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A CMP-sialic acid transporter cloned from *Arabidopsis thaliana*

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Abstract—Sialylation of glycans is ubiquitous in vertebrates, but was believed to be absent in plants, arthropods, and fungi. However, recently evidence has been provided for the presence of sialic acid in these evolutionary clades. In addition, homologs of mammalian genes involved in sialylation can be found in the genomes of these taxa and for some *Drosophila* enzymes, involvement in sialic acid metabolism has been documented. In plant genomes, homologs of sialyltransferase genes have been identified, but there activity could not be confirmed. Several mammalian cell lines exist with defects in the sialylation pathway. One of these is the Chinese hamster ovary cell line Lec2, deficient in CMP-sialic acid transport to the Golgi lumen. These mutants provide the possibility to clone genes by functional complementation. Using expression cloning, we have identified an *Arabidopsis thaliana* nucleotide sugar transporter that is able to complement the CMP-sialic acid transport deficiency of Lec2 cells. The isolated gene (At5g41760) is a member of the triose-phosphate/nucleotide sugar transporter gene family. Recombinant expression of the gene in yeast and testing in vitro confirmed its ability to transport CMP-sialic acid.

Keywords: CMP-sialic acid; CMP-Kdo; Nucleotide sugar transporter; Golgi transport

1. Introduction

Sialic acids are essential for mammalian development.[1] In plants and invertebrates, it is generally believed that these residues are absent. Over the last decades, however, there are several publications reporting the presence of sialic acid in *Drosophila*,[2–4] fungi,[5] and several plant species[6–8] including *Arabidopsis thaliana*.[9] Nevertheless, trials to prove the existence of a functional sialylation pathway by recombinant production of mammalian proteins in plants were so far not successful.[10] On the other hand, three homologous genes of mammalian sialyltransferases have been identified in the genome of *Arabidopsis* and support the idea that sialic acid is metabolized by plants. In contrast to a homologous sialyltransferase from *Drosophila*, for which activity has been confirmed,[11] the activity of these plant transferases has yet to be determined. Since sialyltransferases are Golgi resident type II membrane proteins, activity depends on the supply of CMP-activated sialic acid (CMP-Sia), made by CMP-Sia-synthetase[12,13] in the nucleus, into the compartmental lumen by a CMP-Sia transporter.[14] The latter is a type III protein located in the Golgi membranes of animal cells.[15] Importantly, it is known from mammals, yeast, and protozoa that nucleotide sugar transporters, despite of exhibiting high relation in terms of architecture, show little conservation at primary sequence level.[14] The gene family, referred to in mammals as SLC35 (solute carrier

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family 35)[16] and in plant as TP-NST (triose-phosphate/nucleotide sugar transporter family)[17] is large, comprising over 40 members[18,19] in *Arabidopsis*. Orthologous pairs are difficult to identify between mammalian and plant members of the gene family. We therefore, decided to use a complementation cloning approach to search for a potential CMP-Sia transport protein in *Arabidopsis*. In this attempt we profited from the availability of a CHO cell line (Lec2) deficient in Golgi CMP-sialic acid (CMP-Sia) transport[20] and the fact that nucleotide sugar transporters identified so far can be functionally expressed in heterologous systems[21] (and the literature cited therein). Here we report the cloning of *A. thaliana gene* At5g41760, a TP-NST member that complements the defect in Lec2 cells and after recombinant expression in yeast and testing in vitro was shown to be an active CMP-Sia transporter.

2. Results

The CHO mutant Lec2 is deficient in the transport of CMP-sialic acid[20] and has been used before to clone the mammalian CMP-Sia transporter.[15] As a result of the absence of this transporter, polysialic acid (polySia) is not made in these cells and the presence of this polysaccharide can easily be detected by the monoclonal antibody (mAb) 735.[15,22] To evaluate if *Arabidopsis* expresses a protein able to transport CMP-Sia across the Golgi membrane, we applied a similar strategy of expression cloning as has been used for the mammalian transporter, now using an *Arabidopsis* cDNA library. This library[23] was used before to isolate *Arabidopsis* clones that complemented CHO Lec8,[18] a cell line deficient in UDP-galactose transport.[24]

Using an established sibling selection procedure in which pools of the cDNA library were transfected into mammalian cells,[18,25,26] cDNA pools that rendered Lec2 cells positive for polySia (stained with mAb 735) were further divided into smaller pools until single cDNA clones could be screened. One cDNA clone was isolated in this way and corresponded to the *Arabidopsis* gene At5g41760. The sequence showed about 25% identity with the human nucleotide sugar transporters for CMP-Sia, UDP-galactose, and UDP-*N*-acetylglucosamine. As these three human transporters show a much higher identity among each other, the activity of the newly isolated *Arabidopsis* gene could not have been predicted from the outset. In control experiments the UDP-galactose transporter negative CHO Lec8 cells[24] were not complemented with At5g41760 (data not shown). Using FACS analyses of transiently transfected Lec2 cells, the complementation that was observed by cell surface staining could be confirmed (Fig. 1). This more quantitative measurement demonstrated that the *Arabidopsis* transporter was less potent in complementing Lec2 cells than the mouse CMP-Sia transporter used in parallel as a control. In both populations about 30% cells were transfected, however, the right shift in

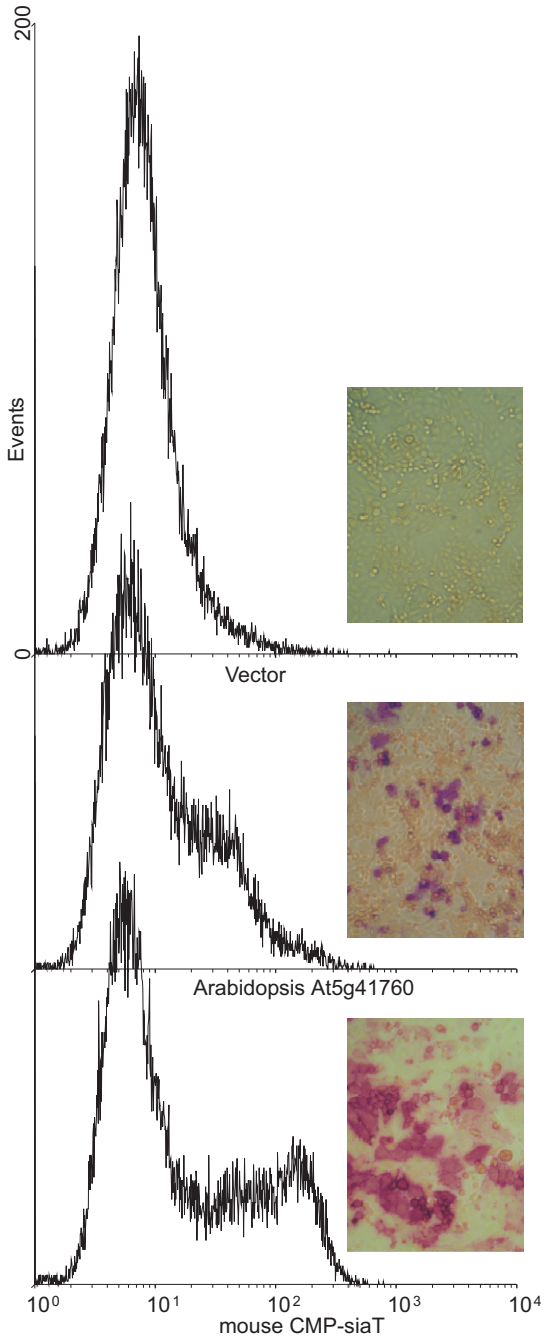

Figure 1. The CMP-Sia transporter deficient cell line Lec2 is complemented by an *Arabidopsis* cDNA. An *Arabidopsis* cDNA library in a mammalian expression vector was transiently expressed in Lec2 cells and by a sibling selection procedure similar as described,[18] a single cDNA clone was identified that was able to complement the genetic defect. Complemented cells are detected by a monoclonal antibody 735[22] against polysialic acid, shown here by FACS and cell surface staining. Lec2 cells were transiently transfected with an empty vector control, the *Arabidopsis* transporter cDNA (At5g41760) and the mouse CMP-Sia transporter.

At5g41760 transfected cells was less pronounced than in cells transfected with the murine gene.

To directly show transport activity of the *Arabidopsis* CMP-Sia transporter, the cDNA was subcloned into a yeast expression vector and the protein expressed in *Saccharomyces cerevisiae*. Golgi vesicles were isolated and in comparison to vesicles isolated from *S. cerevisiae* transfected with the murine transporter tested in an in vitro assay with radioactively labeled CMP-Sia. Yeast has a low background for most nucleotide sugar transport activities and is commonly used to assay transport activities by heterologous expression.[14,21,27,28] As shown in Figure 2, the *Arabidopsis* CMP-Sia transporter showed significant and reproducible activity with CMP-Sia as a substrate, but again notably less than the CMP-Sia transporter from mouse. This experiment confirmed the observed moderate in vivo activity.

3. Discussion

By two methods, complementation and in vitro assaying of recombinant expressed protein, it is shown here that the cDNA clone At5g41760 isolated from *Arabidopsis* is a nucleotide sugar transporter capable to transport CMP-Sia. Compared to the mouse gene clone At5g41760 showed reduced activity in both test systems. Nevertheless, the measured activity represents genuine transport as we have never been able to measure any significant activity in these systems with other transporters. Among the tested genes there have been eleven human, all twelve cloned *Caenorhabditis elegans* and two *Arabidopsis* nucleotide sugar transporters previously shown to be specific for UDP-galactose transport.[18] Still, the question remains if CMP-Sia transport represents the physiological function of the isolated *Arabidopsis* transporter. Even so there are indications that sialic acid can be part of plant glycoconjugates,[6–9] the possibility exists that the newly isolated gene mainly transports CMP-Kdo (ketodeoxyoctonate). Kdo is undoubtedly present in plants[29] and the enzyme which activates Kdo to CMP-Kdo (the CMP-Kdo-synthetase) has also been identified in plants.[30] Although Kdo is structurally different from sialic acid, the two sugars have several common properties. Both are negatively charged and with 8 and 9 C-atoms in Kdo and sialic acid, respectively, large sugars. Most important, however, Kdo and sialic acid are unique among the monosaccharides used in plant and animal in that they are activated with CMP.[12,13] While CMP-Sia is synthesized in the nucleus of mammalian cells, CMP-Kdo synthesis is most likely to occur in the ER of plant cells because the CMP-Kdo-synthetase has been predicted to harbor an ER targeting signal.[29] So far we have been unable to perform transport assays with CMP-Kdo. This nucleotide sugar is extremely labile and not available in radioactively labeled form. An in situ production system of CMP-Kdo as described for the testing of bacterial Kdo transferases[31] may be suited to resolve this problem in the future.

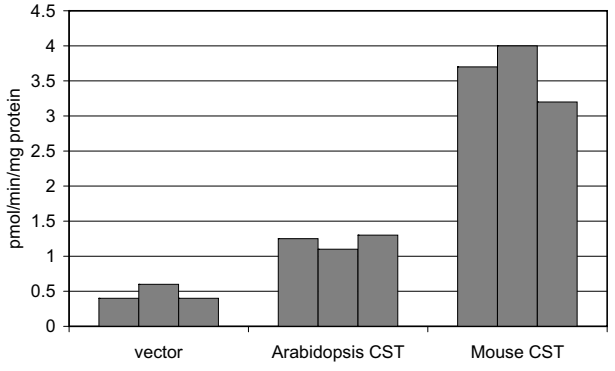

Figure 2. Transport activity of the *Arabidopsis* CMP-Sia transporter. The *Arabidopsis* CMP-Sia transporter was expressed in *Saccharomyces cerevisiae*. Golgi enriched vesicles were isolated and assayed for CMP-sialic acid transport. Activity was compared to the mouse CMP-Sia transporter.[15] Transport assays were done in triplicate using different Golgi preparations represented by individual bars.

Despite of the ongoing debate about the existence of sialic acid in plants, we show here that plants have a transporter with the capability to transport activated sialic acids into the Golgi apparatus, a feature which is of major importance toward the application of plant as factories for the expression of mammalian proteins. Introduction of a transporter gene might thus not be required for the production of sialylated glycoconjugates. On the other hand, this study also shows that the CMP-Sia transport efficiency obtained in mammalian and yeast cells with the plant derived gene is lower than obtained with the mammalian ortholog. Whether this effect accounts to the fact that the plant gene naturally transports a different substrate (e.g., CMP-Kdo) or in both mammalian and yeast cells is expressed under suboptimal conditions, needs further investigation. With this gene in hands, we now have options to engineer and optimize CMP-Sia transport in plants.

4. Experimental

4.1. Expression cloning

The *Arabidopsis* cDNA library has been described[23] and the procedure for expression cloning is the same as in Ref. 18 except for the detection method. For this, antibody 745 was used. Lec2 cell line 6B2[15] grown in α-MEM (Biochrom AG, Berlin) supplemented with 10% fetal bovine serum (Biochrom AG) was used as host. Pools or clones of the cDNA library were transfected into 6B2 cells using Metafectane (Biontex, München, Germany). After two days, cells, grown in 6 well plates, were fixed with 1.5% glutaraldehyde, incubated with the

antibody 735,[22] followed by HRP-conjugated goat-anti-mouse (Jackson immunoresearch), and detected by tyramide signal amplification using biotin-tyramide,[32] streptavidine-alkaline-phosphatase (Caltag), and Fast-Red (Sigma) as a chromogenic substrate.

4.2. FACS analyses

Fluorescence activated cell sorting (FACS) was performed by labeling transiently transfected cells with antibody 735, followed by a secondary FITC-labeled goat anti mouse antibody (Sigma). 10,000 cells were analyzed by cytofluorometry (FACScan, Becton-Dickinson, Heidelberg, Germany).

4.3. In vitro CMP-Sia transport assay

Both the *Arabidopsis* clone At5g41760 and the mouse CMP-Sia transporter[15] were cloned EcoRI/XhoI and EcoRI/XbaI, respectively, in the yeast expression vector pYES/NT-C (Invitrogen). The resulting constructs express the complete open reading frames of the transporters, with an additional N-terminal His-V5 epitope tag to allow the control of expression. Yeast cells were transformed using the lithium acetate method provided by Invitrogen. After transformation, yeast cells were cultured on selective medium containing 0.67% Bacto-yeast nitrogen base without amino acids but supplemented with L-leucine, L-histidine, L-tryptophan, L-lysine, adenine, and 2% glucose. For the induction of expression, cells were grown to A600 0.8 in 2% raffinose, then supplemented with 2% galactose, and cultured for another 3 h.

The subcellular fractionation of yeast cells and the in vitro transport assays were performed essentially as described.[21,33] Golgi-rich fractions were obtained by centrifugation of isolated vesicles at 10,000*g* for 10 min (to remove the endoplasmic reticulum rich fraction) and 100,000*g* for 1 h (Golgi). The 100,000*g* pellet was carefully resuspended in lysis buffer (0.8 mL/g of cells). Protein concentrations were determined using the BCA™ kit (Pierce).

For transport assays, equal volumes (50 μL of each) of 2 mM radioactive CMP-Sia (CMP-[9-^{3}H]NeuAc, obtained from Perkin–Elmer Life Sciences and diluted with cold CMP-NeuAc from Kyowa Hakko Kogyo Co. Ltd, Tokyo, Japan) (3000 dpm/pmol) in assay buffer (10 mM Tris–HCl, pH 7.0; 0.8 M sorbitol; 2 mM $MgCl_2$) and vesicle preparation (equivalent to 75–100 μg of protein) were incubated for 30 s at 30 °C and further processed as described.[21] Golgi vesicles from yeast cells transformed with an empty vector were used to measure endogenous transport. Transport values for each construct were calculated as pmol/min/mg of protein.

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References

1. Schwarzkopf, M.; Knobeloch, K. P.; Rohde, E.; Hinderlich, S.; Wiechens, N.; Lucka, L.; Horak, I.; Reutter, W.; Horstkorte, R. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 5267–5270.
2. Roth, J.; Kempf, A.; Reuter, G.; Schauer, R.; Gehring, W. J. *Science* **1992**, *256*, 673–675.
3. Aoki, K.; Perlman, M.; Lim, J. M.; Cantu, R.; Wells, L.; Tiemeyer, M. *J. Biol. Chem.* **2007**, *282*, 9127–9142.
4. Koles, K.; Lim, J. M.; Aoki, K.; Porterfield, M.; Tiemeyer, M.; Wells, L.; Panin, V. *Glycobiology* **2007**, *17*, 1388–1403.
5. Wasylnka, J. A.; Simmer, M. I.; Moore, M. M. *Microbiology* **2001**, *147*, 869–877.
6. Bourbouze, R.; Akiki, C.; Chardonloriaux, I.; Percheron, F. *Carbohydr. Res.* **1982**, *106*, 21–30.
7. Mayer, F. C.; Dam, R.; Pazur, J. H. *Arch. Biochem. Biophys.* **1964**, *108*, 356–357.
8. Onodera, K.; Hirano, S.; Hayashi, H. *Agr. Biol. Chem.* **1966**, *30*, 1170–1172.
9. Shah, M. M.; Fujiyama, K.; Flynn, C. R.; Joshi, L. *Nat. Biotechnol.* **2003**, *21*, 1470–1471.
10. Lerouge, P.; Cabanes-Macheteau, M.; Rayon, C.; Fischette-Lainé, A. C.; Gomord, V.; Faye, L. *Plant Mol. Biol.* **1998**, *38*, 31–48.
11. Koles, K.; Irvine, K. D.; Panin, V. M. *J. Biol. Chem.* **2004**, *279*, 4346–4357.
12. Kean, E. L.; Münster-Kühnel, A. K.; Gerardy-Schahn, R. *Biochim. Biophys. Acta* **2004**, *1673*, 56–65.
13. Münster-Kühnel, A. K.; Tiralongo, J.; Krapp, S.; Weinhold, B.; Ritz-Sedlacek, V.; Jacob, U.; Gerardy-Schahn, R. *Glycobiology* **2004**, *14*, 43R–51R.
14. Gerardy-Schahn, R.; Oelmann, S.; Bakker, H. *Biochimie* **2001**, *83*, 775–782.
15. Eckhardt, M.; Mühlenhoff, M.; Bethe, A.; Gerardy-Schahn, R. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 7572–7576.
16. Ishida, N.; Kawakita, M. *Pflugers Arch.* **2004**, *447*, 768–775.
17. Ward, J. M. *Bioinformatics* **2001**, *17*, 560–563.
18. Bakker, H.; Routier, F.; Oelmann, S.; Jordi, W.; Lommen, A.; Gerardy-Schahn, R.; Bosch, D. *Glycobiology* **2005**, *15*, 193–201.
19. Knappe, S.; Flügge, U. I.; Fischer, K. *Plant Physiol.* **2003**, *131*, 1178–1190.
20. Deutscher, S. L.; Nuwayhid, N.; Stanley, P.; Briles, E. I.; Hirschberg, C. B. *Cell* **1984**, *39*, 295–299.
21. Ashikov, A.; Routier, F.; Fuhlrott, J.; Helmus, Y.; Wild, M.; Gerardy-Schahn, R.; Bakker, H. *J. Biol. Chem.* **2005**, *280*, 27230–27235.
22. Frosch, M.; Gorgen, I.; Boulnois, G. J.; Timmis, K. N.; Bitter-Suermann, D. *Proc. Natl. Acad. Sci. U.S.A* **1985**, *82*, 1194–1198.
23. Bakker, H.; Lommen, A.; Jordi, W.; Stiekema, W.; Bosch, D. *Biochem. Biophys. Res. Commun.* **1999**, *261*, 829–832.

24. Deutscher, S. L.; Hirschberg, C. B. *J. Biol. Chem.* **1986**, *261*, 96–100.
25. Bakker, H.; Friedmann, I.; Oka, S.; Kawasaki, T.; Nifant'ev, N.; Schachner, M.; Mantei, N. *J. Biol. Chem.* **1997**, *272*, 29942–29946.
26. Münster, A. K.; Eckhardt, M.; Potvin, B.; Mühlenhoff, M.; Stanley, P.; Gerardy-Schahn, R. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 9140–9145.
27. Berninsone, P.; Eckhardt, M.; Gerardy-Schahn, R.; Hirschberg, C. B. *J. Biol. Chem.* **1997**, *272*, 12616–12619.
28. Berninsone, P. M.; Hirschberg, C. B. *Curr. Opin. Struct. Biol.* **2000**, *10*, 542–547.
29. York, W. S.; Darvill, A. G.; Mcneil, M.; Albersheim, P. *Carbohydr. Res.* **1985**, *138*, 109–126.
30. Royo, J.; Gomez, E.; Hueros, G. *J. Biol. Chem.* **2000**, *275*, 24993–24999.
31. Gronow, S.; Brabetz, W.; Brade, H. *Eur. J. Biochem.* **2000**, *267*, 6602–6611.
32. Speel, E. J.; Hopman, A. H.; Komminoth, P. *Methods Mol. Biol.* **2006**, *326*, 33–60.
33. Segawa, H.; Kawakita, M.; Ishida, N. *Eur. J. Biochem.* **2002**, *269*, 128–138.