

# Isolation and Characterization of Plant *N*-Acetylglucosaminyltransferase I (*GntI*) cDNA Sequences. Functional Analyses in the Arabidopsis *cgl* Mutant and in Antisense Plants<sup>1</sup>

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We report on the isolation and characterization of full-length cDNA sequences coding for *N*-acetylglucosaminyltransferase I (GnTI) from potato (*Solanum tuberosum* L.), tobacco (*Nicotiana tabacum* L.), and Arabidopsis. The deduced polypeptide sequences show highest homology among the solanaceous species (93% identity between potato and tobacco compared with about 75% with Arabidopsis) but share only weak homology with human GnTI (35% identity). In contrast to the corresponding enzymes from animals, all plant GnTI sequences identified are characterized by a much shorter hydrophobic membrane anchor and contain one putative *N*-glycosylation site that is conserved in potato and tobacco, but differs in Arabidopsis. Southern-blot analyses revealed that *GntI* behaves as a single-copy gene. Northern-blot analyses showed that *GntI*-mRNA expression is largely constitutive. Arabidopsis *cgl* mutants deficient in GnTI activity also possess *GntI* mRNA, indicating that they result from point mutations. *GntI*-expression constructs were tested for the ability to relieve the GnTI block in protoplasts of the Arabidopsis *cgl* mutant and used to obtain transgenic potato and tobacco plants that display a substantial reduction of complex glycan patterns. The latter observation indicates that production of heterologous glycoproteins with little or no antigenic glycans can be achieved in whole plants, and not in just Arabidopsis, using antisense technology.

In the Golgi apparatus of both plant and animal cells, *N*-acetylglucosaminyltransferase I (GnTI) initiates the formation of complex *N*-linked glycans on secretory glycoproteins that are derived from high-Man glycan precursors synthesized in the endoplasmic reticulum (Kornfeld and Kornfeld, 1985; Kaushal et al., 1988). Early blocks in glycan synthesis in the endoplasmic reticulum are fatal as shown by mutants selected for tunicamycin resistance in the yeast *Saccharomyces cerevisiae* (Barnes et al., 1984), which demonstrates that *N*-glycosylation of proteins is an essential step in eukaryotic cells. At the single cell level, several pleiotropic mutants with defects in Golgi-resident glycan trimming and modification enzymes were isolated by screening mutagenized mammalian cell lines for surviving exposure to specific plant lectins, of which the *lec1* mutation represents a defect in GnTI (for review, see Stanley, 1984). At the whole-organism level, most of these mutations were lethal (e.g. *GntI*-knockout mice die at mid-gestation; Ioffe et al., 1996). This is probably because in mammals, glycoprotein glycans exhibit essential functions such as terminal sialic acid residues are involved in recognition events at the cell surface (Varki, 1997), terminal

Man-6-P modification mediates in targeting of acid hydrolases to lysosomes (Dahms et al., 1989), and carbohydrate moieties also play a role in protein sorting to the apical membrane in epithelial cells (Scheiffele et al., 1995).

The isolation and characterization of GnTI-deficient Arabidopsis *cgl* mutants provided the first evidence that the viability of whole plants can be maintained if small *N*-linked glycoprotein glycans of uniform *N*-acetylglucosamine (GlcNAc)<sub>2</sub>Man<sub>5</sub> structure are present (von Schaewen et al., 1993). Recently, however, Fitchette-Lainé et al. (1997) detected complex glycans with mammalian-type Lewis-a (Le<sup>a</sup>, terminal GalFuc-GlcNAc) modification in higher plants, except in some members of the Cruciferae family like Arabidopsis, and postulated a special role for this epitope in the cell-to-cell communications of most gymnosperms, monocots, and dicots. Hence, it was unclear whether plant species other than Arabidopsis would tolerate the loss of complex glycan modification imposed by, for instance, antisense suppression of the gene encoding GnTI.

To assess *GntI* expression and to devise gene-silencing strategies in higher plants, we isolated several plant-specific *GntI*-cDNA sequences: one from Arabidopsis to allow for further characterization of Arabidopsis *cgl* mutants that are deficient in GnTI-enzyme activity, and those from potato (*Solanum tuberosum* L.) and tobacco (*Nicotiana tabacum* L.) with the goal of generating transgenic solanaceous plants with reduced GnTI levels. The observations indicate

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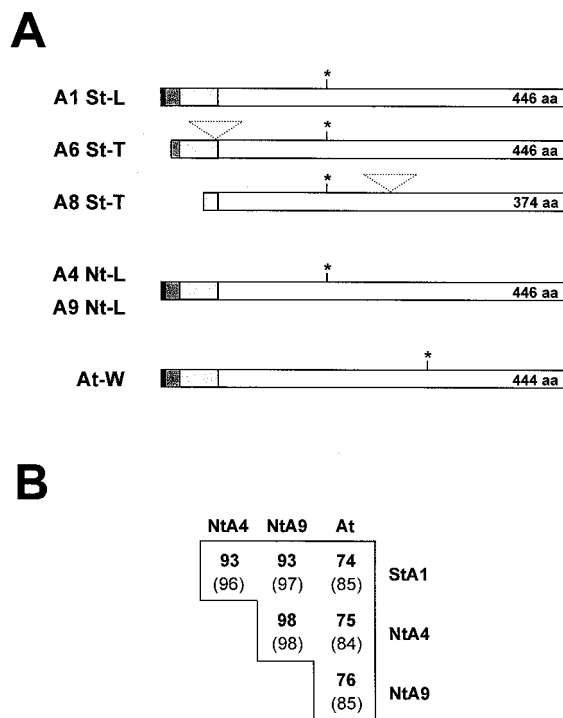
that although *GntI* is present as a single-copy gene in all three plant species investigated, regulation of its expression seems to be complex. *GnTI*-deficient Arabidopsis *cgl* mutant plants also proved useful in expression and complementation analyses. We demonstrate that a drastic reduction of complex-glycan modification by stable antisense suppression of *GntI* did not interfere with the viability of either potato or tobacco transformants, suggesting that the production of therapeutic glycoproteins in agronomically important plant species is feasible.

## RESULTS

### Analysis of the Different Plant *GntI*-cDNA Sequences

Using specific reverse transcriptase (RT)-PCR probes we isolated *GntI*-cDNA sequences from three higher plant species. The characteristics of the deduced *GnTI* primary structures are summarized schematically in Figure 1. From a potato leaf cDNA library, three clones were isolated. Sequence analyses showed that two of the clones were shorter versions of clone A1 (Fig. 1A, A1 St-L). The deduced amino acid sequence of A1 was complete (including 38 bp of 5'-untranslated leader sequence) and matched the general domain structure of a type II membrane protein ( $N_{in}/C_{out}$ ; for review, see Singer, 1990) comprising a short  $NH_2$ -terminal cytoplasmic tail, a single hydrophobic domain (transmembrane anchor), a luminal hinge region, and a large C-terminal catalytic domain. Clone A1 was, therefore, used for further analyses.

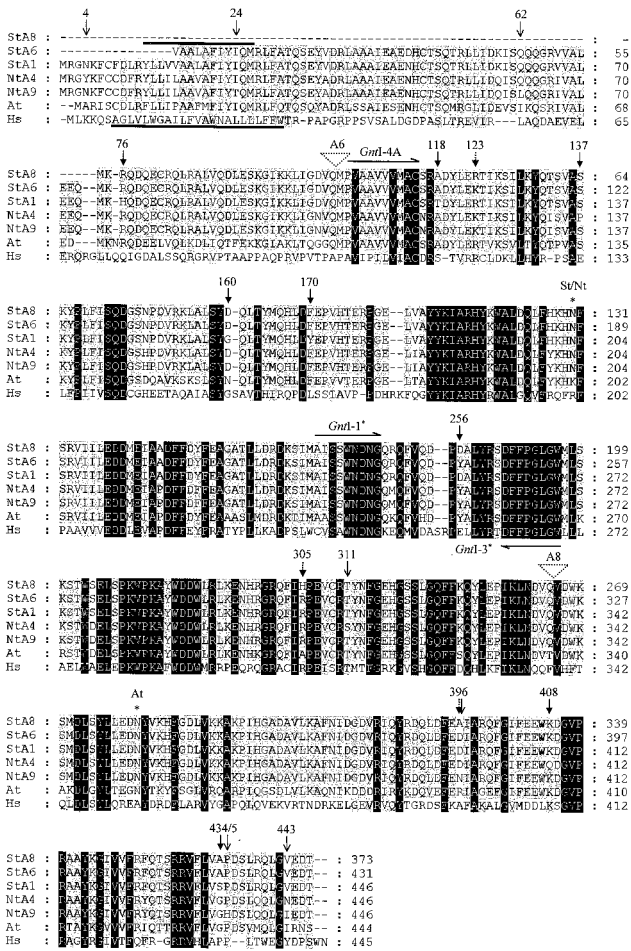
In addition to the above, two 5' incomplete clones were isolated from a potato tuber cDNA library (Fig. 1A, A6/A8 St-T). Compared with the A1 leaf sequence, tuber cDNA A6 lacked 15  $NH_2$ -terminal amino acids and contained an insertion of 103 bp (between DV<sup>104</sup>Q and <sup>105</sup>M, position numbers refer to A1, compare Fig. 2) that matched class II-intron characteristics (data not shown). Tuber cDNA A8 also contained an intron of 106 bp (between DV<sup>338</sup>Q and <sup>339</sup>V, Fig. 2) with an imperfect 5'-splice junction (GC instead of GT, data not shown). Compared with the leaf A1 and tuber A6 cDNA sequences, A8 displayed a completely unrelated 5' region, starting 6 bp upstream of Met codon <sup>74</sup>M in A1 (5'-[*EcoRI*/*NotI*-cDNA linker]-CTG CTC TTG AAG GTG CAC TGT GTT CTC CGG TAA CAA ATG AAG . . . ; the deviating 5' sequence is shown in italics, the in-frame stop codon is highlighted in bold, and the Met codon is underlined). The encoded isoform thus lacked the  $NH_2$ -terminal transmembrane anchor, plus most of the hinge region (Fig. 1A), which should indicate a cytosolic location of the encoded protein. The two tuber sequences, A6 and A8, differed in four amino acid positions from each other (position numbers refer to A1: <sup>123</sup>K↔R, <sup>256</sup>Y↔D, <sup>305</sup>R↔H, <sup>396</sup>D↔A), and in five residues from the A1 leaf sequence (<sup>76</sup>R↔H, <sup>118</sup>A↔T, <sup>160</sup>D↔G, <sup>170</sup>F↔Y, <sup>434</sup>A↔S; see Fig. 2). The



**Figure 1.** Characteristics of the isolated plant *GntI*-cDNA clones. A, Schematic representation of the deduced polypeptide sequences of the isolated plant *GntI*-cDNA clones. Potato leaf (A1 St-L) and tuber clones (A6 and A8 St-T), tobacco leaf (A4 and A9 Nt-L), and the assembled cDNA clone from Arabidopsis whole-plant tissue (At-W). Color code: black, cytosolic region; dark gray, membrane anchor; light gray, stalk region; white box, catalytic domain. The *N*-glycosylation sites are indicated by asterisks. Intron sequences in A6 and A8 are depicted as triangles. B, Homology matrix of the deduced amino acid sequences of the complete plant *GntI*-cDNA clones. Paired alignments were conducted with the GAP option of the Genetics Computer Group software package (Devereux et al., 1984). Percentage values for identical positions are shown in bold and those for similar ones in brackets. St, *S. tuberosum* (potato); Nt, *N. tabacum* (tobacco); At, Arabidopsis.

3'-untranslated regions (UTRs) of the three potato cDNA sequences differed more so. The coding regions shared 98.6% to 99.3% identity at the nucleotide level (starting from common Met codon <sup>74</sup>M in A1). Comparison between the 3'-UTRs of the two tuber cDNAs A6/A8, and tuber cDNA A8/leaf cDNA A1 gives similar values (93.3% and 93.6% identity, respectively), whereas tuber cDNA A6 and leaf cDNA A1 differed most (89.4% identity; data not shown).

Two complete but different *GntI*-cDNA sequences were isolated from a tobacco leaf library (Fig. 1A, A4/A9 Nt-L). A4 displayed a short 5'-untranslated leader (only 26 bp with one mismatch compared with 112 bp in A9) but a much longer 3'-UTR (469 bp compared with 256 bp in A9). Except for 15 bp downstream of the stop codon, the two 3'-UTRs of A4 and A9 differ (91.4% identity compared with 98.75% identity in the coding regions). In a manner similar to the potato leaf and tuber sequences, there were nine



**Figure 2.** Alignment of the deduced plant *GntI*-polypeptide sequences with human *GntI*. Amino acid residues identical in all sequences are highlighted by a black background, those conserved in most of the sequences are marked by a gray background. Hydrophobic regions in the membrane-anchor domains are indicated by a bold line above (plant *GntI*) and underneath (human *GntI*) the NH<sub>2</sub>-terminal sequence block. Primer binding sites are shown as arrows above (sense primers) and underneath (antisense primer) the sequence blocks. Sites of introns in tuber clones A6 and A8 are indicated by triangles. The N-glycosylation consensus sites in the plant sequences are marked by asterisks. St, *S. tuberosum* (potato); Nt, *N. tabacum* (tobacco); At, Arabidopsis (Arabidopsis); Hs, *Homo sapiens* (man). Arrows indicate positions of amino acid differences described in the text. [→] A6 and A8 compared with A1; [←] A6 compared with A8; [↔] A4 compared with A9.

amino acid differences between the polypeptides of the A4 and A9 tobacco leaf clones (<sup>4</sup>Y↔N, <sup>24</sup>I↔T, <sup>62</sup>Q↔L, <sup>137</sup>P↔S, <sup>311</sup>S↔T, <sup>396</sup>D↔N, <sup>408</sup>Q↔K, <sup>435</sup>P↔H, <sup>443</sup>N↔I; see Fig. 2), compared with over 30 differences detected between the leaf sequences of the two solanaceous species (potato A1 compared with tobacco A4 and A9; EMBL accession nos. AJ249878 and AJ249882; Fig. 2). However, in the amphidiploid tobacco genome these differences are thought to be a relic of two separate genomes after hybridization of the two diploid species *Nicotiana sylvestris* and *Nicotiana tomentosiformis* (Narayan, 1987).

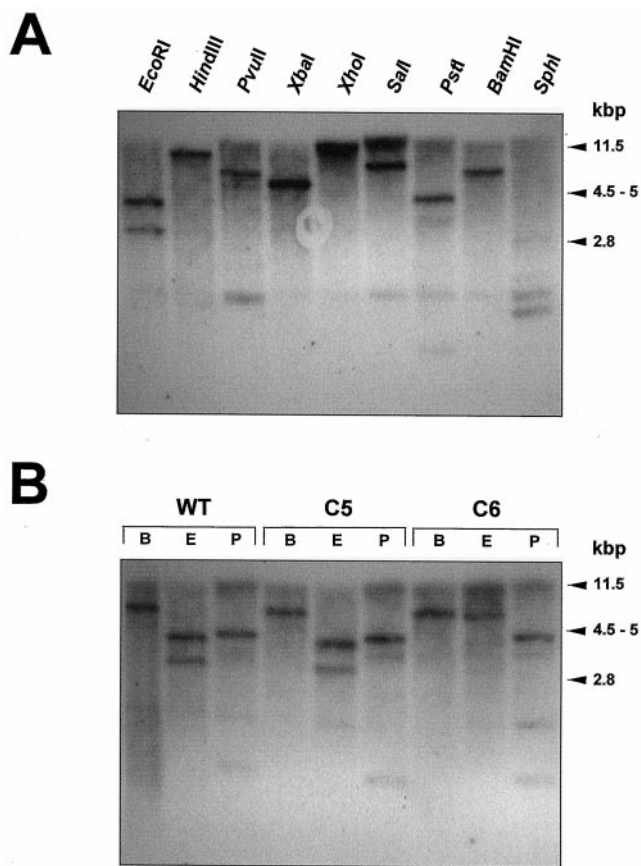
Several *GntI*-cDNA clones with incomplete 5' ends were isolated from a whole-plant Arabidopsis library. The clone with the longest insert was completed by assembly with the missing 5' region (including 116 bp of 5'-untranslated leader sequence) obtained through vector-insert PCR (see "Materials and Methods"; Fig. 1A, At-W).

Figure 1B shows that the overall homologies shared between Arabidopsis *GnTI* and the two solanaceous species (about 75% identity and 85% similarity), as well as between potato and tobacco (93% identity and 96% similarity), and within tobacco (98% identity and 98% similarity), were within the range of values previously determined for other enzymes of primary and secondary plant metabolism (for review, see Wendt et al., 1999, and refs. therein). In contrast, homologies were extremely low, in the range of only 33% to 36% identity and 57% to 59% similarity (data not shown), compared with known animal *GnTI* sequences from man (Kumar et al., 1990), mouse (Pownall et al., 1992), rat (Fukada et al., 1994), and the worm *Caenorhabditis elegans* (Wilson et al., 1994). This is obvious in the alignment of the plant sequences with the human protein, although conserved amino acid blocks are also present (Fig. 2). Compared with human *GnTI*, the NH<sub>2</sub>-terminal transmembrane anchor of the plant sequences is characterized by a much shorter hydrophobic region (15 amino acids compared with 23 amino acids in human *GnTI*; Kumar et al., 1990), which is flanked by conserved positively charged Arg residues on both sides. In addition, all plant *GnTI* sequences identified to date contain one putative N-glycosylation site, which is the same in potato and tobacco (<sup>203</sup>NFS), but differs in context and position from the one in Arabidopsis (<sup>351</sup>NYT).

**Estimation of *GntI* Gene Copy Number in Potato and Arabidopsis**

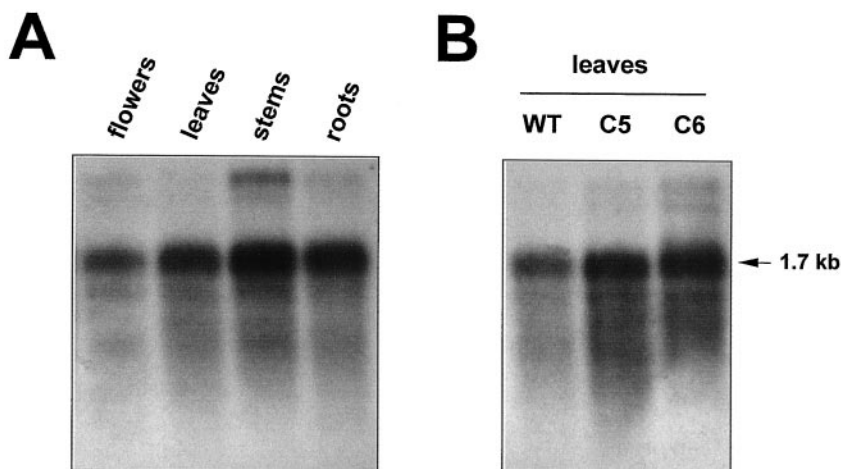
In light of the differences registered between the potato leaf- and tuber-cDNA sequences, we assessed *GntI* gene copy numbers by Southern-blot analyses using the respective plant-specific hybridization probes. With most restriction enzymes employed on genomic DNA, only one band lit up with potato and two with tobacco, which indicated that in the allotetraploid potato genome, *GntI* is present as single-copy gene (data not shown). Figure 3A shows the result obtained with genomic DNA from Arabidopsis: Five of the nine hexamer cutters give rise to a single hybridization signal. The two allelic Arabidopsis *cgl* mutants, C5 and C6, displayed differences in their *EcoRI* restriction patterns (Fig. 3B), which is in agreement with the fact that they result from independent mutation events (von Schaeuwen et al., 1993).





**Figure 3.** Assessment of *Gntl*-copy number in Arabidopsis. Total DNA was isolated from Arabidopsis leaves and 10- $\mu$ g aliquots were digested overnight with the restriction enzymes (100 units of each) indicated above the blots. Samples were concentrated by sodium acetate-ethanol precipitation prior to separation in a 0.7% (w/v) agarose gel and subjected to Southern-blot analyses. A fragment comprising the entire Arabidopsis *Gntl* cDNA was radioactively labeled and used as hybridization probe. Sizes of the molecular mass standard ( $\lambda$ PstI) are indicated. A, Southern-blot analysis of Arabidopsis wild type; B, comparison of the restriction patterns in Arabidopsis wild type and in *cgl* mutant lines, C5 and C6. B, BamHI; E, EcoRI; P, PstI.

**Figure 4.** Analysis of *Gntl*-mRNA expression in Arabidopsis wild type and the *cgl* mutant. Poly(A<sup>+</sup>) mRNA was isolated from 250  $\mu$ g of total RNA and subjected to northern-blot analysis. The radioactively labeled hybridization probe was the same as described for Southern-blot analyses (Fig. 3). A, Different tissues of Arabidopsis wild-type plants; B, leaf samples of Arabidopsis wild type (WT) and Arabidopsis *cgl* mutants C5 and C6. The approximate size of the Arabidopsis *Gntl* mRNA is indicated.

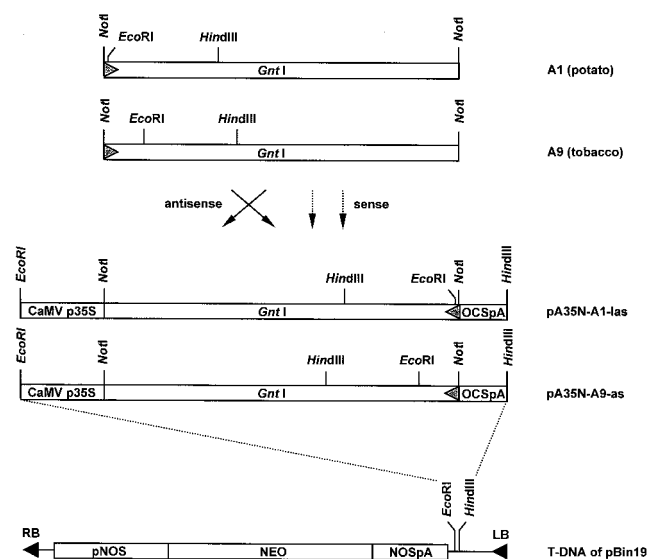


### Analysis of *Gntl*-mRNA Expression

Northern-blot analyses conducted with total RNA indicated that in most plant tissues *Gntl* expression is low and largely constitutive (data not shown). Detection improved upon using poly(A<sup>+</sup>) mRNA, which is shown here for Arabidopsis. The highest *Gntl*-mRNA levels were detected in stems and the lowest was in flowers, whereas comparable expression levels are present in leaf and root tissue (Fig. 4A). In leaves of the two Arabidopsis *cgl* mutant lines, C5 and C6, *Gntl* mRNA was also present. The elevated steady-state mRNA levels in the mutants compared with those in wild-type leaves (Fig. 4B) suggest that in the Arabidopsis *cgl* mutants, *Gntl*-mRNA expression is up-regulated to compensate for missing GnTI activity.

### Complementation Test of *Gntl* Expression Constructs

Complementation of the complex glycan defect in the Arabidopsis *cgl* mutant by a cDNA coding for human GnTI has been reported previously (Gomez and Chrispeels, 1994). To check for functionality of the isolated cDNA sequences prior to generating transgenic plants, cauliflower mosaic virus (CaMV)-35S promoter-driven potato and tobacco *Gntl*-expression cassettes were constructed (Fig. 5). Both potato and tobacco sense and antisense constructs were used for transient expression analyses in protoplasts isolated from leaf tissue of back-crossed "non-stainer" progeny of Arabidopsis *cgl* mutant C5 (von Schaewen et al., 1993). The immunoblot in Figure 6 shows that, compared with a control mock-transformed with phytohemagglutinin (PHA), restoration of complex-glycan staining on high  $M_r$  secretory proteins (compare dotted bracket on the left side of the panel) occurred exclusively with constructs carrying the *Gntl*-cDNA sequence in the sense, but not in the antisense orientation. Strong labeling of bands in the range of 36 to 45 kD was also observed, but only in protoplast samples cultivated for more than 48 h (Fig. 6, lanes 1-5) and not in



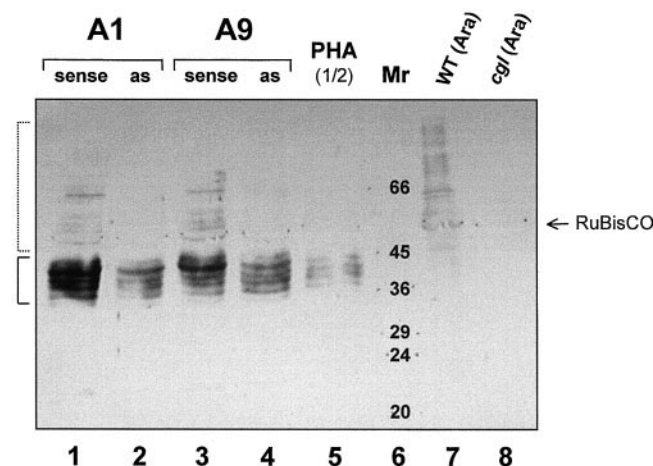
**Figure 5.** Cloning scheme of potato and tobacco *GntI*-sense and -antisense constructs. The complete potato and tobacco *NotI*-cDNA fragments (A1, 1,660 bp; A9, 1,720 bp) were inserted between CaMV-35S promoter (CaMV p35S, approximately 550 bp) and octopine synthase termination sequences (OCSpA, approximately 200 bp) in plant expression vector pA35N (see "Materials and Methods"). Clones with *GntI*-cDNA inserts in sense and antisense orientation, respectively (indicated by arrows), were used for complementation tests (Fig. 6). The *EcoRI*-*HindIII* fragments comprising the entire plant expression cassettes of constructs pA35N-A1-las and pA35N-A9-as, respectively, were inserted into binary vector pBin19 (Bevan, 1984) and used for the generation of transgenic potato and tobacco plants. LB/RB, Left and right T-DNA borders, respectively; pNOS, nopaline synthase promoter driving the neomycin phosphotransferase gene (NEO); NOSpA, nopaline synthase termination sequences.

whole-leaf extracts of *Arabidopsis* wild-type (Fig. 6, lane 7) or *cgl* mutant plants (Fig. 6, lane 8). The labeling of the cultivated protoplasts was most likely due to hemicellulose cell wall precursors of the xyloglucan type (for review, see Hayashi, 1989) synthesized in the Golgi and bound to proteins cross-reacting with the complex glycan antiserum. Staining increased with incubation time (data not shown) and signal strengths were always higher in the sense samples compared with antisense or mock-transformed control samples (Fig. 6; compare sense constructs in lanes 1 and 3 with antisense constructs in lanes 2 and 4, and with the mock-transformed PHA control in lane 5). Relief of the GnTI block in protoplasts transformed with *GntI*-sense constructs (Fig. 6, lanes 1 and 3) resulted in stronger labeling of a subset of the low  $M_r$  bands, indicating that some of the hemicellulose cell wall precursors are bound to glycoproteins containing glycans subject to complex glycan modification.

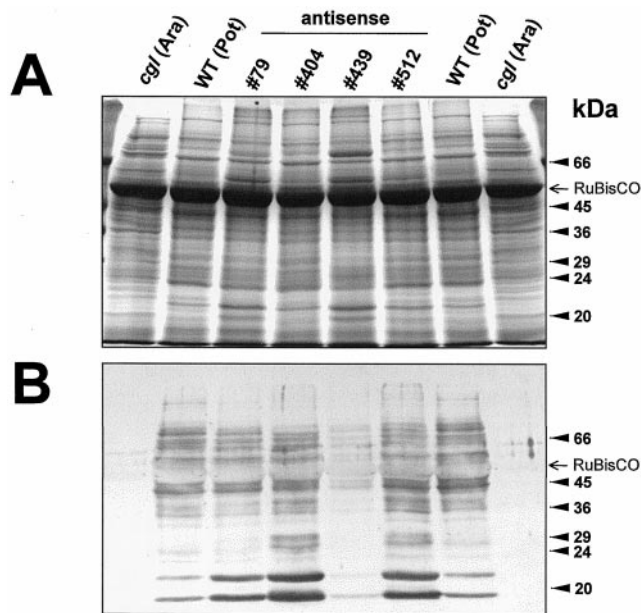
#### Analysis of *GntI* Suppression in Transgenic Plants

Upon verification of the potato and tobacco *GntI*-expression cassettes in the complementation test de-

scribed above, binary vector constructs were generated (Fig. 5) and used for *Agrobacterium tumefaciens*-mediated delivery to leaf discs of the corresponding homologous plant species (i.e. tobacco-antisense constructs for tobacco and potato-antisense constructs for potato). After regeneration of transgenic plants, the extent of *GntI* suppression was assessed indirectly by immunoblot analyses using the complex glycan antiserum on leaf extracts of the primary transformants ( $T_0$  potato,  $n = 512$ ;  $T_0$  tobacco,  $n = 120$ ). In tissue culture, initially four (nos. 79, 404, 439, and 512) of the potato and three (nos. 1, 19, and 20) of the tobacco transformants showed substantial reduction in complex glycan staining compared with untransformed controls. After transfer of these candidates to soil and further propagation, immunoblot analysis was repeated with 3- to 4-week-old plants. The observations are shown for potato. Transformant number 439 repeatedly scored best for reduction of complex glycan formation (Fig. 7), indicating stability of transgene



**Figure 6.** Complementation test of potato and tobacco *GntI*-expression constructs in the *Arabidopsis cgl* mutant. Protoplasts were isolated from 3- to 4-week-old C5 plants and transformed with plasmid DNA using the polyethylene glycol method. Subsequent cultivation was for 115 h at 25°C in the dark. Afterward, cells were chilled on ice, pelleted, and prepared for SDS-PAGE along with leaf samples harvested from *Arabidopsis* wild-type and *cgl* mutant plants. The western blot was stained with Ponceau S to check for comparable transfer and protein amounts (not shown) and developed with a complex glycan antiserum (Laurière et al., 1989). Lanes 1 through 5, Protoplast samples transformed with plasmid DNA of the following plant expression constructs: 1, potato *GntI* (A1) sense; 2, potato *GntI* (A1) antisense; 3, tobacco *GntI* (A9) sense; 4, tobacco *GntI* (A9) antisense; and 5, mock-transformed control (modified PHA-L; von Schaewen et al., 1993). Note that only one-half of the amount used in lanes 1 through 4 was loaded in lane 5 (PHA 1/2). Lane 6, Molecular-mass standard (Sigma SDS VII-L). Lanes 7 and 8, Whole-leaf control samples (50 µg of total protein each): 7, *Arabidopsis* wild type (WT); and 8, *Arabidopsis* mutant C5 (*cgl*). Molecular masses are given in kD. The position of Rubisco (large subunit) is indicated by an arrow. High molecular mass signals ( $\geq 45$  kD bands) resulting from relief of the GnTI block in *cgl* protoplasts are marked by a dotted bracket on the left side of the panel. For origin of low molecular mass signals (36–45 kD bands) marked by a solid bracket, see explanation in the text.



**Figure 7.** Analysis of complex glycan patterns in leaf protein extracts of potato wild-type and selected antisense plants growing in tissue culture. A, Coomassie-stained SDS-gel reference (12% [w/v] polyacrylamide, 75  $\mu$ g of total protein per lane); B, corresponding western blot developed with a complex glycan antiserum. WT (Pot), Untransformed potato wild type; *cgl* (Ara), Arabidopsis *cgl* mutant C5; numbers 79 through 512 (antisense), selected potato transformants. The positions of the molecular mass standard (Sigma SDS VII-L) and of Rubisco (large subunit) are indicated.

expression in this line. It is surprising that within the selected transformants, the extent of complex glycan reduction varied in leaves of different ages and developmental states. Figure 8 shows that antisense suppression of complex glycan formation progresses from primary to fully developed leaves in our strongest (no. 439) and weakest (no. 512) transformants. Thus, the *GntI*-antisense effect is much more pronounced in mature (source) compared with young (sink) leaves.

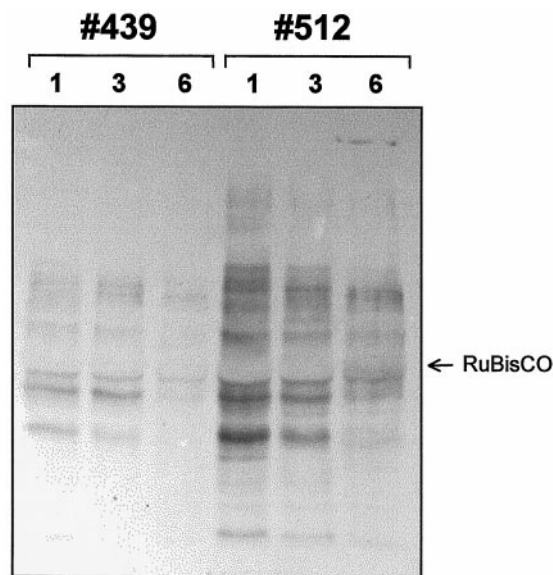
## DISCUSSION

### Characteristics of the Different Plant *GntI*-cDNA Sequences

We report the isolation and characterization of several cDNA clones from three plant species encoding *GntI*, the enzyme that initiates complex-type Asn-linked glycoprotein modification in the Golgi compartment of higher eukaryotes. A greater number of *GntI*-cDNA sequences with significant differences were obtained from potato leaf and tuber libraries of the same cultivar than were obtained for the two leaf cDNAs isolated from tobacco. The intron sequences identified in the two tuber *GntI* sequences indicate that they are derived from precursor mRNA molecules. The different 5' region in tuber cDNA A8 could result from an imperfectly spliced and 5' incomplete pre-mRNA, or it could indicate that *GntI* is also

present in the plant cytosol. Although the putative start codon in the A8 sequence (<sup>74</sup>M in A1, Fig. 2) does not fully comply with the Kozak rules (.ACCATGG..; Kozak, 1989), i.e. positions  $-3$  and  $+4$  should be purines (shown in bold, ACAAATGAAG), position  $-3$  matches the consensus found in a compilation of 79 plant sequences (ACCAATGGCT; Joshi, 1987). In this context, it is noteworthy that an O-linked glycan structure has been identified on a plant nuclear-pore complex protein containing terminal GlcNAc residues preceded by a spacer of several hexoses (Heese-Peck et al., 1995). In contrast, on cytosolic glycoproteins of animals, GlcNAc residues are directly O-linked to the polypeptide backbone at alternatively phosphorylated sites (Hart et al., 1996). Thus, in plants a cytosolic GlcNAc transferase seems to exist. However, whether or not this activity is encoded by the A8 cDNA remains to be shown.

Compared with human *GntI*, the significantly shorter hydrophobic membrane anchor that is flanked by conserved Arg residues on both sides in all plant *GntI* sequences most likely reflects differences in Golgi-membrane structure and *GntI*-retention mechanisms between animals and plants. The fact that the position of the single N-glycosylation site in the plant *GntI* sequences is only conserved among the two solanaceous species, but not in Arabidopsis, argues against their actual use in planta, or at least indicates that they do not play a role other than modulation of the physicochemical properties of the polypeptide (e.g. stabilization of protein conformation; Olden et al., 1985).



**Figure 8.** Progression of complex glycan reduction in terminal leaves of potato *GntI*-antisense plants after growing for 4 weeks in soil. Western-blot analysis was analogous to Figure 7 (only that a 10% [w/v] polyacrylamide gel was used). Numbers 1, 3, and 6 refer to leaves counted from the top of the respective potato plants. Number 439, strongest; number 512, weakest potato *GntI*-antisense plant (compare Fig. 7). The position of Rubisco (large subunit) is indicated by an arrow.



### Evidence for Complex Regulation of *GntI*-Gene Expression

The presence of two *GntI* genes in tobacco is consistent with the amphidiploid nature of the genome. The recently published GntI sequence from tobacco (Strasser et al., 1999) is extremely similar to the A4 cDNA (one amino acid substitution at <sup>408</sup>K↔Q, plus some base changes in the 3'-UTR) and differs in eight amino acid positions from cDNA A9. It is interesting that the differences between the two tobacco *GntI*-cDNA sequences lie in between those values determined for the potato leaf and tuber cDNAs (coding regions without introns starting from common Met codon <sup>74</sup>M in the complete sequences: 98.6% ← 98.75% → 99.3%, and 3'-UTRs: 89.4% ← 91.4% → 93.6%). Despite the dissimilarities in *GntI*-cDNA sequences isolated from different potato tissues, Southern-blot analyses indicate that there is only one *GntI* locus present in all higher plant species investigated to date. For Arabidopsis, this was already suggested by the allelic character of the two independent *cgl*-mutant lines C5 and C6 (von Schaewen et al., 1993).

Due to the amphidiploid nature of the genome, two slightly different *GntI* copies are present in tobacco that are represented by the two cDNA clones A4 and A9 isolated from a tobacco leaf library of the same variety (for assessment of *GntI*-copy numbers in tobacco, see Strasser et al., 1999). However, differences in tissue-specific expression or additional GntI variants could also be created by means of differential splicing. For example, a murine sialyltransferase gene is transcribed from at least three distinct promoter regions that are active in different tissues. Thus, a family of mRNAs with differing 5'-UTRs, but with identical protein-coding domains, is generated from the same gene (Lo and Lau, 1999).

Tissue-specific expression resulting from alternative splicing of the primary transcript has recently also been reported for a single-copy housekeeping gene in Arabidopsis (encoding enoyl-acyl carrier protein reductase; Boer et al., 1999). Another explanation for the differences observed between the potato leaf and tuber *GntI* cDNAs would be nuclear precursor mRNA editing (for review, see Keller et al., 1999). De-amination of adenosine to inosine bases, similar to that which occurs in primary transcripts of Glu receptor channels in mammals (for review, see Seeburg et al., 1998), could create alternative splice sites (as suggested by the imperfect 5'-splice junction of the intron in A8), or give rise to GntI enzymes with slightly different amino acid composition, and thus enlarge the functional repertoire of the genome. Although a full understanding of the underlying mechanism(s) requires comparison with the corresponding genomic sequence, the differences between potato leaf- and tuber-*GntI* cDNAs provide circumstantial evidence that regulation of *GntI*-gene expres-

sion in plants is complex. Also, the fact that one of our tobacco *GntI*-cDNA sequences almost perfectly matches one described in a recent report (Strasser et al., 1999) argues strongly against them being merely the result of copy errors acquired during cDNA synthesis.

### Molecular Analyses Employing Arabidopsis *cgl* Mutants

According to our northern-blot analyses, *GntI*-mRNA expression in plant tissues is largely constitutive, as has been reported for mammal (Kumar et al., 1990; Pownall et al., 1992; Fukada et al., 1994). This is not surprising for a Golgi-resident glycosyltransferase that belongs to the cellular housekeeping enzymes. Our observation that *GntI* mRNA is produced despite undetectable GntI activity in the two Arabidopsis *cgl* mutants, C5 and C6, is consistent with the mutagen employed (ethyl methanesulfonate is known to predominantly create point mutations; von Schaewen et al., 1993). Moreover, the elevated steady-state mRNA levels detected in the two GntI-deficient Arabidopsis *cgl* mutants suggest that *GntI* expression can be up-regulated on demand. A similar phenomenon has been reported for mutant mammalian cell lines with glycosylation defects that show up-regulation or induction of glycosyltransferase genes to alleviate the effects caused by the mutations (Stanley et al., 1996).

The complementation test of the *GntI* plant expression constructs in protoplasts of the Arabidopsis *cgl* mutant proved that the complete potato and tobacco *GntI*-cDNA sequences encode active enzymes. Besides the expected labeling of bands in the high molecular mass range ( $\geq 45$  kD), labeling was enhanced in the range of 36 to 45 kD, but only upon long-term cultivation of protoplasts. This can be explained by a bona fide cross-reaction of protein-bound cell wall precursors massively synthesized in the Golgi of protoplasts that attempt to regenerate functional cell walls. It is known that hemicellulose of the xyloglucan-type constitutes about 20% in the primary cell wall of young growing dicotyledonous plants (Hayashi, 1989). Thus, it is feasible that the xyloglucan-carbohydrate moieties also bind antibodies of the complex glycan antiserum, which is largely an anti-Xyl/Fuc serum (Laurière et al., 1989). Signal strengths were always higher in sense compared with antisense or mock-incubated control samples. This shows that in protoplasts of the Arabidopsis *cgl* mutant, transiently expressed potato and tobacco GntI enzymes act on components bound to these cell wall precursors, indicating that some of them are glycoproteins that acquire complex glycans due to relaxation of the GntI block.

### Developmental Progression of the *GntI*-Antisense Effect in Transgenic Plants

Subsequent use of the antisense constructs to generate transgenic plants resulted in the identification of potato and tobacco transformants with substantial reduction of complex glycan modification on their secretory glycoproteins. The phenomenon was more pronounced in fully developed source leaves compared with immature sink leaves. A comparable progression or gradient of the antisense effect from young to mature leaves was observed in tobacco following a similar antisense approach for suppressing genes encoding cytosolic Glc-6-P dehydrogenase (C. Lange, R. Hauschild, and A. von Schaewen, unpublished data) and cytosolic isocitrate dehydrogenase (S. Galvez and M. Hodges, personal communication). This was initially interpreted to result from some tissue specificity or sink-source response of the CaMV-35S promoter. However, as we now report similar observations for antisense suppression of *GntI*, it seems that the phenomenon may be associated with genes involved in primary metabolism (i.e. housekeeping genes), which are highly expressed in growing tissues (see Fig. 3 for *GntI* expression in Arabidopsis stem tissue and von Schaewen et al., 1995 for mRNA levels of cytosolic Glc-6-P dehydrogenase).

It is probable that a constant level of antisense message produced from the CaMV-35S promoter in the transgenic plants does not suffice for complete suppression of the corresponding endogenous genes in fast growing organs or metabolic sink situations. This is a potentially important observation that might explain the normal behavior of the potato and tobacco *GntI*-antisense plants under controlled growth conditions since it has been suggested that the Lewis-a epitope detected on complex-type glycans of most higher plant species—except in Arabidopsis and members of the Cruciferae family—might not only play a role in cell-to-cell recognition events in animals, but also in plants (Fichette-Lainé et al., 1997). For a final judgement on this, specific plant-pathogen interaction experiments are necessary. Palacpac et al. (1999) recently demonstrated by a gain-of-function approach that expression of human  $\beta$ -1,4-galactosyltransferase also results in deficiency of Xyl and Fuc residues on plant complex glycans carrying novel terminal Gal residues. However, since the entire study was performed with undifferentiated tobacco BY-2 callus cells, viability of transgenic plants expressing human  $\beta$ -1,4-galactosyltransferase remains to be shown.

Our observations demonstrate that effective reduction of GnTI activity can be achieved in mature tissues of solanaceous plants by means of *GntI*-mediated gene silencing. Thus, the resulting deficiency in highly immunogenic complex-type glycoprotein glycans is tolerated in plant species other than Arabidopsis. This now paves the way for the

production of therapeutic glycoproteins in transgenic, agronomically important plant species carrying minimal compatible *N*-glycans of uniform GlcNAc<sub>2</sub>Man<sub>5</sub> structure, a methodology previously reported for mammalian cell lines (Stanley, 1989).

## MATERIALS AND METHODS

### Plant Material

The following soil-grown plants were used in the experiments described: potato (*Solanum tuberosum* L. var Désirée), tobacco (*Nicotiana tabacum* L. var Wisconsin 38), Arabidopsis var Columbia, and Arabidopsis var Columbia *cgl*-mutant lines, C5 and C6 (von Schaewen et al., 1993).

### Oligonucleotide Primers

Commercial sequencing primers KS and SK (Stratagene, Heidelberg) were used for cDNA-clone analyses. The following designed oligonucleotide primers were ordered from MWG BioTech (Ebersberg, Germany).

#### *GntI*-1\*

Degenerate *GntI*-specific sense primer deduced from animal *GntI* sequences (human, rat, mouse, *Caenorhabditis elegans*, and *Xenopus laevis*), corresponding to region <sup>239</sup>AISSWNDNG in potato GnTI; 26-mer, 5'-TG(CT) G(CT) I (AT)(GC) I GCI TGG (AC) A(CT) GA(CT) AA(CT) GG-3'.

#### *GntI*-3\*

Degenerate *GntI*-specific antisense primer deduced from animal *GntI* sequences (human, rat, mouse, *C. elegans*, and *X. laevis*), corresponding to region <sup>266</sup>DFFPGLGW in potato GnTI; 24-mer, 5'-CCA ICC IT(AG) ICC (AGCT) G(GC) (AG) AA (AG) AA (AG) TC-3'.

#### *GntI*-4A

Degenerate *GntI*-specific sense primer, including two 5'-restriction sites (*Hind*III, underlined; *Bam*HI, in bold), corresponding to region <sup>106</sup>PVAAVVVMAC in potato GnTI; 48-mer, 5'-ATC GGA AAG CTT **GGA TCC** CCA GTG GCC(A/G) GCT GTA GTT GTT ATG GCT TGC-3'.

#### *pACT*-Sense

Vector-specific primer based on the 5' region in the multiple cloning site of two-hybrid vector pACT (Durfee et al., 1993), introducing two restriction sites (*Bam*HI, underlined; *Xho*I, in bold); 27-mer, 5'-ATC TGG AAT TCG GAT **CCT CGA GGC** CAC-3'.

#### *GntI*-Bio

*GntI*-specific antisense primer with *Pst*I restriction site (underlined) and 5'-biotin modification (designed for 5' amplification of Arabidopsis *GntI* cDNA sequences), based



on region <sup>111</sup>MACSRADYL in Arabidopsis *GnTI*; 27-mer, bio5'-TTT CAA GAT AGT CTG CAC GAC TGC AGG-3'.

### cDNA Synthesis and PCR Amplification (RT-PCR)

RT of poly(A<sup>+</sup>) mRNA from total plant RNA was performed as by Graeve et al. (1994), modified by von Schaeuwen et al. (1995).

Standard PCR reactions were run essentially as described by Graeve et al. (1994) using 30 to 50 pmol of each primer, 200 μM dNTPs, and 2 units of *Taq*-DNA polymerase per 50 μL reaction in the buffer supplied (Angewandte Geotechnologie Systeme, Heidelberg).

### Cloning of Species-Specific *GntI*-PCR Products

To obtain optimal hybridization probes for the subsequent plant cDNA-library screenings, degenerate primers corresponding to highly conserved regions in animal *GnTI* sequences were used to amplify plant-specific *GntI*-cDNA fragments from potato and tobacco leaf RNA. The resulting approximately 90-bp RT-PCR products were gel-purified using the Qiaex II Gel Extraction Kit (Qiagen, Hilden, Germany), subcloned in *EcoRV* digested pBSK (Stratagene) by standard procedures (Sambrook et al., 1989), introduced into RbCl-competent (Hanahan, 1983) *Escherichia coli* XL1-Blue cells (Stratagene), and sequenced. The deduced primary structures showed similarity to known *GnTI* sequences from animals (→ Q(R/M)QFVQDP(D/Y)ALYRS ← primers depicted as arrows, homologous amino acid residues underlined). The potato fragment was used as a radiolabeled probe for screening the corresponding leaf- and tuber-cDNA libraries. After the complete amino acid sequence was deduced from potato, degenerate primer *GntI*-4A was designed and used in combination with *GntI*-3\* to amplify longer specific cDNA fragments (492 bp) from Arabidopsis and tobacco RNA. RT-PCR products of the appropriate sizes were gel-purified, subcloned in pGEM-T (Promega, Mannheim, Germany), sequenced, and used as described above.

### Labeling of cDNA Probes

For Southern and northern analyses and for screening of cDNA libraries, *GntI*-cDNA fragments were liberated by restriction digests, gel-purified, and radioactively labeled essentially as described in von Schaeuwen et al. (1995), using <sup>32</sup>P(dCTP) and the HexaLabel DNA Labeling Kit (MBI Fermentas, St. Leon-Rot, Germany).

### Isolation of *GntI* cDNA Clones

Screening of potato leaf- and tuber-cDNA, and tobacco leaf-cDNA libraries constructed in λ ZAP II (Stratagene) with *EcoRI/NotI* adapters (Pharmacia, Freiburg, Germany), and of a whole plant Arabidopsis cDNA library constructed in λ Uni-ZAP (Stratagene) with 5'-*EcoRI* and 3'-*XhoI* cDNA ends, was by standard procedures (Sambrook et al., 1989) using radiolabeled specific *GntI*-cDNA probes

obtained from the appropriate species (described above). In vivo excision of the phagemid clones identified followed the instructions in the Stratagene manual.

### Vector-Insert PCR

Amplification of the 5' end of the Arabidopsis *GntI* sequence was conducted by vector-insert PCR according to Tillmann and Eschrich (1998) using a cDNA library constructed in λACT *XhoI* (Durfee et al., 1993) after quantitative conversion to pACT phagemid DNA. In a final volume of 40 μL, the initial reactions contained different amounts of plasmid DNA, 25 nmol primer *GntI*-bio, dNTPs (200 μM each), and 2 units of *Pfu*-DNA polymerase (Stratagene) in the buffer supplied. Incubation was for 2 min at 95°C followed by 30 cycles for 30 s at 95°C and 3 min at 70°C. Isolation of biotinylated DNA was with 25 μL of Dynabeads M-280 Streptavidin (Dyna, Hamburg, Germany) following the protocol of the supplier. Subsequent PCR conditions were as described above using aliquots of biotinylated DNA as template in combination with primers pACT-sense and *GntI*-bio. Amplified fragments of ≥300 bp were gel-purified, digested with *XhoI* and *PstI*, ligated to the respective ends in pBluescript SK (Stratagene), and cloned in *E. coli* XL1-Blue cells (Stratagene). The open reading frame of the clone with the longest insert (440 bp) contained the complete 5' end of Arabidopsis-*GntI*, and served as the template for PCR amplification with vector primer SK (25-mer) and *GntI*-bio. To assemble the full-length cDNA sequence, the obtained fragments were digested with *XbaI* and *SpeI*, ligated to the respective sites in 5' incomplete Arabidopsis *GntI* cDNA clone 10.2-1, and transformed into *E. coli* XL1-Blue cells. The resulting complete Arabidopsis *GntI* cDNA clone, termed pBSK-Ara*GntI*-full, was used for further analyses.

### DNA-Sequence Analysis

Sequence reactions were conducted as described in Wenderoth et al. (1997). Double stranded sequence analyses of the *GntI* cDNAs were conducted by a commercial sequencing service (MWG BioTech, Ebersberg, Germany). The data were analyzed with appropriate programs of the Genetics Computer Group (Madison, WI) software package (Devereux et al., 1984). Sequence alignments were performed using CLUSTAL W version 1.7 (Thompson et al., 1994) and GeneDoc (Nicholas and Nicholas, 1997) programs.

### Nucleic Acid Analyses

For preparation of nucleic acids, tissue of 3- to 4-week-old plants was frozen in liquid nitrogen. Genomic DNA and total RNA were isolated using a RNA/DNA Midi Kit (Qiagen). Poly(A<sup>+</sup>) mRNA was purified from 250 μg of total RNA using the Oligotex mRNA Mini Kit (Qiagen) and separated in a denaturing agarose gel following standard techniques (Sambrook et al., 1989).

Southern analyses of genomic DNA and preparation of RNA samples for northern analyses were performed as described previously (von Schaeuwen et al., 1995). DNA

fragments were transferred to Qiabrane Plus Nylon membranes (Qiagen) and denatured RNA to Hybond N Nylon membranes (Amersham/USB, Braunschweig, Germany). Nucleic acids were immobilized on the blots by UV-cross linking and probed with radioactively labeled full-length cDNA fragments of the corresponding *GntI*-cDNA clones. Filters were washed three times for 30 min at 68°C with 1× SSC (Sambrook et al., 1989) containing 0.1% (w/v) SDS.

### Generation of Binary Vector Constructs for Plant Transformation

To allow for direct insertion of *NotI* digested cDNA sequences, the expression cassette of pA35S (Höfte et al., 1991) was modified by inserting a *NotI* linker (8-mer, NE-Biolabs, Bad Schwalbach, Germany) into the filled *SalI* site of the multiple cloning region, yielding plant expression vector pA35N. Subsequently, the full-length *NotI* cDNA fragment of potato clone A1 (1,660 bp) was introduced into pA35N. The resulting clones were analyzed for insert orientation with respect to the flanking CaMV-35S promoter and octopine synthase termination sequences, and termed pA35N-A1-s (for sense) and pA35N-A1-las (for long antisense), respectively. The cloning strategy for the tobacco-cDNA fragments was analogous. Insertion of the *NotI*-cDNA fragment of tobacco clone A9 (1,717 bp) into pA35N resulted in constructs pA35N-A9-s and pA35N-A9-as. The constructs were tested for complementation of the complex glycan defect in protoplasts of the *Arabidopsis cgl* mutant. Upon verification of their functionality, the complete expression cassettes were obtained by partial digests and introduced as *EcoRI-HindIII* fragments (2,415 bp for A1 and 2,472 bp for A9, respectively) into the T-DNA region of binary vector pBin19 (Bevan, 1984; compare Fig. 5). The resulting binary constructs were transformed into *Agrobacterium tumefaciens* strain GV2260 (Deblaere et al., 1985) using the freeze-thaw method described by Höfgen and Willmitzer (1988). Purified plasmid DNA of single transformants was checked by restriction digests prior to using selected clones for cocultivation with sterile potato and tobacco leaf discs.

### Transient Expression in Protoplasts of the *Arabidopsis cgl* Mutant

Transient expression of pA35N-*GntI*-sense and -antisense constructs in protoplasts of *Arabidopsis cgl* mutant C5 was performed as described previously (von Schaewen et al., 1990) with the modification that 30 µg of plasmid DNA were used to transform 10<sup>6</sup> protoplasts.

### Leaf-Disc Transformation and Regeneration of Transgenic Plants

Transgenic plants were regenerated in tissue culture under continuous kanamycin selection (100 µg mL<sup>-1</sup> for potato and 200 µg mL<sup>-1</sup> for tobacco). *A. tumefaciens*-mediated transformation of leaf explants was essentially as

described by Rocha-Sosa et al. (1989) for potato and as given by Faske et al. (1997) for tobacco.

### Protein Determination, SDS-PAGE, and Immunoblot Analyses

Estimation of protein concentrations, SDS-PAGE, and subsequent immunoblot analyses were conducted as described by Graeve et al. (1994) with the modification that protein extraction buffer without SDS was used (von Schaewen et al., 1993). Blots were developed using a complex glycan antiserum as described by von Schaewen et al. (1993). A fresh batch of antiserum with essentially the same characteristics as described previously (Laurière et al., 1989) was prepared by a commercial service lab (Bio-Science, Göttingen, Germany) using PHA-L (Sigma, Deisenhofen, Germany) for injection into rabbits.

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