Molecular basis of *N*-acetylglucosaminyltransferase I deficiency in *Arabidopsis thaliana* plants lacking complex N-glycans

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GnTI (*N*-acetylglucosaminyltransferase I) is a Golgi-resident enzyme essential for the processing of high-mannose to hybrid and complex N-glycans. The *Arabidopsis thaliana cgl* mutant lacks GnTI activity and as a consequence accumulates oligomannosidic structures. Molecular cloning of *cgl* GnTI cDNA revealed a point mutation, which causes a critical amino acid substitution ($Asp^{144} \rightarrow Asn$), thereby creating an additional N-glycosylation site. Heterologous expression of *cgl* GnTI in insect cells confirmed its lack of activity and the use of the N-glycosylation site. Remarkably, introduction of the $Asp^{144} \rightarrow Asn$ mutation into rabbit GnTI, which does not result in the formation of a new N-glycosylation site, led to a protein with strongly reduced, but still detectable enzymic activity. Expression of Asn^{144} rabbit GnTI in *cgl* plants could partially restore complex N-glycan formation. These results indicate that the complete deficiency of GnTI activity in *cgl* plants is mainly due to the additional N-glycan, which appears to interfere with the proper folding of the enzyme.

Key words: *Arabidopsis thaliana*, baculovirus, *N*-acetylglucosaminyltransferase I, N-glycan, N-glycosylation, UDP-GlcNAc.

INTRODUCTION

In eukaryotes, a highly conserved multistep pathway leads to the covalent modification of newly synthesized cell-surface and secreted proteins with N-glycosidically linked oligosaccharides. The process of N-linked protein glycosylation is initiated in the ER (endoplasmic reticulum) by the transfer of a $Glc_3Man_9GlcNAc_2$ precursor to asparagine residues in the sequence Asn-Xaa-Ser/Thr of the nascent polypeptide. Subsequently, this glycan is subjected to extensive trimming by glycosidases in the ER and in the Golgi apparatus, which results in the formation of oligomannosidic N-linked glycans. The latter structures undergo further processing in the Golgi apparatus and are ultimately converted into hybrid and complex type oligosaccharides [1]. The formation of hybrid and complex N-glycans is initiated by the Golgi-resident glycosyltransferase, GnTI (*N*-acetylglucosaminyltransferase I; EC 2.4.1.101). GnTI transfers a GlcNAc residue from UDP-GlcNAc in β1,2-linkage to the acceptor substrate $Man_5GlcNAc_2$ [Manα1-6(Manα1-3)Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc] to produce $GlcNAcMan_5GlcNAc_2$, which is a prerequisite for the subsequent action of all other processing enzymes. In the absence of GnTI, glycoprotein-bound N-glycans are therefore entirely of the oligomannosidic type. Early steps of protein N-glycosylation, including the action of GnTI, are conserved between mammals and plants, whereas the ultimate processing steps are different. Plants produce complex N-glycans with β1,2-xylose and α1,3-fucose linked to the conserved core oligosaccharide, which may also carry terminal Lewis^a structures [2].

Whereas oligomannose structures are found in all eukaryotes, hybrid and complex N-glycans are characteristic of multicellular organisms. In fact, complex N-glycans play an important role in mammalian development. Deficiency of GnTI is embryonally lethal in mice and the lethality is accompanied by several morphologic and developmental abnormalities [3,4]. For plants, it is generally assumed that hybrid and complex N-glycans are not required for the normal development. The *Arabidopsis thaliana cgl* mutant, which lacks GnTI activity and as a consequence does not contain processed N-linked oligosaccharides, is viable, fertile and shows no obvious phenotype under standard growth conditions [5]. By overexpression of human GnTI in the *cgl* mutant, production of complex N-glycans could be restored, which shows that the defect in the *cgl* mutant is solely due to the lack of GnTI activity [6]. However, the nature of the mutation responsible for the complete deficiency of GnTI activity in *cgl* plants has not been identified yet. In the present study, we present the molecular basis of the *cgl* mutation and establish its effects on structure and function of the enzyme.

EXPERIMENTAL

Plant material

A. thaliana wild-type plants (ecotype Columbia) and *cgl* line C5#5, which was obtained from the Nottingham Arabidopsis Stock Centre (stock number: N6192), were grown in soil in a greenhouse with a 16 h light/8 h dark cycle at a temperature of 22 °C.

Cloning of *A. thaliana* GnTI cDNAs

Total RNA was isolated from 3 week old *A. thaliana* wild-type and *cgl* seedlings using TRIzol® (Invitrogen). Reverse transcriptase reactions were performed with 500 ng of total RNA using oligo(dT) primers and avian myeloblastosis virus reverse transcriptase (Promega). PCR was performed with Pfu (*Pyrococcus furiosus*) DNA-polymerase (Promega) and GnTI-specific primers

Abbreviations used: CTS, cytoplasmic-transmembrane-stem; endo H, endo-β-*N*-acetylglucosaminidase H; ER, endoplasmic reticulum; GnTI, *N*-acetylglucosaminyltransferase I; MALDI–TOF, matrix-assisted laser-desorption ionization–time-of-flight; Man_3-octyl, Manα1-6(Manα1-3)Manβ-1-*O*-octyl; $Man_5GlcNAc_2$, Manα1-6(Manα1-3)Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc; Man_5-GP, $Man_5GlcNAc_2$-glycopeptide; PNGase F, peptide N-glycosidase F.

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AthGnT1f (5′-GATCTGGTTGTTTGTCGTCGAT-3′) and AthGnT2r (5′-TGGAGAAGTACATGTTTTGCA-3′). The resulting PCR product was subcloned using a Zero Blunt Topo PCR cloning kit (Invitrogen), sequenced and then used as template for the construction of the expression vectors. DNA sequencing was performed in a thermocycler using the BigDye Terminator 3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, U.S.A.) and an ABI Prism 3100 Genetic Analyzer. The presence of the single point mutation was also detected in PCR products derived from genomic DNA, which was isolated from *cgl* plants using a GenElute Plant genomic DNA kit (Sigma).

Construction of baculovirus vectors

cDNA fragments encoding the putative catalytic domains of *A. thaliana* wild-type and *cgl* GnTI were amplified using the primers AthGnT24 (5′-GCGCGAGCTCCAGATGCCTGTGGCTGCTGTAG-3′) and AthGnT25 (5′-GCGCGGTACCGCATCAGGAATTTCGAATTCC-3′), digested with *Sac*I–*Kpn*I restriction enzymes and ligated into the corresponding sites of the baculovirus transfer vector pVT-Bac-His [7]. Recombinant proteins encoded by this vector contain a cleavable signal peptide, six consecutive histidine residues and an enterokinase cleavage site at the N-terminus and are thus expected to be secreted by baculovirus-infected insect cells. The pVT-Bac-His vector encoding the catalytic domain of rabbit GnTI was kindly supplied by H. Schachter (Hospital for Sick Children, Toronto, Canada). The D144N ($Asp^{144} \rightarrow Asn$) mutation was introduced into rabbit GnTI cDNA using a PCR-based site-directed mutagenesis method following the instructions provided with the Quik Change kit (Stratagene). Briefly, the fragment encoding the catalytic domain of wild-type rabbit GnTI was excised from pVT-Bac-His with *Bam*HI–*Kpn*I restriction enzymes and cloned into vector pQE30 (Qiagen, Chatsworth, CA, U.S.A.). The pQE30-rabbit GnTI construct was used as a template for site-directed mutagenesis using Turbo-Pfu DNA-polymerase (Stratagene) and mutagenic primers GnTID144N-1 (5′-GTCAGCCAGAACTGTGGGCATGAG-3′) and GnTID144N-2 (5′-CTCATGCCCACAGTTCTGGCTGAC-3′). The template was removed by *Dpn*I digestion and the newly created vector pQE30-D144N was recovered by transformation into DH5α bacterial cells. Subsequently, the GnTI sequence was excised from pQE30-D144N with *Sac*I–*Kpn*I and transferred into pVT-Bac-His. After the final cloning step, all the expression constructs were subjected to DNA sequencing to rule out any artificial mutations.

Expression of GnTI in insect cells

All GnTI constructs were expressed in *Spodoptera frugiperda* Sf21 cells as described previously [8]. Cells and conditioned media were harvested and subjected to enzymic analysis and immunoblotting.

Purification of recombinant GnTI from insect cells

Culture supernatants (50 ml) of Sf21 cells infected with the respective baculoviruses were cleared by centrifugation and dialysed twice against 2 litres of 10 mM sodium phosphate buffer (pH 7.0), 40 mM NaCl, 0.02 % NaN_3. A supernatant containing 20 mM imidazole and 10 % (v/v) glycerol was loaded on to a 5 ml column of Chelating Sepharose (Amersham Biosciences) charged with Ni^{2+} ions, equilibrated in the same buffer. After successive washes with 40 and 80 mM imidazole, the enzyme was eluted with 250 mM imidazole in dialysis buffer. After concentration by ultrafiltration, the concentrate was analysed by SDS/PAGE and silver staining. The GnTI content of the sample was estimated by densitometric analysis of the stained gel using BSA as a standard and ImageQuant software (Molecular Dynamics). The total protein content was determined using the bicinchoninic acid protein assay method (Pierce) and BSA as a standard.

GnTI activity assays

GnTI activity assays were performed as described in [8] using as acceptor substrates either 0.5 mM Man_3-octyl [Manα1-6(Manα1-3)Manβ-1-*O*-octyl; Toronto Research Chemicals] or 0.25 mM $Man_5GlcNAc_2$-glycopeptide (prepared from *Aspergillus oryzae* α-amylase; [9]), and as donor substrate 0.1 mM UDP-[^{14}C]GlcNAc (3000–4000 c.p.m./nmol; Amersham Biosciences). After incubation at 37 °C for 1 h, reactions were stopped by the addition of 0.5 ml of 20 mM sodium tetraborate containing 2 mM EDTA. The radioactive product was isolated by anion-exchange chromatography and quantified by liquid-scintillation counting as described in [9]. GnTI activity (1 unit) corresponds to 1 μmol of reaction product formed per minute. Kinetic parameters were determined for purified GnTI proteins by a series of reciprocal velocity–substrate plots at five different concentrations of the substrates.

Immunoblot analysis and endoglycosidase digestion

Cell lysates, culture supernatants of infected Sf21 cells or purified proteins were subjected to SDS/PAGE (12.5 % gel) under reducing conditions. Separated proteins were either stained according to the Bio-Rad silver-staining method or blotted on to Hybond-ECL® nitrocellulose membrane (Amersham Biosciences), blocked with 5 % (w/v) non-fat skimmed milk in PBS and detected with a 1:5000 dilution of mouse anti-Xpress (anti-enterokinase cleavage site) monoclonal antibody (Invitrogen). The detection was performed after the incubation in a 1:10 000 dilution of a horseradish peroxidase-conjugated goat anti-mouse antibody (Sigma) with SuperSignal West Pico Chemiluminescent substrate (Pierce).

Enzymic deglycosylation with PNGase F (peptide N-glycosidase F) was essentially performed as described in [10]. Briefly, cell lysates (15 μg of protein) or purified enzymes (2 μg) were denatured in the presence of 0.5 % (w/v) SDS and 50 mM 2-mercaptoethanol for 5 min at 95 °C. The samples were subsequently diluted to a final concentration of 50 mM Tris/HCl (pH 8.0) and 20 mM EDTA containing 1.25 % (v/v) Nonidet P40. Samples were divided into two aliquots. One portion served as control and the other was digested with 0.2 unit PNGase F (Roche) for 16 h at 37 °C. Digestion of endo H (endo-β-*N*-acetylglucosaminidase H) was performed in a similar manner. After denaturation, samples were diluted to a final concentration of 75 mM sodium citrate (pH 5.5) containing 1.25 % (v/v) Triton X-100, 50 μg/ml E-64 and 1 mM PMSF. endo H (5 m-units; Roche) was added and the reaction was incubated for 16 h at 37 °C. The deglycosylation reactions were stopped by precipitation with cold methanol, the dried pellets were resuspended in SDS/PAGE sample buffer and analysed by SDS/PAGE and immunoblotting as described above.

Generation of transgenic *A. thaliana* lines

A cDNA fragment encoding the putative CTS (cytoplasmic-transmembrane-stem) region of *A. thaliana* GnTI was amplified by PCR using Pfu DNA-polymerase (Promega) with the primers AthGnT31 (5′-ATTCTAGATGGCGAGGATCTCGTGTGA-3′) and AthGnT30 (5′-GATCACAGCCTGTCCACCTTGAGTGAGTT-3′). Fragments encoding the catalytic domains of rabbit wild-type and D144N GnTI were amplified using the primers RbGnT11 (5′-GGTGGACAGGCTGTGATCCCCATCCTGG-3′) and

RbGnT12 (5′-ATTCTAGATTAAGTCCAACTAGGATCATAG-3′). The PCR products were purified from the gel using a Wizard gel purification kit (Promega) and used as megaprimers for overlap extension PCR [11]. The chimaeric GnTI DNA sequence was amplified using primers AthGnT31 and RbGnT12. Fragments of the expected size were gel-purified and subcloned using the Zero Blunt Topo PCR cloning kit (Invitrogen). The fragments were excised using *Xba*I and cloned into the plant expression vector pPT2, which was derived by removing the β-glucuronidase (GUS) gene from vector pZGA22 [12]. In the vector pPT2, the chimaeric GnTI proteins will be expressed from the strong constitutive cauliflower mosaic virus 35S promoter. The correct assembly of the chimaeric GnTI cDNAs was confirmed by DNA sequencing. The *Agrobacterium tumefaciens* strain UIA143 containing the pMP90 plasmid [13] was used to transform *A. thaliana cgl* plants by the floral dip procedure [14]. Seeds were selected on MS-medium (M5519; Sigma–Aldrich) [15] containing 100 mg/l kanamycin. The integration of the heterologous GnTI sequences was confirmed by PCR from genomic DNA using primers AthGnT31 and RbGnT12. Immunoblotting of proteins extracted from transgenic plants was essentially performed as described previously [16] using an anti-horseradish-peroxidase antibody, which specifically binds complex N-glycans, containing β1,2-xylose and core α1,3-fucose residues.

Preparation of N-linked glycans and MALDI–TOF (matrix-assisted laser-desorption ionization–time-of-flight) MS

Fresh rosette leaves (500 mg) were grounded 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. Glycopeptides were enriched from the supernatant by cation-exchange chromatography and gel filtration as described previously [17]. N-glycans were subsequently released from glycopeptides with PNGase 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, Santa Fe, NM, U.S.A.) linear time-of-flight mass spectrometer capable of dynamic extraction using 2,5-dihydroxybenzoic acid as the matrix.

RESULTS

Analysis of total N-glycans from *A. thaliana cgl* plants

To confirm the absence of complex N-glycans in *cgl* plants, we analysed total N-glycans isolated from protein extracts of leaves by MALDI–TOF-MS. As expected, all complex N-glycans are absent in *A. thaliana cgl* protein extracts and instead a major peak of $Man_5GlcNAc_2$ N-glycans (74.5 %) is present (Table 1). These results clearly confirm the complete lack of any GnTI activity in the *cgl* line.

Molecular basis of the *cgl* mutation

As judged from Northern-blot analysis, GnTI transcripts from *cgl* plants are of the same size as wild-type *A. thaliana* GnTI mRNA, indicating that a point mutation or small deletion accounts for the defect [18]. To elucidate the molecular basis of the *cgl* mutation, we amplified GnTI cDNA prepared from wild-type and *cgl* plants by PCR using primers that flank the coding region. The PCR products from both wild-type and *cgl* plants were of the expected size of 1.4 kb (results not shown). The coding sequence of *A. thaliana* wild-type GnTI was identical with the published sequence [19]. The *cgl* GnTI coding sequence was identical with

Table 1 MS analysis of N-glycans from *A. thaliana* leaves

Relative amounts of total N-glycans detected in *A. thaliana* wild-type and *cgl* plants after MALDI–TOF-MS analysis are shown. Results are means for three independent experiments. See [17] for structural details. n.d., not detectable.

m/z (M + Na)+	Compound	Wild-type (%)	*cgl* (%)
Complex-type structures with fucose and/or with xylose			
1065.7	$Man_3XylGlcNAc_2$ (MMX)	1.6	n.d.
1137.0	$GlcNAcMan_3GlcNAc_2$ (GnM/MGn)	1.4	n.d.
1212.1	$Man_3XylFucGlcNAc_2$ (MMXF)	26.4	n.d.
1269.1	$GlcNAcMan_3XylGlcNAc_2$ (GnMX/MGnX)	1.5	n.d.
1415.5	$GlcNAcMan_3XylFucGlcNAc_2$ (GnMXF/MGnXF)	15.0	n.d.
1472.1	$GlcNAc_2Man_3XylGlcNAc_2$ (GnGnX)	1.4	n.d.
1618.5	$GlcNAc_2Man_3XylFucGlcNAc_2$ (GnGnXF)	26.6	n.d.
Sum		73.9	
Oligomannosidic structures			
933.8	$Man_3GlcNAc_2$ (Man3)	n.d.	n.d.
1096.0	$Man_4GlcNAc_2$ (Man4)	n.d.	1.1
1258.4	$Man_5GlcNAc_2$ (Man5)	10.8	74.5
1420.2	$Man_6GlcNAc_2$ (Man6)	6.1	7.1
1582.4	$Man_7GlcNAc_2$ (Man7)	2.7	7.2
1744.5	$Man_8GlcNAc_2$ (Man8)	3.5	6.7
1907.1	$Man_9GlcNAc_2$ (Man9)	3.0	3.4
Sum		26.1	100.0

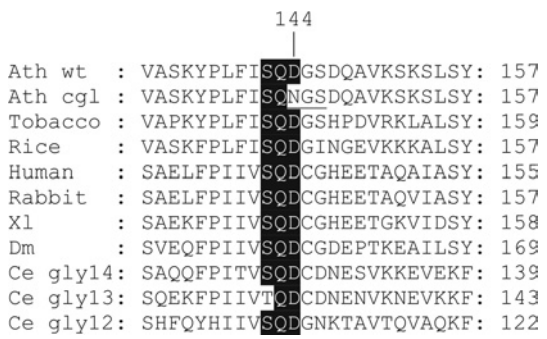

Figure 1 Alignment of selected GnTI amino acid sequences

The sequence surrounding the Ser-Gln-Asp (SQD) motif is shown. Ath wt, *Arabidopsis thaliana* wild-type; Ath cgl, *Arabidopsis thaliana cgl* mutant; Xl, *Xenopus laevis*; Dm, *Drosophila melanogaster*; Ce, *C. elegans*. The alignment was done using the MegAlign sequence analysis tool from the DNAstar software package. The conserved SQD motif is shaded in black. The newly created N-glycosylation site is underlined in the cgl sequence. Asp^{144} is conserved in GnTI sequences across species.

the wild-type sequence except that nucleotide G430 is changed to A430. This single point mutation results in the amino acid substitution of aspartic acid at position 144 with asparagine and thus disrupts the conserved SQD motif present in all GnTI sequences characterized so far (Figure 1). In addition, the D144N mutation creates a new potential N-glycosylation site. The single point mutation was also detected in genomic DNA amplified from *cgl* plants.

Recombinant *cgl* GnTI displays no detectable enzymic activity

To confirm that *A. thaliana cgl* GnTI indeed lacks enzymic activity and to investigate whether the new N-glycosylation site is utilized, we expressed both wild-type *A. thaliana* and *cgl* GnTI in insect cells using the baculovirus expression system. Soluble forms of wild-type and *cgl* GnTI were fused to a leader sequence containing a cleavable signal peptide, a His_6-tag and an enterokinase cleavage site. Recombinant virus was used to infect

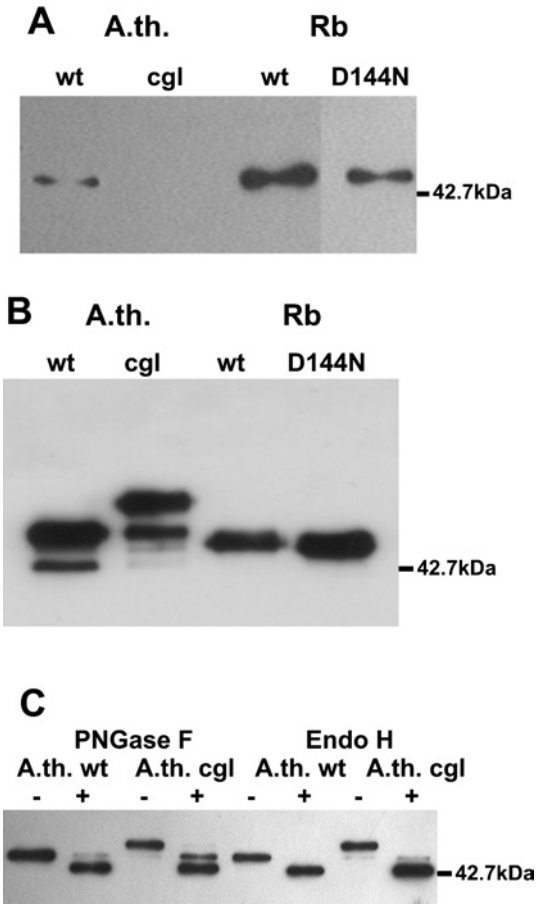

Figure 2 Expression of recombinant GnTI in insect cells

Conditioned media (**A**) and cell lysates (**B**) of Sf21 cells expressing the catalytic domain of GnTI from *A. thaliana* wild-type (A.th. wt), *A. thaliana cgl* (A.th. cgl), rabbit wild-type (Rb wt) and rabbit D144N (Rb D144N) GnTI were analysed by immunoblotting with antibodies to the enterokinase cleavage site. No reaction was observed with lysates from uninfected or mock-infected cells. (**C**) Endoglycosidase digestion of insect cell extracts expressing *A. thaliana* wild-type (A.th. wt) or *cgl* (A.th. cgl) GnTI. Proteins were incubated either with PNGase F or endo H and were detected by immunoblotting.

Spodoptera frugiperda Sf21 insect cells. Expression of wild-type *A. thaliana* GnTI was monitored in cell lysates and conditioned media by immunoblotting. Although a single band was visible in the conditioned media (Figure 2A) two protein bands were detected in cell lysates (Figure 2B). The secreted enzyme and the major intracellular form were found to be 1–2 kDa larger than anticipated, providing evidence for the presence of an N-glycan. This indicates that the single N-glycosylation site present at position 351 of wild-type *A. thaliana* GnTI is almost quantitatively occupied. In contrast with wild-type GnTI the *cgl* protein was detected exclusively in cell lysates (Figures 2A and 2B). Two bands were visible, with the major form being slightly larger than glycosylated wild-type GnTI. This argues for the additional use of the newly generated N-glycosylation site at position 144. To confirm the presence of N-glycans, the heterologously expressed proteins were subjected to endoglycosidase treatment. On digestion with either PNGase F or endo H a shift in the mobility of the major form of wild-type *A. thaliana* GnTI was visible (Figure 2C), which demonstrates the presence of a predominantly oligomannosidic N-glycan. Endoglycosidase digestion of *cgl* GnTI also led to co-migration of the major form of the protein with the non-glycosylated wild-type enzyme (Figure 2C), confirming the presence of two N-glycans of the high-mannose type, one linked to Asn^{144} and the other attached to Asn^{351}. In contrast, secreted wild-type *A. thaliana* GnTI was sensitive to PNGase F, but not endo H (results not shown), thus carrying a complex N-glycan as is typical for glycoproteins which have passed through the Golgi apparatus. Since high-mannose N-glycans are mostly found on ER-resident glycoproteins, the exclusive presence of such structures on *cgl* GnTI indicates that this protein is quantitatively retained within the ER.

Table 2 GnTI activity and protein content in crude lysates and supernatants

GnTI activity was determined with Man_5-GP (Man_5) as acceptor substrate. The GnTI protein content of the samples was assessed by immunoblotting with antibodies to the enterokinase cleavage site present in the recombinant proteins and is given as the percentage of the total amount of GnTI protein detected in the individual cultures. Results are means for duplicate determinations. n.d., not detectable.

	GnTI activity (Man_5) [nmol · h^{-1} · (mg of total protein)$^{-1}$]		GnTI protein content (%)	
	Lysate	Supernatant	Lysate	Supernatant
A. thaliana wild-type	3.8	102	72	28
A. thaliana cgl	1.6	n.d.	100	n.d.
Rabbit wild-type	17.9	2059	18	82
Rabbit D144N	2.2	n.d.	30	70
Mock infected	1.6	n.d.	n.d.	n.d.

Crude cell lysates and conditioned media were assayed for GnTI activity *in vitro* using a radiometric assay and Man_5-GP ($Man_5GlcNAc_2$-glycopeptide) as acceptor substrate. Cell lysates from Sf21 cells expressing wild-type *A. thaliana* GnTI as well as conditioned media thereof showed significant amounts of GnTI activity. The specific activity in cell lysates was 3.8 (nmol of product formed) · h^{-1} · (mg of total cellular protein)$^{-1}$. More than 95 % of wild-type *A. thaliana* GnTI activity was found in the conditioned media [102 nmol · h^{-1} · (mg of total cellular protein)$^{-1}$]. Interestingly, the cell lysate contained 72 % of the total enzyme protein as estimated by immunoblotting, demonstrating that a large fraction of the intracellular enzyme is catalytically inactive (Table 2). In contrast, *cgl* GnTI displayed no detectable activity in the media and the activity in the cell lysates was as low as the background from endogenous insect GnTI in mock-infected cells [1.6 nmol · h^{-1} · (mg of total cellular protein)$^{-1}$]. Essentially the same results were obtained when the samples were assayed with Man_3-octyl, another standard GnTI acceptor substrate (results not shown). Attempts were made to isolate *cgl* GnTI from culture supernatants by means of affinity chromatography, exploiting the His_6-tag of the recombinant protein. However, the purified fractions did not contain any GnTI protein as determined by silver staining and immunoblots, which corroborates the initial finding that *cgl* GnTI is not secreted. In contrast, wild-type *A. thaliana* GnTI could be readily purified from the respective supernatants. The specific activity of purified wild-type *A. thaliana* GnTI with Man_5-GP and Man_3-octyl as acceptor substrates was found to be 3.42 units/mg and 0.09 unit/mg respectively. The K_m (app) values for Man_5-GP, Man_3-octyl and UDP-GlcNAc were calculated to be 0.140, > 3.0 and 0.050 mM respectively.

D144N rabbit GnTI displays weak enzymic activity

The results presented above indicate that the inactivity of *A. thaliana cgl* GnTI may either be due to the additional N-glycosylation site or the D144N mutation. The alteration of the highly

Table 3 Kinetic analysis of purified recombinant GnTI

GnTI activity was determined with either Man_5-GP (Man_5) or Man_3-octyl (Man_3) as acceptor substrates. K_m values were deduced from double-reciprocal plots at five different concentrations of the substrates. All values represent the means ± S.D. for 2–3 independent experiments. n.a., not analysed.

	K_m (app) (mM)		
Source	Man_5	Man_3	UDP-GlcNAc (Man_5)
Rabbit wild-type	0.190 ± 0.017	0.784 ± 0.091	0.080 ± 0.022
Rabbit D144N	1.284 ± 0.038	n.a.	0.258 ± 0.030
A. thaliana wild-type	0.140 ± 0.023	> 3.0	0.050 ± 0.01

conserved SQD motif might change the affinity for the donor substrate UDP-GlcNAc, since it is known from the crystal structure of rabbit GnTI that this motif interacts with the uracil ring of the donor substrate [20]. To address this question, the *cgl* mutation was introduced into rabbit GnTI by site-directed mutagenesis. For the rabbit enzyme, the substitution of aspartic acid at position 144 with asparagine does not create a new N-glycosylation site (see Figure 1). The introduced mutation was confirmed by DNA sequencing and the catalytic domain of the protein was expressed in insect cells as described above. Both wild-type rabbit GnTI and the D144N variant could be detected in crude cell lysates and conditioned media as a single band (Figure 2). The secretion of D144N GnTI was only marginally reduced when compared with wild-type rabbit GnTI. Neither polypeptide showed a mobility shift on PNGase F digestion (results not shown), confirming the absence of N-glycans. For D144N GnTI, only marginal enzymic activity could be detected in crude cell lysates using Man_5-GP as substrate [2.2 nmol · h^{-1} · (mg of total cellular protein)$^{-1}$ compared with 1.6 nmol · h^{-1} · (mg for mock-infected cells)$^{-1}$], whereas the GnTI activity of the corresponding culture supernatant was below the detection limit (Table 2). The specific activity of wild-type rabbit GnTI was found to be 17.9 nmol · h^{-1} · mg^{-1} in cell lysates and 2059 nmol · h^{-1} · mg^{-1} in conditioned media under the same assay conditions. This indicates that D144N rabbit GnTI displays a strongly reduced enzymic activity when compared with the wild-type protein. Both GnTI forms were purified from conditioned media to investigate their enzymic properties in more detail (Table 3). Purified D144N GnTI displayed low, but clearly detectable levels of enzymic activity. The calculated specific activity of the purified D144N protein was 0.35 % of wild-type rabbit GnTI (0.02 unit/mg versus 5.7 units/mg using Man_5-GP as acceptor substrate). The D144N mutation reduced the affinity of rabbit GnTI towards the donor substrate UDP-GlcNAc [3.2-fold increase in K_m (app)] and also increased the K_m (app) for Man_5-GP (6.8-fold).

Expression of rabbit D144N GnTI in *cgl* plants

Transgenic plants were generated to investigate whether rabbit D144N GnTI could compensate for the lack of GnTI activity in the *cgl* line. Transformation constructs contained either the catalytic domain (amino acids 106–447) of wild-type or D144N rabbit GnTI fused to the CTS region (amino acids 1–102) of *A. thaliana* GnTI under the control of the strong constitutive cauliflower mosaic virus 35S promoter. The fusion to the autologous *A. thaliana* CTS region was made to facilitate correct Golgi targeting and retention. Transgenic plants were selected by means of resistance to kanamycin, and successful genomic integration of the heterologous GnTI genes was confirmed by PCR (results not shown). Positive lines were analysed by immunoblots using antibodies which bind to β1,2-xylose- and α1,3-fucose-

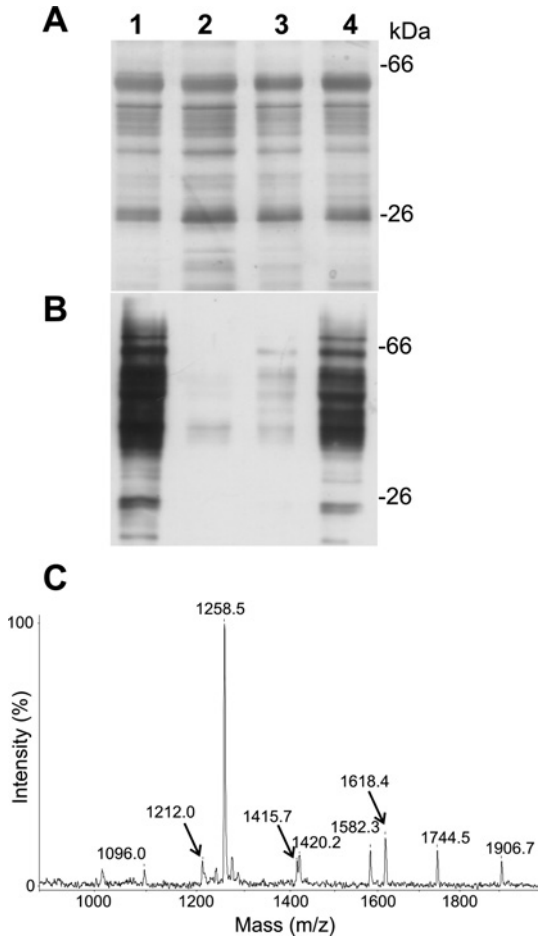

Figure 3 Complementation of the *A. thaliana cgl* mutant by wild-type and D144N rabbit GnTI

Proteins from leaves were separated by SDS/PAGE, silver stained (**A**) and detected by immunoblotting using antibodies directed against β1,2-xylose and α1,3-fucose containing N-glycans (**B**). 1: *A. thaliana* wild-type plants, untransformed; 2: *cgl* plants, untransformed; 3: *cgl* plants expressing D144N rabbit GnTI; 4: *cgl* plants expressing wild-type rabbit GnTI. (**C**) MALDI–TOF MS analysis of *cgl* plants expressing D144N rabbit GnTI. Peaks representing complex N-glycans are marked with arrows (see Table 1 for the corresponding structures).

containing complex N-glycans. *Cgl* plants containing the wild-type rabbit GnTI construct displayed a staining comparable with *A. thaliana* wild-type plants. Transgenic *cgl* plants expressing D144N rabbit GnTI showed significant, albeit weak staining (Figure 3), indicating that the expression of the latter is capable of partially complementing the GnTI deficiency of *cgl* plants. This was confirmed by MALDI–TOF-MS analysis (Figure 3C), which revealed that 15.8 % of the endogenous N-glycans are of the complex type.

DISCUSSION

In the present study, we show that *cgl* GnTI differs from the wild-type *A. thaliana* enzyme at a single position (D144N), with the affected residue being part of the highly conserved SQD motif. X-ray crystal-structure analysis of wild-type rabbit GnTI indicated that Asp^{144} is involved in the binding of the donor substrate UDP-GlcNAc and the metal ion required as a cofactor. The observed reduced affinity of D144N rabbit GnTI for the donor substrate UDP-GlcNAc demonstrates that the mutation clearly affects nucleotide sugar binding. The loss of the negative charge

of Asp^{144} is expected to perturb a key hydrogen bond required for the stabilization of the enzyme–substrate complex. It has been suggested that the catalytic mechanism of GnTI is based on an ordered sequential reaction in which UDP-GlcNAc binds first to the enzyme and then to the $Man_5GlcNAc_2$ acceptor. The product, $GlcNAcMan_5GlcNAc_2$, is subsequently released and UDP leaves last [21]. This reaction mechanism is supported by data derived from the crystal structure of rabbit GnTI [20] as well as by a quantum mechanical model [22]. Interestingly, the binding of UDP-GlcNAc leads to certain conformational changes within the enzyme, which are necessary for the creation of the acceptor-binding site. Our finding that the mutation of a residue involved in binding of the donor substrate results in a concomitant increase in the K_m (app) value for the acceptor substrate clearly corroborates these data.

Two additional mutants have been described with weak GnTI activity [23]. These Chinese-hamster ovary cell mutants (Lec1A) displayed increased K_m (app) values for $Man_5GlcNAc_2$ and UDP-GlcNAc [24,25]. One mutation, R303W, appears to destabilize a structural element critical for catalysis. The other mutation, D212N, is part of the conserved EDD motif, which, similar to Asp^{144}, has critical interactions with UDP-GlcNAc and the metal ion [23]. Hence it is intriguing to observe that the D144N and D212N mutations exert similar effects on the catalytic properties of the enzyme.

The D144N substitution is not sufficient to explain the complete lack of GnTI activity in *A. thaliana cgl* plants. We expressed *cgl* GnTI as a soluble protein in insect cells and found the enzyme to be quantitatively retained within the cells. Endoglycosidase treatment of recombinant *cgl* GnTI showed that Asn^{144} is modified with an N-linked oligosaccharide. These experiments also revealed that both *cgl* GnTI N-glycans are of the oligomannosidic type, a typical hallmark of ER-resident proteins. This indicates that recombinant *cgl* GnTI is trapped within the ER and hence does not reach later compartments of the secretory pathway. Therefore the attachment of an oligosaccharide at position Asn^{144} seems to interfere with the correct folding of GnTI and as such accounts for the complete deficiency of GnTI activity in *A. thaliana cgl* plants.

So far two point mutations, C123R and G320D, have been described that completely inactivate mammalian GnTI [26,27]. The Gly^{320} seems to be crucial for the proper binding of acceptor substrates as judged from the crystal structure of rabbit GnTI. The other amino acid, Cys^{123}, is not located in the active site of the enzyme. This suggests that the C123R substitution rather interferes with proper folding of the protein, resembling the D144N mutation of *A. thaliana cgl* GnTI in this respect.

In a recent study, it was shown that the reduction of GnTI activity below 3 % of the wild-type level did not significantly affect the formation of complex N-glycans in *Nicotiana benthamiana* plants [28]. In the present study, we have expressed a mutant form of GnTI with an even lower residual activity (0.35 %) in *cgl* plants, which was sufficient for the generation of detectable amounts of complex N-glycans. This corroborates the finding that physiological GnTI levels are far higher than required to act on all glycoproteins passing through the Golgi assembly line.

The importance of hybrid and complex N-glycans for mammals is manifested by the embryonic lethality of GnTI knockout mice as well as by the essential role of GnTI in neuronal viability [3,4,29]. In contrast, little is known about the function of these oligosaccharides in lower multicellular organisms. In *Caenorhabditis elegans*, GnTI null mutations resulted in no obvious phenotype under normal environmental conditions [30]. Hence, GnTI activity seems dispensable for normal nematode development, as previously shown for *A. thaliana*. However, *cgl* plants are hypersensitive to certain kinds of stress [5] and exhibit a slightly longer flowering period [31]. The *cgl* line expressing the rabbit D144N GnTI variant contains intermediate levels of complex N-glycans and may thus provide a valuable tool to investigate further the physiological roles of these oligosaccharides in plants.

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