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TITLE:

Transport of UDP-galactose in plants: Identification and functional characterization of AtUTr1, an *Arabidopsis thaliana* UDP-galactose/UDP-glucose transporter.

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ABSTRACT

The synthesis of non-cellulosic polysaccharides and glycoproteins in the plant cell Golgi apparatus requires UDP-galactose as substrate. The topology of these reactions is not known, although the orientation of a plant galactosyltransferase involved in the biosynthesis of galactomannans in fenugreek is consistent with a requirement for UDP-galactose in the lumen of the Golgi cisternae. Here we provide evidence that sealed, right-side-out Golgi vesicles isolated from pea stems transport UDP-galactose into their lumen and transfer galactose, likely to polysaccharides and other acceptors. In addition, we identified and cloned *AtUTr1*, a gene from *Arabidopsis thaliana* that encodes a multitransmembrane hydrophobic protein similar to nucleotide sugar transporters. Northern analysis showed that *AtUTr1* is indeed expressed in Arabidopsis. *AtUTr1* is able to complement the phenotype of MDCK ricin resistant cells, a mammalian cell line deficient in transport of UDP-galactose into the Golgi. *In vitro* assays using a Golgi enriched vesicle fraction obtained from *Saccharomyces cerevisiae* expressing AtUTr1-mycHis is able to transport UDP-galactose but also UDP-glucose. AtUTr1-mycHis does not transport GDP-mannose, GDP-fucose, CMP-sialic acid, UDP-glucuronic acid or UDP-xylose when expressed in *S. cerevisiae*. AtUTr1 is the first transporter described, able to transport UDP-galactose and UDP-glucose. Thus AtUTr1 may play an important role in the synthesis of glycoconjugates that contain galactose and glucose in Arabidopsis.

INTRODUCTION

UDP-galactose is a substrate used in the synthesis of non-cellulosic polysaccharides and glycoproteins. The incorporation of galactose into these macromolecules is catalyzed by galactosyltransferases, which are thought to be localized in the Golgi apparatus (1). A galactosyltransferase from fenugreek involved in the synthesis of galactomannans has been recently cloned (2). The information derived from the primary sequence of this protein suggests that this enzyme is a membrane bound protein with its catalytic site facing the lumen of the Golgi cisternae. This orientation is similar to galactosyltransferases involved in protein glycosylation of animal cells. Since it is likely that transfer of galactose to non-cellulosic polysaccharides and glycoproteins takes place in the lumen of the Golgi apparatus, transport of UDP-galactose should be required to fulfill this process.

UDP-galactose transporters have been described in animal cells, Drosophila and yeast (3-7). Studies in MDCK-cells and yeast mutants deficient in transport of UDP-galactose into the Golgi lumen have shown that they play important roles in galactosylation of proteins, proteoglycans and sphingolipids (8-10). Complementation of mutants allowed the cloning of putative UDP-galactose transporter genes from human and *S. pombe* (6,10). Through PCR-based approaches and screening of libraries, murine, hamster and Drosophila cDNAs were also isolated (6,7,11). Expression of these genes in *S. cerevisiae* followed by *in vitro* transport assays into Golgi vesicles confirmed that the gene products actually transport UDP-galactose (12). These genes encode proteins with a molecular mass around 35 kDa; their hydrophobicity plots predict that they have 8 to 10 transmembrane domains.

Despite the potential importance of UDP-galactose transporter in the synthesis of cell wall components, a UDP-galactose transport activity has not been characterized in the plant Golgi apparatus, and a gene encoding for a UDP-galactose transporter has not yet

been identified. Here we report, that sealed Golgi vesicles isolated from pea stems transport UDP-galactose and transfer galactose to endogenous acceptors. In addition, we cloned a cDNA from *A. thaliana*, named *AtUTr1* for *Arabidopsis thaliana* UDP-galactose Transporter 1, that was functionally characterized by complementing a Golgi UDP-galactose transporter mutant, and by expressing it in *S. cerevisiae* and measuring transport in vitro of nucleotide sugars in Golgi enriched vesicles. The results show that *AtUTr1* encodes a nucleotide sugar transporter able to transport both UDP-galactose and UDP-glucose, while it does not use other nucleotide sugars tested. Transport of UDP-galactose into plant Golgi vesicles supports a model in which galactosylation of polysaccharides and other acceptors such as lipids occurs in the lumen of the Golgi apparatus, and the *AtUTr1* gene product from Arabidopsis could be involved in that process.

MATERIALS AND METHODS.

Radiolabelled Substrates: UDP-[^{3}H]galactose (12Ci/mmol), UDP-[^{3}H]glucose (21Ci/mmol) GDP-[^{3}H]fucose (17.3 Ci/mmol), UDP-[^{3}H]xylose (10 Ci/mmol), CMP-[^{3}H]N-acetylneuraminic acid (32.8 Ci/mmol), GDP-[^{3}H]mannose (18.9 Ci/mmol), Na-[^{3}H]acetate (4.1 Ci/mmol) and [^{3}H]deoxyglucose (29.8 Ci/mmol) were purchased from NEN-DuPont. [^{3}H]UDP-glucuronic acid was enzymatically prepared as described by Orellana and Mohnen (13).

Isolation of Golgi Vesicles from Pea Stems: A Golgi-enriched vesicle fraction was isolated from the third internode of 7 days-old etiolated pea seedlings as described previously by Muñoz et al. (14). The orientation and integrity of these vesicles was confirmed measuring latency of the lumenal enzymes UDPase and xyloglucan α,1-2 fucosyltransferase (15).

Proteolysis of the Golgi vesicles: Proteolysis was carried out as described by Wulff et al. (15). Briefly, vesicles were treated with proteinase K (40 ng per 1 µg of Golgi vesicles protein) for 30 min at 30ºC. The reaction was stopped by adding 1 mM PMSF and the samples were kept on ice until they were used in the UDP-galactose uptake assays.

Measurements of UDPase using native gels: The lumenal UDPase activity was determined as described by Orellana et al. (16). Briefly, Golgi vesicles were solubilized using 1.5% Triton X-100. Samples were separated in a 10% polyacrylamide gel and the activity was measured in the presence of 3 mM UDP and 1.5 mM $Pb(NO_3)_2$. The inorganic phosphate released during the reaction formed an insoluble complex with lead, which is then visualized with 1% $(NH_4)_2S$.

UDP-galactose uptake assays in pea stems Golgi vesicles: Golgi vesicles were incubated with 1 µM UDP-[^{3}H]galactose in a medium containing 0.25 M sucrose, 10 mM Tris-HCl pH 7.5 and 1 mM $MgCl_2$ (STM buffer). To stop the reaction, the vesicles were diluted in cold STM buffer and filtered through 0.7 µm glass fiber filters. Subsequently the filters were washed with additional 10 volumes of cold STM buffer, dried, and the radioactivity determined by liquid scintillation counting.

Transport of UDP-galactose in pea stem Golgi Vesicles: Transport of UDP-galactose was measured using a modification of the method described by Perez and Hirschberg (17). Golgi vesicles (1 mg of protein) were incubated in STM buffer at 25ºC with 1 µM UDP-[^{3}H]galactose (sp. activity 788 cpm/pmol). The final volume was 0.4 ml. After 5 min the reaction was stopped by dilution using 4 ml of cold STM-buffer, and the vesicles were immediately separated from the incubation medium by centrifugation at 100,000 x *g* for 40 min. The vesicles pellet was surface washed and resuspended in 500 µl of water. The sample was brought to 70% ethanol and kept on ice for 30 min. After centrifugation at 10,000 x *g* for 15 min, the radioactivity associated with the 70% ethanol-insoluble material was determined by liquid scintillation spectrometry (T_{Ps}). The 70% ethanol-soluble material was dried, resuspended in 100 µl of water and extracted with 100µl of chloroform/methanol 5:1. The water-soluble UDP-[^{3}H]galactose (S_t), and the radioactivity present at the organic phase plus the insoluble fraction that remained at the interface, were determined (T_{P+L}). The amount of UDP-galactose within the vesicles (S_i) was calculated by subtracting the estimated amount of radioactive UDP-[^{3}H]galactose outside the vesicles (S_o) from S_t as described by Perez and Hirschberg (17). To calculate the amount of substrate outside the vesicles (S_o), the concentration of substrate in the medium was multiplied by the volume outside the vesicles in the pellet (V_o) which was calculated using the non-penetrator

[^{3}H]acetate. The total volume in the pellet (V_t) was calculated using [^{3}H]deoxyglucose which penetrates the vesicles. Thus the internal volume (V_i), required to estimate the internal concentrations of substrates, was determined by subtracting V_o from V_t.

Cloning of *AtUTr1*: *AtUTr1* cDNA was amplified from the *Arabidopsis thaliana* cDNA library pFL61 (American Type Culture Collection) using Platinum Pfx polymerase (Gibco-BRL) and primers flanking the coding region, designed from the genomic sequence. The upstream primer was TCTAGGATCCTAATGGAGGTCCATGG CTCC and contained a BamHI restriction site (underlined sequence). The downstream primer was ATGAGCGGCCGCCTTCCACTCTTTTGCTTC and contained a NotI site (underlined sequence). A single amplification product of the expected size (1 kb) was obtained. After verification by sequencing, the product was digested with BamHI and NotI and cloned in the mammalian expression vector pcDNA3.1-mycHis version A (Invitrogen) to generate pcDNA-AtUTr1-mycHis. For expression in *S. cerevisiae*, a vector derived from p426GPD (18) was engineered to contain a mycHis tag as follows. A 237 bp fragment containing the mycHis tag was amplified from pcDNA3.1-mycHis using the reverse and T7 primer, digested with BamHI and ligated to BamHI/SmaI digested p426GPD to generate p426GPD-mycHis. The 1 kb PCR product containing *AtUTr1* cDNA was digested with BamHI and NotI, and ligated to BamHI/Not I digested p426GPD-mycHis vector to generate p426GPD-AtUTr1-mycHis.

Yeast Transformation and Subcellular Fractionation: *S. cerevisiae* strain RSY255 (*MATa, ura3-52, ura3-52, leu2-3,112*) transformed with the URA plasmids p426GPD-mycHis and p426GPD-AtUTr1-mycHis were grown at 30ºC in liquid medium containing 0.67% yeast nitrogen base, 2% glucose and SCM-URA. Transformation with the plasmids was done using lithium acetate and following standard procedures. *S. cerevisiae*

transformed with the p426GPD-mycHis and p426GPD-AtUTr1-mycHis plasmids were grown and spheroplast were prepared using Zymolyase100T as described by Berninsone et al. (19). Cells were then disrupted by pipetting the cells suspension up and down and then centrifuged successively at 450 x *g*, 10,000 x *g* and 100,000 x *g* to obtain pellet fractions P_1, P_2 and P_3. The last fraction, P_3, was enriched in Golgi apparatus-derived vesicles (20).

Expression in yeast of AtUTr1-mycHis determined by western blot analysis: The expression of AtUTr1 in microsomal membranes was monitored by western blotting using PDF membranes and a c-myc monoclonal antibody (Santa Cruz Biotechnology).

Nucleotide Sugar Transport Assays in Yeast Golgi-Enriched Vesicles: Transport assays were performed as described by Berninsone et al. (19). Yeast Golgi–enriched vesicles (1 mg of protein from fraction P3) were incubated at 30ºC in the presence of the radioactive nucleotide sugar to be tested. After 3 min the vesicles were separated from the incubation medium by centrifugation at 100,000 x *g* for 50 min. The pellet was washed and resuspended in 4% perchloric acid and the total acid-soluble radioactive nucleotide sugars (S_t) were determined. The amount of radioactivity inside the vesicles (S_i), was calculated by subtracting the estimated amount of radioactive nucleotide sugar outside the vesicles (So) from the St.

Stable Transfection of MDCK cells and Ricin Sensitivity Assay: MDCK and MDCK-RCA^r (21) cells were grown in complete medium (MEM, 10 % FCS and antibiotics) at 37ºC. MDCK-RCA^r cells were transfected with pcDNA3-mycHis and pcDNA-AtUTr1-mycHis using lipofectamin (GIBCO Life Thecnologies, Grand Island, NY)). Transfectants were selected using complete medium containing 0.8 mg/ml geneticin (G418). Geneticin-resistant colonies were cloned and resistance to ricin was determined by plating 2000 cells/well in 24-well plates using complete medium containing 0.1 µg/ml RCAII (EY

Laboratories, San Mateo, CA). After 7 days of exposure to RCAII at 37ºC, surviving cells were determined by staining with 1% methylene blue in 50 % methanol.

RNA Isolation and Northern Blots: Total RNA was extracted from two weeks old Arabidopsis seedlings using Trizol (Gibco-BRL, Gaithersburg, MD) and poly-A+ RNA was isolated using oligo(dT) cellulose (MessageMaker mRNA Isolation System; Life Technologies). Total RNA (40 µg) and poly A+ RNA (2.5 µg) were fractionated by electrophoresis in an agarose gel containing formaldehyde and capillary transferred to nylon membranes (Hybond N+; Amersham) using 10X SSC. A ^{32}P-radiolabeled DNA probe was prepared from the *AtUTr1* cDNA fragment using a random priming oligolabelling kit (Amersham). Hybridization and all the other procedures were done as described by Orellana et al. (22).

RESULTS.

Topology of galactosylation, and transport of UDP-galactose into Golgi vesicles.

To investigate the topology of galactosylation and transport of UDP-galactose into the plant Golgi apparatus we analyzed the uptake of UDP-[^{3}H]galactose into sealed, right-side-out pea stems Golgi vesicles using a filtration assay. Uptake of UDP-[^{3}H]galactose was higher at 25ºC than at 0ºC, and decreased close to background levels when vesicles where heat-inactivated before the assay (Fig. 1). These results suggest that a protein-mediated process was involved in the uptake of UDP-galactose. Permeabilization of the vesicles prior to the assay using low concentrations of Triton X-100, did not change significatively the uptake of UDP-[^{3}H]galactose, suggesting that little UDP-[^{3}H]galactose was free in the lumen, and likely [^{3}H]galactose was transferred to endogenous acceptors that remained associated to the permeabilized vesicles. Treatment of the vesicles with Proteinase K, caused a decrease in the uptake of UDP-galactose (Fig.2). This treatment did not affect the activity of a lumenal Golgi UDPase (16) measured in native gels. As seen in figure 2B, the activity band detected in Golgi vesicles was still present in proteinase K-treated vesicles, while this activity band disappeared when Triton X-100-permeabilized Golgi vesicles were treated with the protease. This result suggest that proteinase K treatment did not alter the integrity of Golgi vesicles, therefore a protein (or proteins) located in the cytosolic face of the Golgi membrane would be required for the uptake and/or the transfer to acceptors. Permeabilization of the proteinase-K treated vesicles led to an increase in the incorporation of radioactive substrate into the Golgi vesicles, indicating that galactosyltransferases were still active and suggesting that, like the UDPase, these galactosyltransferases were located in the lumen. Proteinase K treatment of vesicles already permeabilized decreased the uptake of UDP-[^{3}H]galactose in all conditions, suggesting that proteolysis affected the

Fig.1

Fig.2

lumenal galactosyltransferases. These results suggested that galactosyltransferases are located in the lumen of the Golgi apparatus and UDP-galactose should be transported into Golgi vesicles. The addition of DIDS (4,4´-diisothiocyanatostilbene-2-2´-disulfonic acid), a known inhibitor of nucleotide sugar and anionic transporters (23), decreased the uptake of UDP-galactose supporting the idea that transport of UDP-galactose takes place in the plant Golgi apparatus (Fig.1).

To provide additional evidence that pea stems Golgi vesicles are able to transport UDP-galactose we used the method described by Perez and Hirschberg (17) that allows the estimation of the amount of UDP-galactose located inside the vesicles (S_i) as well as its concentration ([Si]) within the vesicles upon incubation of Golgi vesicles with UDP-[^{3}H]galactose. Table 1 shows that content of UDP-[^{3}H]galactose within the pea vesicles (S_i) was higher at 25ºC than at 0ºC, suggesting that transport of UDP-[^{3}H]galactose was temperature dependent. Incubation of Golgi vesicles with UDP-[^{3}H]galactose resulted in a significant transfer of radioactivity into 70% ethanol-insoluble material, suggesting that [^{3}H]galactose was transferred to polysaccharides. The 70% ethanol soluble material was subjected to chloroform-methanol partitioning, where part of the radioactive material became insoluble and other part became soluble in the organic phase, suggesting that [^{3}H]galactose was transferred to glycolipids and glycoproteins. Transfer to acceptors was also temperature dependent (Table 1).

Table 1

Identification of a putative UDP-galactose transporter gene in *Arabidopsis thaliana*

The results shown above, suggest that UDP-galactose transporters are present in the plant Golgi apparatus. Thus, to identify plant genes encoding for a putative UDP-galactose transporter we searched the *A. thaliana* database, for sequences similar to UDP-galactose transporter genes already described in animal cells and yeast (3, 4, 24). When we begun this

work the Arabidopsis genome was not completely sequenced, and at that time, by using the TBLASTN algorithm we identified a gene (currently annotated as At2g02810) that showed high sequence similarity (54% similarity at the protein level) to the human UDP-galactose Transporter related l gene (hUGTrel1) (24). Later on, when the Arabidopsis genome was completely sequenced, we found other sequences (shown below) that have similarity to UDP-galactose transporters genes, however, the first gene identified remained as the most similar to hUGTrel1. We named this gene *AtUTr1* for *Arabidopsis thaliana* UDP-galactose Transporter 1 (Fig. 3A). The predicted protein has an estimated molecular weight of 36,942 daltons, and hydropathy analyses predict 8 putative transmembrane domains. Interestingly, comparison of the hydrophobicity plots of the AtUTr1 and hUGTrel1 proteins showed that they are highly similar (Figure 3B). Fig.3

To confirm that *AtUTr1* is indeed expressed in Arabidopsis we performed Northern analysis using total RNA and poly A+ RNA obtained from 2 weeks-old seedlings. A single band of 1.58 kb was detected in all cases (Fig.4). Fig.4

***AtUTr1* complements a MDCK mutant cell line deficient in UDP-galactose transport into the Golgi.**

To test the ability of AtUTr1 to transport UDP-gal, we cloned *AtUTr1* in the pcDNA3-myc mammalian expression vector and then we stably transfected it in the mutant cell line MDCK-RCA^r. The primary defect observed in this mutant is an impairment in transport of UDP-galactose into the Golgi apparatus, which results in reduced availability of UDP-galactose for the lumenal galactosyltransferases. This mutant cell line tolerates 10 times higher concentrations of the lectin ricin (RCA) than do wild type cells (8,21). Ricin has a cytotoxic effect that depends on the binding to galactosyl residues and the ricin-resistance phenotype of the mutant correlates with a pleitropic deficiency in galactosylation

of glycoproteins and sphingolipids. Transfection with *AtUTr1* resulted in changes in the morphology and rate of growth of the transfectant, resembling the wild type cells instead of the mutant (not shown). Addition of ricin at 0.1 µg/ml for 7 days had toxic effects both on the mutants transfected with *AtUTr1* and the wild type cells (Fig.5), however, this concentration of ricin showed no visible toxicity on the non-transfected mutant nor the mutant transfected with the vector alone. These results suggest that expression of AtUTr1 in MDCK-RCA^r cells restores galactose addition into glycoconjugates by complementing the deficiency of transport of UDP-galactose into the Golgi. Fig.5

***AtUTr1* encodes a protein that transports UDP-galactose and UDP-glucose.**

To confirm that *AtUTr1* encodes for a nucleotide sugar transporter, and also to study the specificity of the transporter, we expressed a mycHis epitope tagged version of the protein in the yeast *S. cerevisiae*, an organism that has been used for the heterologous expression and functional analysis of other nucleotide sugar transporters genes such as the CMP-sialic acid and UDP-galactose transporters (12, 25). Western blot analysis using antibodies against the myc epitope showed that the microsomal fraction obtained from yeast transformed with an expression vector containing AtUTr1-mycHis expressed a protein of 30 kDa (Fig.6). This molecular weight was lower than the expected molecular mass predicted from the primary sequence of the protein plus the mycHis epitope (41 kDa). However, a difference between the predicted molecular mass and the apparent one was also found in other cases of heterologous expression of nucleotide sugar transporters in yeast (25). Fig.6

To determine the substrate specificity of AtUTr1-mycHis using an independent approach, we expressed AtUTr1-mycHis in *S. cerevisiae* and obtained Golgi vesicle enriched-fractions from yeast expressing AtUTr1-mycHis and from cells transformed with

the empty vector. The latency of GDPase activity in both of these vesicles preparations (26) was above 85% indicating that vesicles were right-side-out and sealed. Transport of UDP-galactose was significantly higher into Golgi vesicles isolated from yeast expressing AtUTr1-mycHis than in vesicles isolated from yeast transformed with the vector alone (Fig.7). In contrast, transport of CMP-sialic acid, GDP-mannose, GDP-fucose, UDP-glucuronic acid, and UDP-xylose was not significantly different in vesicles obtained from the yeast expressing AtUTr1-mycHis, and the control. We also found that expression of AtUTr1-mycHis significantly increased transport of UDP-glucose into the vesicles, indicating that AtUtr1 is also able to transport UDP-glucose. No UDP-glucose epimerase activity was detected in the yeast Golgi vesicles expressing AtUTr1 (not shown), ruling out the possibility that UDP-glucose transport activity resulted from UDP-galactose formation. The increase in the transport of UDP-galactose and UDP-glucose by vesicles isolated from yeast expressing AtUTr1 was 4 fold and 2.3 fold over the control respectively. However, when the absolute values (fmol/mg/3 min) transported into the vesicles were compared, the amount of UDP-glucose was higher than the amount of UDP-galactose. This result suggest that AtUTr1 prefers UDP-glucose as substrate, however, the presence in yeast Golgi vesicles of an endogenous mechanism involved in transport of UDP-glucose, and not UDP-galactose (19, 27), may contribute to obtain a higher increment in transport of UDP-glucose when AtUTr1 is expressed in yeast. This would not allow us to establish a direct comparison of the net amount of UDP-galactose and UDP-glucose transported by AtUTr1. Then, these results indicate that *AtUTr1* encodes a nucleotide sugar transporter that uses both UDP-galactose and UDP-glucose as substrates.

Fig.7

DISCUSSION

Different lines of evidence suggest that Golgi vesicles isolated from pea stems are able to transport UDP-galactose. Studies of UDP-galactose uptake, along with controlled proteolysis of Golgi vesicles, indicated that a protease sensitive factor is required for galactosylation. The use of DIDS, a known inhibitor of nucleotide sugar and anionic transporters, caused a decreased in UDP-galactose uptake. Finally, UDP-galactose transport studies, using a well-standardized procedure, indicated that UDP-galactose is transported into the lumen of Golgi vesicles. On the other hand, incubation of Golgi vesicles with UDP-galactose resulted in a significant transfer of galactose into endogenous acceptors, and a significative portion was likely to be polysaccharides. It is known that UDP-galactose is a substrate for the synthesis of different non-cellulosic polysaccharides (xyloglucan, galactomannans, rhamnogalacturonan I and II), proteoglycans such as arabinogalactans, and glycoproteins (28). However, until now only one galactosyltransferase involved in polysaccharide biosynthesis has been cloned. This enzyme from *Trigonella foenum-graecum L*, participates in galactomannan biosynthesis, and its coding sequence predicts a type II membrane bound protein, with its catalytic domain oriented towards the lumen of the Golgi apparatus (2). Mammalian galactosyltransferases are also membrane bound proteins with their catalytic domains facing the lumen of the Golgi apparatus (29). Then, in order to have a normal galactosylation process, the topological arrangement of the galactosyltransferases make necessary the transport of UDP-galactose from the cytosol into the Golgi lumen. Therefore, it is likely that synthesis of galactose-containing cell wall polysaccharides as well as the galactosylation of other acceptors (i.e. glycoproteins and lipids) that takes place in the plant Golgi lumen would depend on the transport of UDP-galactose.

Studies in mammalian cells, drosophila and yeast indicate that multiple transmembrane proteins are responsible for the transport of UDP-galactose in the Golgi apparatus. In order to identify UDP-galactose transporters in plants, we searched the *A. thaliana* database for sequences homologous to UDP-galactose transporters from other organisms. This analysis led us to identify *AtUTr1*, a gene that in Northern analysis of RNA from Arabidopsis seedlings appears as a single band, and whose sequence predicts a multitransmembrane domain protein. Two lines of evidence indicate that AtUTr1 is able to transport UDP-gal. First, a MDCK mutant cell line deficient in transport of UDP-galactose into the Golgi, that is highly tolerant to the cytotoxic effect of the lectin ricin, becomes sensitive to ricin when *AtUTr1* is stably transfected in these cells. This result suggests that AtUTr1 is able to restore UDP-galactose transport into the Golgi apparatus of these cells. Berninsone et al. (19) have recently shown that SQV7, a *Caenorhabditis elegans* protein that transports UDP-galactose as well as UDP-glucuronic acid and UDP-N-acetylgalactosamine, was also able to complement the MDCK-RCA^r cell line. Therefore, to evaluate directly both the ability to transport UDP-galactose and the specificity of AtUTr1, our second approach was to express the gene in the yeast *S. cerevisiae*, and to measure transport of nucleotide sugars into a Golgi enriched fraction. In this heterologous expression system, AtUTr1 was able to transport UDP-galactose and UDP-glucose but not other nucleotide sugars such as CMP-sialic acid, GDP-mannose, GDP-fucose, UDP-glucuronic acid and UDP-xylose. Although the net amount of UDP-glucose transported into vesicles expressing AtUTr1 was higher than the amount of UDP-galactose, the presence of an endogenous transport activity for UDP-glucose, and not UDP-galactose, makes difficult to establish a direct comparison in the substrate preference of AtUTr1. The reason is that nucleotide sugar transporters are antiporters that exchange nucleotide sugars

with nucleoside monophosphate (30). Since UDP-glucose is endogenously transported (19, 27), it is likely that UMP formed in the vesicles from UDP-glucose, may stimulate the transport of UDP-glucose by AtUTr1. On the other hand, under normal conditions UDP-galactose is not transported, then it is unlikely that UMP may be formed. Therefore, despites the ability of AtUTr1 to transport UDP-galactose, the lack of UMP, may affect the efficiency of AtUTr1 to transport UDP-galactose in yeast Golgi vesicles. In summary, our results confirm that AtUTr1 is a nucleotide sugar transporter that uses both UDP-galactose and UDP-glucose as substrates indicating that is a nucleotide sugar transporter with a dual specificity.

Recently, Kainuma et al (5) showed that HUT1, a gene from *S. cerevisiae*, homologous to *AtUTr1* and hUGTrel1, is able to transport UDP-galactose but not UDP-glucose. Moreover, the overexpression of HUT1 in yeast estimulated the galactosylation of cell surface molecules synthesized at the Golgi apparatus, suggesting that HUT1 is able to transport UDP-galactose into the Golgi lumen, however, immunofluorescence studies and genetic analysis suggest that HUT1 is located at the endoplasmic reticulum in yeast (31). Although AtUTr1 is able to transport UDP-galactose, its actual subcellular localization is not known yet. The fact that *AtUTr1* complements a mutant mammalian cell line deficient in transport of UDP-galactose into the Golgi, suggests that at least some of the expressed protein is localized in the Golgi apparatus of the transfected cells. Moreover, upon heterologous expression in yeast we measured UDP-galactose/UDP-glucose transport in a membrane fraction enriched in Golgi vesicles, suggesting that some of the transporter may be located at the Golgi apparatus in yeast. Future studies using specific antibodies against AtUTr1 and immunoelectronmicroscopy will help to determine what is the actual localization of AtUTr1 in Arabidopsis.

UDP-galactose transporters from humans, *S. pombe*, hamster and mice show high conservation in their sequences. Moreover, they share some consensus sequences where key aminoacid residues have been shown to be essential for the transport of UDP-galactose (6). Although AtUTr1 also transports UDP-gal, we do not find these conserved sequences in the protein. SQV7, a *C. elegans* nucleotide sugar transporter that transports UDP-galactose as well as UDP-N-acetyl galactosamine and UDP-glucuronic acid as substrates, does not have the primary sequence motifs found in the mammalian UDP-galactose transporters either (32). *AtUTr1* is the first gene found in the Arabidopsis database to show homology to UDP-galactose transporters already described in other organisms. The completion of the genome sequence of Arabidopsis allowed the identification, using TBLASTN analysis, of at least five other genes present in the Arabidopsis genome that encode for putative UDP-galactose transporters (Figure 8). These genes have different degree of sequence similarity to UDP-galactose transporter genes from other species. However, the level of aminoacid identity is not an indicator of their substrate specificity (33); therefore functional expression of these genes will be necessary to test whether they encode for UDP-galactose transporters or they have other specificities.

Figure 8

The physiological relevance of nucleotide sugar transport into the Golgi has been demonstrated in yeast, nematodes, protozoa, Drosophila, mammalian cell lines and humans. In each of these organisms, impairment of nucleotide sugar transport into the Golgi apparatus results in a deficiency of the corresponding sugar in glycoconjugates which can in turn produce dramatic phenotypes (3, 32, 34-37). In plants, the transport of UDP-galactose into the Golgi apparatus is likely to be a critical step to ensure the proper synthesis of galactose-containing polysaccharides such as xyloglucan, galactomannans, rhamnogalacturonans, as well as glycoproteins. Thus, impairment of a transporter such as

AtUTr1 may decrease the transport of UDP-galactose into the Golgi apparatus and affect their synthesis, leading to phenotypic changes in cell wall architecture. AtUTr1 is also able to transport UDP-glucose, and it has been already shown that UDP-glucose is transported into the lumen of the Golgi apparatus in plants (14), therefore mutations in *AtUTr1* may also affect the synthesis of glucose-containing polysaccharides.

This is the first description of a UDP-galactose/UDP-glucose transporter. Along with the recent description of an Arabidopsis GDP-mannose transporter (38), this is the first step towards a deeper insight into the molecular mechanisms and functions of nucleotide sugar transporters in plants. The identification of these genes will now allow us to generate and analyze mutants in order to determine the role of nucleotide sugar transporters in cell wall biosynthesis *in planta*.

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FIGURE LEGENDS

Figure 1: Uptake of UDP-galactose by pea stems Golgi vesicles. Golgi vesicles (100 µg of protein) were incubated with 1µM UDP-[^{3}H]galactose for 5 min under different conditions: 0ºC, incubation in an ice bath; 25ºC, incubation at 25ºC; +Tx-100, incubation at 25 ºC in the presence of 0.1% Triton X-100; 100ºC, vesicles boiled for 5 min prior to the assay; DIDS, incubation in the presence of 20 µM DIDS. The reaction was stopped by diluting 10-fold with STM buffer, and filtering immediately. Filters were dried, and the radioactivity was counted using liquid scintillation. Results are mean ± SD.

Figure 2: UDP-galactose uptake into Golgi vesicles is sensitive to protease treatment. A.- Golgi vesicles were incubated with (+) and without (-) proteinase K in the presence (+) and absence (-) of 0.1% Triton X-100 as described in the Materials and Methods section. These vesicles were immediately used in UDP-[^{3}H]galactose uptake experiments,

performed in the absence (white bars, intact vesicles) or presence (black bars, permeabilized vesicles) of 0.1% Triton X-100. **B.-** To check the integrity of the vesicles we determined the activity of a lumenal UDPase using native gels. Upon electrophoresis in a 10% polyacrylamide gel the UDPase activity was detected using 3 mM UDP. The arrowheads indicate the position of the lumenal Golgi UDPase. Proteinase K treatment in Triton X-100 permeabilized Golgi vesicles produced proteolytic fragments still active which are shown with a bracket. Treatment with Triton X-100 alone had no effect on the mobility of the UDPase activity (not shown).

Figure 3: Comparison of the primary sequence and hydrophobicity plot of AtUTr1 and hUGTrel1. A.- The protein primary sequence predicted for *AtUTr1* and hUGTrel1 were compared using CLUSTAL and then depicted using Shadebox. **B.-** Kyte-Doolittle hydropathy plots of AtUTr1 and hUGTrel1. The accession number for AtUTr1 is AY115566.

Figure 4: *AtUTr1* is expressed in *Arabidopsis thaliana*. Total RNA and poly A+ RNA isolated from 2 weeks-old seedlings were fractionated in a 1.5% agarose-formaldehide gel, blotted to a nylon membrane as described in methods, and hybridized with the *AtUTr1* cDNA labelled with ^{32}P. Lane 1 contains 40 μg of total RNA. Lane 2 contains 2.5 μg of purified polyA+.

Figure 5: *AtUTr1* makes MDCK-RCA^r cells sensitive to the lectin ricin. Cells were grown at 37ºC for 7 days in the presence (+) and absence (-) of 0.1 μg/ml of ricin (RCA II). Then the culture medium was removed and the surviving cells were stained using 1% methylene blue in 50 % methanol. MDCK, wild type cells; RCA^r, MDCK cell line mutant deficient in transport of UDP-galactose transport into the Golgi; Control, MDCK-RCA^r

cells stably transfected with the vector pcDNA.3-mycHis; AtUTr1, MDCK-RCA^r cells stably transfected with pcDNA3-AtUTr1-mycHis.

Figure 6: AtUTr1-mycHis is expressed in *Saccharomyces cerevisiae* and found in a microsomal fraction. Microsomes were prepared from yeast transformed both with the vector alone (p426GPD-mycHis) (Lane 1) or the vector containing *AtUTr1* (Lane 2). Proteins were detected by western blot using an anti-myc monoclonal antibody.

Figure 7: AtUTr1 expressed in yeast transport UDP-galactose and UDP-glucose: A subcellular fraction enriched in Golgi vesicles was isolated from both yeast transformed with the vector alone (open bars) or the vector containing *AtUTr1* (black bars). Vesicles were incubated with 0.1 µM of different radiolabelled nucleotide sugar for 3 min at 30ºC and the amount of nucleotide sugars transported (S_i) was determined as described in Methods. Determinations were done using triplicates and the standard error is depicted. **A.-** Transport of UDP-galactose, UDP-glucuronic acid, UDP-xylose, GDP-fucose and CMP-sialic acid. **B.-** Transport of GDP-mannose and UDP-glucose.

Figure 8: The *Arabidopsis thaliana* genome has several putative UDP-galactose transporters. A search using TBLASTN, reveals that six Arabidopsis genes encode for proteins that belong to a family of proteins (AtUTr) that have sequence similarity to UDP-galactose transporters from human (huUGalT1, huGalT2 and huGTrel1), *S. pombe* (spGms, spHUT1), *S. cerevisiae* (scHUT1) and *C. elegans* (SQV7). **A.-** Shows a tree based on the aminoacid sequence of UDP-galactose transporters obtained with the CLUSTAL W program. **B.-** The structural characteristics of the different AtUTr genes and the deduced proteins is shown. (&) The comparison of aminoacid identity between *AtUTr1* (At2g02810) and the others sequences was performed using the program SIM at www.expasy.org. (*) accession number defined by the Arabidopsis Genome Initiative (AGI).

T°	Solutes within vesicles S_i (pmol/mg)	Transfer to 70 % ethanol insoluble acceptors. T_{Ps} (pmol/mg)	Transfer to acceptors at the organic phase + the aqueous/organic interface. T_{P+L} (pmol/mg)	Total transport $(S_i+T_{Ps}+T_{P+L})$ (pmol/mg)	Concentration of solutes in the vesicles $[S_i]$ (µM)	Concentration of solutes in incubation medium $[S_m]$ (µM)	Latency of vesicles (%)
0 °C	0.38 ± 0.11	1.12 ± 0.11	0.74 ± 0.15	2.25 ± 0.30	0.06 ± 0.02	0.97 ± 0.02	89
25 °C	1.62 ± 0.31	9.54 ± 0.89	4.57 ± 0.13	15.73 ± 1.07	0.25 ± 0.05	0.90 ± 0.04	

Table 1: Transport of UDP-galactose into Pea stems Golgi vesicles. Golgi vesicles (1 mg) were incubated with 1 µM UDP-[^{3}H]galactose for 5 min in a final volume of 0.4 ml. The reaction was stopped diluting with 4 ml of cold STM (0.25M sucrose, 10 mM Tris/HCl pH 7.5, 1 mM $MgCl_2$) and immediately centrifuged at 100.000 x *g*. The amount of UDP-[^{3}H]galactose transported into the lumen of the vesicles (S_i), the amount of [^{3}H]galactose transferred into 70 % ethanol insoluble acceptors (T_{Ps}) and to acceptors at the organic phase plus the aqueous/organic interface (T_{P+L}) are shown. To estimate the concentration of UDP-galactose in the vesicles [S_i] the volume V_o and V_i were determined as described in methods. [S_m] correspond to the concentration of UDP-galactose in the medium once the reaction is finished

Figure 1

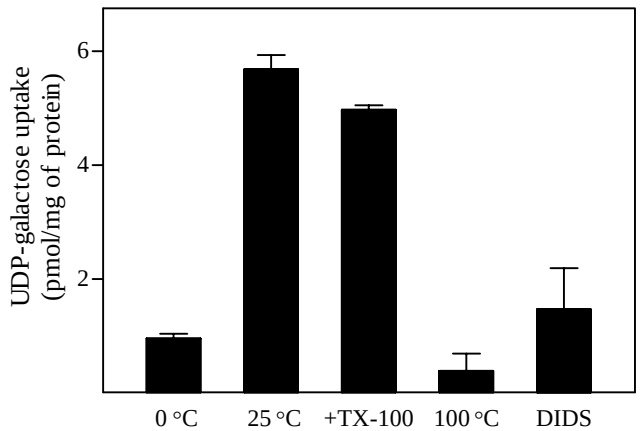

UDP-galactose uptake
(pmol/mg of protein)
6
4
2
0 °C
25 °C
+TX-100
100 °C
DIDS

Figure 2

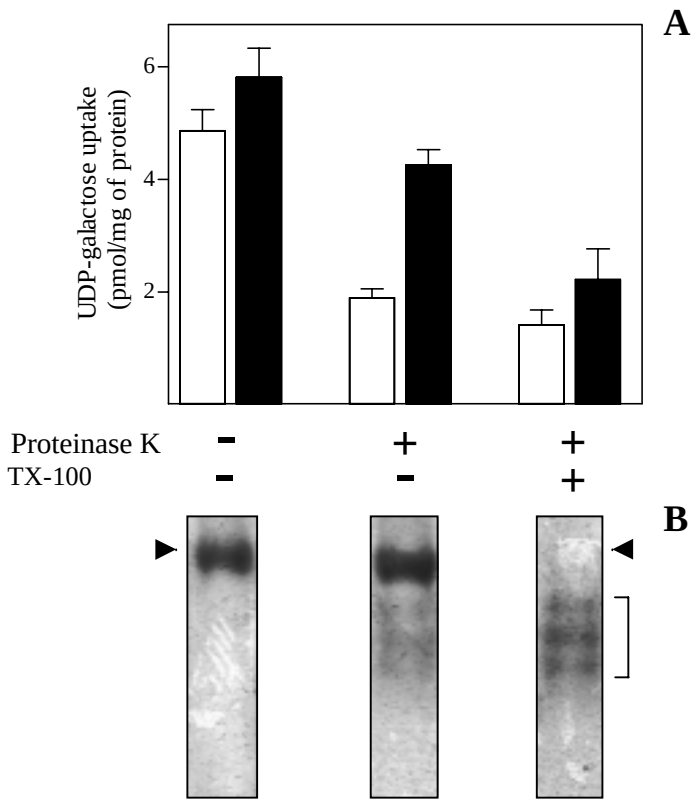
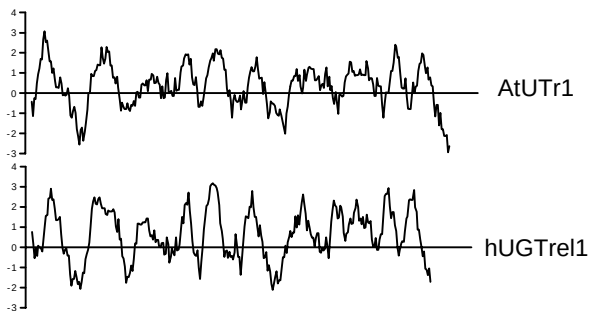

A
UDP-galactose uptake
(pmol/mg of protein)
6
4
2
Proteinase K
TX-100
-
-
+
-
+
+
B

Figure 3

A

```
AtUTr1      1 MEVHGSGFRRILLLALCISGIWSAYIYQGVLQETLS
hUGTrel1    1 MASSSSLVPDRLRLPLCFLGVFVCYFYYGILQEKIT

AtUTr1     37 TKRFGP--DEKRFEHLAFLNLAQSVVCLIWSYIMIK
hUGTrel1   37 RGKYGEGAKQETFTFALTLVFIQCVINAVFAKILIQ

AtUTr1     71 LWSNAGNGGAPWWTYWSAGITNTIGPAMGIEALKYI
hUGTrel1   73 FFDTARVDRTRSWLYAACSISYLGAMVSSNSALQFV

AtUTr1    107 SYPAQVLAKSSKMIPVMLMGTLVYGIRYTFPEYMCT
hUGTrel1  109 NYPTQVLGKSCKPIPVMLLGVTLLKKKYPLAKYLCV

AtUTr1    143 FLVAGGVSIFALLKTSSKTISKLAHPNAPLGYALCS
hUGTrel1  145 LLIVAGVALFMYKP---KKVVGIEEHTVGYGELLLL

AtUTr1    179 LNLAFDGFTNATQDSIASRYPKTEAWDIMLGWNLWG
hUGTrel1  178 LSLTLDGLTGVSQDHVRAHY-QTGSNHMMLNINLWS

AtUTr1    215 TIYNIIYWFGIPQGIGFEAIQFCKLHPEAAWDILKY
hUGTrel1  213 TLL--LGWGILFTGELWEFLSFAERYPAIIYNILLF

AtUTr1    251 CICGAVGQNFIFMTISNFGSLANTTIITTRKFVSIV
hUGTrel1  247 GLTSALGQSFIFMTVVYFGPLTCSIITTRKFFTIL

AtUTr1    287 VSSVWSGNPLSLKQWGCVSWVFGGLAYQIYLKWKKL
hUGTrel1  283 ASVILFANPISPMQWVGTVLVFLGLCLDAKFGKGAK

AtUTr1    323 QRVEKKKQKS
hUGTrel1  319 KTSH------
```

B



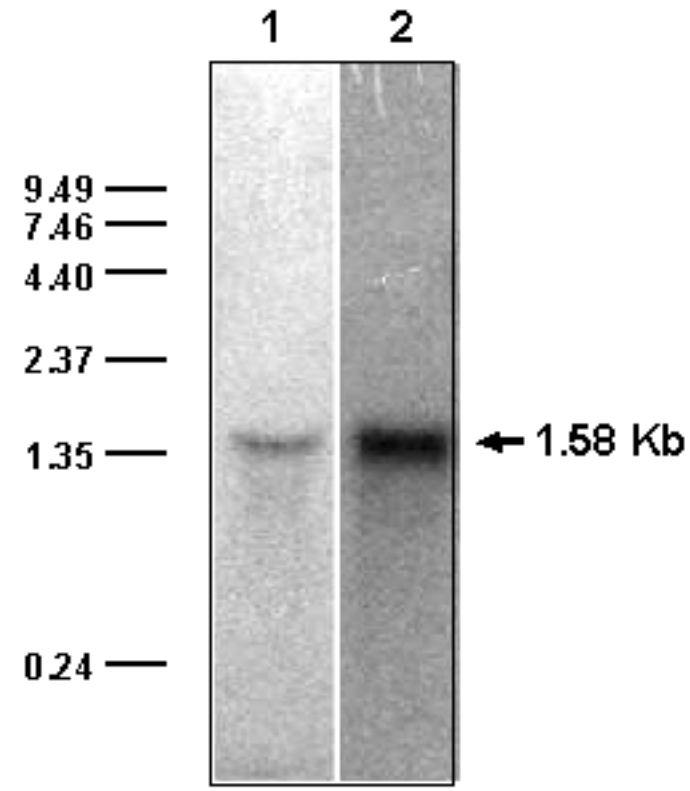

1
2
9.49
7.46
4.40
2.37
1.35
0.24
1.58 Kb

FIGURE 5

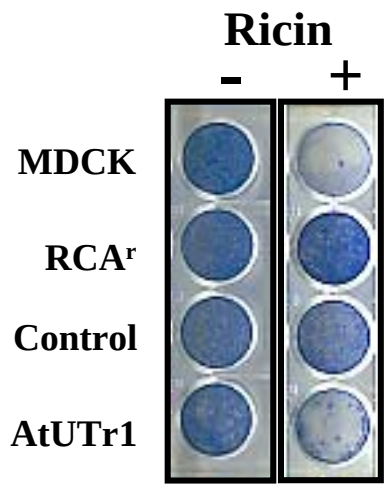


FIGURE 6

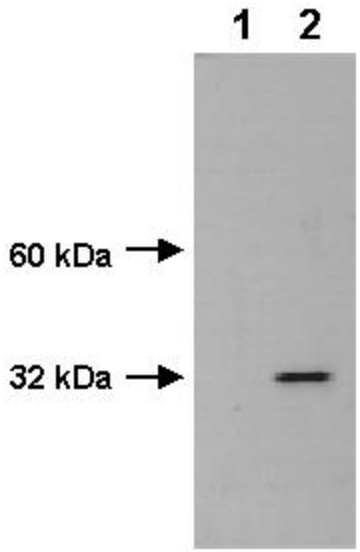


Figure 7

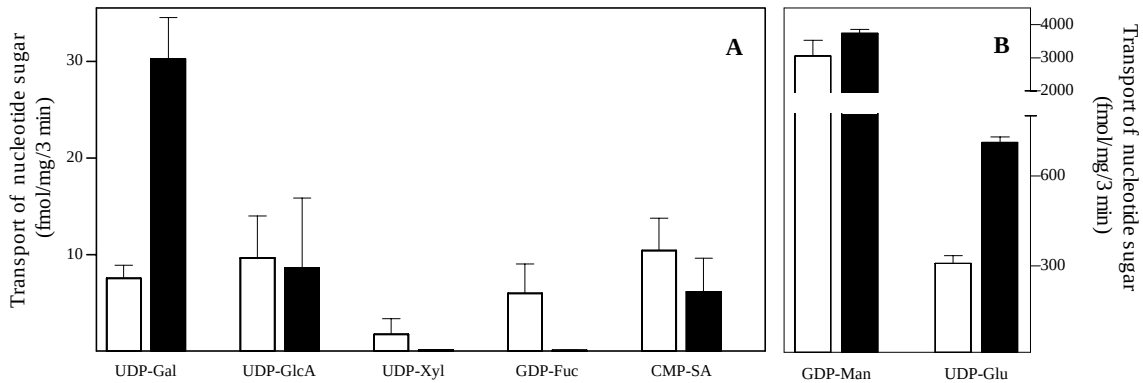
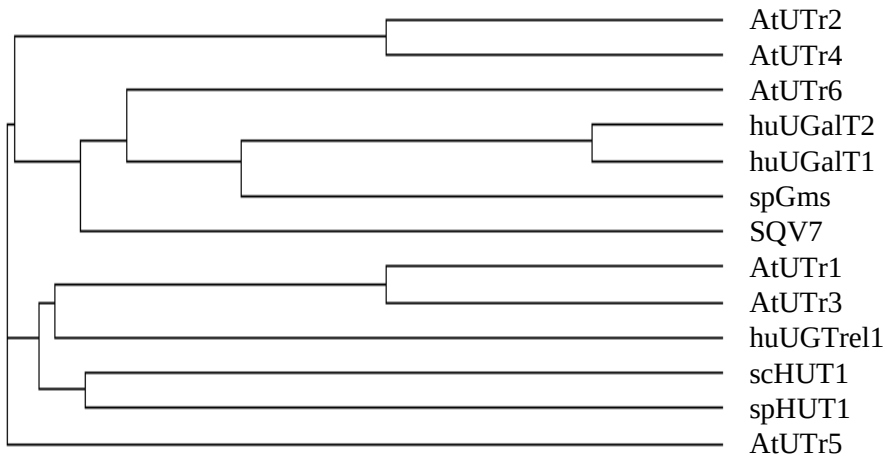

Transport of nucleotide sugar
(fmol/mg/3 min)
30
20
10
A
UDP-Gal
UDP-GlcA
UDP-Xyl
GDP-Fuc
CMP-SA
B
4000
3000
2000
600
300
GDP-Man
UDP-Glu
Transport of nucleotide sugar
(fmol/mg/3 min)

Figure 8

A



B

	Accession number*	Chromosome	Introns	Mr (kDa)	% Identity[&]
AtUTr1	At2g02810	II	6	36.9	100
AtUTr2	At4g23010	IV	6	40.3	26.6
AtUTr3	At1g14360	I	6	36.7	87
AtUTr4	T12C24.13	I	6	40.5	26.9
AtUTr5	At3g46180	III	8	38.4	28.3
AtUTr6	At3g59360	III	14	50.3	26