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Targeting of Active Sialyltransferase to the Plant Golgi Apparatus

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Glycosyltransferases in the Golgi apparatus synthesize cell wall polysaccharides and elaborate the complex glycans of glycoproteins. To investigate the targeting of this type of enzyme to plant Golgi compartments, we generated transgenic Arabidopsis plants expressing α-2,6-sialyltransferase, a glycosyltransferase of the mammalian *trans*-Golgi cisternae and the *trans*-Golgi network. Biochemical analysis as well as immunolight and immunoelectron microscopy of these plants indicate that the protein is targeted specifically to the Golgi apparatus. Moreover, the protein is predominantly localized to the cisternae and membranes of the *trans* side of the organelle. When supplied with the appropriate substrates, the enzyme has significant α-2,6-sialyltransferase activity. These results indicate a conservation of glycosyltransferase targeting mechanisms between plant and mammalian cells and also demonstrate that glycosyltransferases can be subcompartmentalized to specific cisternae of the plant Golgi apparatus.

INTRODUCTION

The Golgi apparatus is a central organelle in the secretory pathway of plant cells. Proteins initially inserted into the endoplasmic reticulum (ER) are sorted here into a range of vesicles for transport onward to the vacuole, plasma membrane, and cell wall. Moreover, the enzymes that synthesize a wide range of carbohydrate structures are localized in the membranes of the Golgi apparatus. Pectins and hemicelluloses, which are cell wall matrix polysaccharides, are manufactured by transfer of sugars from sugar nucleotide precursors by these glycosyltransferases (Gibeaut and Carpita, 1994). The O- and N-linked glycans of glycoproteins become elaborated by similar sugar transfer reactions as they move through the Golgi apparatus (Dupree and Sherrier, 1998). After the high-mannose core is trimmed, *N*-acetylglucosamine residues are added to the N-linked glycans. They may then acquire fucose, xylose, or galactose residues (Fitchette-Laine et al., 1994). The final complex glycan structure is similar to that of some mammalian N-linked glycoproteins; however, it may contain β-1,2-linked xylose and α-1,3-linked fucose. There have been no reports of the addition of terminal sialic acid residues. These differences lead to the high antigenicity of some plant proteins and are a barrier to the medical use of recombinant proteins produced in plants (Jenkins et al., 1996). Therefore, it is important to understand the specificity and regulation of the Golgi glycosyltransferases so that we can apply this knowledge to a wide range of biological and biotechnological processes.

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The plant Golgi apparatus consists of many stacks of membrane cisternae distributed throughout the cell. This distribution is distinct from that of the mammalian Golgi. The number of cisternae in each stack varies between three and eight, depending on the cell type, secretory status, and species (Zhang and Staehelin, 1992). Current models of the plant Golgi apparatus suggest that the stack is compartmentalized, with different enzymes of cell wall synthesis and glycan modification in the *cis*-, medial, and *trans*-cisternae (Moore et al., 1991; Fitchette-Laine et al., 1994). Such compartmentation could reduce the specificity needed in each sugar transfer reaction and also could alter the type of structures synthesized, for example, by separating early intermediates from the enzymes that catalyze the final additions. However, in the absence of any cloned plant Golgi membrane–localized enzymes, evidence for these compartments in the plant Golgi apparatus has been based on localization of the polysaccharide and glycoprotein products (Moore et al., 1991; Lynch and Staehelin, 1992; Zhang and Staehelin, 1992; Fitchette-Laine et al., 1994; Sherrier and VandenBosch, 1994). This work supports a model of compartmentation of products, but a definition of distinct Golgi compartments on the basis of enzyme localization has not been achieved (Dupree and Sherrier, 1998).

The Golgi glycosyltransferases involved in the synthesis of a variety of glycans and polysaccharides have been identified from yeasts, worms, flies, and mammals (Narimatsu, 1994; Campbell et al., 1997). Although they show little sequence similarity, most are type II membrane proteins with a short cytosolic N-terminal tail, a single transmembrane domain (TMD), and a luminal catalytic domain (Paulson and

Colley, 1989). Each enzyme is apparently specific for a nucleotide sugar and a narrow range of acceptor substrates. Given the broad range of origins and functions of these characterized enzymes, it is likely that the plant Golgi glycosyltransferases have a similar structure.

Despite the flow of membrane lipids and proteins through the secretory pathway, the glycosyltransferases are retained in the Golgi apparatus. The necessary signals for targeting to the mammalian and yeast Golgi compartments have been localized to the TMD and neighboring polypeptide regions of the enzymes (Munro, 1991; Nilsson et al., 1991; Swift and Machamer, 1991; Wong et al., 1992; Colley, 1997). Targeting to the plant Golgi apparatus has not yet been investigated because of the absence of any cloned genes for plant glycosyltransferases.

In this work, we directly addressed the question of targeting to the plant Golgi apparatus. The mammalian enzyme α-2,6-sialyltransferase (ST) has a clearly defined TMD Golgi targeting signal (Weinstein et al., 1987; Munro, 1991, 1995b; Wong et al., 1992). We reasoned that if the mechanisms of targeting to the plant Golgi apparatus are related to those in animal cells, then this enzyme might be retained in the Golgi apparatus when expressed in plant cells. We show here that the protein is effectively retained in the plant Golgi apparatus. Moreover, it is localized to distinct Golgi subcompartments. This suggests a conservation of retention signals and indicates that mechanisms exist to compartmentalize glycosyltransferases within the plant Golgi stack. We also show that the enzyme is catalytically active, which supports the idea that it is possible to modify glycosylation activities of plant cells.

RESULTS

Generation of Transgenic Plants Expressing Epitope-Tagged ST

The enzyme ST is retained in the *trans*-Golgi cisternae and *trans*-Golgi network (TGN) of mammalian cells (Roth et al., 1985). We chose to express this glycosyltransferase to investigate the plant Golgi apparatus because its targeting has been well studied. Furthermore, plants apparently lack sialic acid, and expression of this enzyme was unlikely to be deleterious. To enable simple detection of the enzyme, we used a construct encoding ST with a C-terminal Myc epitope tag. It has been shown that epitope tagging at the C terminus does not affect targeting of ST in mammalian cells (Munro, 1991; Yang and Storrie, 1998). The Myc epitope has also been used successfully for biochemical localization of the plasma membrane H^+-ATPase in plants (DeWitt and Sussman, 1995). To give high levels of expression in all cells, the construct was driven by the constitutive cauliflower mosaic virus 35S promoter.

After Agrobacterium-mediated transformation of Arabidopsis by vacuum infiltration, plants were screened for expression of the foreign glycosyltransferase by using immunoblot analysis of leaf proteins probed with A14 rabbit anti-Myc antibodies. Two plants expressing a protein of 48 kD, which is the expected size of glycosylated ST with the Myc epitope tag, were selfed to produce the homozygous lines ST2/11 and ST4/10. Immunoblot analysis of leaf protein extract from these lines (Figure 1A) demonstrated that the antibody specifically recognizes ST. The minor band of ~43 kD is most likely due to lesser glycosylation of ST rather than a proteolytic product, because ST contains three potential glycosylation sites and is glycosylated in mammalian cells (Weinstein et al., 1987).

Biochemical Analysis of ST Subcellular Localization

Callus cultures induced from roots of Arabidopsis yield relatively homogeneous cells that are amenable to biochemical

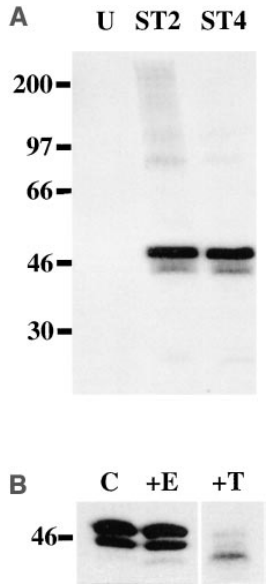

Figure 1. Immunoblot Analysis of Rat ST Expressed in Transgenic Arabidopsis Plants.

(A) Specific detection of ST in leaf extracts of transgenic plants with the A14 anti-Myc antibody. ST2/11 (ST2) and ST4/10 (ST4) are two homozygous independently transformed lines. U is the untransformed control.

(B) Analysis of the glycosylation state of ST expressed in ST2/11 callus. ST is largely resistant to digestion by endo-β-*N*-acetylglucosaminidase (Endo H), shown by the small amount of protein that shifts to the lower molecular weight. C is the untreated control; +E is the Endo H–treated sample. The lower molecular weight form is greatly increased in cells treated with the glycosylation inhibitor tunicamycin (+T). Molecular mass markers are indicated at left in kilodaltons.

and microscopic analysis (Gomez and Chrispeels, 1994; T.A. Prime, D.J. Sherrier, and P. Dupree, unpublished data). Callus lines were induced from the transgenic plants and maintained in liquid culture.

Transit of ST through the secretory pathway in the callus cells was investigated by endo-β-*N*-acetylglucosaminidase H (Endo H) digestion of proteins. This enzyme cleaves the chitobiose core of high-mannose and hybrid N-linked oligosaccharides of glycoproteins, but it does not recognize the complex glycans after processing by Golgi-localized enzymes (Maley et al., 1989; Gomez and Chrispeels, 1993). In the callus cells, the majority of ST had a mobility of 48 kD, with a lesser form of ~43 kD, as shown in Figure 1B. The majority of ST was resistant to Endo H, because there was only a slight increase in a minor 40-kD band after digestion. We confirmed that this band corresponds to unglycosylated protein by treatment of callus with tunicamycin, a drug that prevents addition of the high-mannose glycan (Duksin and Mahoney, 1982) in the ER. After 40 hr, most of the ST was unglycosylated, and the higher molecular weight forms were much reduced (Figure 1B). Therefore, the resistance to Endo H indicates that the glycoprotein had been processed by the Golgi mannosidases. Heterologous expression of membrane proteins can result in aggregation or misfolding of the protein in the ER, as was found when ST was expressed in yeast (Krezdorn et al., 1994). The resistance to Endo H suggested that the majority of the ST is not retained in the ER when expressed in these plant cells.

To investigate the subcellular localization in more detail, we separated the membrane compartments of the callus cells by equilibrium linear sucrose density gradient centrifugation. The distribution of ST and several organelle markers is compared in Figure 2. The fractions containing ST were identified by immunoblot analysis. ST was found to peak in fractions 6 and 7, which corresponds to 28 to 32% (w/w) sucrose. Very little ST was detected in fraction 5, whereas a significant but minor proportion was present in fractions 8 and denser.

We were next interested to determine whether the ST cofractionated with potential Golgi markers. Latent UDPase activity is a widely accepted Golgi marker, although the enzyme has not been localized by microscopy (Quail, 1979). The graph in Figure 2 shows that the latent UDPase activity peaked in fractions 6 and 7 at a density similar to that previously found for Golgi membranes from Arabidopsis callus (Gomez and Chrispeels, 1994). The distribution was strikingly similar to that of ST. A second potential Golgi marker is the group of glycoproteins recognized by the JIM84 antibody. These glycoproteins are present in the Golgi apparatus; in some species and cells, they are also found in the plasma membrane (Horsley et al., 1993; Henderson et al., 1994; Evans et al., 1997). We investigated whether this group of proteins cofractionated with the ST. The fractionation profile was very similar to that of both the latent UDPase activity and ST, suggesting that in these callus cells, all of these proteins are in the same compartment.

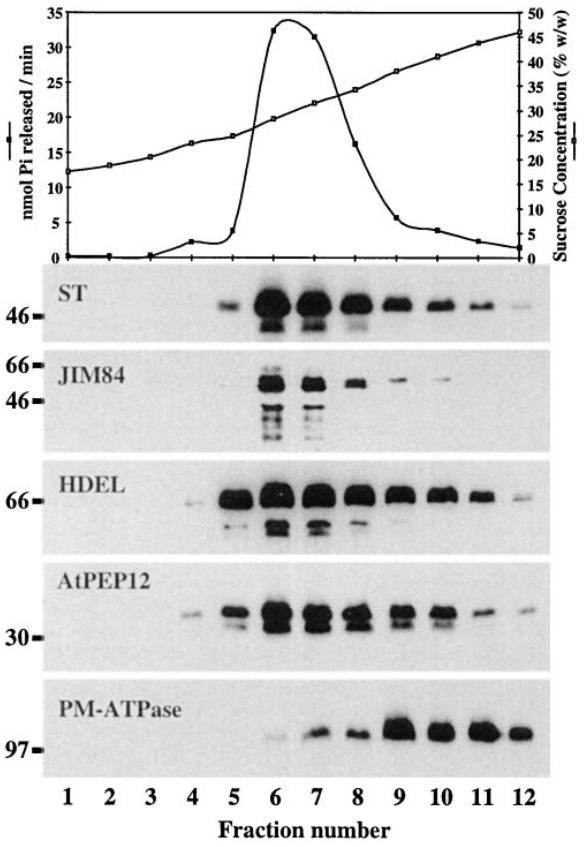

Figure 2. Biochemical Localization of ST by Subcellular Fractionation of ST-Expressing Callus.

Cellular membranes initially were sedimented onto a 16/48% sucrose cushion before flotation through a 25 to 50% linear sucrose gradient, and the fractions were subjected to immunoblot analysis and UDPase activity assay. Latent UDPase activity peaked sharply in fractions 6 and 7 (graph), as did ST and the glycoproteins detected by the JIM84 antibody. ER proteins detected with an anti-HDEL antibody (HDEL), AtPEP12, and the plasma membrane H^+-ATPase (PM-ATPase) fractionated differently. Molecular mass markers are indicated at left in kilodaltons.

Proteins from other organelles were identified in the fractions from the sucrose density gradient. The luminal ER proteins, detected with anti-HDEL antibodies (Napier et al., 1992), were present in the same fractions as ST, but the profile was slightly different, and more ER proteins were present in fraction 5. The biochemical fractionation did not show clear separation of ER and Golgi proteins, although Endo H digestion clearly indicated that the ST is not in the ER. AtPEP12 is a recently described marker of a post-Golgi, potentially prevacuolar compartment (da Silva Conceição et al., 1997). Immunoblot analysis using an antibody raised against this protein showed that it is present in a wider range of sucrose density fractions than is ST. To assess

whether the plasma membrane has a significantly different fractionation profile from ST, we used antibodies that recognize a plasma membrane H^+-ATPase (Morsomme et al., 1996). The plasma membrane equilibrated at much denser sucrose fractions, peaking in fractions 9 through 12. In conclusion, this biochemical analysis is consistent with a Golgi compartment localization of ST.

Localization of ST by Immunofluorescence

Complete separation of organelles by biochemical fractionation is not possible. To provide further evidence for Golgi localization of the glycosyltransferase, ST in root cells was visualized by confocal and conventional fluorescence light microscopy. As shown in Figure 3A, the immunofluorescent labeling was punctate, with abundant lozenge-shaped structures throughout the cell cytoplasm. In untransformed plants, only nonspecific background labeling was observed (data not shown). There is no widely accepted Golgi-specific labeling pattern. However, using JIM84 labeling of glycoproteins, Horsley et al. (1993) found the Golgi labeling in onion and maize cells to be a very similar punctate pattern. We were unable to colocalize the JIM84 epitopes and ST in our root tissue, probably because the JIM84 epitopes are not sufficiently expressed in Arabidopsis roots. To our knowledge, there are no other suitable Golgi markers available.

To test further the identification of these structures as Golgi stacks, we investigated their sensitivity to brefeldin A (BFA). This drug has variable effects on the morphology of the Golgi apparatus, causing clustering or vesiculation, depending on the conditions (Satiat-Jeunemaitre and Hawes, 1992, 1994; Driouich et al., 1993; Satiat-Jeunemaitre et al., 1996; Robinson et al., 1997; Staehelin and Driouich, 1997).

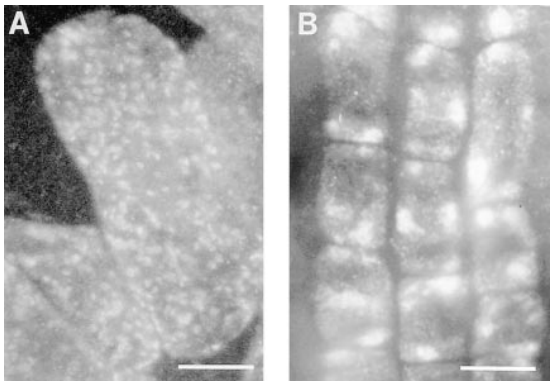

Figure 3. Immunofluorescent Labeling of ST in Root Cells.

(A) Punctate labeling throughout the cytoplasm of a large root cell visualized by confocal microscopy.

(B) Clustering of ST-labeled structures in several small cells after BFA treatment and visualized by conventional light microscopy. Bars in **(A)** and **(B)** = 10 μm.

When the plants were incubated in 50 μg/mL BFA for 1 hr, the ST-labeling pattern changed. A few large structures rather than the numerous dispersed small punctate spots were seen (Figure 3B). This indicates that ST–Myc localized to a BFA-sensitive compartment, which is consistent with a Golgi localization.

Immunogold Electron Microscopic Localization of ST to the Golgi Apparatus

In the absence of a compartmental marker that could be used to correlate the biochemical fractionation and light microscopy, we supported the results by immunogold electron microscopy. As shown in Figure 4, significant numbers of gold particles were observed over cisternae and membranes closely associated with the Golgi stack in conventionally fixed callus tissue. There was no significant labeling of mitochondria and ER (Figure 4A) or the cell wall and vacuole (Figure 4B). In sections of untransformed plant material, which were used as controls, there was no significant labeling (data not shown). Therefore, this glycosyltransferase is targeted specifically to the Golgi apparatus and associated membranes in plant cells.

Inspection of the labeled stacks indicated that the gold particles were asymmetrically present over the Golgi apparatus (Figures 4A and 4B). To determine the distribution of ST, we quantified gold labeling from 50 Golgi apparatus profiles. We were unable to derive a consistent morphological distinction between TGN and the *trans*-most cisterna of a stack. In many cases, the *trans*-most membrane was partially attached to the stack and therefore had characteristics intermediate between a TGN and *trans*-cisterna, as has been seen previously (Staehelin and Moore, 1995; Dupree and Sherrier, 1998). The *trans*-most membranous structure of each Golgi apparatus was therefore defined as T, and adjacent cisternae were defined as −1, −2, and so forth, progressing toward the *cis* side. As shown in Figure 5, this analysis revealed a very clear distribution of gold label within a subset of Golgi membranes. Most of the label was found in the *trans*-most two membranes, demonstrating a subcompartmentation of this glycosyltransferase within the Golgi apparatus.

Activity of the Foreign Glycosyltransferase

The targeting of the ST to the Golgi apparatus suggested that the protein is properly folded for export from the ER. Because we are unaware of any reports of either ST activity or the existence of sialic acid residues in plant cells, we investigated whether the expressed foreign protein conferred ST activity on these plants. Cell membranes were collected from untransformed and transformed callus lines and assayed for ST activity by incubation with CMP–^{3}H-sialic acid together with asialofetuin acceptor (Carey and Hirschberg,

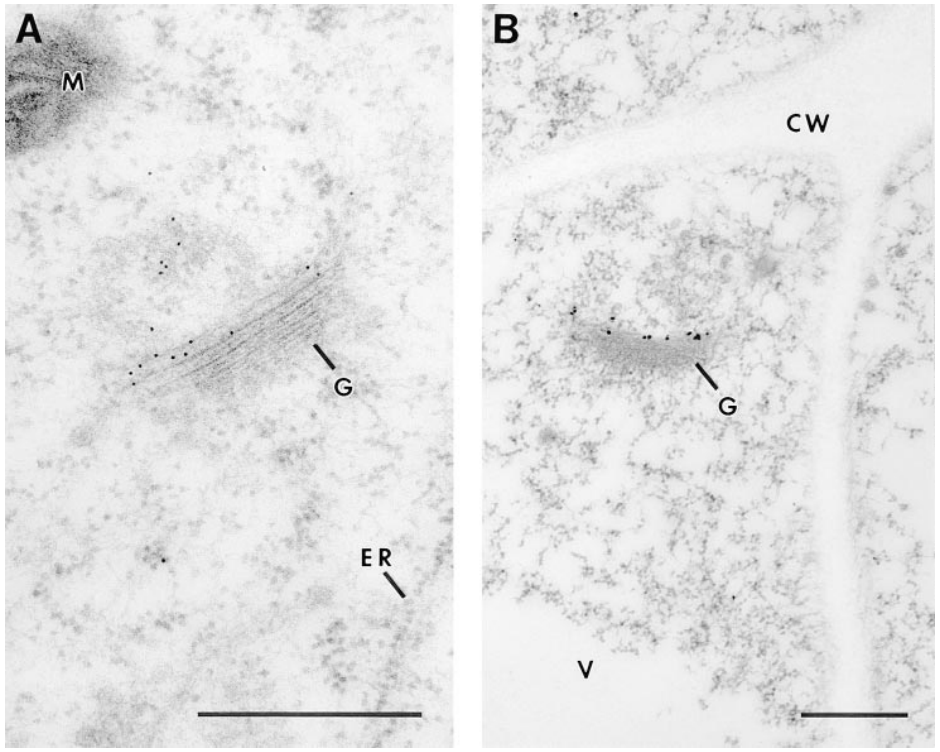

Figure 4. Localization of ST to *trans*-Membranes of the Plant Golgi Apparatus by Immunoelectron Microscopy.

(A) ST labeling is confined to the Golgi (G) and absent from the mitochondria (M) and ER.
(B) ST labeling is confined to the Golgi and absent from the cell wall (CW) and vacuole (V). Bars in **(A)** and **(B)** = 0.5 μm.

1980). As illustrated in Figure 6, significant levels of incorporation were catalyzed by both ST2/11 and ST4/10 lines. The addition of Triton X-100 to the membranes significantly stimulated incorporation, indicating high latency of the enzyme. This demonstrates that the ST enzyme has the native topology with a luminal catalytic domain. Membranes from untransformed callus cells incorporated no more sialic acid than a boiled membrane control (data not shown). These results indicate that there is no detectable endogenous ST activity in wild-type plant cells but that a significant change in glycosylation activity was achieved by expression of the mammalian glycosyltransferase.

DISCUSSION

Golgi glycosyltransferases transfer sugar residues onto glycoproteins and polysaccharide precursors as they pass along the secretory pathway. Despite this forward movement, these enzymes are retained within the plant Golgi apparatus. We examined the targeting and compartmentation of this type of protein by localizing a Myc epitope–tagged foreign glycosyltransferase in transgenic plants. The enzyme is targeted to the *trans*-most membranes of the Golgi apparatus. This suggests that Golgi targeting signals and mechanisms are conserved between plants and animals. It also shows that glycosyltransferases can be retained within a distinct subcompartment of the plant Golgi apparatus.

To localize the protein by immunocytochemistry and biochemical fractionation, we took advantage of the Myc epitope–tagging approach. Although a tag might affect targeting of the protein, this tag has been demonstrated not to affect targeting of the ST protein in mammalian cells (Munro, 1991; Yang and Storrie, 1998). It has proven especially difficult to localize membrane proteins in plants by immunodetection and electron microscopy, perhaps because of low expression levels and because fixation conditions that give good structural preservation often destroy antigenicity of plant antigens. The Myc epitope has been used successfully elsewhere in plants (DeWitt and Sussman, 1995), but immunogold labeling and electron microscopy remained problematic. We found here that the rabbit antibody A14 gave good specific labeling with little background immunoreactivity to endogenous plant proteins. Furthermore, the antigenicity of the Myc epitope tag was not destroyed by the fixatives required for reasonable structural preservation or by the high temperature needed for polymerization of the embedding resin.

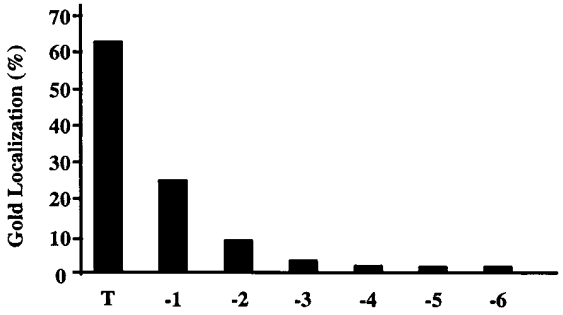

Figure 5. Quantification of Immunogold Labeling.

Gold particles over the *trans*-most membrane of the Golgi apparatus (T) and cisternae in a *cis*-ward direction (−1 to −6) were counted from 50 individual Golgi stacks.

The precise targeting of a mammalian enzyme to the plant Golgi apparatus indicates a conservation of targeting signals and therefore probably the conservation of mechanisms between the kingdoms. Previously, the complementation of the Arabidopsis *N*-acetylglucosaminyltransferase I mutant (*cgl*) by the human transferase indicated that some active foreign glycosyltransferase activity must reach the plant Golgi apparatus, but evidence of specific retention was not obtained (Gomez and Chrispeels, 1994). Here, rat ST has been localized by biochemical fractionation, and this localization was supported by immunocytochemistry. Although some ST enzyme might escape the Golgi apparatus and reach the plasma membrane or vacuole, we found no significant immunogold label at these membranes, suggesting effective retention.

The signals for retention of this ST in the mammalian Golgi apparatus have been localized to the TMD and flanking sequences (Weinstein et al., 1987; Munro, 1991, 1995b; Wong et al., 1992). The length of the TMD is particularly important, because replacement of the 17 hydrophobic amino acids with a 23–amino acid TMD resulted in plasma membrane localization (Munro, 1991). Although we have not formally proven that the same characteristics of the protein are essential for retention in plants, we consider it unlikely that a "cryptic" signal could be recognized. Whereas an ER localization might result from protein misfolding (Hammond and Helenius, 1995; Pedrazzini et al., 1997) and an absence of organelle targeting information might result in delivery to the plasma membrane or vacuole, as occurs in mammalian or yeast membrane proteins, respectively, the precise targeting to a Golgi subcompartment seen here is highly specific. Therefore, it is probable that similar properties of the glycosyltransferase result in targeting in both mammalian and plant cells.

Golgi enzyme retention mechanisms in animals and yeast may entail so-called kin recognition or result from segregation of proteins into membrane domains of different bilayer thickness and lipid composition. The kin recognition model proposes that Golgi enzymes form heteroligomers within a compartment. These oligomers would be too large to enter the vesicles moving forward through the Golgi apparatus or onward to the plasma membrane (Nilsson et al., 1994). It is difficult to interpret our results in terms of the kin recognition model (Pelham, 1998) because the plant Golgi apparatus is unlikely to have close relatives of ST. The bilayer thickness model suggests that a concentration gradient of cholesterol and glycolipids from the ER to the plasma membrane of mammalian cells could cause the thickness of the lipid bilayer to increase. Golgi proteins would be unable to progress across or beyond the Golgi stack due to their relatively short TMDs (Munro, 1995a, 1995b, 1998). Indeed, the levels of phytosterols and the glycolipid glucosylceramide have been found to be higher in plant plasma membrane fractions than ER-enriched membrane preparations (Hartmann and Benveniste, 1987; Lynch, 1993). Electron microscopy studies also have long suggested that the bilayer thickness changes across the plant Golgi stack (Grove et al., 1968). Our results would therefore be consistent with a change in lipid composition between the *trans*-Golgi and the plasma membrane, preventing the ST from entering the vesicles that leave the TGN. It will be interesting to see whether plant Golgi proteins have short TMDs like their mammalian and yeast counterparts.

The localization of ST specifically to the *trans*-most cisternae of the plant Golgi apparatus reveals that the Golgi glycosyltransferases can be subcompartmentalized within the stack. Current models of glycosylation of proteins and polysaccharides suggest that the *cis*-, medial, and *trans*-cisternae might possess different glycosyltransferases. However, these suggestions are based mainly on immunogold labeling of the corresponding glycosylation products and separation of enzyme activities by fractionation of tissue homogenates (Dupree and Sherrier, 1998). The model is also supported by recent findings that the soluble cytosolic pro-

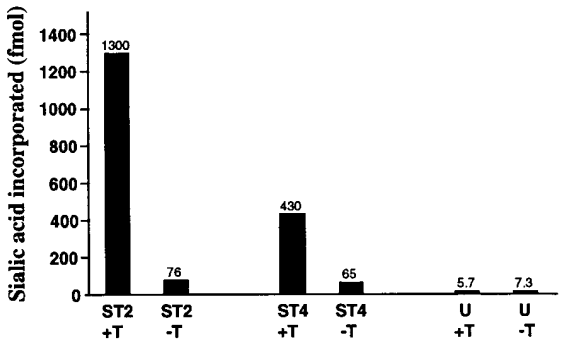

Figure 6. Activity of ST in the Transgenic Plants.

Membranes of ST2/11 (ST2) and ST4/10 (ST4), when supplied with the asialofetuin acceptor, incorporated sialic acid from CMP–^{3}H-sialic acid. Triton X-100 (+T) greatly stimulated this activity over nonpermeabilized membranes (−T). Membranes of the untransformed control (U) showed only nonspecific incorporation.

tein RGP1, which is thought to be involved in polysaccharide synthesis, is preferentially associated with the *trans*-Golgi membranes (Dhugga et al., 1997). By demonstrating that ST is retained within *trans*-Golgi membranes, it is clear that the plant Golgi stack should not be considered to be a single biochemical compartment.

In mammals and yeast, three distinct compartments have been described: a *cis*-Golgi network or early compartment, a medial compartment, and a *trans*/TGN or late compartment. In mammalian cells, ST is localized to the *trans*-cisternae and the TGN. The plant TGN has a quite different morphology from that of mammals. The former is less extensive and more reminiscent of a cisterna. It is often defined as a membrane separate from the stack, perhaps with a more complex morphology or associated clathrin-coated vesicles (Dupree and Sherrier, 1998). We were unable to distinguish morphologically the plant TGN structures with our preservation procedures. Membranes associated with the stack that we believe to be the TGN were often labeled (Figure 6). It is also possible that this compartment might be contiguous with the AtPEP12 post-Golgi compartment (da Silva Conceição et al., 1997), but this must be verified by double localization. However, a labeled TGN structure was not present near all Golgi stacks. In cases in which the Golgi apparently has no TGN, it is thought that the *trans*-most cisterna might take on the function (Staehelin and Moore, 1995). Our data support a model of similarity between the TGN and *trans*-cisternae, because they suggest an overlap in protein composition. Indeed, owing to the morphological similarity of the TGN and cisternae in some algae and plant cells, it has been suggested that the *trans* compartment might extend many cisternae into the stack (Mellman and Simons, 1992). However, we found almost 90% of the label on the *trans*-most two membranes (Figure 5), suggesting that this compartment extends only one or two cisternae into the stack in these cells. ST will now provide a valuable *trans*-Golgi marker for further studies on compartmentation of Golgi enzymes in Arabidopsis.

We demonstrated that ST is inserted into the plant membranes with the normal type II topology and is active when supplied with substrates. Previously, it has been shown that the mammalian Golgi *N*-acetylglucosaminyltransferase I could glycosylate endogenous plant proteins, but targeting was not investigated (Gomez and Chrispeels, 1994). Our data show that targeting conservation is sufficient to retain ST effectively, and we saw no evidence of saturation of the retention mechanisms. In contrast to the situation in yeast, in which expression of Golgi glycosyltransferases resulted in mislocalization to the ER (Krezdorn et al., 1994), the ability to retain this active enzyme suggests that it may become possible to modify glycosylation of proteins or polysaccharides within the plant Golgi. Plants will be useful expression systems for glycoproteins because complex N-linked glycans appear not to be essential in Arabidopsis (von Schaewen et al., 1993). To obtain sialylation in plants, the enzymes of CMP–sialic acid synthesis and the Golgi sugar nucleotide transporter will have to be coexpressed. Modification of glycosylation activities of the Golgi is potentially important not only for medical biotechnological purposes (Jenkins et al., 1996) but also as a tool to investigate the role of plant glycan structures and cell wall polysaccharides. By ectopically expressing glycosyltransferases, these carbohydrates could be modified, and any effect on development or cell wall properties could be investigated.

METHODS

Construction of c-Myc Epitope–Tagged α-2,6-Sialyltransferase Plasmid

Molecular cloning techniques were performed as described by Sambrook et al. (1989). A 1.3-kb fragment encoding Myc-tagged α-2,6-sialyltransferase (ST) and delineated by HindIII and SmaI from SMH3 (Munro, 1991) was cloned under the control of the cauliflower mosaic virus 35S promoter in PNJK12 (Kilby et al., 1995), which is a derivative of pGA482 (An, 1986). The construct was selected in *Escherichia coli* DH5α and in *Agrobacterium tumefaciens* by tetracycline resistance and conferred kanamycin resistance on plant transformants. The construct was subsequently transferred into Agrobacterium strain C58C1 (van Larebeke et al., 1974) by triparental mating.

Plant Culture, Growth, and Transformation

The *Arabidopsis thaliana* ecotype Columbia plants used for the transformation experiments were cultured hydroponically on corrugated plastic boards in an aerated nutrient solution (2 mM $MgSO_4$, 2 mM $Ca[NO_3]_2$, 50 μM Fe-EDTA, 70 μM H_3BO_4, 14 μM $MnCl_2$, 0.5 μM $CuSO_4$, 1 μM $ZnSO_4$, 0.2 μM $NaMoO_4$, 10 μM NaCl, 0.01 μM $CoCl_2$, 5 mM KNO_3, and 2.5 mM KH_2PO_4). The plants were grown at 20°C with 16 hr of light. The total light intensity of the three fluorescent tubes and one Grolux (Sylvania) tube was 80 μmol m^{-2} sec^{-1}. In addition, a UV-A 351 fluorescent tube (Q Panel Co., Cleveland, OH), suspended 30 cm above the rosettes, was turned on for 60 min daily both before and up to 7 days after vacuum infiltration.

Transgenic plants were generated by Agrobacterium-mediated transformation according to Bechtold et al. (1993) with modifications. Infiltration duration was 30 min at 30 torr. Transformants were selected on Murashige and Skoog media (Murashige and Skoog, 1962) supplemented with 30 mg/L kanamycin and 8 g/L agar. Homozygous lines, giving 100% kanamycin-resistant progeny, were selected after selfing for two generations. Initiation and maintenance of liquid callus culture were performed according to Blackhall (1992), with modifications. The callus cultures were cultured at 25°C in 12 hr of subdued light and shaken at 110 rpm to provide aeration. To prevent glycosylation, we added 10 μg/mL tunicamycin (Fluka Biochemicals, Buchs, Switzerland) to the growth medium 40 hr before harvesting.

Biochemical Analysis

Fractions enriched in specific subcellular membranes and proteins were purified from callus culture cells according to Gomez and

Chrispeels (1994), with modifications. In brief, one volume of culture cells was homogenized with two volumes of 12% sucrose made in 100 mM Tris-Cl and 1 mM EDTA buffer, pH 7.8 (STE), using a polytron with a 94 PTA 10EC head attachment (Kinematica, Lichfield, UK) for two pulses of 7 sec each before centrifuging twice at 2200*g* for 5 min. The supernatant was overlaid on a 5-mL 48% STE and 3-mL 16% STE two-step sucrose cushion. After 5 hr of centrifugation at 90,000*g* at 4°C in a Beckman SW28 swing-out rotor (High Wycombe, UK), the "total" membrane floating above the 48% STE was collected. This was adjusted to 60% sucrose and then underlaid below a 20 to 45% STE linear gradient. Fourteen 2-mL fractions were collected after centrifugation for 24 hr with the same conditions.

The plant proteins were resolved using a 10% polyacrylamide gel according to Laemmli (1970) and subsequently electroblotted onto nitrocellulose according to Towbin et al. (1979). The primary antibodies used for immunoblot analysis were anti-Myc, A14 (Santa Cruz Biotechnologies, Santa Cruz, CA; 1:1000); anti-AtPEP12 (da Silva Conceição et al., 1997; 1:3000); anti-H^+–ATPase, Castor V (Morsomme et al., 1996; 1:5000); and anti-HDEL, 2E7 (Napier et al., 1992; 1:4). The secondary antibody was horseradish peroxidase–conjugated goat anti–rabbit or anti–mouse antibody (Bio-Rad; 1:3000). Detection was performed by enhanced chemiluminescence.

Latent UDPase activity was assayed according to Nagahashi and Kane (1982), with modifications, using $MnCl_2$ rather than $MnSO_4$ as well as including 0.5 mM NaN_3 and 100 μM NaMo in the reaction. A volume of 25 μL of individual membrane fractions was used in each assay, which was stopped after a 20-min incubation period by the addition of 100% (w/v) trichloroacetic acid to a final concentration of 10%. The amount of inorganic phosphate released was measured according to Ames (1966).

ST activity was assayed according to Carey and Hirschberg (1980). In brief, 50 μL of cell total membrane from the sucrose step gradient for each assay was incubated at 37°C for 30 min with 150 μL of reaction mix (1.5 μL [0.3 μCi] CMP–^{3}H-sialic acid [New England Nuclear Life Sciences, Boston, MA], 50 μL [10 mg/mL] asialofetuin [Sigma], and 98.5 μL buffer [33 mM Na_3PO_4, 100 mM NaCl, pH 7.5, and 0.2% Triton X-100]). The reaction was stopped by adding 1 mL of cold 1% phosphotungstic acid (PTA) (Sigma) in 0.5 N HCl and left on ice for 15 min. The precipitate was pelleted and rinsed three times with fresh 1% PTA/0.5 N HCl. The pellet was dissolved in 1 mL of 1 N NaOH and transferred to a scintillation vial containing 0.5 mL of water, 0.3 mL of 4 N HCl, and 18 mL of Ecoscint H scintillation fluid (National Diagnostics, Hull, UK). Radioactivity was counted on a Beckman scintillation counter.

Immunocytochemistry

Immunofluorescent labeling of the foreign protein in root cells of the transgenic plants was performed according to Goodbody and Lloyd (1994), with modifications. All solutions were made in microtubule stabilization buffer (MTSB; 50 mM Pipes, 5 mM EGTA, and 5 mM $MgSO_4$, pH 6.9). Four-day-old seedlings were fixed in 4% formaldehyde for 60 min and digested using 10% Driselase (Sigma) for 15 min before permeabilization with 10% DMSO and 0.4% Nonidet P-40 for 15 min. An additional blocking step was performed by incubating the seedlings in 1% BSA and 0.1% normal goat serum (Sigma). Antibody incubations were both performed for 1 hr followed by three rinses in the blocking solution after the primary incubation and in buffer only after the secondary incubation. The primary anti-Myc antibody was 9E10, and the secondary antibody was Cy3-conjugated goat anti–mouse antibody (Jackson Labs, Luton, UK). Imaging was conducted on a Bio-Rad confocal microscope or a Zeiss Axioskop light microscope (Welwyn, UK). The confocal micrograph in Figure 3A is a composite of eight sections of a total thickness of 40 μm. Brefeldin A (BFA)–treated plants were processed in the same way after 1-hr incubation in 50 μg/mL BFA in MTSB.

Callus material was processed for electron microscopy according to VandenBosch et al. (1995), except that Pipes buffer was used. Sections 90 nm thick were cut using a Reichert-Jung UltraCut E microtome (Vienna, Austria) and collected on polyvinyl Formvar (Agar Scientific, Stansted, UK)–coated nickel grids. Immunogold labeling using anti-Myc, A14 as the primary antibody and 10-nm gold-conjugated goat anti–rabbit antibody (British Biocell, Cardiff, UK) as the secondary antibody was according to standard protocols (Herman and Melroy, 1990). Sections were counterstained in aqueous uranyl acetate and alkaline lead citrate before they were visualized on a Philips 300 transmission electron microscope (Cambridge, UK).

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