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Arabidopsis thaliana expresses multiple Golgi-localised nucleotide-sugar transporters related to GONST1

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Abstract Transport of nucleotide-sugars across the Golgi membrane is required for the lumenal synthesis of a variety of essential cell surface components, and is mediated by nucleotide sugar transporters (NSTs) which are members of the large drug/metabolite superfamily of transporters. Despite the importance of these proteins in plants, so far only two have been described, GONST1 and AtUTr1 from *Arabidopsis thaliana*. In this work, our aim was to identify further Golgi nucleotide-sugar transporters from *Arabidopsis*. On the basis of their sequence similarity to GONST1, we found four additional proteins, which we named GONST2, 3, 4 and 5. These putative NSTs were grouped into three clades: GONST2 with GONST1; GONST3 with GONST4; and GONST5 with six further uncharacterized proteins. Transient expression in tobacco cells of a member of each clade, fused to the Green Fluorescent Protein (GFP), suggested that all these putative NSTs are localised in the Golgi. To obtain evidence for nucleotide sugar transport activity, we expressed these proteins, together with the previously characterised GONST1, in a GDP-mannose transport-defective yeast mutant (*vrg4-2*). We tested the transformants for rescue of two phenotypes associated with this mutation: sensitivity to hygromycin B and reduced glycosylation of extracellular chitinase. GONST1 and GONST2 complemented both phenotypes, indicating that GONST2, like the previously characterized GONST1, is a GDP-mannose transporter. GONST3, 4 and 5 also rescued the antibiotic sensitivity, but not the chitinase glycosylation defect, suggesting that they can also transport GDP-mannose across the yeast Golgi membrane but with a lower efficiency. RT-PCR and analysis of Affymetrix data revealed partially overlapping patterns of expression of *GONST1–5* in a variety of organs. Because of the differences in ability to rescue the *vrg4 -2* phenotype, and the different expression patterns in plant organs, we speculate that GONST1 and GONST2 are both GDP-mannose transporters, whereas GONST3, GONST4 and GONST5 may transport other nucleotide-sugars *in planta*.

Keywords *Arabidopsis thaliana* · GDP-mannose transporter · Golgi apparatus · GONST · Plant cell wall

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Introduction

The plant Golgi apparatus is an organelle that is specialised for glycosylation. Most non-cellulosic cell wall polysaccharides are synthesised there, and proteins and lipids are modified by the addition of carbohydrate side chains. The substrates for the synthesis of these glycans are sugars that are activated by the addition of a nucleotide (nucleotide-sugars), such as GDP-mannose, GDP-fucose, UDP-galactose, UDP-glucose and UDP-xylose (Hirschberg et al. 1998; Reiter and Vanzin 2001). Current evidence suggests that most enzymes involved in nucleotide-sugar synthesis are located in the cytosol (Coates et al. 1980; Bonin et al. 1997), although a few inter-conversion pathways are duplicated in the Golgi lumen (Harper and Bar-Peled 2002; Burget et al. 2003).

Two topological models have been proposed for the synthesis of non-cellulosic plant polysaccharides (Gibeaut 2000). Multifunctional glycosyltransferase enzymes that span the Golgi membrane may use cytosolic nucleotide-sugars and release the polysaccharide product on the lumenal side of the membrane. This translocation activity would be similar to that of cellulose synthases at the plasma membrane. Candidates for such non-cellulosic polysaccharide synthases include the cellulose synthase-like (Csl) proteins (Richmond and Somerville 2001; Dhugga et al. 2004), but the topology of these proteins has not yet been experimentally demonstrated. In addition, glycosyltransferases with a single membrane-spanning region and a lumenal catalytic domain transfer a sugar from a nucleotide-sugar onto the acceptor glycan chain. The catalytic domains of several such enzymes have been shown to face the lumen of the Golgi apparatus in plants, yeast and animals (Gibeaut 2000; Keegstra and Raikhel 2001; Sterling et al. 2001). A nucleotide-sugar transporter (NST) in the Golgi membrane is required to supply these enzymes with nucleotide-sugar substrates from cytosolic sources. Similarly, NSTs are required for lumenal glycosylation of glycoproteins and glycolipids. Given the diversity of nucleotide-sugars and the scale of biosynthetic processes in the Golgi apparatus, substantial nucleotide-sugar transport across the Golgi membrane is likely to be necessary.

Recently, the first two plant Golgi NSTs were characterised from *Arabidopsis thaliana*: GONST1 is capable of transporting GDP-mannose (Baldwin et al. 2001), while AtUTr1 transports UDP-galactose and UDP-glucose (Norambuena et al. 2002). Genes encoding NSTs have also been cloned from a wide variety of other organisms (reviewed by Gerardy-Schahn et al. 2001; Jack et al. 2001; Martinez-Duncker et al. 2003). These NSTs are proteins with multiple membrane-spanning domains, and appear to function as antiporters, exchanging nucleoside-monophosphate for specific nucleotide-sugars (Capasso and Hirschberg 1984). Many are thought to be monospecific, but a few are able to transport more than one nucleotide-sugar (Hong et al. 2000; Norambuena et al. 2002). Given their roles in providing the substrates for glycosyltransferases, NSTs could regulate glycan synthesis via substrate-level control. For example, mutants in the *Saccharomyces cerevisiae* Golgi GDP-mannose transporter VRG4 are unable to mannosylate proteins and glycolipids effectively (Dean et al. 1997) and the *Leishmania donovani* GDP-mannose transporter mutant *lpg2* is defective in the mannosylation of cell surface lipophosphoglycan, which occurs in the Golgi (Ma et al. 1997).

NSTs belong to the drug/metabolite superfamily of transporters. In plants, these include the plastidic triose-phosphate translocator (TPT) and related phosphate translocator proteins (Knappe et al. 2003). Nucleotide-sugars are known to be transported across plastid membranes by unrelated translocators (Shannon et al. 1998), but it remains possible that as yet undiscovered plastidic NST/TPT-related proteins are also required.

Although only two plant NSTs have been characterised to date, it is highly probable that a large number of transporters, perhaps with multiple nucleotide sugar specificities, are necessary to transport the variety of nucleotide-sugars across the Golgi membrane. Here, we investigate the localisation and demonstrate the transport activity of four novel homologues of GONST1 from *A. thaliana*. Our results suggest the existence in the Golgi apparatus of multiple NSTs with different biochemical specificities and patterns of expression.

Materials and methods

Plant growth and transformation

Arabidopsis thaliana (cv. 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).

Isolation of cDNAs and construction of expression plasmids

To identify *Arabidopsis* GONST1 homologues, protein and nucleotide databases were searched with BLAST or TBLASTN using the amino acid sequence of GONST1 as the query (At2g13650; Baldwin et al. 2001). cDNAs encoding GONST2, 3, 4 and 5 were cloned by RT-PCR using a commercially available kit, according to the manufacturer's instructions (Invitrogen) from RNA purified from different *Arabidopsis* organs: GONST2 from callus, GONST3 and GONST4 from rosette leaves and GONST5 from siliques. The *attB* -flanked PCR products for recombination in the GATEWAY cloning system entry vector pDONR201 (Invitrogen) were generated using the *attB1* sequence 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTGACC-3′ and the *attB2* sequence 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTA-3′ in each primer pair. The primers used for *GONST* amplification were the following: for *GONST1*, attB1-ATGAAATTGTACGAACACGAT and attB2-GGACTTCTCCCTCATTTT; for *GONST2*, attB1-ATGTCTGCCGTGAAACTG and attB2-TGACATTTTAGCTCTGGCA; for *GONST3*, attB1-ATGTCGACGAATGATGAG and attB2-TAGTTTCTCTTCTGATTTCA; for *GONST4*, attB1-ATGTCGTCCTCTCGATTC and attB2-TACAACAGAAGCTAGTTTC, and for *GONST5* attB1-ATGGAGGAAGGAAGTCTG and attB2-AATTTTACTCTCGAGTTTATC. The cDNA sequences of *GONST2–5* have been deposited in the EMBL/GenBank database; their Accession Nos. are listed in the legend to Fig. 1.

For expression in the *Saccharomyces cerevisiae vrg4-2* strain, the cDNA of each *GONST* gene was recombined into the yeast destination vector, pYES-DEST52,

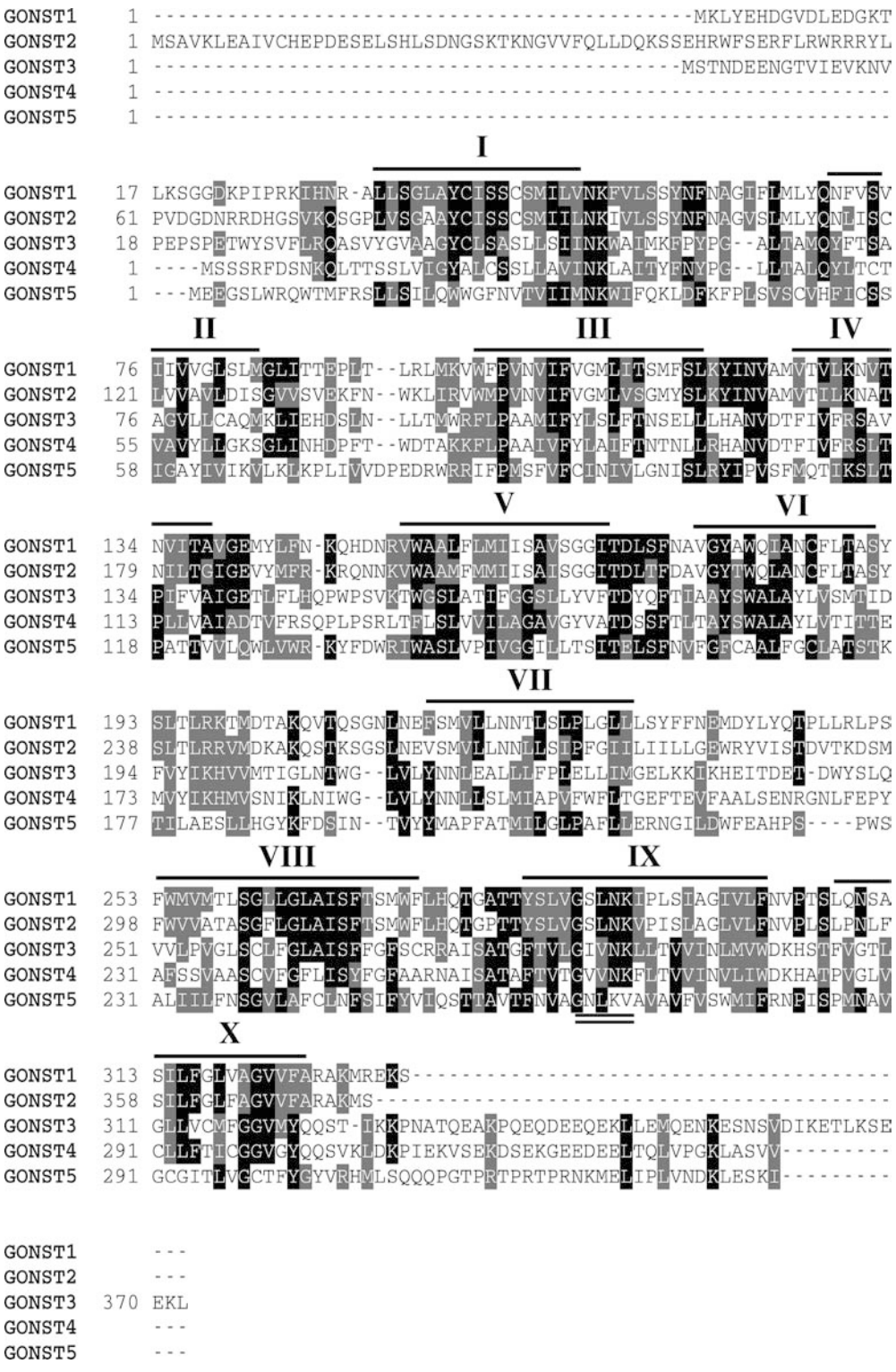

Fig. 1 Alignment of GONST1 homologues from *Arabidopsis thaliana*. The locations of the predicted ten membrane-spanning domains are indicated by the *Roman numerals* (I-X). The position of the GXLNK motif in GONST1 and GONST2 is marked with a *double underline*. Amino acid identities are *boxed in black* and conservative amino acid substitutions are *boxed in grey*. Amino acids are numbered beginning with the first amino acid of each of the proteins. The EMBL Accession Nos. of the corresponding cDNA are: *GONST2*, AJ551325; *GONST3*, AJ551326; *GONST4*, AJ551327; *GONST5*, AJ551328

according to the manufacturer's instruction manual (Invitrogen).

For expression in plants, *GONST1*, *GONST4* and *GONST5* were amplified by PCR from the corresponding GATEWAY entry vectors to introduce unique *Xba* I and *Sal* I restriction sites at the 5′ and 3′ end, respectively (underlined in the primer sequences). The primers used were the following: for *GONST1*, 5′-CAGGACGTCTAGATGAAATTGTACGAACACGATGGAG-3′ and 5′-CATGACCGTCGACTTGGACTTCTCCCTCATTTTGGCTCTGGC-3′; for *GONST4*, 5′-CAGGACGTCTAGATGTCGTCCTCTCGATTCGATTCAAAC-3′ and 5′-CATGACCGTCGACTTTACAACAGAAGCTAGTTTCCCCGG-3′, and for *GONST5*, 5′-CAG-

GACGTCTAGATGGAGGAAGGAAGTCTGTGGC-G-3′ and 5′-CATGACCGTCGACTTAATTTTACTC-TCGAGTTTATCATTAAC-3′. Each gene was then cloned into the binary vector pVKH18-EN6 (Batoko et al. 2000) for *Agrobacterium* -mediated transformation and transient expression in planta. The resulting recombinant vectors were designated pVKH18-EN6::-GONST1, pVKH18-EN6::GONST4 and pVKH18-EN6::GONST5. In these plasmids each gene was inserted in frame at the 5′-end of the *GFP5* gene (Haseloff et al. 1997).

DNA sequencing and sequence analysis

DNA sequence analysis was performed at the Sequencing Facility of the Department of Biochemistry (University of Cambridge) using sequencers (Models 377 and 373, Applied Biosystems) and Big Dye (Applied Biosystems) termination reactions. Sequences were analysed with the Wisconsin Package version 10.3 (Accelrys).

Hydrophobic sequences were determined using the program TMPRED (http://www.ch.embnet.org/software/TMPRED_form.html).

Predicted molecular weights, pI and the *N* -glycosylation sites (Wagh and Bahl 1981) were calculated using web servers (http://www.up.univ-mrs.fr/~wabim/d_abim/compo-p.html and http://www.cbs.dtu.dk/services/NetNGlyc/). The phylogenetic tree of the NSTs and TPT proteins was constructed using the program T-Coffee (http://www.ch.EMBnet.org; Notredame et al. 2000). Sequences were aligned and trimmed of all poorly conserved sequence that was improperly aligned. To investigate clustering with GDP-mannose transporters, trees were also constructed including Vrg4p (Dean et al. 1997), *Candida glabrata* Vrg4 (Nishikawa et al. 2002a), *C. albicans* Vrg4 (Nishikawa et al. 2002b) and Lpg2 (Ma et al. 1997). GONST amino acid sequences were aligned using ClustalW (http://www.ebi.ac.uk/clustalw) and BOXSHADE 3.21 programs (http://www.ch.embnet.org/software/BOX_form.html). Amino acid sequence identities were compared using DNAMAN software package (Lynnon Biosoft).

RT-PCR and Affymetrix data analysis of *Arabidopsis* GONST gene expression

Duplicate samples of total RNA (isolated from flowers, siliques, stems of 6-week-old plants, callus from 4-day-old plants, rosette leaves and roots from 3-week-old plants) were prepared according to Brusslan and Tobin (1992). Aliquots (1 μg) of total RNA were reverse transcribed according to the enzyme manufacturer's instructions (Invitrogen). RT-PCRs were carried out using the following gene-specific sense and antisense primers, located just upstream and downstream of the start and stop codons, respectively: for *GONST1*, 5′-TTAGAGGATCCTAGGTCTTAGCTTTGCAATG-3′ and 5′-GAGAGAATTCGGACTTCTCCCTCATT-TGG-3′; for *GONST2*, 5′-CGCGAATTCGAGGG AACTTTTCTGCGATT-3′ and 5′-CGCGAATTCCA-CAATCCGGCTTGGAAAAT-3′; for *GONST3*, 5′-GA-AGGATCCGTCGAAAGCTAATGAGTCAC-3′ and 5′-GAAGGATCCCTGTAAGCTCCAACCACTAT-3′; for *GONST4*, 5′-CGCAAGCTTGCCTTCCTCTAAT-TAGTCTC-3′ and 5′-CGCAAGCTTAGGCTGCAA-AACAATGACAC-3′; and for *GONST5*, 5′-CGC-TCTAGACTTTTGGATCCTTCTCTGAC-3′ and 5′-CGCTCTAGAGCGTTCAATAGTCTAGTGAC-3′. Controls without RT were also performed. For PCR amplification 2 μl of the RT mix was used. The PCR was carried out in a reaction volume of 20 μl, containing 1× Buffer with $MgCl_2$ (Roche Diagnostics), 10 μM of each primer and 10 mM dNTPs. The PCR program was as follows: 5 min at 94°C for the hot start, held at 80°C to add 0.7 U of High Fidelity Taq DNA polymerase (Roche), followed by 10 cycles of 15 s at 94°C, 30 s at the optimal annealing temperature (50°C), and 90 s at 72°C; then 30 cycles of 15 s at 94°C, 30 s at 61°C, and 90 s at 72°C, followed by 10 min at 72°C. One-tenth of the amplified product was used as the template for nested PCR reactions, following the same protocol as above, and using the GATEWAY primers listed above. The PCR program was as follows: 5 min at 94°C for the hot start, held at 80°C to add 0.7 U of High Fidelity Taq DNA polymerase (Roche), followed by 20 cycles of 15 s at 94°C, 1 min at the optimal annealing temperature (54°C), and 1 min at 72°C; then 10 cycles of 15 s at 94°C, 1 min at 60°C, and 1 min at 72°C, followed by 10 min at 72°C.

Affymetrix data for the expression of *GONST1–5* in *A. thaliana* cv. Columbia were obtained from the Nottingham *Arabidopsis* Stock Centre (NASC, University of Nottingham, UK; http://arabidopsis.info). The datasets used were: A1-WillA-Con (for callus data), A3-Rente-WS2-Control_SLD (for seedling data), Yap_A1-AMF (for root data), A2-Greco-WT (for rosette leaf data), DT002_ATH1_pollen (for pollen data), Millenaar_A1-Mill-Air-Rep1 (for stem data) and Vizcay Barrena_A3-Wilson-lea (for flower data). A gene was considered expressed if designated present by the Affymetrix algorithm (http://www.affymetrix.com/support/technical/technotes/statistical_reference_guide.pdf) in the majority of replicates.

Plant transient transformation and confocal microscopy

Four-week-old *Nicotiana tabacum* SR1 (cv. Petit Havana) greenhouse plants grown at 24/18°C on a 16 h/8 h light/dark cycle, were used for *Agrobacterium*-mediated transformation and transient expression (Batoko et al. 2000) by infection with a bacterial culture with an OD_{600} value of 0.2. Transformed leaves were analysed 72 h after infection of lower epidermal cells. Where indicated, Brefeldin A (BFA; Sigma-Aldrich; stock solution: 10 mg/ml in DMSO) was used at a concentration of

10 μg/ml. The stock solution was kept at 20°C and a working solution was prepared fresh just prior to use. Leaf segments were kept in BFA for 1 h before analysis and observation under the microscope. Samples were mounted on a slide with the BFA solution.

Confocal imaging was performed using an upright Zeiss META LSM 510 Laser Scanning Microscope and a 63× water-immersion objective. For imaging co-expression of YFP and GFP constructs, excitation lines of an argon ion laser of 458 nm for GFP and 514 nm for YFP were used alternately, with line switching using the multi-track facility of the microscope. The fluorescence was detected using a 458/514 nm dichroic beam splitter and a 475–525 nm bandpass filter for GFP and a 560–615 nm bandpass filter for YFP. Post-acquisition image processing was performed with the LSM 5 Image Browser (Zeiss) and Adobe PaintShop Pro 7.0 software.

Yeast strains and growth conditions

The wild-type *S. cerevisiae* strain RSY255 (*MATα* ura3-52 leu2-211; from R. Schekman) and the *vrg4-2* mutant NDY5 (*MATα* ura3-52 leu2-211 vrg4-2; Poster and Dean 1996) were grown at 30°C in either synthetic complete (SC) medium (Sherman 1991) or YP medium (1% yeast extract, 2% bactopeptone), supplemented with 2% sugar (glucose or galactose). The *vrg4-2* strain was transformed by the lithium acetate procedure (Agatep et al. 1998). Transformants were selected on SC-uracil medium. Hygromycin B sensitivity was assayed by supplementing YP plates with hygromycin B (30 μg/ml), as described by Dean (1995). Wild-type and transformed *vrg4-2* strains were streaked at a concentration of 2 OD/ml and plates were photographed 2 days after streaking.

Fractionation of yeast and immunoblotting

Yeast strains were grown in SC medium supplemented with 2% raffinose, until the cultures reached a density of 0.4 OD and then transferred to SC medium supplemented with 2% galactose and 1% raffinose until 1–2 OD. Yeast cells were disrupted as described previously (Baldwin et al. 2001), except that total membranes were extracted by centrifugation at 100,000× *g* for 3 h and resuspended in 10 mM TRIS-HCl (pH 7.5) and 1 mM EDTA. Protein concentrations were determined by the bicinchoninic acid method according to the manufacturer's instructions (Pierce Biotechnology). Samples were heated for 10 min at 70°C in Laemmli buffer, fractionated by 10% SDS-PAGE and transferred to a nitrocellulose membrane by electroblotting (Laemmli 1970; Towbin et al. 1979). The anti-V5 antisera were used according to the manufacturer's protocol (Invitrogen). Antibody binding was detected using goat anti-mouse IgG conjugated to horseradish peroxidase (1:2000 dilution; Bio-Rad) and the ECL Plus reagent kit (Amersham Biosciences).

Isolation of extracellular chitinase and analysis of its chitin binding capacity

Native extracellular chitinase was purified from yeast grown to stationary phase in YP medium supplemented with galactose, essentially as described previously (Kuranda and Robbins 1991; Abeijon et al. 1993). Chitin used in binding experiments was prepared from purified chitin (Sigma) which had been boiled in 1% SDS-1% β-mercaptoethanol, and then extensively washed with water. Washed chitin (0.2 g) was added to the culture supernatants from wild-type and transformed *vrg4-2* strains (200 ml). The suspension was mixed overnight on a rotary shaker at 4°C. The chitin was then collected by centrifugation, and washed three times with an ice-cold solution containing 0.8% NaCl, 0.02% KCl, 0.12% Na_2HPO_4, and 0.02% NaH_2PO_4. The washed pellet was suspended in Laemmli buffer and heated at 100°C for 10 min to dissociate bound chitinase. Then chitinase samples corresponding to 20 ml of culture medium were fractionated by 6% SDS-PAGE, and the gel was stained with colloidal Coomassie blue G250, according to Prime et al. (2000). A scanned 16-bit image of the gel was analyzed for the migration of the chitinase band using Gene Tools software (Syngene).

Results

Identification of GONST1 homologues

To identify candidate Golgi NSTs in *Arabidopsis*, the protein and nucleotide sequence databases were searched for proteins closely related to GONST1. We selected four predicted proteins for further study, which we named GONST2, 3, 4 and 5, based on their higher sequence similarity to GONST1, particularly in the predicted nucleotide-sugar binding region (Gao et al. 2001). cDNAs encoding these proteins were cloned by RT-PCR from RNA isolated from various *Arabidopsis* organs. *GONST1* has six introns (Baldwin et al. 2001), and alignment of the cDNAs with the corresponding genomic sequences revealed the presence of several introns in *GONST2* and *GONST5* (Table 1). However, both *GONST3* and *GONST4* were found to lack introns.

The predicted amino acid sequence of GONST2 was similar at the N-terminus to that of the annotated protein At1g07290, but differed substantially from it at the C-terminus. GONST3, 4 and 5 sequences were identical to the ones predicted (At1g76340, At5g19980 and At1g21870, respectively). The sequences of the GONST proteins show significant identity over their entire length, except that GONST2 has an N-terminal sequence extension (Fig. 1). Overall sequence identity between the five GONST proteins ranges from 12 to

Table 1 Characteristics of the GONST proteins

Protein	Accession No. (MIPS code)	No. of introns in gene	No. of amino acids	Predicted molecular weight (kDa)	pI	No. of putative N-glycosylation sites
GONST1	At2g13650	6	333	36.8	9.8	2
GONST2	At1g07290	8	375	41.6	10.3	2
GONST3	At1g76340	-	372	41.7	4.9	2
GONST4	At5g19980	-	341	37.4	7.7	-
GONST5	At1g21870	4	341	38.8	10.3	3

Table 2 Amino acid identities of the GONST proteins

Protein	Degree of sequence identity (%)				
	GONST1	GONST2	GONST3	GONST4	GONST5
GONST1	100	60.9	15.9	17.5	19.5
GONST2		100	15.6	16.3	16.7
GONST3			100	35	11.9
GONST4				100	17.3
GONST5					100

61% (Table 2). The GONST proteins have calculated molecular weights ranging from 36.8 to 41.7 kDa and, with the exception of GONST3, all the proteins have a basic isoelectric point (Table 1). Apart from GONST4, all the GONST proteins have putative *N* -glycosylation sites. However, the locations of these sites are not conserved and most lie within probable membrane domains, suggesting that they may not be used. Hydrophobicity analysis of the GONST protein sequences revealed that they are highly hydrophobic (Fig. 1), which is consistent with their presumed roles as membrane-spanning transporters. Since the available algorithms are not able accurately to predict membrane-spanning regions in proteins with multiple transmembrane domains (TMDs), we used the alignment between all the GONST-related proteins to identify conserved hydrophobic regions. As predicted for GONST1 (Baldwin et al. 2001), this analysis suggested that they each contain 10 TMDs (Fig. 1). Thus, the sequence analysis indicated that GONST2–5 were good candidate NSTs suitable for further investigation.

The putative GONST proteins segregate into different clades

Golgi NSTs and plastidic TPT-related proteins are part of the drug/metabolite superfamily of transporters. To investigate their relationship to other NST/TPT-related proteins in *Arabidopsis* we generated a phylogenetic tree for GONST1–5, together with further homologues in the *Arabidopsis* database. We included the AtUTr1 NST family (Norambuena et al. 2002) and some phosphate-translocator-related proteins (Knappe et al. 2003).

This analysis indicated that the five GONST proteins can be grouped into three distinct clades, perhaps reflecting a specialisation for the transport of different substrates. GONST2 clustered with GDP-mannose transporters, such as GONST1 and *S. cerevisiae* Vrg4p (Fig. 2). Since GONST2 possesses a putative nucleotide-sugar-binding GXLNK motif region almost identical to that in GONST1, we hypothesized that it may be another Golgi GDP-mannose transporter.

GONST3 and GONST4 clustered together but outside this clade, and are the next most-similar proteins encoded in the *Arabidopsis* genome. Their relationship to GONST1, GONST2 and AtUTr proteins suggested that GONST3 and 4 could also be Golgi NSTs. GONST3 and 4 were recently classified in NST subfamily L, separately from GONST1 in subfamily K (Martinez-Duncker et al. 2003).

GONST5 is in a distinct clade of seven previously uncharacterised proteins (Fig. 2). Recently, Knappe

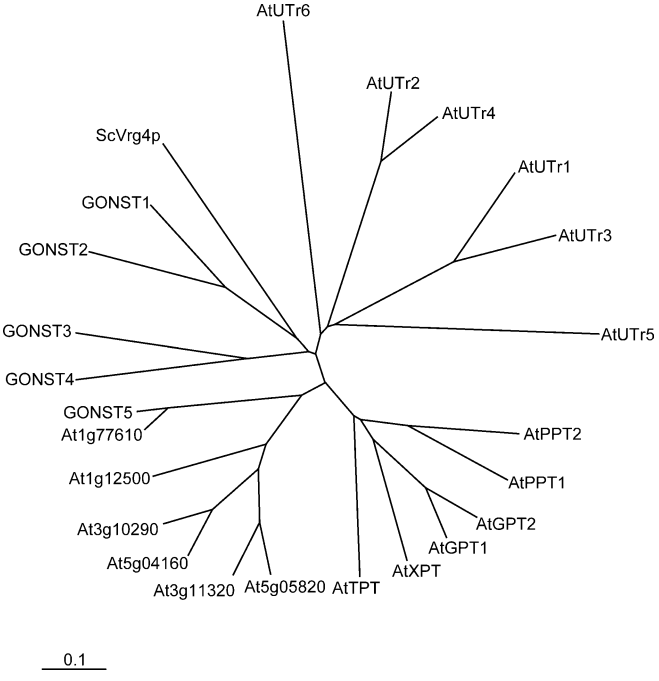

Fig. 2 Sequence relationships between GONST1–5 and previously described *Arabidopsis* NST and TPT-related proteins. A phylogenetic tree was constructed based on the amino acid sequences of GONST1–5 and all the known and putative NSTs and TPT-related proteins in *Arabidopsis*. The Accession Nos. of the sequences used were: ScVrg4p, U15599; AtUTr1, At2g02810; AtUTr2, At4g23010; AtUTr3, At1g14360; AtUTr4, At1g12600; AtUTr5, At3g46180; AtUTr6, At3g59360; AtTPT, At5g46110; AtXPT, At5g17630; AtGPT1, At5g54800; AtGPT2, At1g61800; AtPPT1, At5g33320; AtPPT2, At3g01550). The 0.1 scale bar represents 0.1 amino acid substitutions per site

et al. (2003) classified this clade as KV/A/G, based on the amino acids found after a conserved lysine in these and other NST/TPT-related proteins. Based on their divergence from known Golgi NSTs and plastidic transporters, we hypothesized that these proteins might have nucleotide sugar or other transport activities in the Golgi, plastid or elsewhere in the cell.

GONST1-GFP, *GONST4-GFP* and *GONST5-GFP* fusions are localised in the Golgi apparatus

To determine whether these novel GONST proteins are localised in the Golgi apparatus, as would be predicted of NSTs required for cell wall polysaccharide synthesis, we generated fusions with the Green Fluorescent Protein (GFP) and transiently expressed them in tobacco leaf epidermal cells.

Previously we have shown that GONST1-YFP is localised to the Golgi apparatus when transiently expressed in onion epidermal cells (Baldwin et al. 2001). To determine GONST1 localisation upon transient expression in tobacco epidermal cells, we first expressed GONST1-GFP together with the Golgi marker AtERD2-YFP (Boevink et al. 1998; Brandizzi et al. 2002b), and analysed the fluorescence with a laser scanning confocal microscope. As expected, we found a high level of colocalisation of the two proteins in small punctate structures typical of the Golgi apparatus (Fig. 3c, arrowhead), which were highly motile in the cytoplasm (not shown). We also co-expressed GONST1-GFP with the Golgi marker sialyltransferase-YFP (ST-YFP) (Wee et al. 1998; Brandizzi et al. 2002a), and also GONST1-GFP with GONST1-YFP, and we obtained a similarly high level of colocalisation in each case (not shown). These results indicated that GONST1-GFP and GONST1-YFP fusions are localised to the Golgi when transiently expressed in tobacco epidermal cells.

To investigate the localisation of GONST1 homologues in the cell, we also visualised the subcellular distribution of GONST4-GFP and GONST5-GFP in vivo after transient expression in tobacco epidermal cells. The punctate distribution of fluorescence resembled the pattern seen with the GONST1-GFP fusion (Fig. 3a, d, g). No GFP fluorescence was detected in the plastids (not shown). To determine whether the proteins were actually localised in the Golgi apparatus, we co-expressed the GONST4-GFP or GONST5-GFP fusions with GONST1-YFP. The GONST4-GFP and GONST5-GFP fusions showed substantial colocalisation with the GONST1-YFP (Fig. 3f and i, arrowheads), indicating that the GONST4 and 5 fusions are targeted to the same organelle as GONST1. In some cells, there were also some smaller motile structures that contained GONST-GFP fusions (Fig. 3a, d and g; arrows). The number of these smaller structures increased with expression level, suggesting they could be an artifact of over-expression. Figure 4 shows cells co-expressing GONST4-GFP or GONST5-GFP with GONST1-YFP that were treated with Brefeldin A (BFA). All the labelled structures were sensitive to BFA treatment, and, in the presence of the drug, the fluorescence pattern became similar to that of the endoplasmic reticulum. Together, their colocalisation with Golgi markers and their sensitivity to BFA indicate that GONST4 and GONST5 are localised to the Golgi in plant cells. We were unable, despite repeated attempts with various expression vectors, to express GONST2-GFP or GONST3-GFP in tobacco epidermal cells, suggesting they are not amenable to localisation by fusion to GFP.

Expression of GONST proteins in *S. cerevisiae*

To determine if any of the newly identified GONST proteins have NST activity, we expressed them in mutant yeast cells that are defective in nucleotide sugar transport. Before embarking on the functional characterization, we first tested whether the proteins could be successfully expressed in yeast.

Previously, we had shown that GONST1 could complement a *vrg4-2* yeast strain (Baldwin et al. 2001). *vrg4-2* strains are deficient in the ability to transport GDP-mannose because of a mutation in the GXLNK motif of Vrg4p (Dean et al. 1997; Gao et al. 2001) and consequently show abnormal sensitivity to hygromycin B (Dean 1995). Although it is not understood precisely how the hygromycin sensitivity arises, since a substantial number of cell wall mannan synthesis mutants acquire this phenotype, it is clear that it is a consequence of defects in mannosylation in the Golgi (Dean 1995).

GONST2–5 cDNAs were cloned into pYES-DEST52, a *GAL1* -inducible, C-terminally V5-tagged yeast expression vector. *GONST1* in pYES-DEST52 was used as a positive control, and the pYES-DEST-52 vector alone was used as a negative control (empty vector control). We were unable to transform and express all the GONST proteins in the *vrg4-2 dpm1* strain JPY26 used previously (Baldwin et al. 2001), so we transformed the *vrg4-2 DPM1* strain NDY5, which displays normal glycosylation activity in the ER.

After growth in galactose-containing medium to induce expression, membranes were prepared from the wild-type and transformed *vrg4-2* strains. Membrane proteins were resolved by SDS-PAGE, blotted onto a membrane filter and probed with anti-V5 antisera. Figure 5 shows that the antiserum detected multiple specific bands in lanes containing membrane fractions from *vrg4-2* transformed with the *GONST1*, *2*, *3*, *4* and *5* constructs. These bands were absent in the empty-vector control and in the wild-type strain (Fig. 5) and also absent when the cells were grown in glucose-containing medium (not shown). The apparent molecular weight of the major band detected in each transformant (~30–50 kDa) was similar but not identical to the predicted molecular weight of the GONST proteins (36.8–41.7 kDa; Table 1). These results were confirmed using antiserum raised against the second C-terminal epitope,

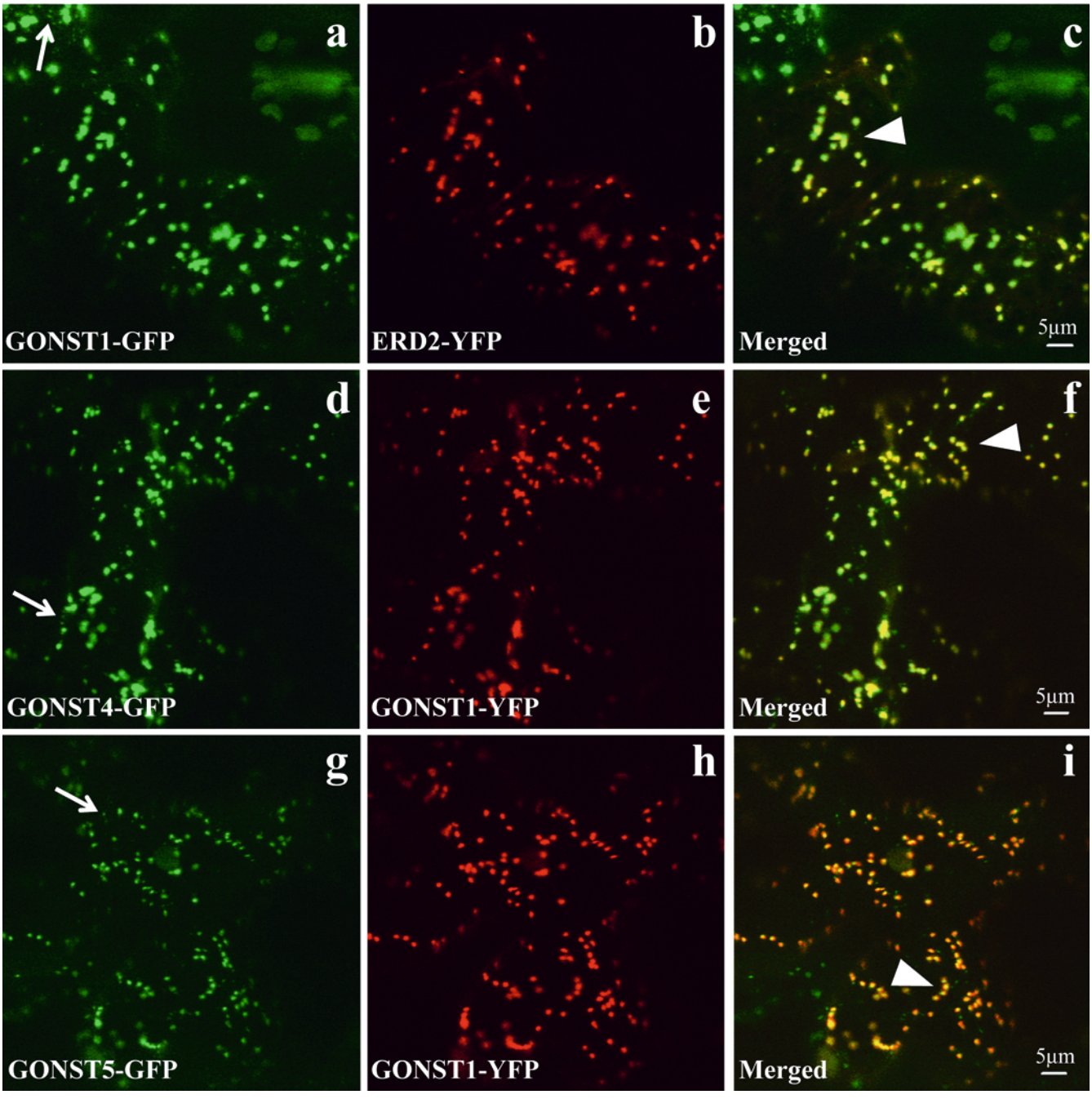

Fig. 3a–i Localisation of GONST-GFP fusions to the Golgi apparatus. Confocal scanning laser micrographs of a lower epidermis cell of a *N. tabacum* leaf. **a** GONST1-GFP. **b** Golgi-localised AtERD2-YFP. **c** Merged GONST1-GFP and AtERD2-YFP. **d** GONST4-GFP. **e** GONST1-YFP. **f** Merged GONST4-GFP and GONST1-YFP. **g** GONST5-GFP. **h** GONST1-YFP. **i** Merged GONST5-GFP and GONST1-YFP. Note the extensive overlap of the GONST-GFP proteins (*green*) and the Golgi marker YFP fluorescence (*red*), which appears *yellow* (*arrowhead*) in the merged images. Some GONST-GFP fluorescence is also identifiable in small structures near the Golgi bodies (*arrow*)

6×His (data not shown). Moreover, antiserum raised against the N-terminal segment of GONST1 (Baldwin et al. 2001) also recognized the same bands in fractions from *vrg4-2* cells expressing GONST1 (data not shown), demonstrating that GONST1 was not truncated at either the N- or C-terminus**.** Differences between predicted and apparent molecular weights have been observed when other hydrophobic NSTs have been expressed in heterologous systems (Berninsone et al. 1997; Norambuena et al. 2002). Thus, we believe that the hydrophobicity of the GONST proteins is the reason for the aberrant mobility. The bands with lower mobility detected by the antisera may correspond to multimeric forms of these proteins, which did not disaggregate under these electrophoresis conditions. Treatment with Endoglycosidase H to remove any *N* -linked glycans did

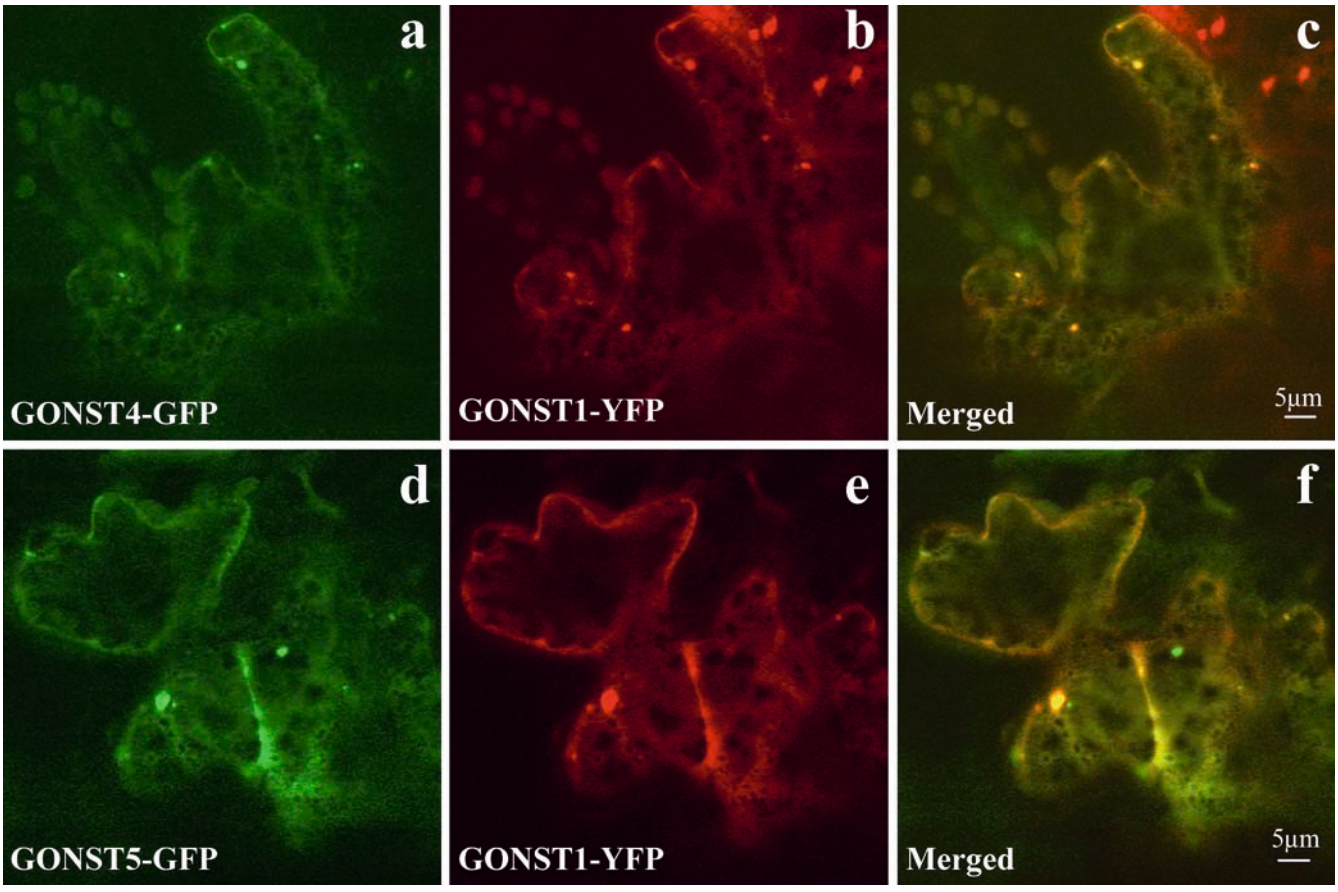

Fig. 4a–f The punctate structures containing GONST4-GFP, GONST5-GFP fusions and Golgi-localised GONST1-YFP are sensitive to treatment with Brefeldin A. **a** GONST4-GFP. **b** GONST1-YFP. **c** Merged GONST4-GFP and GONST1-YFP. **d** GONST5-GFP. **e** GONST1-YFP. **f** Merged GONST5-GFP and GONST1-YFP

not alter the mobility of the GONST proteins (data not shown), indicating that in yeast the proteins are not *N* -glycosylated. In summary, all the GONST proteins were expressed in a galactose-inducible fashion and targeted to the membranes of the *vrg4-2* mutant strain, indicating that these transformed strains were suitable for functional analysis.

Functional analysis of GONST proteins in *S. cerevisiae*

To investigate nucleotide sugar transport activity of the GONST proteins, we first examined their ability to complement the hygromycin sensitivity of the *vrg4-2* mutant. On glucose- and galactose-containing media without the antibiotic, all the yeast strains grew (Fig. 6a, b). In the presence of the antibiotic, only the wild-type strain grew on control glucose-containing medium (Fig. 6c). Interestingly, when their expression was induced with galactose, all five GONST proteins complemented the sensitivity of the *vrg4-2* mutant to hygromycin B (Fig. 6d). These results indicate that lumenal mannosylation in the yeast Golgi is stimulated by the expression of these proteins, and suggest that all the GONST proteins are indeed nucleotide sugar transporters.

Although these data indicate that GDP-mannose is a substrate for all the GONST proteins, the divergence of their sequences suggested that some may have substrate preferences different from that of GONST1. To inves-

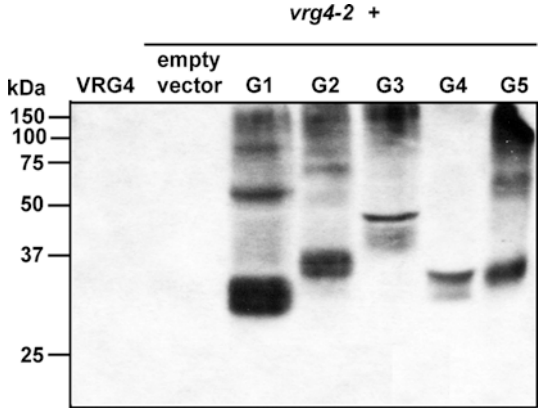

Fig. 5 Expression of GONST1, 2, 3, 4 and 5 in the *vrg4-2* strain of *S. cerevisiae*. Proteins (10 μg) from a total membrane fraction prepared from wild-type yeast (VRG4), *vrg4-2* transformed with pYES-DEST52 (empty vector) or *vrg4-2* transformed with pYES-DEST52 containing *GONST1, 2, 3, 4* or *5* (G1–G5) were resolved by SDS-PAGE and subjected to western analysis using anti-V5 antiserum

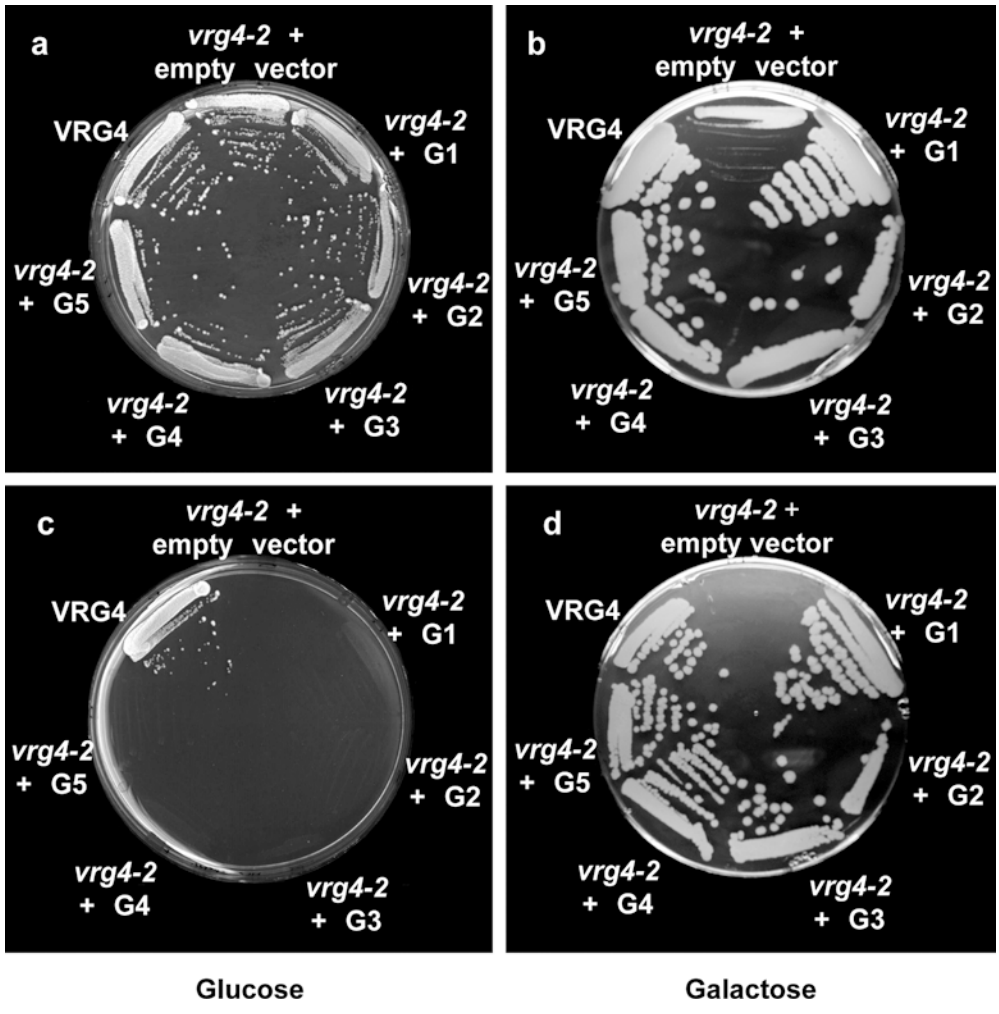

Fig. 6a–d Expression of all five GONST proteins complements the hygromycin B sensitivity of *vrg4-2* cells. Yeast strains, as in Fig. 5, were grown at 30°C. **a** YPglucose (repressing medium). **b** YPgalactose (inducing medium). **c** YPglucose + 30 μg/ml hygromycin B. **d** YPgalactose + 30 μg/ml hygromycin B

tigate whether they could all mediate substantial rates of GDP-mannose transport, we tested the ability of each GONST protein to complement the defect in protein glycosylation seen in the mutant by assaying the electrophoretic mobility of extracellular chitinase. Chitinase is a highly mannosylated protein, whose mobility on gels depends on the length of the *O*-linked manno-oligosaccharide chains which are extended in the Golgi apparatus using lumenal GDP-mannose (Kuranda and Robbins 1991). Wild-type and transformed *vrg4-2* strains were grown to saturation in induction medium (YPgalactose) and extracellular chitinase was isolated from the medium. Immunoblot analysis using anti-V5 antisera revealed that all five transformed yeast strains expressed GONST proteins in these cultures (data not shown). In agreement with previous findings (Dean et al. 1997), chitinase secreted from the empty-vector control migrated with a higher mobility (Rf = 0.8–0.9) than that secreted from the wild-type strain (VRG4, Rf = 0.65–0.75), as a consequence of its under-glycosylation (Fig. 7). In contrast, a proportion of the chitinase from mutant yeast cells expressing GONST1 or GONST2 migrated with a lower mobility corresponding to that of fully glycosylated chitinase. Chitinase secreted from *vrg4-2* expressing GONST3, 4 or 5 migrated similarly to the under-glycosylated form expressed in the empty-vector control. These results indicated that GONST1 and GONST2 mediated sufficient transport of GDP-mannose across the Golgi membrane to rescue the chitinase glycosylation defect, whereas GONST3, 4 and 5 did not, suggesting that the latter transporters may have other substrate specificities.

Expression of the *GONST* genes in *Arabidopsis*

To determine where the *GONST* genes are expressed in *Arabidopsis*, RT-PCR analysis was performed on RNA extracted from various organs. As shown in Table 3, *GONST* expression is widespread, but shows some organ specificity. *GONST1* and *GONST4* were expressed in all the organs analysed. The ubiquitous expression of *GONST1* found here is consistent with our earlier northern analysis and *GONST1* promoter::GUS fusion assays (Baldwin et al. 2001). *GONST2* and *GONST3* were detected in all organs except roots, whereas *GONST5* was expressed only in siliques, flowers and rosette leaves. Analysis of Affymetrix data for *GONST*

gene expression in roots, callus, rosette leaves and stems revealed a similar trend of expression, supporting the conclusions based on the RT-PCR survey (Table 3). However, *GONST2* and *GONST5* expression was not detected in Affymetrix data for any organs, suggesting that the level of expression of these genes is lower than the detection threshold of this method. Interestingly, in the Affymetrix pollen data, no signal was detected from any *GONST* genes, indicating little or no expression in this cell type (data not shown). The partially overlapping expression patterns suggest that there is functional redundancy among the GONST proteins or that they may have different transport specificities.

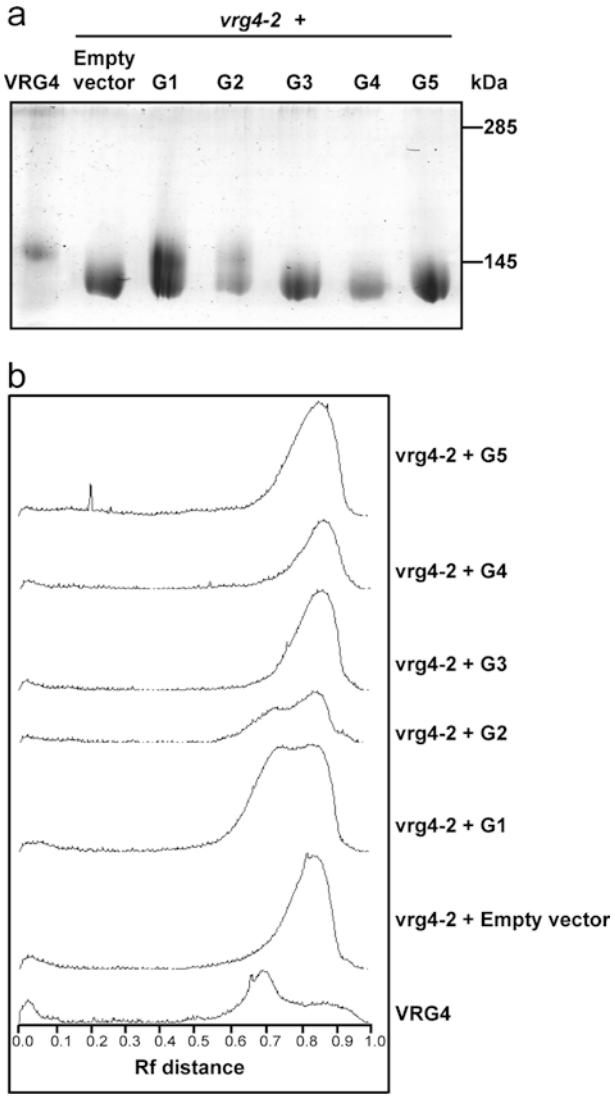

Fig. 7a, b *GONST1* and *GONST2* complement the glycosylation phenotype of extracellular chitinase of the *vrg4-2* strain of *S. cerevisiae*. Yeast strains were grown to saturation in inducing medium (YPgalactose) and chitinase was isolated from the media. Purified chitinase, resolved by SDS-PAGE, was stained with Coomassie G250 (**a**) and its migration was analysed using Gene Tools software (**b**)

Discussion

The completion of the genome sequence of *Arabidopsis* has allowed the identification of multiple genes present in the genome that encode putative transporters. Here we have studied four proteins, GONST2–5, which show similarity to GONST1, a GDP-mannose transporter located in the Golgi (Baldwin et al. 2001). Localisation and functional expression studies in yeast indicated that these proteins are likely to be Golgi-localised nucleotide sugar transporters.

We propose that the GONST proteins have ten TMDs (Fig. 1). Although only six to eight TMDs have been predicted in other NSTs (Gao and Dean 2000; Norambuena et al. 2002; Knappe et al. 2003), our prediction is in agreement with that of Jack et al. (2001), who studied the family of NSTs that includes GONST1 and Vrg4p. We looked for hydrophobic domains that are conserved among these homologous proteins, since their membrane topology is probably identical. There are ten of these domains, leading to the prediction that there are ten membrane-spanning domains in GONST1–5. In this model the putative GDP-mannose binding domain of GONST1 (GXLNK, Gao et al. 2001) is located within the ninth TMD, refining our original model (Baldwin et al. 2001). In our current prediction, this domain is close to a cytosolic loop, as required for the binding of cytosolic GDP-mannose, and may line an aqueous channel through the Golgi membrane. Recently, in a survey of TPT homologues that included some NSTs, Knappe et al. (2003) identified a second potential substrate-binding site. The equivalent domain in the GONST proteins contains conserved NK residues that are C-terminal to the first TMD (Fig. 1). In our model this would be on the lumenal side of the Golgi membrane and could be involved in binding or antiport of nucleoside-monophosphate from the Golgi lumen.

Table 3 RT-PCR analysis/ Affymetrix analysis of *GONST* gene expression in different *Arabidopsis* organs

Tissue	Gene[a]				
	GONST1	*GONST2*	*GONST3*	*GONST4*	*GONST5*
Stem	+/+	+/−	+/+	+/+	−/−
Silique	+/−	+/−	+/−	+/−	+/−
Flower	+/+	+/−	+/+	+/+	+/−
Root	+/+	−/−	−/+	+/+	−/−
Callus	+/+	+/−	+/+	+/+	−/−
Rosette leaves	+/+	+/−	+/+	+/+	+/−

[a](+), gene expression detected; (−), gene expression not detected

GONST1 was the first NST to be successfully localised to the Golgi apparatus in plants, based on the expression of a fusion with YFP. Here, GFP fusions with GONST4 and GONST5 were also localised in the Golgi apparatus when transiently expressed in tobacco cells. The punctate structures colocalised with GONST1-YFP fluorescence, were highly mobile, and were sensitive to BFA. Since GONST2 is very similar to GONST1 and GONST3 is closely related to GONST4 (Fig. 2), we believe that GONST2 and GONST3 are also very likely to be targeted to the Golgi apparatus. This is supported by their NST activity in the Golgi when expressed in yeast (Figs. 6 and 7). GONST5 is a member of a family of seven previously uncharacterised proteins. This family was recently named KV/A/G by Knappe et al. (2003), and shows more distant similarity to previously characterised NSTs and TPTs. The demonstration here that GONST5 is localised to the Golgi apparatus suggests that other members of this family may also be Golgi-localised nucleotide-sugar transporters.

All five GONST proteins complemented the hygromycin B sensitivity of the *vrg4-2* yeast strain, which is deficient in GDP-mannose transport. Since the sensitivity arises from defective mannosylation in the yeast Golgi lumen as a result of inadequate transport of GDP-mannose, the recovery of hygromycin B resistance indicates that all these proteins can transport this nucleotide sugar across the Golgi membrane in yeast. We therefore propose that, like GONST1, GONST2–5 are Golgi nucleotide sugar transporters. This view is supported by the demonstration that, again like GONST1, GONST4 and 5 are localised in the Golgi apparatus *in planta*. GONST1 and GONST2 appear to have a greater ability to transport GDP-mannose, because these two transporters also rescued the defect in glycosylation of secreted, extracellular, chitinase in the Golgi lumen. The typical consensus sequence (GXLNK) found in previously studied GDP-mannose transporters is conserved in GONST1 and GONST2, whereas this sequence is less conserved in the other GONST proteins.

Why might *Arabidopsis* express so many proteins that are able to transport GDP-mannose across Golgi membranes? *In planta*, these NSTs may transport alternative or multiple substrates. Many NSTs are thought to be specific for a single nucleotide-sugar (Hirschberg et al. 1998). Recently, however, several NSTs from animals (Berninsone et al. 2001; Segawa et al. 2002) and AtUTr1 from *Arabidopsis* (Norambuena et al. 2002) have been shown to transport more than one nucleotide-sugar. Indeed, Lpg2 in *L. donovani* is a GDP-mannose transporter that is known to be multispecific, as it is also capable of transporting GDP-arabinose and GDP-fucose (Hong et al. 2000). Hence, it is possible that GONST3, 4 and 5 may have additional nucleotide-sugar transport activities *in planta*. The most likely alternative substrates are the similar nucleotide sugars GDP-fucose (Wulff et al. 2000), GDP-glucose, and GDP-L-galactose, which are used in the synthesis of plant glycans (Zablackis et al. 1996). Since expression levels of the GONSTs vary in yeast, it is not possible to measure accurately the relative GDP-mannose transport activities of GONST1–5 in the yeast vesicles. Furthermore, since yeast metabolises GDP-mannose far more efficiently than other nucleotide sugars, comparison of rates of transport of GDP-mannose and other nucleotide sugars is difficult. By reconstituting GONST proteins into artificial liposomes we hope in the future to determine their substrate specificities. A further possible explanation for the presence of multiple genes encoding NSTs in the genome is that the transporters have different expression patterns. Indeed, the expression survey suggested there are some organ-specific expression differences (Table 3) between the *GONST* genes. However, since *GONST1* is expressed in most cell types (Baldwin et al. 2001), there is clearly some overlap of expression of the different genes. The transporters might therefore be partially redundant, as a consequence of relatively recent gene duplications (The Arabidopsis Genome Initiative 2000) or perhaps they are incorporated into different polysaccharide-synthesizing complexes (Seifert et al. 2002).

Analyses of mutants that are defective in nucleotide-sugar transport into the Golgi have demonstrated the physiological relevance of NSTs in yeast, nematodes, protozoa, *Drosophila*, mammalian cell lines and humans. In each of these cases, mutations in NSTs result in a loss of the corresponding sugar in glycoconjugates, which can in turn produce dramatic phenotypes (Descoteaux et al. 1995; Poster and Dean 1996; Herman and Horvitz 1999; Lübke et al. 2001; Segawa et al. 2002). Similar studies in plants have not yet been reported. The transport of GDP-mannose into the Golgi apparatus could be required for the proper synthesis of mannose-containing hemicelluloses (Baldwin et al. 2001; Handford et al. 2003). However, the recent identification of a β1–4 mannan synthase in guar suggests that cytosolic GDP-mannose may be used for synthesis of this polysaccharide (Dhugga et al. 2004). Alternatively, mannose might be added to glycosylceramides and glycosylinositol phosphoryl ceramides. The identification and characterization of these GONSTs will allow us to generate and analyse mutants, to determine their substrate specificity, and to investigate the role of NSTs in glycoconjugate biosynthesis *in planta*.

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