibited GnTI activity comparable to wildtype plants (100% ±28). However 19 lines showed a significant reduction (<50%), and 10 revealed even activity below 15%. To obtain homozygous individuals these plants were selected for further propagation into T2 generation. All of them showed equal or reduced reduction of GnTI activity (3–15% residual activity in *in vitro* assays) compared to their parental plants. Noteworthy, one line, 34S/2, displayed a GnTI reduction below the detection limit (Table 1). This line was selected for further analysis and propagated into T3 generation. All progeny exhibited the same amount of downregulation indicating the

Table 1. GnTI and GnTII *in vitro* activities in wildtype plants and line 34S/2

	Specific GnTI activity nmol h^{-1} mg^{-1}	*Relative GnTI activity %*	*n*	*Specific GnTII activity nmol h^{-1} mg^{-1}*	*Relative GnTII activity %*	*n*
N.b. wildtype	2.75 ± 0.77	100 ± 28	18	2.18 ± 0.55	100 ± 25	5
N.b. 34S/2	≤0.08	<3	13	2.05 ± 0.43	94 ± 21	5
A.th. wildtype	3.47					
A.th. cgl	≤0.08					

The mean specific activity plus SD is shown. All enzymatic assays were done in duplicate. $n =$ the number of independent experiments (analysis of either different individuals or replications from one plant; for 34S/2 data from plants of the T2 and T3 generation are shown). Equal amounts of microsomal preparations were used for GnTI and GnTII activity measurements. The GnTI activity of *A. thaliana* wildtype and the GnTI mutant cgl are shown as controls.

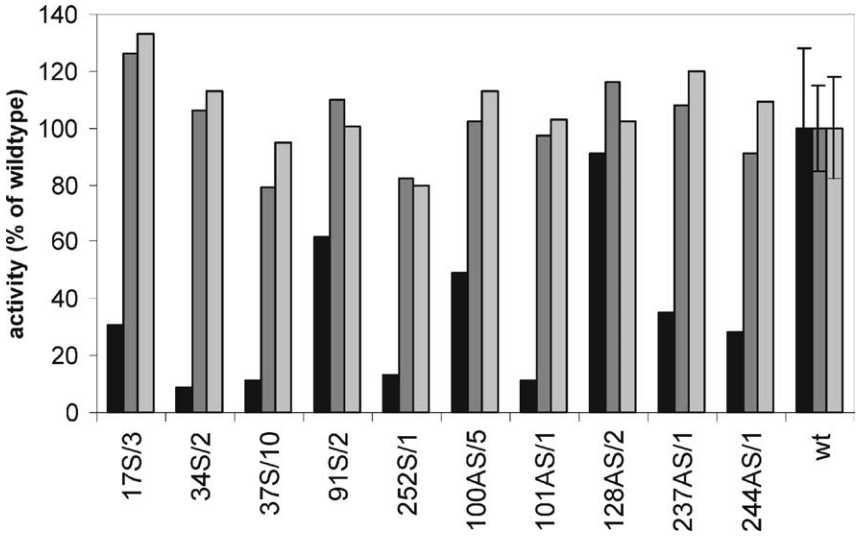

Figure 3. Comparison of representative GnTI, β-*N*-acetylglucosaminidase and α-mannosidase activities in selected transgenic T1 lines (S: sense line; AS antisense line). Black bars: GnTI activity; dark grey bars: α-mannosidase activity, light grey bars β-*N*-acetylglucosaminidase activity.

stability and inheritance of GnTI silencing. The residual GnTI activity as measured by *in vitro* assays in all T2 and T3 34S/2 plants was always below 0.08 nmol h^{-1} mg^{-1} of microsomal protein, which corresponds to less than 3% compared to that of wildtype plants. Activities of the control α-mannosidase and β-*N*-acetylglucosaminidase were comparable in line 34S/2 and wildtype plants. Furthermore, to show that other enzymes of the N-glycosylation pathway were not affected, GnTII activity was measured using $GlcNAcMan_3GlcNAc_2$-pyridylamine as acceptor and UDP-GlcNAc as donor substrate. The product, $GlcNAc_2Man_3GlcNAc_2$-pyridylamine, was analysed by reversed phase HPLC. The amount of GnTII activity in line 34S/2 was essentially the same as in wildtype plants (Table 1). The reduction of GnTI activity was detected in 5, 7 and 10 week old 34S/2 plants, which indicates a stable inhibition during these developmental stages of the *N. benthamiana* plants. Taken together these data obtained by direct measurement of GnTI activity showed the specific and significant reduction of GnTI in our transgenic plants and in particular in line 34S/2. Line 34S/2 and all other transgenic lines developed completely like wildtype plants and showed no obvious phenotype under standard growth conditions.

Analysis of N-glycans from endogenous glycoproteins

By immunological means it was investigated whether the drastic reduction of GnTI activity has an influence on the N-glycan spectrum of endogenous proteins. Antibodies specific for β1,2-xylose/core α1,3-fucose containing N-glycans were employed for immunoblotting. Initially, protein extracts from all 112 transgenic T1 lines were screened. However, no significant changes in signal intensity could be observed. The same result was obtained for line 34S/2, where protein extracts from leaves in different developmental stages (e.g. primary to fully developed leaves) and different tissues of transgenic T2 and T3 lines were investigated (Figure 4).

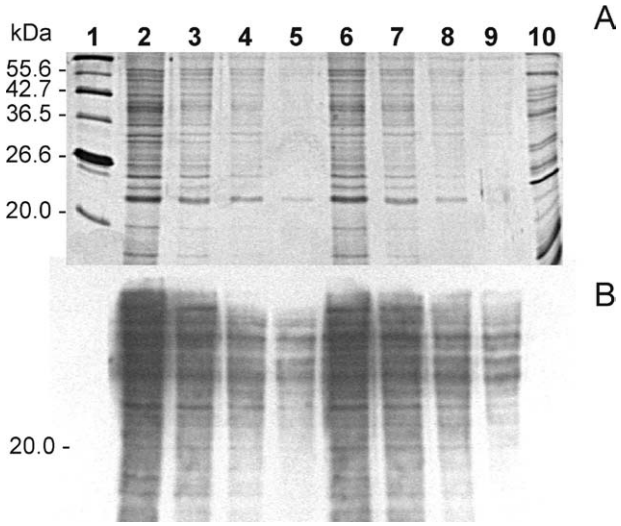

Figure 4. Analysis of total soluble protein extracts from roots of wildtype and 34S/2 T3 plants. Protein extracts were subjected to 12% SDS-PAGE and silver-stained (A) or transferred and probed with anti-horseradish peroxidase antibodies (B). Lane 1: protein marker; lanes 2–5: 1:1 dilutions of protein from wildtype plants; lanes 6–9: 1:1 dilutions of protein from line 34S/2; lane 10: control: 6 μg protein extract from *A. thaliana cgl* mutant, which does not produce complex N-glycans [30].

Mass spectrometric analysis was performed from two selected transgenic plants (34S/2, 101AS/1) to determine *N*-glycan profiles in detail. The mass spectrum of the *N*-glycans derived from wildtype proteins showed a major peak of $GlcNAc_2Man_3XylFucGlcNAc_2$ ($[M+Na]^+ = 1618$)

Table 2. Relative amounts of total N-glycans detected in wildtype and 34S/2 plants

m/z (M + Na)$^+$	*Structure*	*Wildtype (%)*	*34S/2 (%)*
Complex-type structures with fucose and/or with xylose			
1065.7	$Man_3XylGlcNAc_2$ (MMX)	1.8 (0.6–3.0)	4.4 (0.0–12.8)
1212.1	$Man_3XylFucGlcNAc_2$ (MMXF)	14.8 (11.8–17.1)	18.1 (7.7–28.7)
1269.1	$GlcNAcMan_3XylGlcNAc_2$ (GnMX/MGnX)	3.1 (3.0–3.3)	3.1 (0.0–7.5)
1415.5	$GlcNAcMan_3XylFucGlcNAc_2$ (GnMXF/MGnXF)	10.5 (9.1–11.9)	10.0 (8.2–12.2)
1472.1	$GlcNAc_2Man_3XylGlcNAc_2$ (GnGnX)	3.3 (2.4–3.9)	3.1 (1.6–6.1)
1618.5	$GlcNAc_2Man_3XylFucGlcNAc_2$ (GnGnXF)	49.5 (40.9–58.6)	41.1 (22.1–56.9)
2235.0	(GF)(GF)XF[a]	1.2 (0.5–2.0)	1.0 (0.0–1.0)
	Sum	84.2 (81.7–87.4)	80.8 (67.9–87.8)
Oligomannosidic structures			
1258.4	$Man_5GlcNAc_2$ (Man5)	2.5 (1.0–5.7)	5.1 (1.2–11.4)
1420.2	$Man_6GlcNAc_2$ (Man6)	2.4 (1.7–3.0)	3.1 (1.3–3.8)
1582.4	$Man_7GlcNAc_2$ (Man7)	3.2 (2.5–3.9)	3.3 (1.5–4.5)
1744.5	$Man_8GlcNAc_2$ (Man8)	5.5 (4.1–6.6)	5.4 (1.3–8.6)
1907.1	$Man_9GlcNAc_2$ (Man9)	2.2 (1.3–2.8)	2.3 (0.7–4.0)
	Sum	15.8 (12.6–18.3)	19.2 (12.2–32.1)

The peaks of MALDI-TOF spectra from wildtype (*n = 4*) and line 34S/2 (*n = 5*) were quantified and the mean values as wall as the range (in parenthesis) are shown. Each of the spectra represented an independent experiment.
[a]Lewis A containing structure (Galβ1-3(Fucα1-4)GlcNAcβ1-2Manα1-6(Galβ1-3(Fucα1-4)GlcNAcβ1-2Manα1-3)(Xylβ1-2) Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc)

accompanied by $GlcNAcMan_3XylFucGlcNAc_2$ ($[M+Na]^+ = 1415$), $Man_3XylFucGlcNAc_2$ ($[M+Na]^+ = 1212$) and minor components (Table 2). In contrast to the expected accumulation of $Man_5GlcNAc_2$ structures in GnTI silenced plants only a slight increase was detected (Figure 5, Table 2). More than 80% of the total N-glycans were still of complex-type as in the wildtype plants.

Discussion

In this work we wanted to investigate the feasibility of influencing protein N-glycosylation in *N. benthamiana* plants by reducing the expression of the key enzyme GnTI. The oligosaccharide structure created by GnTI serves as a substrate for the synthesis of complex and hybrid N-glycans. Thus we hypothesised, a decrease in the GnTI expression level would lead to a shift from core containing complex-type N-glycans to oligomannosidic N-glycans containing mainly $Man_5GlcNAc_2$. Using post-transcriptional gene silencing we obtained several lines with reduced endogenous GnTI mRNA and reduced GnTI activity and one line, 34S/2, displayed a reduction of enzyme activity even below the detection limit of the *in vitro* assay. However, neither MALDI-TOF analyses of total N-glycans nor immunoblots using antibodies specific for β1,2-xylose/core α1,3-fucose containing N-glycans displayed substantial differences compared to wildtype plants. This result was rather unexpected, in particular since Wenderoth and von Schaewen [5] claimed a significant reduction of complex N-glycans by means of GnTI suppression using antisense constructs in potato and tobacco. By immunoblot analysis using a complex N-glycan antiserum it was shown that one out of 512 potato lines displayed a clearly

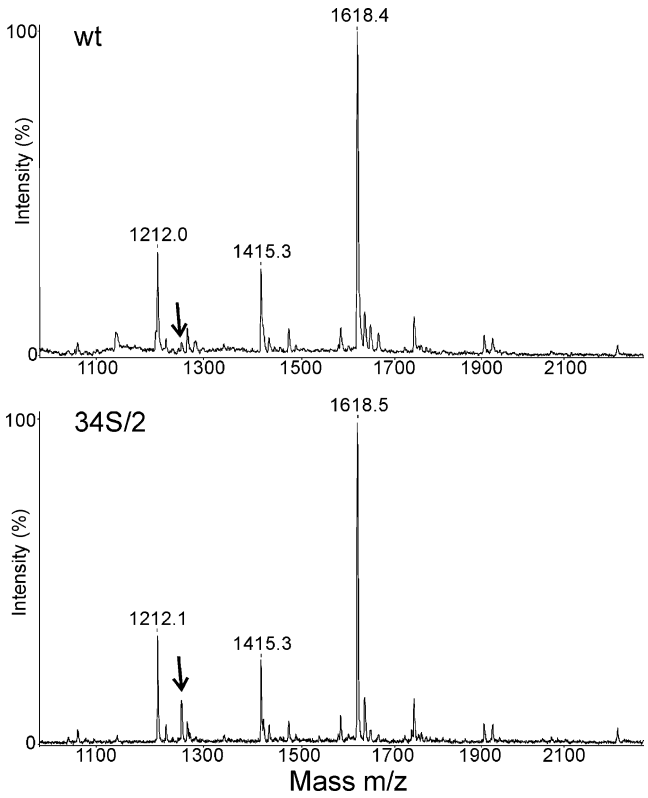

Figure 5. MALDI-TOF mass spectra of total N-glycans derived from a representative wildtype plant (wt) and a transgenic 34S/2 T3 plant are shown. The three major peaks representing complex-type N-glycans with β1,2-xylose and core α1,3-fucose are indicated. The $Man_5GlcNAc_2$ peak (*m/z* = 1258) is highlighted by an arrow.

reduced and reproducible staining compared to wildtype plants. However, additional experiments to show that this line harbours a reduced GnTI activity and indeed produces higher amounts of the expected $Man_5GlcNAc_2$ structures were not performed. The analysis by immunoblotting using antisera against complex N-glycans can also be misleading since the binding of the antibodies to the glycans can be inhibited by the presence of other sugar residues. In our laboratories we have recently shown that binding of rabbit anti-horseradish peroxidase serum to glycoproteins containing β1,2-xylose and core α1,3-fucose is prevented by terminal GlcNAc residues [23].

How can we explain the unexpected finding that the N-glycans were not significantly changed although we detected a substantial reduction of GnTI enzyme activity? One explanation would be the presence of an alternative pathway with either a second GnTI-like enzyme or with other enzymes that can act without the previous action of GnTI. Although *N. benthamiana* contains like tobacco two GnTI isoforms [3] these are highly homologous and would thus both be a target of the homology-dependent gene silencing mechanism. In addition the highly specific GnTI assay using the physiological $Man_5GlcNAc_2$ substrate would also detect other non-homologous GnTI enzyme activities acting on this acceptor. To test the presence of possible GnTI related enzymes $Man_3GlcNAc_2$ was utilised as acceptor substrate. However, neither in wildtype nor in 34S/2 mutants we were able to measure any conversion of the substrate (data not shown). This highly excludes the presence of GnTI isoforms with different substrate specificity as described for *C. elegans* [24]. From previous studies in our laboratories we know that, unlike animal GnTI enzymes, plant GnTI does not utilise $Man_3GlcNAc_2$ substrate very efficiently [3, and unpublished]. For enzymes that act further downstream in the N-glycan biosynthesis pathway it is well documented that they can not bypass GnTI action and utilise substrates like $Man_5GlcNAc_2$ or $Man_3GlcNAc_2$ to produce the observed complex N-glycans [25–29]. Moreover *A. thaliana* plants that are devoid of GnTI activity accumulate $Man_5GlcNAc_2$ without any complex N-glycans [30]. All these data do not favour an alternative N-glycosylation pathway in plants and clearly demonstrate the fundamental role of GnTI in plants as a prerequisite for the production of hybrid and complex N-glycans.

The second more likely explanation for our results is the presence of surplus GnTI activity. Normal levels of GnTI activity may at least under standard laboratory conditions, be far higher than required to act on every glycoprotein passing through the Golgi assembly line. Support for this hypothesis comes for example from the analysis of Lec1A CHO cell mutants [31,32]. Lec1A CHO glycosylation mutants have due to point mutations within the coding sequence a structurally altered GnTI enzyme with reduced affinity for acceptor as well as donor substrate. While Lec1A isolates displayed under standard assay conditions only 2% relative GnTI activity, considerable amounts of complex N-glycans were produced. Moreover, an RNAi approach to downregulate GnTI in *C. elegans* did not produce any phenotype [33] and overexpression of GnTI either in mammalian cells [31] or in plants [5,6] did not result in an increased amount of complex N-glycans.

In contrast to mammals where GnTI knockout mutants are not viable this enzyme seems to be dispensable in *A. thaliana* since GnTI mutants do not seem to have any obvious phenotype [30] indicating different roles of complex N-glycans in mammals and plants. Line 34S/2 is viable and does not show any morphological phenotype under standard growing conditions. However, the unusual high-number of primary transformants that did not produce seeds still raises the question, whether these sterility is linked to the knockdown of GnTI to levels where it affects the N-glycan structures. It can not be ruled out that a complete knockout of GnTI *in N. benthamiana* plants might have a different phenotype than in *A. thaliana*. This phenotype might be due to differences in the amount of Lewis A structures. Total N-glycans from *N. benthamiana* contain detectable amounts of Lewis A structures (see Table 2), whereas in mass spectra derived from total N-glycans of *A. thaliana* no Lewis A epitopes are detectable [34,35]. The identification of other plant species with a GnTI knockout and further characterisation of GnTI silencing lines should help to unambiguously identify the role of complex N-glycans in plants.

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