

Identification and Characterization of GONST1, a Golgi-Localized GDP-Mannose Transporter in Arabidopsis

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Transport of nucleotide sugars across the Golgi apparatus membrane is required for the luminal synthesis of a variety of plant cell surface components. We identified an Arabidopsis gene encoding a nucleotide sugar transporter (designated GONST1) that we have shown by transient gene expression to be localized to the Golgi. GONST1 complemented a GDP-mannose transport-defective yeast mutant (*vrg4-2*), and Golgi-rich vesicles from the complemented strain displayed increased GDP-mannose transport activity. GONST1 promoter:: β -glucuronidase studies suggested that this gene is expressed ubiquitously. The identification of a Golgi-localized nucleotide sugar transporter from plants will allow the study of the importance of this class of proteins in the synthesis of plant cell surface components such as cell wall polysaccharides.

INTRODUCTION

In recent years, the realization of the crucial role of the plant cell surface in growth and development has triggered a renaissance in cell wall research (Roberts, 2001). Much of the renewed interest in this field stems from the potential to alter cell wall composition, which would be of major importance in the food and timber industries. As such, studies of cell wall biosynthesis are crucial. Cellulose synthesis occurs on the plasma membrane, whereas noncellulosic polysaccharides are synthesized in the Golgi apparatus (Dupree and Sherrier, 1998). The protein backbone of cell surface-associated glycoproteins is made in the endoplasmic reticulum (ER) and subsequently transferred to the Golgi apparatus for the addition of glycan moieties. Therefore, the plant Golgi apparatus is an organelle specialized for sugar transfer to polysaccharides and glycoproteins (Fincher et al., 1983) and also possibly for the modification of glycolipids (Fujino et al., 1985; Dupree and Sherrier, 1998).

Noncellulosic polysaccharides and cell surface-associated glycoproteins contain a number of different sugars, such as galactose, arabinose, fucose, xylose, mannose, rhamnose, glucose, galacturonic acid, and glucuronic acid. The glycosyltransferases responsible for the synthesis of these glycans recognize specific donor and acceptor substrates. Recently, several genes encoding glycosyltransferases involved in hemicellulose or glycoprotein synthesis

have been cloned (Keegstra and Raikhel, 2001). These genes encode type II single transmembrane domain (TMD) proteins resembling the Golgi glycosyltransferases studied in yeast and animal cells. The catalytic domains of these enzymes in yeast and animals have been shown to face the lumen of the Golgi apparatus (Gibeaut, 2000). A similar topology has been demonstrated for all of the plant glycosyltransferases studied to date (Keegstra and Raikhel, 2001).

Glycosyltransferases use sugars that are activated by the addition of a nucleotide (nucleotide sugars) for donation of the sugar moiety. Most sugars, such as galactose and xylose, are linked to the nucleotide UDP, whereas mannose and fucose are attached to GDP (Seitz et al., 2000). Current evidence suggests that the enzymes for nucleotide sugar synthesis are located in the cytosol (Coates et al., 1980; Bonin et al., 1997). This presents an issue of topology, because both the Golgi glycosylation reactions and their products are luminal. Therefore, it has been proposed that nucleotide sugar transporters (NSTs) provide substrates for the lumenally orientated glycosyltransferases, and such activities have been detected in plant Golgi membranes (Muñoz et al., 1996; Neckelmann and Orellana, 1998; Wulff et al., 2000).

Genes encoding NSTs have been cloned recently from a wide variety of organisms, including human (Eckhardt et al., 1996), *Kluyveromyces lactis* (Abejón et al., 1996), *Leishmania donovani* (Ma et al., 1997), *Saccharomyces cerevisiae* (Dean et al., 1997), and *Caenorhabditis elegans* (Berninson et al., 2001). These membrane-spanning transporter proteins appear to function as antiporters, exchanging nucleoside monophosphate for specific nucleotide sugars (Capasso and Hirschberg, 1984). Given their role in providing the substrate

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for glycosyltransferases, NSTs are a potential control point for glycan synthesis via substrate-level control. For example, mutants in the yeast Golgi GDP-mannose transporter Vrg4p are unable to mannosylate proteins and glycolipids effectively (Dean et al., 1997), and the *L. donovani* GDP-mannose transporter mutant *lpg2* is defective in the mannosylation of cell surface lipophosphoglycan (Ma et al., 1997). The identification and cloning of plant Golgi NSTs will be essential for understanding their role in regulating substrate levels for the luminal glycosyltransferases. In this article, we present the identification and characterization of a gene encoding a plant Golgi nucleotide sugar transporter (*GONST1*) and demonstrate that the protein encoded by *GONST1* is able to transport GDP-mannose.

RESULTS

Molecular Cloning and Preliminary Characterization of *GONST1*

To identify candidate Golgi NST genes, the peptide sequences of the *S. cerevisiae* Vrg4p and *L. donovani* LPG2 GDP-mannose transporters were used to search for Arabidopsis expressed sequence tags (ESTs) that might encode homologous proteins. The sequences of two ESTs, 133F12T7 and 139F21T7, both derived from the same gene, showed significant similarity to the C terminus of these proteins. To determine the complete sequence of the gene, the insert from 139F21T7 was used to screen a bacterial artificial chromosome (BAC) genomic library, and the partial sequence of positive BAC clones from chromosome 2 was determined. By using specific primers designed from this genomic sequence, a cDNA was amplified by polymerase chain reaction (PCR) from an Arabidopsis seedling cDNA library. Alignment of the cDNA with the genomic sequence revealed the presence of six introns and seven exons. The predicted peptide sequence is similar to that of the recently deposited predicted protein At2g13650 from the Arabidopsis Genome Sequencing Project, except that there are two additional exons in the cDNA we isolated. The cDNA encodes a predicted protein of ~37 kD, and we designated the protein Golgi nucleotide sugar transporter 1 (*GONST1*; Figure 1A). Two putative *N*-glycosylation sites (amino acid residues 131 to 133 and 221 to 223), based on the consensus glycosylation sequence (NXS/T; Wagh and Bahl, 1981), also are present in the predicted amino acid sequence. Hydrophobicity analysis predicted that the protein contains between eight and 10 TMDs, consistent with a role as a membrane-spanning transporter (Figure 1B). The protein has ~25% identity with both Vrg4p and LPG2, and the homology extends over the entire sequence (Figure 1C). Importantly, each protein contains a conserved motif containing the amino acids GXLNK. In yeast, the GALNK motif has been

shown to be involved in the binding of GDP-mannose by Vrg4p (Gao et al., 2001). Thus, the protein encoded by this cDNA showed all of the characteristics expected of an NST.

A preliminary investigation of *GONST1* expression was performed to ascertain the size and abundance of the *GONST1* transcript. Figure 2 shows an RNA gel blot of poly(A)⁺ RNA from callus and bolt tissue of Arabidopsis hybridized with an antisense RNA probe made from *GONST1*. A strongly hybridizing transcript of ~1.2 kb was observed in callus tissue, which is consistent with the length of the cDNA isolated, and a lower level of hybridization to bolt mRNA also was observed. In addition, faint hybridization to a transcript of the same size was observed in leaf mRNA (data not shown). Thus, the RNA gel blots suggested a low level of expression of *GONST1* throughout the plant body.

Functional Analysis of *GONST1* Expressed in *S. cerevisiae*

Because *GONST1* shows significant homology with Vrg4p and LPG2, we examined whether *GONST1* might complement the *S. cerevisiae* strain JPY26 3d (*vrg4-2*). This strain is deficient in the ability to transport GDP-mannose because of a mutation in the *VRG4* gene (Dean et al., 1997; Gao et al., 2001) and shows both abnormal resistance to vanadate and sensitivity to hygromycin B (Ballou et al., 1991; Dean, 1995), probably as a result of altered cell wall mannan. The *GONST1* cDNA cloned in a yeast integrating vector, pRS306 (pSc-*GONST1*), was used to transform the *vrg4-2* mutant. On synthetic complete (SC) medium, *vrg4-2* and the transformants grew marginally slower than the wild-type *VRG4* strain (Figure 3A). Figure 3B shows that *GONST1* complemented the vanadate resistance phenotype of *vrg4-2*, because the transformant, like the *VRG4* wild-type strain (JPY25 6c), was sensitive to 5 mM vanadate. Furthermore, *GONST1* could complement the hygromycin B sensitivity of the mutant *vrg4-2* strain (Figure 3C), because the transformant, as well as the wild-type strain, grew in the presence of hygromycin B. The vanadate sensitivity and hygromycin B resistance conferred by complementation of the yeast GDP-mannose transporter mutant with *GONST1* suggest that the protein encoded by this gene has the ability to transport GDP-mannose.

To determine where *GONST1* was being expressed in the transformed yeast strain, a rabbit polyclonal antiserum was raised against a synthetic peptide corresponding to the 14 predicted N-terminal amino acids of this protein. Figure 4A shows that the anti-*GONST1* antiserum detected a protein that was present in the 125,000g (P3) membrane fraction of the transformed yeast strain but that was absent from the same fraction of wild-type *VRG4* and *vrg4-2* mutant yeast. The protein detected by the antiserum has a mass of ~37 kD, consistent with the predicted size of *GONST1*. To prepare a membrane fraction enriched in *GONST1* for transport studies, membranes were separated by differential centrifugation into a larger organelle P2 (7,800g pellet) fraction,

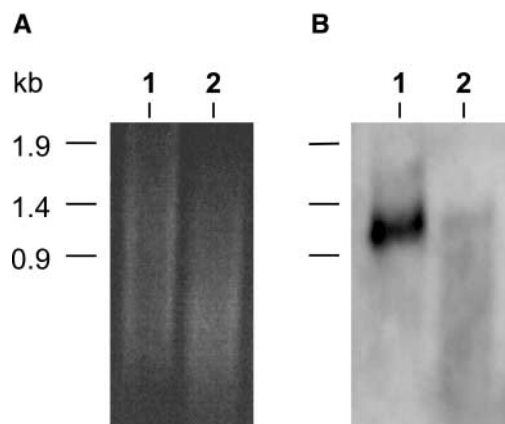


Figure 2. RNA Gel Blot Probed with *GONST1*.

(A) Ethidium bromide-stained RNA gel. Lane 1, 2 μg of callus poly(A)⁺ Arabidopsis RNA; lane 2, 2 μg of bolt poly(A)⁺ Arabidopsis RNA.

(B) Corresponding RNA gel blot hybridized with digoxigenin-labeled, single-stranded RNA probe of *GONST1*.

which was likely to contain ER membranes, and a smaller vesicle P3 (125,000g pellet) fraction, which was likely to be Golgi rich (Horazdovsky and Emr, 1993; Dean and Poster, 1996). Proteins from the P2 and P3 pellets of the transformant were probed with the anti-GONST1 antiserum (Figure 4B) and an antiserum against Anp1p (Figure 4C), a known Golgi-localized protein (Jungmann and Munro, 1998). Both GONST1 and Anp1p were shown to be enriched in the P3 fraction, consistent with GONST1 being targeted correctly to the Golgi apparatus in the transformed yeast strain.

Because GONST1 was present in the yeast Golgi-rich fraction, we used this subcellular fraction to measure GDP-mannose uptake. To avoid the interference produced by the activity of dolichol phosphomannose synthase (Dpm1p) that may mask the measurement of GDP-mannose transporter activity (Abeijon et al., 1989), we used both wild-type *VRG4* and *vrg4-2* strains that harbor a mutation in the *DPM1* gene, resulting in a 90 to 95% decrease in Dpm1p activity (Orlean et al., 1988). Golgi-rich vesicles were prepared from strains *VRG4*, *vrg4-2*, and *vrg4-2* expressing the GONST1 protein and incubated with GDP-¹⁴C-mannose. As shown in Figure 5, the quantity of GDP-mannose incorporated into the Golgi-rich vesicles of *vrg4-2* yeast expressing the GONST1 protein was almost 10-fold greater than that incorporated into vesicles of the untransformed *vrg4-2* mutant. This result strongly suggests that GONST1 is indeed a GDP-mannose transporter.

Targeting of the GONST1-YFP Fusion Protein to the Golgi Apparatus

The subcellular localization of GONST1 was investigated in plants by using a fusion protein consisting of GONST1 fused

at its C terminus to a modified green fluorescent protein (GFP) named YFP. Fusion of C-terminal tags such as GFP, myc, or hemagglutinin does not affect the localization of NSTs in other organisms (Descoteaux et al., 1995; Ma et al., 1997; Gao and Dean, 2000; Lühn et al., 2001), and GFP does not alter the targeting of the Golgi xylosyltransferase in plants (Essl et al., 1999). The chimeric protein (GONST1-YFP) was expressed transiently in onion epidermal cells after biolistic transformation. Two days after bombardment, many cells displayed a punctate pattern of YFP fluorescence, consistent with a Golgi-localized protein (Figures 6A and 6C). The diameter of the spots was 1 μm , similar to the size of the Golgi apparatus in tobacco cells (Dupree and Sherrier, 1998).

To test further the identification of these fluorescent structures as Golgi stacks, we investigated their sensitivity to brefeldin A (BFA) and colocalization with mitochondria and actin components of the cortical cytoskeleton (Figures 6B,

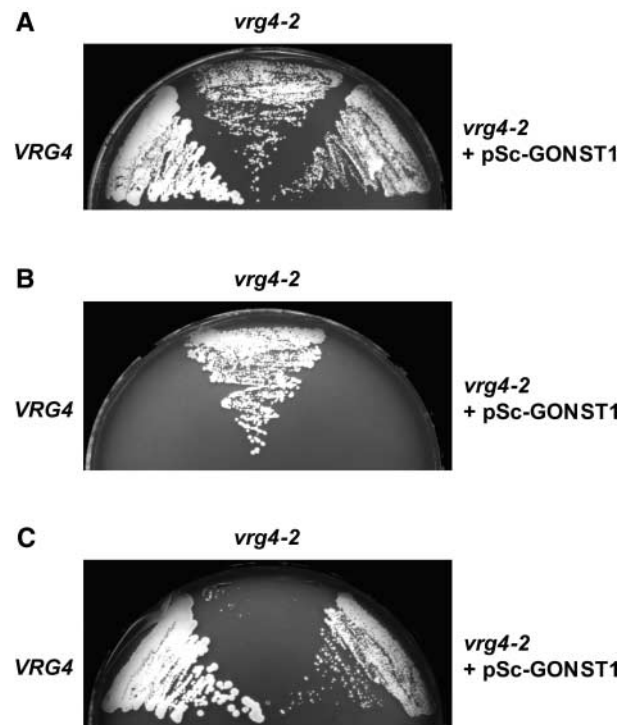


Figure 3. Functional Complementation of *vrg4-2* yeast by pSc-GONST1.

Yeast strains JPY25 6c (*VRG4*), JPY26 3d (*vrg4-2*), and JPY26 3d transformed with pSc-GONST1 (*vrg4-2* + pSc-GONST1) were grown on agar plates, with and without supplements, at 30°C.

(A) SC + 0.5 M KCl medium.

(B) SC + 0.5 M KCl medium supplemented with 5 mM sodium orthovanadate.

(C) SC + 0.5 M KCl medium supplemented with 500 $\mu\text{g}/\text{mL}$ hygromycin B.

6D, and 6E). BFA is known to have a variety of effects on the morphology of the Golgi apparatus, causing either clustering or vesiculation, depending on the conditions and tissue studied (Satiat-Jeunemaitre and Hawes, 1992, 1994; Driouch et al., 1993; Satiat-Jeunemaitre et al., 1996; Robinson et al., 1997; Staehelin and Driouch, 1997; Wee et al., 1998; Essl et al., 1999). When transformed epidermal peels were incubated in 100 $\mu\text{g}/\text{mL}$ BFA for 2 hr, the pattern of YFP fluorescence within the cells changed dramatically. Rather than the small, numerous punctate bodies noted previously, a few large, aggregated fluorescent structures were observed (Figure 6B). This indicated that the GONST1-YFP fusion protein was localized in a BFA-sensitive compartment, which strongly supports Golgi localization. To exclude the possibility that the punctate fluorescence was mitochondrial in origin, the mitochondria were labeled with MitoTracker Orange (Molecular Probes, Eugene, OR) for 10 min. Mitochondria and GONST1-YFP fluorescence did not colocalize (Figure 6E).

Several recent publications have demonstrated that the plant Golgi apparatus moves on an actin/ER network (Boevink et al., 1998; Nebenfuhr et al., 1999). We used a GFP-mouse talin construct (Kost et al., 1998) to label actin in conjunction with our GONST1-YFP to investigate any colocalization of these structures. The punctate bodies containing the GONST1 protein were associated with and streamed along actin components of the cortical cytoskeleton (Figure 6D; a time lapse movie is available at <http://www.bio.cam.ac.uk/proteomics/GONST1plusactin.mov>). These data are consistent with previous studies showing the Golgi apparatus interacting with the actin network. Although we cannot formally exclude the possibility that the YFP fusion causes targeting of GONST1 to the Golgi apparatus, we consider this highly unlikely. The observed punctate distribution of the GONST1-YFP fluorescence, the effect of BFA, the lack of colocalization with mitochondria, the mobility on the actin cytoskeleton, and the functional data from yeast indicate that GONST1 is a Golgi-localized GDP-mannose transporter.

β -Glucuronidase (GUS) Histochemical Analyses of *GONST1* Expression

To investigate the expression of *GONST1* in plants, we transformed *Arabidopsis* with a *GONST1* promoter::GUS fusion protein (pGONST1::GUS). Histochemical staining for GUS in whole seedlings (Figure 7A) demonstrated that GUS activity was ubiquitous throughout the plant body, with slightly higher levels of expression being observed in the vascular tissues. Staining of *Arabidopsis* inflorescences demonstrated that all floral organs plus the pedicels were stained evenly (Figure 7B). In RNA gel blot experiments, it was observed that the *GONST1* transcript was expressed most abundantly in callus tissue (Figure 2). With this in mind, we made a line of transgenic callus derived from the GONST1-GUS *Arabidopsis* to confirm that histochemical staining correlated with the results observed in the RNA gel

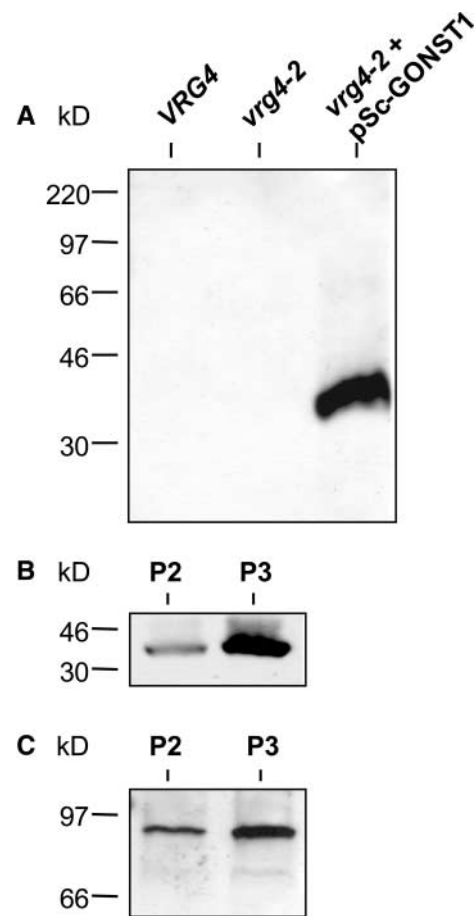


Figure 4. The GONST1 Protein Is Expressed in *vrg4-2* Yeast Transformed with pSc-GONST1 and Is More Abundant in the Golgi-Rich (P3) Fraction.

(A) Protein (5 μg) of the P3 fractions prepared from yeast strains JPY25 6c (*VRG4*), JPY26 3d (*vrg4-2*), and JPY26 3d transformed with pSc-GONST1 (*vrg4-2* + pSc-GONST1) were resolved by SDS-PAGE and subjected to protein gel blot analysis using the anti-GONST1 antiserum.

(B) and (C) The P2 and P3 fractions of JPY26 3d transformed with pSc-GONST1 were resolved and subjected to protein gel blot analysis using the anti-GONST1 antiserum (B; 7.5 μg of protein) or antiserum against Anp1p, a yeast Golgi marker protein (C; 5 μg of protein).

blot experiments. Histochemical staining of the transgenic callus demonstrated intense staining of the callus tissue (Figure 7C) within 30 to 60 min of exposure to the GUS substrate, which was significantly faster than the time required to stain the intact plant tissue. These data show that the expression of the GDP-mannose transporter *GONST1* is essentially ubiquitous in these plant tissues.

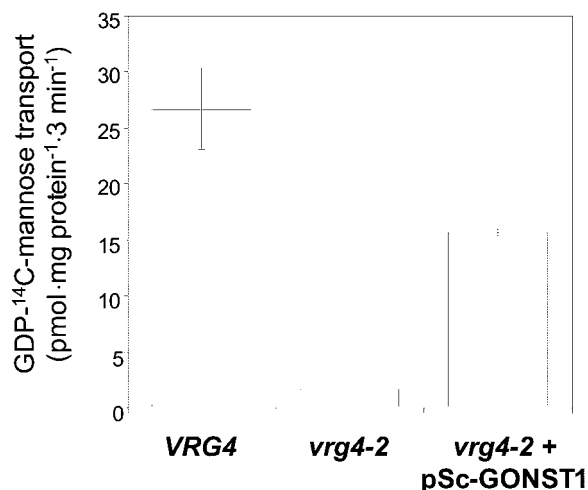


Figure 5. Increased GDP-Mannose Uptake in the Golgi-Rich Vesicle Fraction of *vrg4-2* Yeast Expressing GONST1.

Golgi-rich (P3) vesicles were prepared from yeast strains JPY25 6c (*VRG4*), JPY26 3d (*vrg4-2*), and JPY26 3d transformed with pSc-GONST1 (*vrg4-2* + pSc-GONST1). Membranes were incubated in reaction mix containing 0.18 μ M GDP-¹⁴C-mannose (224.1 mCi/mmol) for 0 or 3 min at 30°C, and reactions were stopped by filtration. GDP-¹⁴C-mannose uptake is calculated as the difference between the two time points. The averages of triplicate assays and standard error bars are shown. The results shown are representative of three independent experiments, and enhanced incorporation of GDP-mannose into the P3 fraction of three other transformants also was observed.

DISCUSSION

Previous work has shown a requirement for the transport of nucleotide sugars across the Golgi membrane for the synthesis of plant cell surface components (Muñoz et al., 1996; Neckelmann and Orellana, 1998; Wulff et al., 2000). In this article, we present the cloning and preliminary characterization of a Golgi-localized nucleotide sugar transporter (*GONST1*) from plants and demonstrate that it is able to transport GDP-mannose across Golgi membranes. *GONST1* shows significant amino acid identity (~25%) to the two previously isolated GDP-mannose transporters, Vrg4p and LPG2 (Figure 1; Dean et al., 1997; Ma et al., 1997). Importantly, all three sequences possess a conserved amino acid motif, GXLNK. In yeast, the GALNK motif has been shown to bind to the GDP moiety of GDP-mannose and thereby facilitate transport of this nucleotide sugar into the Golgi lumen (Gao et al., 2001). Hydrophobicity analysis predicts that the *GONST1* sequence has between eight and 10 TMDs. By comparing the patterns of hydrophobicity of *GONST1* and the functionally related Vrg4p and LPG2, and also of the more distantly related human GDP-fucose transporter (Lühn et al., 2001), we predict that *GONST1* contains 10 TMDs.

This is more than the predicted six to eight TMDs of Vrg4p, but it would place both the N and C termini on the cytosolic face, as has been shown for this yeast GDP-mannose transporter (Gao and Dean, 2000). In both of these models, the putative GDP binding motif (GXLNK) would be in a cytosolic loop, as required for the binding of cytosolic GDP-mannose.

Complementation of the growth phenotypes of the yeast GDP-mannose transporter mutant *vrg4-2* (Dean et al., 1997) indicated that *GONST1* can increase GDP-mannose transport into the yeast Golgi apparatus (Figure 3). By using a GDP-¹⁴C-mannose uptake assay of a yeast Golgi-enriched vesicle fraction, membranes from the *vrg4-2* mutant displayed a severe defect in the ability to transport GDP-mannose, with activity ~5% of that in the wild-type *VRG4* strain, consistent with previous findings (Dean et al., 1997). We showed that vesicles isolated from *vrg4-2* expressing the *GONST1* protein possessed increased GDP-mannose uptake activity to ~60% of the wild-type level (Figure 5). Thus, because *GONST1* was able to complement a yeast mutant deficient in a GDP-mannose transporter, and also increased the uptake of GDP-mannose in yeast Golgi vesicles that expressed the *GONST1* protein, we concluded that *GONST1* is an Arabidopsis NST that uses GDP-mannose as its substrate.

Searches of the Arabidopsis genome database reveal that *GONST1* is a member of a large family of putative Golgi NSTs in Arabidopsis (P. Dupree, unpublished results). One of these, At1g07290, shows ~50% amino acid identity to *GONST1* and possesses the GXLNK motif; therefore, it is a candidate for a second Arabidopsis GDP-mannose transporter. The other sequences show more distant homology and perhaps are translocators of the many other nucleotide sugars required in the Golgi lumen. Indeed, there is a large family of eukaryotic NSTs with divergent sequences and nucleotide sugar specificities (Berninsone and Hirschberg, 2000). To date, the majority of NSTs have been found to be specific for a single nucleotide sugar (Hirschberg et al., 1998). For example, the UDP-*N*-acetylgalactosamine transporter isolated from rat liver transports only this nucleotide sugar (Puglielli et al., 1999). Monospecific transporters would permit simple regulation of luminal nucleotide sugar content through the modulation of expression levels or potential post-translational modifications that may lead to regulation of their activities. However, recently, a small number of transporters have been shown to transport more than one nucleotide sugar (Hong et al., 2000; Berninsone et al., 2001). Interestingly, LPG2 is multispecific, transporting GDP-arabinose and GDP-fucose as well as GDP-mannose (Hong et al., 2000). Hence, it is possible that *GONST1* also may be involved in the transport of other nucleotide sugars, such as GDP-fucose and GDP-glucose, both of which are required for plant glycan synthesis (Piro et al., 1993; Wulff et al., 2000).

The *GONST1*-YFP fusion protein was targeted to the Golgi apparatus, displaying a typical punctate pattern of fluorescence when expressed transiently in onion epidermal cells (Figure 6A). This punctate pattern represented Golgi

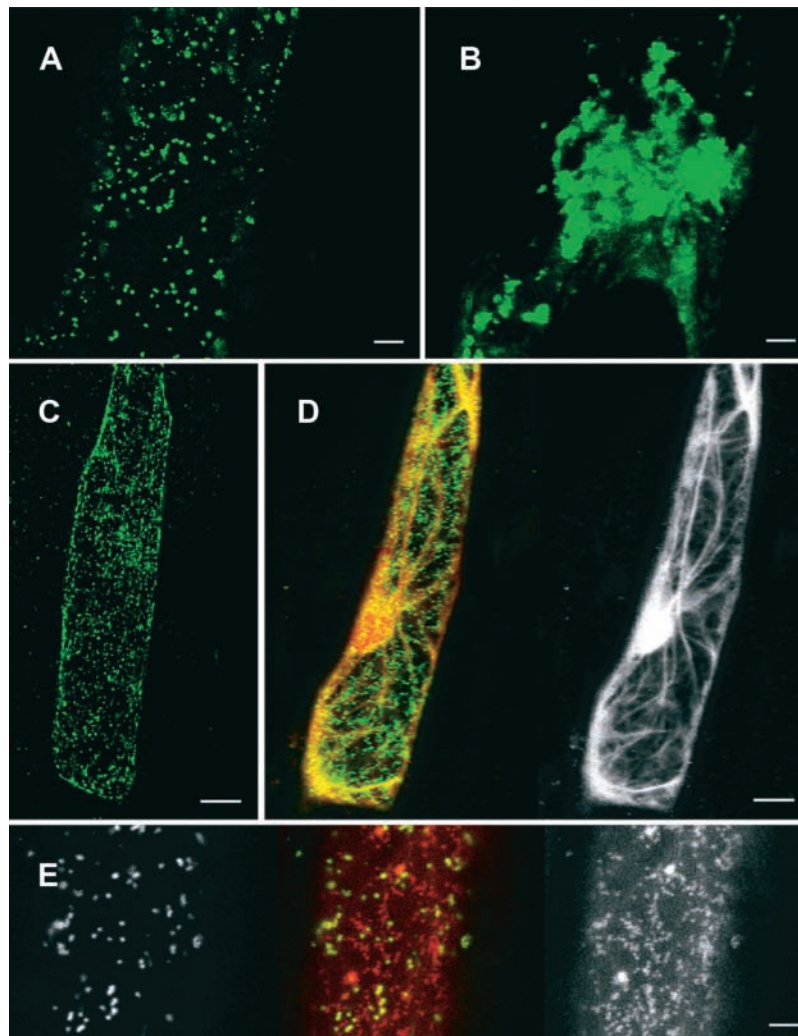


Figure 6. Distribution of GONST1-YFP in Living Onion Epidermal Cells.

(A) Extended focus confocal image through cortical cytoplasm of a single onion cell showing the punctate distribution of fluorescent GONST1-YFP. Bar = 10 μm .

(B) Extended focus image through cortical cytoplasm of a single cell as shown in **(A)** showing clustering of GONST1-YFP fluorescence after BFA treatment. Bar = 10 μm .

(C) Extended focus image of a cell as shown in **(A)**. Bar = 25 μm .

(D) Image of a single onion cell as in **(C)** showing colocalization of the actin cytoskeleton (yellow) and GONST1-YFP (green) (left image) and the actin cytoskeleton alone (right image). Bar = 25 μm .

(E) Localization of mitochondria (red and right image) and GONST1-YFP (green and left image). Bar = 10 μm .

stacks, because BFA treatment caused the fluorescently labeled Golgi apparatus to aggregate (Figure 6B). The Golgi stacks harboring GONST1-YFP also were shown to stream along the actin components of the cortical cytoskeleton (Figure 6D), consistent with previous studies of Golgi streaming (Boevink et al., 1998; Nebenfuhr et al., 1999). It will be interesting to determine by immunoelectron microscopy whether GONST1 is localized to specific cisternae within the Golgi

stack, because the glycosyltransferases in the different cisternae require different substrates (Dupree and Sherrier, 1998). Targeting of NSTs to the Golgi apparatus is an interesting but poorly understood phenomenon. Studies on the yeast GDP-mannose transporter indicate that the N-terminal sequence may facilitate export to the Golgi apparatus and that dimerization of Vrg4p also is necessary (Gao and Dean, 2000). Although we have not fully characterized the targeting

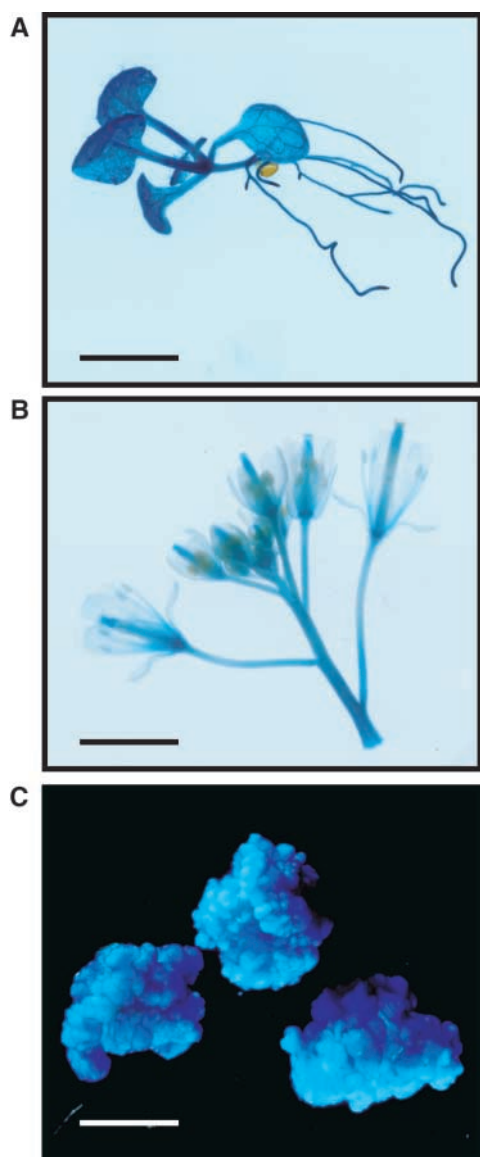


Figure 7. Expression of GONST1-GUS in Arabidopsis.

- (A)** Seedling, stained overnight.
(B) Inflorescence, stained overnight.
(C) Callus, stained for 30 min.
 Bars = 0.5 cm.

of GONST1 in yeast, the localization of GONST1 in the Golgi-rich vesicles of transformed yeast cells (Figures 4B and 4C) further suggests some conservation between plants and yeast of targeting of Golgi NSTs.

What are the roles of a GDP-mannose transporter in plants? Mannose is present in a range of cell wall polysaccharides in a variety of plant species. The major mannose-containing polysaccharides are the mannans, with a back-

bone of β -(1 \rightarrow 4)-linked mannose residues, glucomannans, in which glucose partly replaces the mannose, and the closely related galactomannans, with galactose residues α -(1 \rightarrow 6) linked to the mannan backbone. Galactomannans function as storage polysaccharides in many legume seed endosperms, and similarly, pure mannans are found in the seed of both monocotyledonous and dicotyledonous plant species (Buckeridge et al., 2000). Glucomannan is a major hemicellulose of gymnosperm secondary cell walls and is a significant component of angiosperm secondary walls (Piro et al., 1993). We know little as yet about the presence of mannan in Arabidopsis cell walls, but mannose constitutes 4 to 9% of the neutral sugars present in leaf walls (Reiter et al., 1997), which may be derived from secondary wall mannan. Our expression studies indicate that *GONST1* is expressed throughout the plant (Figure 7), which may suggest that it is required for the synthesis of a variety of mannose-containing macromolecules other than secondary wall mannan. Alternative mannose-containing structures include glucuronomannan, a polymer of very low abundance and unknown function, which has a backbone of α -(1 \rightarrow 4)-linked mannose and β -(1 \rightarrow 2)-linked glucuronic acid. In addition, arabinogalactan proteins have been reported to contain mannose residues (Fincher et al., 1983), and glycolipids also can be decorated with mannose (Fujino et al., 1985). Based on what we know about the synthesis of other polysaccharides and yeast glycolipids, the addition of mannose to these glycans likely occurs in the Golgi apparatus, but this has yet to be investigated in Arabidopsis. *GONST1* is unlikely to be involved in *N*-linked glycan synthesis, because that occurs on a dolichol-linked precursor in the ER (Schenk et al., 2001). If *GONST1* transports other nucleotide sugars such as GDP-fucose, it could be involved in the synthesis of xyloglucan and pectin as well as mannose-containing polysaccharides.

The role of GDP-mannose transport across the Golgi membrane in the substrate control of biosynthesis has been demonstrated in studies of the yeast *Vrg4p* (Dean et al., 1997). In these studies, Dean and colleagues demonstrated that the glycoproteins and glycolipids synthesized in the *vrg4-2* mutant were mannosylated to a much lower level. These studies and those of others (Toma et al., 1996) strongly support the hypothesis that NST activity may be an important control point in glycosylation, via the provision of substrate for the luminal glycosyltransferases (Abeijon et al., 1997). It has been shown that the ratio of glucose and mannose in glucomannan can be altered by varying the GDP-mannose/GDP-glucose concentrations in vitro (Piro et al., 1993). The catalytic domains of all known plant Golgi glycosyltransferases reside within the Golgi lumen (Keegstra and Raikhel, 2001). Therefore, a transporter is required to provide substrates for these enzymes, and we propose that the activity of this transporter might regulate synthesis by limiting substrate to these enzymes. However, the possibility exists that some glycosyltransferases may be multiple membrane-spanning cellulose synthase-like enzymes (Gibeaut, 2000). In this scenario, nucleotide sugars would be delivered di-

rectly to the catalytic site of the enzymes on the cytosolic side of the Golgi membrane, and the growing polysaccharide chain would be extruded into the Golgi lumen (Gibeau, 2000). There is as yet no experimental evidence for this model. Future experiments on Arabidopsis plants with the *GONST1* gene disrupted will enable a distinction to be made between these two models for the incorporation of mannose into various glycans in the plant Golgi apparatus.

METHODS

Plant Growth and Transformation

The *Arabidopsis thaliana* (ecotype Columbia) used in this study was grown according to Wee et al. (1998). Initiation and maintenance of liquid callus cultures were performed according to Prime et al. (2000). Transgenic Arabidopsis lines were generated by *Agrobacterium tumefaciens*-mediated transformation using the floral dipping technique as described by Clough and Bent (1998). Transformants were selected according to Wee et al. (1998).

Yeast Strains and Growth Conditions

Saccharomyces cerevisiae strains JPY25 6c (MAT α *ura3-52 his3- Δ 200 trp1- Δ 901 ade2-101 dpm1*) and JPY26 3d (MAT α *ura3-52 leu2-3 112 ade2-101 vrg4-2 dpm1*) (Dean et al., 1997) were grown on synthetic complete (SC) agar (Sherman, 1991) supplemented with 0.5 M KCl (Poster and Dean, 1996) at 30°C. JPY26 3d transformed with the *URA3*-containing yeast integration plasmid pSc-GONST1 were selected on SC-uracil agar. Hygromycin B and vanadate sensitivity were tested by supplementing SC plates with 500 μ g/mL hygromycin B (Dean, 1995) and 5 mM sodium orthovanadate (Ballou et al., 1991), respectively. Plates were photographed 4 or 5 days after streaking. Yeast strain JPY26 3d was transformed by the lithium acetate procedure (Agatep et al., 1998).

Generation of Constructs

DNA manipulations were performed according to standard protocols (Sambrook et al., 1989). Expressed sequence tag (EST) clones, Texas A&M University bacterial artificial chromosome (BAC) filters, and BAC clones were obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus). The BAC filters were screened using the insert from EST clone 139F21T7 labeled with fluorescein, hybridized, and visualized using the Gene Images module from Amersham Pharmacia. The *GONST1* gene was predicted from the sequence of the positively hybridizing BAC clones T15K7 and T10F5 using GENSCAN (Burge and Karlin, 1997), GRAIL (Uberbacher and Mural, 1991), and NETGENE2 (Hebsgaard et al., 1996). Polymerase chain reaction (PCR) was used to amplify the *GONST1* cDNA from the Arabidopsis ecotype Landsberg cDNA library pFL61 (obtained from the American Type Culture Collection, Rockville, MD) using specific primers designed from the genomic sequence. The upstream primer sequence was 5'-TTAAGAATTCT-AGGTCTTAGCTTTGCAATG-3' and the downstream primer was 5'-

TATATGTCGACTTAGGACTTCTCCCTCATTT-3'. The 1.0-kb PCR fragment corresponding to the *GONST1* cDNA was then ligated into the EcoRI-Sall site of the yeast expression vector p426GPD to create pGONST1.

The construct pGONST1-YFP was created by cloning a PCR-amplified fragment using the upstream primer (5'-TTAGAGGATCCTAGG-TCTTAGCTTTGCAATG-3') and the downstream primer (5'-GAG-AGAATTCGGACTTCTCCCTCATTTGG-3') into the BamHI and EcoRI sites of the binary vector pBIN 35S Knat YFP (a generous gift of Jim Haseloff, University of Cambridge, UK). For the construction of pGONST1::GUS, an \sim 2-kb region of genomic DNA upstream of the *GONST1* translation initiation codon was amplified by PCR from the TAMU BAC clone T10F5 using the upstream primer 113 (5'-GAG-ACCCGGGCTCAAGAACAAGATGAGTGTCGTAAAC-3') located 1868 bp upstream of the translation initiation codon and the downstream primer 114 (5'-TCTCGGATCCGCAAAGCTAAGACCTACCAAAGAAAC-3') located immediately adjacent to the initiation codon. The PCR product was digested with SmaI and BamHI and cloned immediately upstream of the β -glucuronidase (GUS) gene in plasmid pBI 101.2 (Jefferson et al., 1987) to create a transcriptional fusion. For the construction of pGEM4Z-GONST1 for RNA probe synthesis, pGONST1 was digested with KpnI-EcoRI to release the *GONST1* cDNA, and this DNA fragment then was subcloned into pGEM4Z (Promega). For the construction of pSc-GONST1, the *GONST1* cDNA sequence plus the adjoining glyceraldehyde phosphate dehydrogenase promoter were amplified by PCR from pGONST1 using the upstream primer 184 (5'-AATTAACCTCACTAAAGGG-3') and the downstream primer 185 (5'-CCTTCGCTCGACTCAATTGAG-GTCTTCCTCGCTGATTAATTTTGTTCGGACTTCTCCCTCATTTGGC-TC-3'). The resultant PCR fragment was digested and subcloned as a SacI-Sall fragment into the yeast integration vector pRS306 (Sikorski and Hieter, 1989). EST and BAC clones and all constructs were sequenced on Applied Biosystems sequencers (models 377 and 373; Foster City, CA) using big dye terminator reactions.

RNA Isolation and RNA Gel Blot Analyses

Total RNA was isolated as described by Coen et al. (1990). Poly(A)⁺ RNA was isolated from total RNA with the PolyAtract mRNA Isolation System (Promega). RNA gel blotting was performed as described by Sambrook et al. (1989) with 2 μ g of mRNA loaded per lane. The RNA probe synthesized from pGEM4Z-GONST1 was labeled with digoxigenin-11-UTP (Roche) and used according to the kit manufacturer's instructions.

Subcellular Fractionation of Yeast

Yeast strains were grown in MM2 (glucose [2%], yeast nitrogen base without amino acids [0.67%; Difco], uracil [40 mg/L], adenine [59 mg/L], ala, arg, asn, asp, cys, glu, gln, gly, his, *myo*-inositol, ile, lys, met, *p*-aminobenzoic acid, phe, pro, ser, thr, trp, tyr, val [all at 76 mg/L], and leu [380 mg/L]) at 30°C to an OD₆₀₀ of 3 to 5. Yeast cells were fractionated essentially as described previously (Gao et al., 2001) with some modifications. After washing in 10 mM Na₂S₂O₃, cells were resuspended in spheroplast buffer (1.4 M sorbitol, 50 mM potassium phosphate, pH 7.5, 10 mM Na₂S₂O₃, 0.33% β -mercaptoethanol, and 8 units lyticase/OD₆₀₀) and incubated for 2 hr at 37°C. Spheroplasts were pelleted and resuspended in ice-cold membrane buffer (0.8 M sorbitol, 10 mM triethanolamine, pH 7.2, 1 mM EDTA, and 1 mM

phenylmethylsulfonyl fluoride) and lysed by repeated pipetting (20 times) in a wide-bore pipette followed by four strokes in a Dounce homogenizer (Wheaton, Millville, NJ). The lysed cells were centrifuged at 1000g, forming S1 (supernatant) and P1 (pellet) fractions. P1 was lysed another two times by pipetting and homogenization as described above, and the three S1 fractions were pooled. S1 was centrifuged at 5,000g, 7,800g, and 125,000g to obtain fractions P-loose, P2, and P3, respectively. Pellet fractions were resuspended in a small volume of membrane buffer.

Protein Fractionation and Immunoblotting

P2 and P3 fractions were pelleted for 45 min at 100,000g and resuspended in 10 mM Tris-Cl, pH 7.5, and 1 mM EDTA. Protein concentrations were determined by the bicinchoninic acid method according to the manufacturer's instructions (Pierce Chemical Co.). Samples were incubated for 15 min at 37°C in Laemmli buffer containing 2% SDS, and after brief centrifugation, samples were fractionated by 12% SDS-PAGE and transferred to a nitrocellulose membrane by electroblotting (Laemmli, 1970; Towbin et al., 1979). Anti-GONST1 antiserum was raised in rabbits against a synthetic peptide of the 14 N-terminal amino acids (MKLYEHGVDLEDG) and affinity purified by Abcam (Cambridge, UK). When using the anti-GONST1 antiserum, membranes were blocked overnight at 4°C in PBS, 5% milk, and 0.5% Tween 20 followed by incubation in anti-GONST1 antiserum (1:2000 dilution) overnight at 4°C. The anti-Anp1p antiserum (a kind gift of Sean Munro, MRC-LMB, Cambridge, UK) was used as described previously (Jungmann and Munro, 1998). Antibody binding was detected using anti-rabbit antiserum conjugated to horseradish peroxidase (1:250 dilution; Bio-Rad, Hercules, CA) and enhanced chemiluminescence (Wee et al., 1998).

GDP-Mannose Uptake Assay

The Golgi-rich vesicle fraction (P3; 350 µg of protein) was incubated at 30°C in reaction mixture (1 mL) containing 0.3 M sucrose, 30 mM triethanolamine, pH 7.2, 5 mM MgCl₂, 5 mM MnCl₂, and 0.18 µM GDP-¹⁴C-mannose (224 mCi/mmol; DuPont-New England Nuclear). After 3 min, reactions were stopped with 3 mL of ice-cold stop buffer (0.5 M sucrose and 1 mM EDTA) and the Golgi vesicles were immediately washed onto 0.45-µm nitrocellulose filters (25 mm diameter; Whatman) by vacuum filtration (model FH 225V; Hoefer Amersham Pharmacia Biotech, Little Chalfont, UK). The vesicles were washed again with 5 mL of stop buffer and dried, and the filters were transferred to vials containing 5 mL of scintillation fluid (Ecoscint A; National Diagnostics, Atlanta, GA). The radioactivity was counted by scintillation counting. A time 0 assay was used to determine the amount of radioactivity bound nonspecifically to the outside of the vesicles and to the nitrocellulose filters.

Biolistic Transformation

Plasmid DNA (5 µg) was delivered into onion (*Allium cepa*) epidermal cells using tungsten particle bombardment. For experiments in which the actin cytoskeleton and the Golgi apparatus were labeled simultaneously, using constructs pGONST1-YFP and the talin-green fluorescent protein (GFP) construct (PSY C14) (Rees et al., 1990; McCann and Craig, 1997; Kost et al., 1998), respectively, 2.5 µg of each plasmid was bound to the tungsten microprojectiles.

Onion bulbs were purchased from a local market, and inner epidermal peels were placed on agar plates containing 1 × Murashige and Skoog (1962) salts, 30 g/L sucrose, and 2% agar (type IV; Sigma, St. Louis, MO), pH 5.7. Peels were bombarded within 1 hr of transfer to agar plates. Tungsten microprojectiles (1.1 µm; Bio-Rad) were coated with DNA according to the manufacturer's instructions. Microprojectiles were bombarded into the onion epidermal cells using a Biolistic PDS-1000/He system (Bio-Rad) with 900-p.s.i. rupture discs under a vacuum of 28 in Hg. After bombardment, the cells were allowed to recover for 48 hr on agar plates at 22°C in continual light before being viewed under the confocal microscope. To monitor the effects of brefeldin A (BFA) on the distribution of pGONST1-YFP, transformed onion epidermal peels were incubated in 100 µg/mL BFA for 2 hr. The effects of BFA on the distribution of the YFP signal then were monitored as described above. For the MitoTracker experiments, sections of transformed onion epidermal peels were stained with 65 µM CM-H₂ TMROS (MitoTracker Orange, Molecular Probes, Eugene, OR) by incubation in the dark for 10 min before viewing.

Confocal Microscopy

Imaging of GFP, YFP, and MitoTracker fluorescence was performed on a Leica (Wetzlar, Germany) DMRXA confocal laser scanning microscope. Serial optical sections of 0.5 to 2 µm were obtained using an excitation wavelength of 488 nm for GFP and MitoTracker and 514 nm for YFP, with emission signals being collected at the emission maxima of 510, 527, and 576 nm, respectively. A combined extended focus image then was produced from the confocal series using Leica TCS-NT software.

Detection of GUS Activity

Preliminary screening of 59 independently transformed lines for GUS activity was performed using a fluorometric assay as described by Gallagher (1992). Five independent homozygous lines (giving effectively 100% kanamycin-resistant progeny after selfing for two generations of transformants) identified from the GUS assay screen were selected for GUS histochemical analyses. Transgenic Arabidopsis seedlings, inflorescences, and callus from each of the five lines were stained with 1 mM 5-bromo-4-chloro-3-indoyl-β-D-glucuronide (Melford Laboratories, Kings Lynn, UK) in staining buffer (100 mM NaPO₄, pH 7.2, 25 mM EDTA.Na₂, 0.5 mM K₃Fe(CN)₆, 0.5 mM K₄Fe(CN)₆, and 0.1% Triton X-100) for between 30 min and overnight at 37°C and were cleared in 70% ethanol for 48 hr. Stained plant material was photographed using a Nikon (Tokyo, Japan) F camera body attached to a Nikon Multiphot Macro system with Kodak 64 T tungsten film.

Accession Number

The EMBL accession number for the *GONST1* cDNA is AJ314836.

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