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Isolation of a Mutant *Arabidopsis* Plant That Lacks *N*-Acetyl Glucosaminyl Transferase I and Is Unable to Synthesize Golgi-Modified Complex N-Linked Glycans[1,2]

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The complex asparagine-linked glycans of plant glycoproteins, characterized by the presence of β1→2 xylose and α1→3 fucose residues, are derived from typical mannose$_9$(*N*-acetylglucosamine)$_2$ ($Man_9GlcNAc_2$) N-linked glycans through the activity of a series of glycosidases and glycosyl transferases in the Golgi apparatus. By screening leaf extracts with an antiserum against complex glycans, we isolated a mutant of *Arabidopsis thaliana* that is blocked in the conversion of high-manne to complex glycans. In callus tissues derived from the mutant plants, all glycans bind to concanavalin A. These glycans can be released by treatment with endoglycosidase H, and the majority has the same size as $Man_5GlcNAc_1$ glycans. In the presence of deoxymannojirimycin, an inhibitor of mannosidase I, the mutant cells synthesize $Man_9GlcNAc_2$ and $Man_8GlcNAc_2$ glycans, suggesting that the biochemical lesion in the mutant is not in the biosynthesis of high-mannose glycans in the endoplasmic reticulum but in their modification in the Golgi. Direct enzyme assays of cell extracts show that the mutant cells lack *N*-acetyl glucosaminyl transferase I, the first enzyme in the pathway of complex glycan biosynthesis. The mutant plants are able to complete their development normally under several environmental conditions, suggesting that complex glycans are not essential for normal developmental processes. By crossing the complex-glycan-deficient strain of *A. thaliana* with a transgenic strain that expresses the glycoprotein phytohemagglutinin, we obtained a unique strain that synthesizes phytohemagglutinin with two high-mannose glycans, instead of one high-mannose and one complex glycan.

In both plant and animal cells, the biosynthesis of glycoproteins by the secretory pathway is accompanied by the attachment of high-Man glycans to specific Asn residues and the subsequent modification of these glycans by enzymes in the ER, the Golgi apparatus, and the vacuole. Glycosidases and glycosyltransferases convert typical $Glc_3Man_9GlcNAc_2$ glycans first to high-Man glycans and then to cglys that have fewer Man residues and additional sugars such as Fuc, Gal, and Xyl (in plants) or sialic acid (in mammals) (Kornfeld and Kornfeld, 1985; Kaushal et al., 1988; Faye et al., 1989). Glycoproteins can contain a heterogeneous array of glycan structures, and the extent of the Golgi modifications differs for different proteins, for various glycosylation sites on a single protein, and are not necessarily uniform for a single glycosylation site (Rademacher et al., 1988; Sturm, 1991).

The functions of N-linked glycans are poorly understood, in part because glycans do not have a single function (Olden et al., 1985). When N-linked glycans can be shown to have a specific role, such as protecting the polypeptide from proteolytic degradation, it is likely that high-Man glycans can fulfill this role just as well as cglys do. Do these Golgi-modified cglys perhaps have additional roles?

Several glycosylation mutants of mammalian cells, which have a defect in a particular step of protein glycosylation, have been isolated and characterized (see Stanley, 1984, for review). These mutants are defective in biosynthesis of the mature oligosaccharide attached to dolichol PPi or in glycan processing, or show an altered terminal glycosylation pattern. In most cases, the functional consequences of glycosylation mutations were difficult to assess, which may be due to the fact that the mutants are cultured cells and not intact organisms. Some mutant cells defective in N-glycan biosynthesis show a conditional-lethal phenotype, whereas others have defects in intracellular trafficking (Stanley, 1984). In contrast, Chinese hamster ovary cell mutants that lack GnT I activity and, as a consequence, have no complex carbohydrates, are quite healthy and synthesize many glycoproteins that are biologically active, despite their truncated carbohydrates (Kumar et al., 1990). The presence of altered complex cell surface glycans correlates with metastasis and tumorigenicity (Dennis et al., 1987), but a functional relation between these glycan alterations and increased metastatic potential has not yet been shown. Whole mammal glycosylation mutants are very rare and were discovered as human genetic disorders such as HEMPAS, a deficiency in mannosidase II and/or low levels

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[2] The first two authors contributed equally to this paper. A.v.S. isolated the mutants, characterized them, determined differences in phenotype, and carried out the transgenic experiments. A.S. carried out the biochemical analysis of the glycans and determined the enzymic lesion of the mutant.

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Abbreviations: cgly, complex glycan; Dol, dolichol; GnT I, *N*-acetyl glucosaminyl transferase I; PHA, phytohemagglutinin.

of GlcNAc-transferase II (Fukuda, 1990; Fukuda et al., 1990). In these rare cases, mutations in protein glycosylation seem to have severe effects on the viability of the mutants. In plants, no glycosylation mutants at the cell culture level or at the level of an intact organism are known.

We previously generated an antiserum (called cgly serum, hereafter) that is specific for the N-linked cglys found on plant glycoproteins. Most of the antibodies in this serum are directed at the β1→2 Xyl residue of the plant cgly (Laurière et al., 1989). In this paper, we report the isolation of a mutant of *Arabidopsis thaliana* that was found by screening with this serum a large population of mutant plants for those whose extracts did not react with the cgly serum on immuno-dot blots. We obtained two independent mutants that are allelic and whose glycoproteins have primarily $Man_5GlcNAc_2$ glycans. The mutant cells lack the GnT I activity, which catalyzes the first step of cgly biosynthesis. Here, we describe the physiological consequences of this mutation and show that a transgenic *A. thaliana* line that expresses PHA synthesizes this protein with two high-Man glycans rather than with a high-Man glycan and a cgly, as is normally the case (Sturm et al., 1992).

MATERIALS AND METHODS

Ethyl Methanesulfonate Mutagenesis and Identification of *cgl* Mutants

Arabidopsis thaliana var Columbia seeds were mutagenized with ethyl methanesulfonate (Sigma), the seeds were sown in a soil peat mixture at a density of 300 seeds per pot (4 × 4 inches), and M_1 plants were grown at 20°C. The M_2 seeds were collected in separate batches to minimize the number of mutants originating from the same M_1 plant.

M_2 seeds were surface sterilized for 2 min in ethanol and for 10 min in commercial bleach supplemented with 0.15% Tween-20, followed by five 5-min washes in sterile water. Seeds were spread on germination medium (Schmidt and Willmitzer, 1988), subjected to a 3-d cold treatment at 4°C in the dark, and transferred to culture shelves (20°C, 16-h light/8-h dark cycle) for germination. After 1 week, the seedlings were transferred to fresh agar plates along a grid to facilitate identification of putative mutants (24 plants per 100-mm diameter dish). Leaf extracts of 3- to 4-week-old plants were tested with cgly antiserum in immuno-dot blots. Nonstainers were singled out and verified by immunoblot and affinoblot analyses. Leaf explants of mutants were placed on callus-inducing agar (Valvekens et al., 1988). Calli were grown under low-light conditions and transferred regularly to fresh medium to provide sufficient material for biochemical characterization.

Crosses

Immature anthers were removed with forceps, and the pistils were fertilized on the following day. Pollination was repeated on consecutive days until pods developed. For the backcrosses, mutants were emasculated and pollinated with anthers of *A. thaliana* var Columbia plants.

Preparation of Protein Samples and Deglycosylation of Glycoproteins

Leaf material (100 mg 100 μL^{-1}) was homogenized in microfuge tubes with protein extraction buffer containing 0.1% SDS, 50 mM Hepes (pH 7.5), and 2 mM sodium bisulfite. Extracts were cleared by centrifugation (2 × 5 min at maximum speed in a minifuge), and the protein concentration of the supernatant was determined with Protein Assay reagent (Bio-Rad) and BSA as a standard according to the specifications of the manufacturer. Deglycosylation of glycoproteins was performed on protein extracts from leaf tissue after TCA precipitation. Enzymic deglycosylation with endoglycosidase H (Boehringer Mannheim) was performed as described by Trimble et al. (1983). Chemical deglycosylation using trifluoromethanesulfonic acid (Sigma) was as described by Edge et al. (1981).

Immunoblot and Affinoblot Analysis

The M_2 progeny of the ethyl methanesulfonate mutagenesis was screened by immuno-dot blots: Leaf portions were cut from 3- to 4-week-old plants and transferred to the well of a 96-well microtiter plate containing 25 μL of protein extraction buffer. The entire plate was subjected to three freeze-thaw cycles at −80 and 30°C. A 5-μL aliquot of the liquid in each well was transferred to nitrocellulose and allowed to dry completely. The dot blot was blocked in 3% gelatin (Bio-Rad) in Tris-buffered saline (10 mM Tris, 0.5 M NaCl [pH 7.2]) for at least 1 h and developed as an immunoblot using the cgly serum (Laurière et al., 1989). Detection was by goat anti-rabbit immunoglobulin coupled to peroxidase (Bio-Rad) and standard visualization procedures (Harlow and Lane, 1988). For immunoblot analysis of polypeptides, equal amounts of total protein (usually 30 μg) were fractionated by SDS-PAGE and transferred to nitrocellulose, and the membranes were blocked in 3% gelatin/Tris-buffered saline. Detection of glycoproteins on nitrocellulose blots probed with Con A was essentially as described by Faye and Chrispeels (1985). Gelatin was omitted from the incubation buffers and replaced by 0.1% Tween-20 to reduce background staining.

Labeling of Callus Tissue with [^{3}H]Glucosamine or [^{3}H]Fuc

Callus tissue (2 g) was incubated for 3 h in 10 mL of Murashige-Skoog medium without Suc (Murashige and Skoog, 1962). Incubation was done in a Petri dish at 27°C in the dark on a rotating shaker. The liquid was then replaced with 2 mL of fresh medium containing 100 μCi of D-[6-^{3}H]-glucosamine (0.96 TBq $mmol^{-1}$; Amersham) or 100 μCi of L-[5,6-^{3}H]Fuc (1.85 TBq $mmol^{-1}$; Amersham), and the tissue was then incubated for an additional 24 h. In vivo labeling in the presence of 5 mM 1-deoxymannojirimycin was done as described by Vitale et al. (1989).

Assay of GlcNAc-Transferase I Activity

Callus tissue (5 g) was homogenized with a mortar and pestle in 7.5 mL of ice-cold gradient buffer (100 mM Tris/HCl [pH 7.8], 1 mM $MgCl_2$, and 0.5 mM DTT) containing 12%

(w/w) Suc. The homogenate was squeezed through two layers of Miracloth (Calbiochem) and centrifuged for 3 min at 1300*g*. The supernatant (7.5 mL) was layered on a discontinuous Suc gradient containing 1.5 mL of 35% (w/w) Suc under 3.5 mL of 16% (w/w) Suc in gradient buffer in an SW-40 centrifugation tube (Beckman Instruments). The gradient was centrifuged for 120 min at 50,000*g*. The organelle fraction (microsomes) at the 16 to 35% Suc interface was collected (1 mL). The microsomes were diluted with 12.5 mL of gradient buffer and sedimented by centrifugation at 4°C for 90 min at 50,000*g*. The pellet was homogenized with a glass rod in 200 μL of 125 mM Mes buffer adjusted with sodium hydroxide to pH 6.25, containing 0.25% (w/w) Triton X-100, 250 mM Suc, and 125 mM GlcNAc.

Microsomes (40 μL) were mixed with 0.05 μmol of Man_3octyl, 0.5 μCi of UDP-[6-^{3}H]*N*-acetyl-D-glucosamine (NEN), 100 nmol of UDP-*N*-acetyl-D-glucosamine (Sigma), and 250 nmol of ATP (Sigma), each in 2 μL of water, and 2 μL of 250 mM $MnCl_2$. In control experiments, 2 μL of water without Man_3octyl were added. The structure of this small Man_3 glycan is equivalent to that of the Man core of Asn-linked glycans on glycoproteins. The reaction was allowed to proceed at 30°C for 60 min and then stopped by the addition of 0.4 mL of 20 mM sodium borate (pH 9) containing 1 mM EDTA. The mixture was cleared by centrifugation and immediately loaded onto a 1-mL column of AG 1-X8 anion-exchange resin (Bio-Rad) in a Pasteur pipette to separate products from unreacted UDP-[^{3}H]GlcNAc. The column was washed successively three times with 0.8 mL of water. The total eluate was freeze-dried and redissolved in 200 μL of water. The solution was cleared by centrifugation, and an aliquot (10 μL) was counted by liquid scintillation spectrometry. For product identification, 100 μL of the solution were loaded on a C_{18} reverse-phase column (Ultrosphere ODS 5 μm, 4.6 × 250 mm; Beckman Instruments) and eluted with water:acetonitrile (86:14). Fractions (1 mL) were collected and counted by liquid scintillation spectrometry. The column was calibrated with [^{14}C]GlcNAcMan_3octyl. The enzyme assays were run with duplicate samples, which in all cases gave identical results.

Isolation of ^{3}H-Labeled Proteins and Analysis of Glycopeptides and Glycans

For the preparation of ^{3}H-labeled proteins, the callus tissue was washed twice with 10 mL of medium before homogenization with a mortar and pestle in liquid nitrogen. The powder was extracted three times with 8 mL of cold acetone and dried. The powder was suspended in 5 mL of 2% SDS in 100 mM Tris/HCl (pH 7.4) and heated for 5 min in boiling water. After cooling, the insoluble material was removed by centrifugation. Proteins were precipitated from the supernatant at a final concentration of 80% cold acetone. The pellet was washed three times with 80% acetone in water and dried. For analysis of polypeptides by SDS-PAGE and fluorography, an aliquot of the pellet was dissolved in SDS-containing sample buffer.

For the preparation of glycopeptides, the dried pellet was suspended in 100 mM Tris/HCl (pH 8.0), containing 5 mM $CaCl_2$, and digested with Pronase (2 mg mL^{-1}). Conditions for Pronase digestion and purification of glycopeptides by gel-filtration chromatography on Bio-Gel P-4 were as described by Sturm et al. (1987). For the isolation of high-Man-type glycans, the glycopeptides were digested with endoglycosidase H, followed by separation on Con A Sepharose as described by Sturm et al. (1987). High-Man-type glycans were analyzed by gel-filtration chromatography on a calibrated high-resolution gel-filtration column of Bio-Gel P-4 as described by Sturm (1991). Digestion of glycans with α-mannosidase and β-*N*-acetylglucosaminidase was done as described by Sturm et al. (1987).

Arabidopsis Transformation with a Modified PHA-L Gene

The mature protein sequence of the bean lectin PHA-L contains no Met. To facilitate future radioactive labeling experiments, a Leu codon at position 16 was exchanged for one encoding a Met by site-directed mutagenesis (described by Tague et al., 1990). The modified sequence (PHA-M16) was introduced into pA35S via *Xho*I-*Sal*I sites and transferred into the binary vector pDE1001 as described by Höfte et al. (1991), giving rise to construct pD35-M16, which was subsequently introduced into *Agrobacterium tumefaciens* strain LBA4404. To transform *Arabidopsis* plants, seeds were grown under sterile conditions in liquid germination medium with gentle agitation at 20°C and constant light. After 7 d, roots were harvested and transformed with LBA4404:pD35-M16 according to the protocol described by Valvekens et al. (1988). Seeds of the T_1 progeny were germinated on AM agar supplemented with 50 μg mL^{-1} of kanamycin, and strong expressors were identified by immunoblot analysis with anti-PHA antiserum. The amount of PHA in the progeny of transformants PHA-#1 and PHA-#4 was estimated to range between 0.5 and 1% of total protein, respectively. Although we did not check the copy number of PHA-M16 in our transgenic lines, a frequency of about 5% kanamycin-resistant seedlings in the progeny of PHA-#1 indicates that the transgene integrated at least at two independent sites in the *Arabidopsis* genome.

Vacuole Preparation

Isolation of vacuoles prepared from *Arabidopsis* protoplasts was essentially as described by Höfte et al. (1991). BSA (0.5%) was included in the lysis buffer, which resulted in higher yields of clean vacuoles (usually 20–50%). In contrast to the tobacco protocol, the vacuole buffer of the Ficoll step gradient contained 0.6% betaine instead of 0.45 M mannitol as an osmotic stabilizer. This resulted in a better separation of unlysed protoplasts and vacuoles and caused the vacuoles to band at the 4/0% Ficoll interphase.

Assessing the Effect of the *cgl* Mutation on the Phenotype of Plants Grown under Stress

Ten days after germination on agar plates, the seedlings were transplanted to an autoclaved soil/sand mixture at a density of 15 plants per box and allowed to grow for 10 d more under SD (10 h of light/14 h of dark). The boxes with rosette plants were then transferred to one of three different

environmental regimens: (a) light stress: LD (16 h of light/8 h of dark) with 300 μE of light, (b) cold stress: constant illumination at 100 μE at 8°C, and (c) heat stress: LD (16 h of light/8 h of dark) at 100 μE at 30°C. After 3 more weeks of growth, the boxes were photographed, and small (5 mm in diameter) leaf discs were collected (50 per sample) for assay of Chl and protein content, as well as a fresh weight and dry weight determination.

RESULTS

Isolation of *cgl* Mutants

Mutants without cglys (*cgl* mutants) were identified by screening a population of mutagenized M_2 generation plants. Leaf extracts were spotted on nitrocellulose paper (96 per sheet) and developed as immuno-dot blots with an antiserum that is specific for cglys of plant glycoproteins (Laurière et al., 1989). We identified several plants whose extracts contained no epitopes that reacted with this serum. All candidates were retested, and the results were confirmed by immunoblot with the same serum and a Con A affinoblot. By screening approximately 10,000 plants, we obtained three putative *cgl* mutants (C4, C5, and C6) whose extracts produced no reaction with the antiserum. One mutant (C4) was lost during backcrossing (see below). Extracts from C5 and C6 were subjected to SDS-PAGE, followed by affinoblotting with Con A and peroxidase (Faye and Chrispeels, 1985) and immunoblotting with the cgly serum (Fig. 1). The results show that extracts from wild-type *A. thaliana* (W1 and W2) contain numerous proteins that react with the cgly serum (Fig. 1A, lanes 1 and 2) and fewer proteins that bind Con A (Fig. 1B, lanes 1 and 2). In plants, as in animals, most of the high-Man glycans are converted to cglys as the proteins pass through the Golgi apparatus. In the *cgl* mutant plants (C5, C6), there are no proteins that react with the cgly serum (Fig. 1A, lanes 3 and 4) and numerous proteins that react with Con A (Fig. 1B, lanes 3 and 4). A comparison of lanes 5 and 6 with lanes 7 and 8 shows that in the mutants there are additional proteins that react with Con A, as well as proteins that bind Con A more abundantly. These results indicate that in the mutants the processing of glycans may be blocked, and as a result, there are additional Con A-binding sites.

Genetic Analysis of *cgl* Mutants

The initial *cgl* mutant plants obtained were male sterile, and the genetic properties of the mutants were determined after cross-pollinating mutant lines with wild-type Columbia plants. In the F_1 generation, all of the progeny were wild type, indicating that staining is dominant over nonstaining. In the F_2 generation, staining with the cgly serum segregated about 3:1, confirming the recessive nature of the *cgl* trait. Nonstainers from the F_2 generation were backcrossed again with the wild type, and the entire procedure was repeated three more times (four backcrosses). Two of the three mutants were processed in this way; the third mutant was lost early in the procedure.

To test whether the two mutant lines C5 and C6 were allelic, plants carrying these mutations in the homozygous condition were subjected to the following crosses: immature

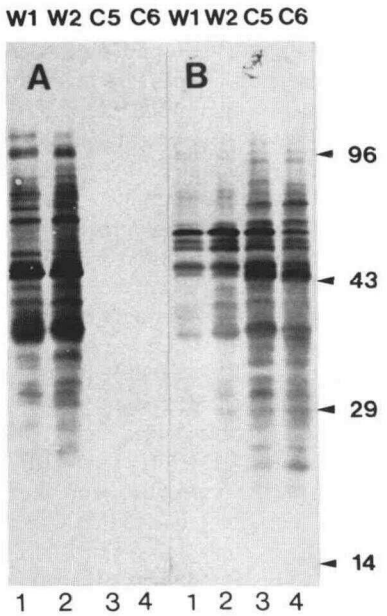

Figure 1. Immunoblot detection of Xyl-containing cglys (A) and affinoblot detection of high-Man glycans (B) of wild-type (W1, W2) and mutant (C5, C6) *Arabidopsis* polypeptides. The proteins in a total leaf extract were separated by SDS-PAGE and transferred to nitrocellulose. Lanes 1 to 4 in A were probed with the cgly antiserum in a peroxidase-coupled reaction; lanes 1 to 4 in B were probed with Con A and peroxidase. Arrowheads on the right indicate the positions of the size markers in kD.

homozygous C6 flowers were emasculated and pollinated with mature anthers of homozygous C5 flowers. Immunodot blot analysis of the progeny revealed 100% nonstainers ($n = 96$), which shows that both mutants carry the defect in the same gene and, thus, are allelic.

Biochemical Analysis of Glycans from *cgl* Mutants

Callus tissue from two wild-type (W1 and W2) and the two *cgl* mutant plants (C5 and C6) were labeled in vivo with [^{3}H]Fuc, and the polypeptides were fractionated by SDS-PAGE. A Coomassie brilliant blue-stained gel (Fig. 2A) shows that both mutant and wild type have identical polypeptide patterns. A fluorograph of this gel (Fig. 2B) shows that the wild type contains a number of fucosylated glycoproteins, but no [^{3}H]Fuc was incorporated in the proteins of the mutant.

Callus tissues from two wild-type (W1 and W2) and the two *cgl* mutant plants (C5 and C6) were labeled in vivo with [^{3}H]glucosamine, total SDS-soluble proteins were digested with pronase, and the products were separated by gel filtration. Purified glycopeptides were subjected to affinity chromatography on Con A Sepharose to separate them into bound and nonbound fractions (Fig. 3). Approximately four-fifths of the glycans from wild-type glycoproteins did not bind to Con A (based on the percentage of radioactivity), whereas one-fifth did bind and was eluted (Fig. 3A); analysis of W1 and W2 gave identical results; therefore, Figure 3 shows only the data for W1. In contrast, proteins synthesized by the two *cgl* mutants had no glycopeptides that failed to

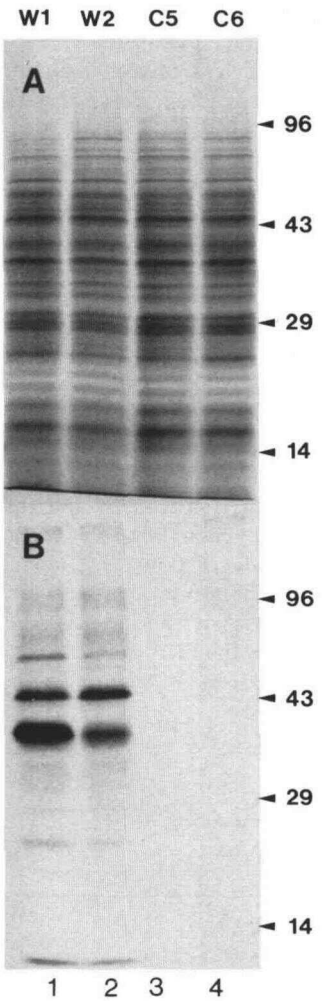

Figure 2. Fluorograph of [^{3}H]Fuc-labeled glycoproteins. Callus tissue was labeled with [^{3}H]Fuc and homogenized. The solubilized polypeptides were separated by SDS-PAGE and stained with Coomassie brilliant blue (A), and a fluorograph was prepared (B). Arrowheads on the right indicate the positions of the size markers in kD.

bind to Con A and a much higher amount that bound (B). Analysis of C5 and C6 gave identical results; therefore, Figure 3 shows only the data for C6.

Total glycopeptide fractions were digested with endoglycosidase H and fractionated on Con A Sepharose, and the bound glycan fraction was analyzed using a high-resolution gel-filtration column. Figure 4 shows the analysis of the endoglycosidase H-released glycans from wild-type callus proteins (B; analysis of W1 and W2 gave identical results, and, therefore, Fig. 4 shows only the data for W1) and from mutant callus proteins (C; analysis of C5 and C6 gave identical results, and, therefore, Figure 4 shows only the data for C6). Fig.4A shows the calibration of the column with the two high-Man-type glycans from phaseolin with nine and seven Man residues (fractions 130 and 138/139, respectively) (Sturm et al., 1987). Glycoproteins from wild-type callus tissue had mainly glycans with eight, seven, and six Man residues. Glycans of the same size were also found attached to glycoproteins from the mutant callus tissues, although the most abundant glycan eluted from the column as a glycan with five Man residues (see fractions 148–150).

The individual glycan peaks shown in Figure 4, B and C, were digested with β-*N*-acetylglucosaminidase. Neither a release of radioactivity nor a shift of the elution positions of the glycans was observed, indicating the absence of terminal GlcNAc residues (data not shown). The digestion of individual glycan peaks with α-mannosidase generated in all cases a small glycan corresponding to one Man residue attached to a GlcNAc, indicating that the glycans consisted exclusively of one core GlcNAc residue and a varying number of Man residues (data not shown).

cgl Mutant Lacks GlcNAc-Transferase Activity

The accumulation of the processing intermediate $Man_5GlcNAc_2$ in the mutant suggests a lesion in GnT I. Therefore, wild-type and mutant callus tissue was analyzed for GnT I activity. Microsomes were isolated and incubated in the presence of Triton X-100 with UDP-[^{3}H]GlcNAc and Man_3octyl, a synthetic substrate for GnT I (Möller et al., 1992). ATP and GlcNAc were added to the assay mixture to inhibit pyrophosphatase and hexosaminidase, respectively. The production of [^{3}H]GlcNAcMan_3octyl by microsomes from wild-type callus tissue was confirmed by reverse-phase HPLC chromatography on a C_{18} column calibrated with [^{14}C]Glc-

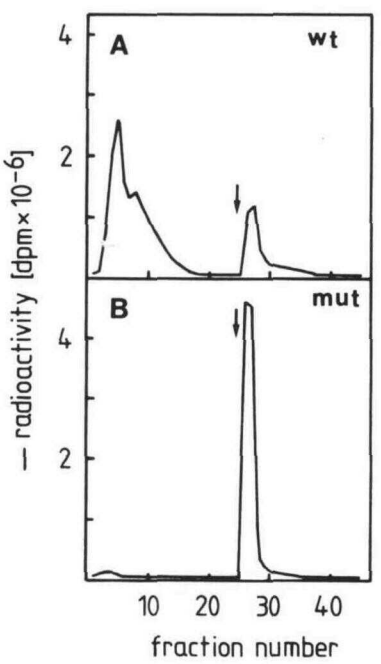

Figure 3. Con A chromatography of glycopeptides from wild-type (wt) (A) and mutant (mut) (B) *Arabidopsis* polypeptides. Proteins labeled in vivo with [^{3}H]glucosamine were digested with pronase, and the glycopeptides were purified by gel filtration on Bio-Gel P-4 and subsequently separated by affinity chromatography on Con A (column size, 1.5 mL of Con A Sepharose; fraction size, 1 mL). The arrows indicate the starting point of elution with 200 mM α-methylmannoside.

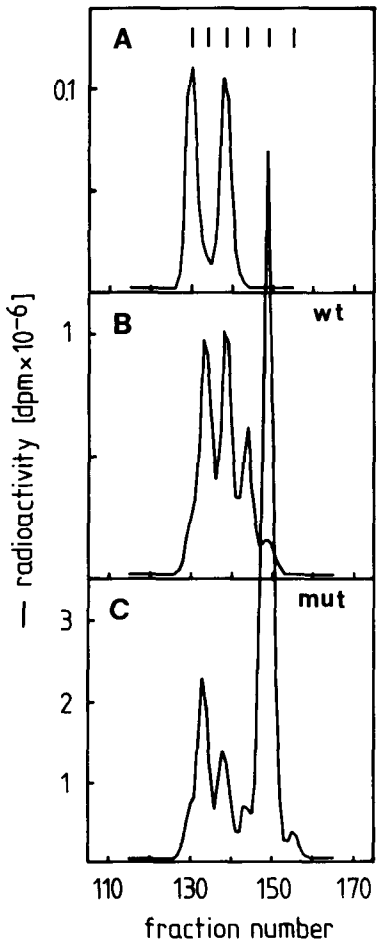

Figure 4. Gel-filtration chromatography of high-Man-type glycans from phaseolin (A) and from polypeptides of wild-type (B) and mutant (C) *Arabidopsis* callus tissue. Proteins labeled in vivo with [^{3}H]glucosamine were digested with pronase. Glycopeptides were purified by gel-filtration chromatography on a short column of Bio-Gel P-4 and digested with endoglycosidase H. High-Man-type glycans were isolated by affinity chromatography on Con A and subsequently separated on a calibrated high-resolution gel-filtration column of Bio-Gel P-4 (column size, 150 × 1 cm; fraction size, 0.5 mL; buffer, 0.1 M acetic acid). The bars in A indicate (from left to right) the elution positions of high-Man glycans with nine, eight, seven, six, five, and four Man residues.

$NAcMan_3$octyl (Fig. 5A). No [^{3}H]$GlcNAcMan_3$octyl was formed by microsomes from the *cgl* mutant callus tissues, confirming the absence of GnT I activity (Fig. 5B).

Assessing the Phenotype

Wild-type and *cgl* mutants were grown side by side under SD (10 h of light) or LD (continuous illumination) conditions in sterile magenta boxes. After 6 weeks of growth, the plants looked identical. In another experiment, 10-d-old plants were transferred to a soil/sand mixture, grown for 10 d more under SD conditions, and then transferred to a heat stress (30°C), cold stress (8°C), and light stress (more than 300 μE) regimen (see "Materials and Methods" for details). All wild-type plants survived the transplantation procedure, but about one-fourth to one-third of the *cgl* plants died as a result of being transplanted. Nevertheless, the *cgl* plants that survived grew as well as the wild-type plants under these stress conditions. The only noticeable difference was that the *cgl* plants grown under heat stress appeared to have necrotic spots (from stray pathogen infection?), whereas the wild-type plants did not. The fresh weight, dry weight, and protein and Chl content of the wild-type and *cgl* strains were similar (Table I).

Analysis of Glycans on the Product of a Transgene

To determine the nature of the glycans on a specific glycoprotein with well-characterized glycans, we examined the glycans on PHA synthesized in the *cgl* background. *A. thaliana* was first transformed with a chimeric gene with the coding sequence of *dlec2* (the gene that encodes PHA-L) in which the expression is driven by the cauliflower mosaic virus 35S promoter. In bean (*Phaseolus vulgaris*) seeds (Sturm et al., 1992) and seeds of transgenic tobacco (Sturm et al., 1988), PHA accumulates in protein storage vacuoles and has a high-Man glycan at Asn^{12} and a cgly at Asn^{60}. To find the subcellular location of the PHA in the leaves of the transgenic *A. thaliana*, we compared PHA levels in total extract, proto-

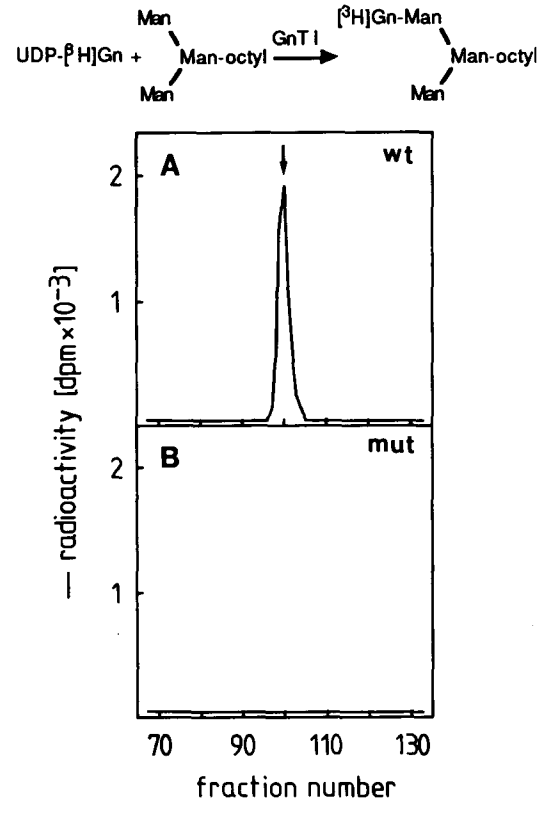

Figure 5. HPLC elution pattern of GnT I product using Man_3octyl as the substrate and UDP-[^{3}H]GlcNAc as the donor for GlcNAc assaying the wild type (wt) (A) and the mutant (mut) (B). HPLC separation was carried out on a reverse-phase C_{18} column using water:acetonitrile, 86:14 (v/v), at 1 mL min^{-1}. The arrow in A indicates the elution position of [^{14}C]$GlcNAcMan_3$octyl.

Table I. *Effect of different stresses on the accumulation of fresh weight, dry weight, Chl, and protein in the wild-type (wt) and the cgl mutant plants*

Fifty leaf discs of 5 mm diameter taken from the rosette leaves of 6-week-old plants were analyzed. All data are means ± SD per 50 leaf discs.

Stress	Strain	Fresh Wt	Dry Wt	Chl	Protein
				mg	
Cold (8°C)	*cgl*	340 ± 0.0	74 ± 14.1	0.405 ± 0.053	0.876 ± 0.001
	wt	348 ± 27.8	76 ± 14.7	0.394 ± 0.004	1.190 ± 0.004
Heat (30°C)	*cgl*	236 ± 6.4	19 ± 1.5	0.215 ± 0.006	0.312 ± 0.003
	wt	235 ± 7.6	19 ± 1.3	0.207 ± 0.007	0.314 ± 0.011
Light (>300 μE)	*cgl*	304 ± 25.5	34 ± 1.0	0.250 ± 0.007	0.480 ± 0.002
	wt	355 ± 7.5	40 ± 3.1	0.268 ± 0.020	0.502 ± 0.007

plasts, and vacuoles. The results (Fig. 6) show equal amounts of PHA in each fraction when the lanes are loaded for equal acid phosphatase or α-mannosidase, which are both vacuolar marker enzymes. We do not know why PHA separates into two bands. One explanation is the loss of some C-terminal amino acids resulting from the action of vacuolar carboxypeptidase. These results are consistent with the conclusion that all of the PHA in these transgenic *Arabidopsis* plants accumulates in the vacuoles. A parallel immunoblot carried out with antibodies to the ER marker protein binding protein shows that the vacuole fraction is free of binding protein and, therefore, is not contaminated with ER (Fig. 6). The equality of the strength of the signal in the two lanes indicates that this fraction is not contaminated by ER.

We subsequently crossed the *A. thaliana* var Columbia strain expressing PHA with both *cgl* mutant strains and scored the selfed progeny for PHA synthesis in the mutant background. The nature of the glycans on PHA in the extracts of wild-type and mutant plants was examined by determining the effect of endoglycosidase H treatment on the mobility of PHA after separation by SDS-PAGE and immunoblotting. The results (Fig. 7) clearly show that treatment with endoglycosidase H of PHA synthesized in the *cgl* background results in a change in the mobility of PHA (lane 5), similar to that obtained by total chemical deglycosylation (lane 1); treatment with endoglycosidase H of PHA synthesized by wild-type plants results in a smaller mobility shift. These results are compatible with the presence of one high-Man glycan on

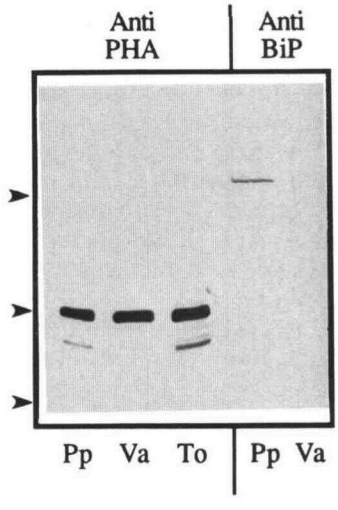

Figure 6. Analysis of the subcellular location of PHA in transformed *A. thaliana*. Vacuoles (Va) were isolated from protoplasts (Pp) of leaf cells, and total extract (To), protoplasts, and vacuoles were assayed for acid phosphatase, a vacuolar marker enzyme. SDS-PAGE gel lanes were loaded with equal amounts of acid phosphatase activity, and after transfer to nitrocellulose paper, the immunoblot was developed with antibodies against PHA and binding protein (BiP). The equality of the strength of the signal in the two lanes indicates that all of the PHA is in the vacuoles. The arrows on the left indicate the positions of the size markers of 18.4 (lower), 29, and 68 kD.

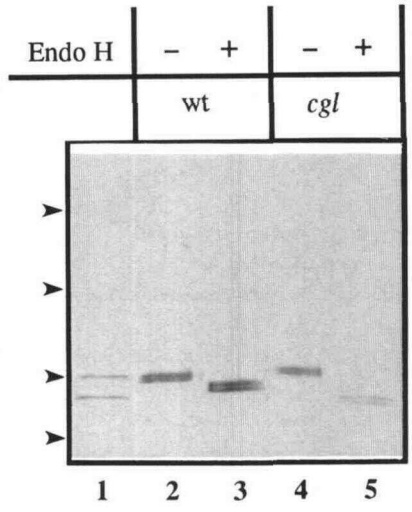

Figure 7. Analysis of the glycans on PHA synthesized by normal (wt) and *cgl* mutant plants. *A. thaliana* extracts were treated with endoglycosidase H, the proteins were separated by gel electrophoresis, and an immunoblot was developed with antibodies against PHA. Lane 1, A mixture of PHA-L and chemically deglycosylated PHA-L. Lanes 2 and 3, PHA synthesized from a transgene in a control *A. thaliana* plant, before and after endoglycosidase H treatment. Lanes 4 and 5, PHA synthesized from a transgene in *cgl* mutant, before and after endoglycosidase H treatment. Notice that the shift in position of PHA corresponds to the removal of one glycan for control and two glycans for *cgl* plants. Arrowheads on the left indicate the positions of the size markers of 18.4 (lower), 29, 43, and 69 kD.

PHA in the wild-type background and two high-Man glycans on PHA synthesized in the *cgl* mutant background.

DISCUSSION

The metabolic block in the *cgl* mutant leads to the accumulation of $Man_5GlcNAc_2$ glycans. From the biosynthesis and processing pathway of plant N-linked glycans (Fig. 8), we can predict the types of mutants we might identify with our cgly serum that is largely an anti-β1→2 Xyl serum (Laurière et al., 1989). Because the addition of Xyl can occur without the action of α-mannosidase II and/or GlcNAc-transferase II (Johnson and Chrispeels, 1987; Tezuka et al., 1992), we did not expect to find α-mannosidase II and GlcNAc-transferase II mutants. Some plant cglys have Xyl but no Fuc, indicating that xylosylation is independent of fucosylation. Thus, we were also unlikely to find mutants in fucosyl-transferase. Recent evidence from mammalian cells indicates the presence of a processing endo-α-mannosidase that can act as a bypass for α-glucosidase activity in glycan processing (Fujimoto and Kornfeld, 1991). If such an enzyme is also present in plant cells, it would exclude the possibility of finding mutants in α-glucosidase I and II. This leaves enzymes involved in the biosynthesis of the high-Man-type oligosaccharide precursor, oligosaccharide transferase, and the glycan-processing enzymes mannosidase I and GnT I as the most likely candidates for our mutant screen. Mutations in the carrier proteins that transport sugar nucleotides across the Golgi membranes could also result in the absence of specific sugars on the cglys.

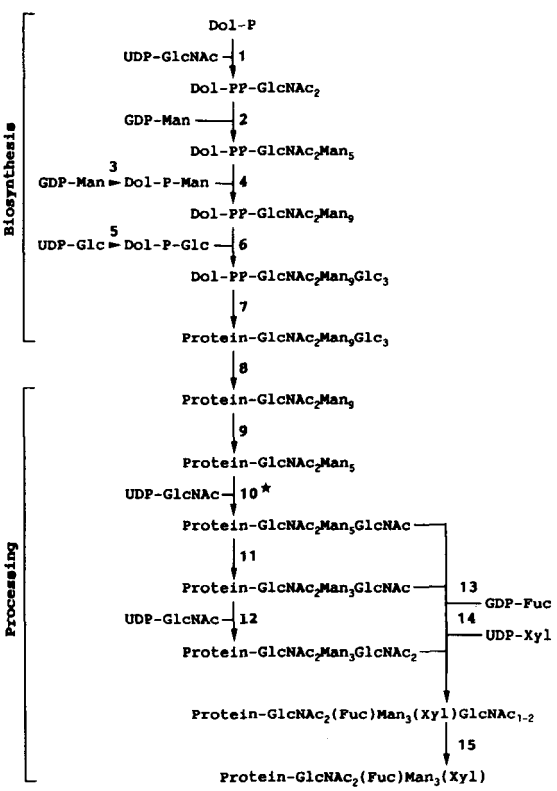

Figure 8. Proposed biosynthesis and processing pathway of a typical N-linked cgly from plants. The enzymes involved are GlcNAc-1-P transferase and GlcNAc-transferase (1), mannosyl-transferase (2), Dol-P-Man synthase (3), Dol-P-mannosyl-transferase (4), Dol-P-Glc sythase (5), Dol-P-glycosyl-transferase (6), oligosaccharide-transferase (7), glucosidase I and II (8), mannosidase I (9), GlcNAc-transferase I (10), mannosidase II (11), GlcNAc-transferase II (12), fucosyl-transferase (13), xylosyl-transferase (14), *N*-acetyl glucosaminidase (15). The enzyme activity 10 (GlcNAc-transferase I), which is missing in the *A. thaliana* mutants C5 and C6, is marked by an asterisk.

The characterization of the *cgl* mutant of *A. thaliana* showed that it is blocked in the processing of N-linked glycans. Analysis of the glycans revealed that they were predominantly of the $Man_5GlcNAc_2$ type, with small amounts of Man_6, Man_7, and Man_8 glycans. In callus of the wild-type *A. thaliana*, most of the glycans are of the complex type with a small portion of Man_6, Man_7, and Man_8 (Figs. 3 and 4). The *cgl* mutant is, therefore, similar to the Lec1 mutants of Chinese hamster ovary cells (Stanley, 1984, 1987). These cells lack GnT I and have predominantly $Man_5GlcNAc_2$ glycans (Li and Kornfeld, 1978; Robertson et al., 1978).

Glycoproteins with a predominance of $Man_5GlcNAc_2$ glycans could be the result of different mutations leading to a biochemical lesion in GnT I, in Dol-P-Man synthase, or in Dol-P-mannosyl transferase (Fig. 8). The biosynthesis of the Glc_3Man_9 $GlcNAc_2$ chains, which are transferred *en bloc* to the nascent polypeptide chains, involves, first, the synthesis of $Man_5GlcNAc_2$-PP-Dol, followed by the stepwise addition of Man residues from Dol-P-Man. A lesion in Dol-P-Man synthase or the Dol-P-mannosyl transferase would arrest the synthesis of glycans at the $Man_5GlcNAc_2$ stage. Such glycans can be glucosylated, however, and transferred to nascent polypeptide chains (Chapman et al., 1979). Removal of the Glc residues by the α-glucosidases yields $Man_5GlcNAc_2$ glycans whose structure is different from the $Man_5GlcNAc_2$ glycans produced by the action of mannosidase I on $Man_9GlcNAc_2$ glycans. Such glycans cannot be cleaved by endoglycosidase H because they lack the α1→3 Man on the α1→6 Man branch. Because the glycans in the mutant can be cleaved by endoglycosidase H, this identifies GnT I as the likely biochemical lesion in the *cgl* mutant. This lesion was confirmed by the direct assay of GnT I in extracts of wild-type and mutant callus tissues.

The mutant plants are able to complete their normal life cycle under optimal growth conditions in the laboratory, as well as under various stress conditions, and they have no obvious phenotype. This result is in contrast to findings in mammals, in which glycosylation defects cause serious diseases in the homozygous condition (Fukuda, 1990; Fukuda et al., 1990). Both wild type and mutants make rosettes and produce a single inflorescence stem. Flowering, seed set, and seed germination also appear normal. These results suggest that cglys are not an essential posttranslational modification on the large number of glycoproteins on which they normally occur. In *Dictyostelium*, the Golgi-enzyme-catalyzed modifications of high-Man glycans involve the addition of intersecting GlcNAc residues, sulfation, and phosphorylation (Freeze, 1992), but inhibition of these processing events by deoxymannojirimycin and swainsonine does not appear to

inhibit cell aggregation and development (Sharkey and Kornfeld, 1991).

The presence of $\beta 1 \rightarrow 2$ xylosyl and $\alpha 1 \rightarrow 3$ fucosyl residues on the glycans of plant glycoproteins makes them unusually antigenic and limits the use of plants for the production of pharmacologically important glycoproteins by recombinant DNA technology and plant transformation. The production of biologically active glycoproteins with minimal carbohydrate heterogeneity is of great interest to the biotechnology industry (Bergh et al., 1988; Goochee et al., 1991). Immunoglobulin genes have recently been expressed in tobacco plants, but the proteins that were produced contained the highly immunogenic glycans typically found on plant glycoproteins (Hein et al., 1991). Mutant cell lines of mammalian cells can be used for the production of glycoproteins with uniform glycans (Stanley, 1989). The experiments reported here open the way to producing proteins with defined glycans in plants.

We tested this possibility by crossing the *cgl* mutant with a strain of *Arabidopsis* that had been transformed with a gene for PHA driven by the cauliflower mosaic virus 35S promoter. PHA, the lectin of the common bean, is a vacuolar glycoprotein with one high-Man and one cgly (Sturm et al., 1992). Nonstaining progeny that also expressed PHA were obtained in the F_2 generation, and these plants synthesized PHA with two high-Man glycans (Fig. 7), demonstrating that mutant plants, such as the one described here, could be used to produce glycoproteins with nonimmunogenic and/or defined glycans.

The functions of glycans, and especially of cglys of plant glycoproteins, are poorly understood. As with mammalian glycoproteins, glycans on plant glycoproteins are necessary for protein stability. When suspension-cultured plant cells are grown in the presence of tunicamycin, many extracellular proteins fail to accumulate in the culture medium and in the cell walls, because the unglycosylated forms are unstable and degraded either just before or just after secretion (Driouich et al., 1989; Faye and Chrispeels, 1989). Such extracellular proteins play important roles in plant development (Cordewener et al., 1991; De Jong et al., 1992). The finding that the *cgl* mutant of *A. thaliana* can complete its normal life cycle indicates that Golgi-modified glycans are not a necessary posttranslational modification for many proteins. Such proteins may need glycans for stability, but these glycans probably do not need to be modified in the Golgi. Indeed, glycoproteins are secreted normally by plant cells and accumulate to normal levels when Golgi processing is inhibited (Driouich et al., 1989). We must, therefore, consider the possibility that cglys are needed only on a small subset of glycoproteins that are synthesized when plants are subjected to a particular biotic or abiotic stress. To uncover the nature of these glycoproteins remains a challenge for the future.

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