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The HUP1 gene product of *Chlorella kessleri*: H^+/glucose symport studied in vitro

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Abstract

An in vitro system was established to measure secondary active transport mediated by plant H^+ symporters. For this purpose plasma membranes of *Schizosaccharomyces pombe* cells transformed with the HUP1 gene coding for the H^+/hexose symporter of *Chlorella kessleri* were fused with cytochrome-*c* oxidase containing proteoliposomes. After energization with ascorbate/TMPD/cytochrome *c* these vesicles built up a protonmotive force of > 130 mV consisting mainly of a membrane potential of > 100 mV (inside negative). Energized vesicles accumulated D-glucose in a pH-dependent way up to 30-fold which was not the case with control vesicles prepared from cells transformed with the plasmid not containing the HUP1 gene. The K_m value for D-glucose uptake was $5 \cdot 10^{-5}$M. The pH-dependence of accumulation was not due to a difference in protonmotive force, but reflected the pH-dependence of the carrier activity, i.e., the accumulation was determined by kinetic and by thermodynamic parameters. In the system both components of protonmotive force $\Delta\psi$ and ΔpH can be manipulated individually, which allows to evaluate to what extent they contribute to sugar accumulation. The results indicate that under certain conditions the internal pH may be a limiting factor for D-glucose accumulation.

Key words: Glucose/proton symporter; Vesicle; Cytochrome-*c* oxidase; *Chlorella*

1. Introduction

Transport studies with membrane vesicles were first introduced by Kaback [1] and intensively applied to investigate molecular details of the *Escherichia coli* lactose/proton symport [2]. H^+-symporters in right side out oriented vesicles can be energized by protonmotive force generated by proton pumping of cytochrome-*c* oxidase. This system has been optimized for bacterial transporters in Koning's laboratory [3] and recently it has also successfully been used to study active amino acid and sugar transport in yeasts [4–7]. So far plant transporters have not been investigated in this way.

Measurements of plant sugar transport in vitro have been reported by Busch [8], Buckhout [9] and Lemoine and Delrot [10]. These authors demonstrated concentrative uptake of sucrose into plasma-membrane vesicles from sugar beets isolated by the aqueous two-phase partitioning method described by Larson [11]. Similar transport studies have been carried out with plasma membrane vesicles from other plants and with other substrates [12]; they all supported the in vivo evidence for proton-coupled transport in plants [13–15].

The inducible glucose/H^+ symporter of the green unicellular alga *Chlorella* has intensively been studied over the years [16–18]. The corresponding gene has been cloned [19] and functionally expressed in *Schizosaccharomyces pombe* on a multicopy plasmid [20,21]. All trials to prepare and purify plasma membranes from *Chlorella* cells with its strong cell walls have been unsuccessful so far. The experimental detour via *S. pombe*, however, and the high expression of the *Chlorella* glucose/H^+ symporter in the plasma membranes of this yeast, made the establishment of an in vitro system feasible. Plant transport proteins would in this way become accessible for studies of mechanis-

Abbreviations: Ph_4P^+, tetraphenylphosphonium ion; TMPD, *N,N,N,N'*-tetramethyl-*p*-phenylenediamine; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; pmf, protonmotive force; Enzyme: Cytochrome-*c* oxidase, ferrocytochrome-*c*:oxidoreductase (EC 1.9.3.1).

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tic details and the model previously proposed for *Chlorella* H^+/glucose symport [22] could in the future be studied without interference of cellular metabolism and compartmentation.

It will be shown, that with the cytochrome-*c* oxidase vesicles containing the H^+-symporter of *Chlorella* a degree of energization can be achieved which is much higher than that obtained in the in vitro plant transporter studies referred to above; hence, also the accumulation of substrate is considerable higher. Moreover the effect of individual components of the protonmotive force can be studied separately. In this paper, this in vitro system has been characterized.

2. Materials and methods

2.1. Materials

The radiolabeled substances D-[U-^{14}C]glucose (288 mCi/mmol), L-[1-^{14}C]glucose (57 mCi/mmol), 3-*O*-methyl[U-^{14}C]glucose (126 mCi/mmol), tetra[^{3}H]phenylphosphonium bromide (33 Ci/mmol) and [1-^{14}C]acetic acid (59 mCi/mmol) were obtained from Amersham Buchler (Braunschweig).

Cytochrome-*c* oxidase isolated from beef heart mitochondria according to Ramirez et al. [23] was a generous gift of J. Ramirez UNAM, Mexico. L-α-Phosphatidylethanolamine (type IX from *E. coli*, approx. 50%), cytochrome *c*, L-glucose, gluconic acid, carbonyl cyanide *m*-chlorophenyl hydrazone were from Sigma: n-octyl β-D-glucoside, tetraphenylphosphonium bromide were from Merck, Darmstadt.

2.2. Strains and growth conditions

A strain of *Schizosaccharomyces pombe* YGS-5 showing no hexose transport activity (kindly supplied by B. Milbrandt and M. Höfer) was transformed by plasmid pEVP11 bearing HUP1 gene coding for uptake protein from *Chlorella* (*S. pombe* TCY 96). The same strain transformed by plasmid without an insert (*S. pombe* TCY 104) was used as a control. Both transformants were grown on rotatory shaker at 29°C in a minimal medium containing 0.67% yeast nitrogen base without amino acids, 2% gluconic acid and mixture of filter-sterilized amino acids and bases consisting of (in mg/l) adenine 17.2, uracil 38.4, histidine 38.4, tryptophan 38.4, arginine 38.4, methionine 38.4, tyrosine 14.4., isoleucine 57.6, phenylalanine 48, valine 57.6, and threonine 57.6. Exponentially growing cells were harvested at A_{578} of 1.0–1.5.

2.3. Isolation of plasma membranes

Plasma membranes were isolated essentially as described by Dufour et al. [24]. The purified membranes were resuspended in 50 mM potassium phosphate, pH 6.3, 20% glycerol and 1 mM EDTA, frozen in liquid nitrogen and kept at −80°C. The purity of plasma membrane fraction was checked as described previously [5]; the contamination by mitochondrial membranes was less than 10%.

2.4. Preparation of liposomes and reconstitution of cytochrome-c oxidase

The liposomes were prepared from L-α-phosphatidylethanolamine (type IX from *E. coli*) by sonifying 25 mg of lipids in 1 ml 50 mM potassium phosphate, pH 6.3, containing 7.5 mg octyl glucoside. After clarifying the solution cytochrome-*c* oxidase (0.8 mg protein) was added and the solution was kept on ice for 10 min. The cytochrome-*c* oxidase containing proteoliposomes were formed after detergent dialysis overnight against total of 5 litre 50 mM potassium phosphate, pH 6.3 (with two changes).

2.5. Fusion of proteoliposomes with yeast plasma membranes

The proteoliposomes were usually mixed with yeast plasma membranes at a ratio of 20:1 (phospholipid/protein) in a final volume of 300–500 μl and 1 mM $MgSO_4$ was added. The suspension was rapidly frozen and stored in liquid nitrogen. Before use the fused membranes were slowly thawed at room temperature and sonified for 5–7 s on ice in an Eppendorf tube with a probe type sonifier B 12 (Branson Sonic Power Company).

2.6. Internal volume

For the estimation of internal vesicle volume, the method described in [6] was used. Radioactive 200 μM D-[^{14}C]glucose was enclosed into the vesicles during the fusion procedure. The fused vesicles were separated from the medium by filtration on cellulose nitrate filters and washed twice with 2.0 ml of 100 mM LiCl. The internal volume calculated after scintillation counting amounted to 1.0 μl/mg phospholipid.

2.7. Assay of sugar uptake

The uptake experiments were done at room temperature in an open magnetically stirred vessel in a final volume of 0.3–0.8 ml with water saturated O_2 flushing over the surface. The vesicles were diluted with 50 mM potassium phosphate of desired pH, spun down for 15 min at 40000 rpm (rotor Ti 60) and resuspended in the same buffer containing 1 mM $MgSO_4$ to a final concentration of 0.2–0.4 mg protein/ml. The energization was started by addition of 20 mM ascorbate, 0.2 mM

tetramethyl-*p*-phenylenediamine and 20 μM cytochrome *c*. At zero time, the radioactively labeled sugar was added at desired concentration. Aliquots of 40–50 μl were withdrawn at intervals, diluted with 2.0 ml of 100 mM LiCl, filtered through 0.22 μm-pore-size cellulose nitrate filters (Schleicher and Schuell) and washed with 2.0 ml 100 mM LiCl. The radioactivity was determined by scintillation counting.

2.8. Determination of the transmembrane electrical potential

The membrane potential (inside negative) was determined from the distribution of tetra-[^{3}H] phenylphosphonium cation (Ph_4P^+) [18] according to the Nernst-Donnan equation:

$$\Delta E = -2.3\frac{RT}{nF}\log\frac{[Ph_4P^+]_{in}}{[Ph_4P^+]_{out}}$$

where $-2.3RT/nF$ represents approx. 59 mV at 25°C.

Experimental conditions were the same as in the uptake experiments with the exception that the membrane filters used were cellulose acetate filters (Schleicher and Schuell); cellulose nitrate filters were found not suitable because Ph_4P^+ strongly binds to these filters. The retention of D-[^{14}C]glucose containing vesicles did not differ significantly with the types of filters.

2.9. Determination of the pH gradient

The transmembrane proton gradient (interior alkaline) was determined from the distribution of [^{14}C]-acetic acid using the Henderson-Hasselbalch equation: $pH = pK_{Ac} + \log[Ac^-]/[HA_c]$, where $pK_{Ac} = 4.75$, $[Ac^-]$ and [HAc] are the concentrations of dissociated and undissociated acetic acid, respectively, assuming that only the undissociated form penetrates the lipid membrane and dissociates within the vesicles according to the ambient pH. The experimental conditions were the same as above.

3. Results and discussion

3.1. Generation of protonmotive force in yeast plasma membranes vesicles

For the characterization of the HUP1 gene product from *Chlorella kessleri* in vitro, the mutant strain *S. pombe* YGS-5 lacking D-glucose transport activity was transformed by a multi-copy plasmid bearing the HUP1 gene [21]. The plasma membranes prepared from such transformants were fused with cytochrome-*c* oxidase proteoliposomes and the resulting vesicles were energized by the electron donor system ascorbate/TMPD/cytochrome *c*. The membrane potential calculated from the accumulation of Ph_4P^+ inside the vesicles (Fig. 1A) amounted to about 100 mV (inside negative). This value was obtained after correction for nonspecific, i.e., '$\Delta\psi$ independent' binding (the amount of Ph_4P^+ found in the vesicles after deenergization). The calculated value is slightly overestimated since the nonspecific '$\Delta\psi$ dependent' Ph_4P^+ binding [25] is not considered. The other component of the protonmotive force, ΔpH (inside alkaline), calculated from the accumulation of acetic acid (Fig. 1B) was about 0.45 which corresponds to 27 mV. Thus the overall protonmotive force in these vesicles amounted to 120–130 mV.

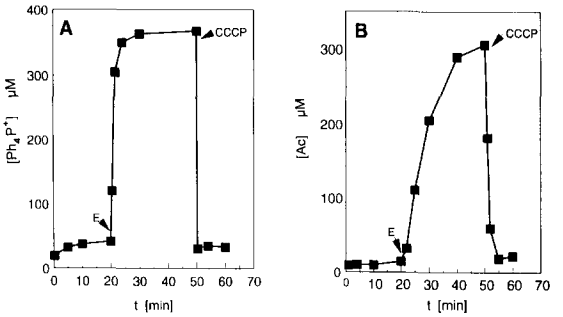

Fig. 1. Tetraphenylphosphonium cation (A) and acetate (B) uptake by vesicles prepared from the membranes of *Schizosaccharomyces pombe* TCY 96 (HUP1 transformed). The vesicles (2.75 mg phospholipid/ml) were incubated in 50 mM potassium phosphate buffer, pH 6.3, 1 mM $MgSO_4$, and energized (at arrow) with ascorbate, TMPD and cytochrome *c* as described in Materials and methods. CCCP was added at final concentration of 25 μM. Concentration of Ph_4P^+ and acetate was 5.66 μM and 111 μM, respectively. $\Delta\psi$ at 30 min after energization amounted to 103 mV and ΔpH to 0.45.

3.2. Uptake of D- and L-glucose into reconstituted plasma membrane vesicles

When D- or L-glucose is added to non-energized vesicles prepared from HUP1 transformed yeast cells a differential uptake of the two stereoisomers is observed (Fig. 2B). D-Glucose is taken up somewhat faster than L-glucose in the first 3 min. This preferential D-glucose uptake indicates that the HUP1 transporter can act as a facilitator in this in vitro system, which contrasts to what is known so far from in vivo analysis of the *Chlorella* hexose transporter [18,22]. Sugar efflux from intact cells under steady state conditions (at high inside concentrations of hexose analogues) proceeds together with protons [26] and could be inhibited by uncouplers [27]. In addition, the typical overshoot experiment possible with bacterial transporters as well as with facilitators of erythrocytes cannot be carried out with *Chlorella* [27].

On energization of the vesicles by the electron donor system (ascorbate, TMPD, cytochrome *c*) a rapid accu-

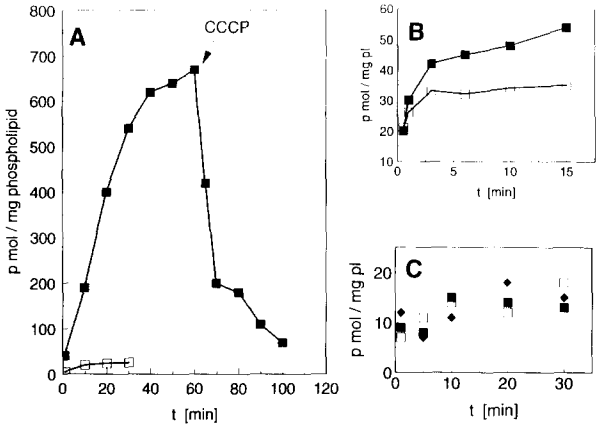

Fig. 2. Uptake of glucose into yeast plasma membrane vesicles. (A) To the vesicles prepared from *S. pombe* TCY 96 membranes 50 μM D-glucose, and ascorbate, TMPD and cytochrome *c* were added at $t = 0$ (■), or ascorbate was omitted (□). Dashed line corresponds to concentration equilibrium. (B) To the same vesicles 63 μM D-glucose (■) or L-glucose (□) were added (non-energized conditions). (C) Uptake of 50 μM glucose into vesicles from plasma membranes of control transformants *S. pombe* TCY 104 (vector without HUP1 insert): (□) D-glucose, non-energized; (■) D-glucose in the presence of ascorbate, TMPD, and cytochrome *c* (energized); (◆) L-glucose in the presence of ascorbate, TMPD and cytochrome *c* (energized). (pl, phospholipid).

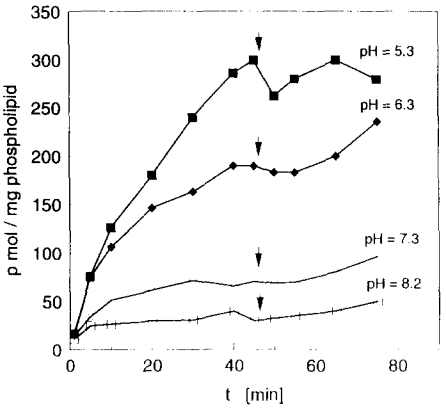

Fig. 3. Accumulation of D-glucose in plasma membrane vesicles from *S. pombe* TCY 96: dependence on external pH. The vesicles were diluted in 50 mM potassium phosphate buffer of the pH indicated, spun down and resuspended in the same buffer to a phospholipid concentration of 7.0 mg/ml. Energization by ascorbate, TMPD and cytochrome *c* started at $t = 0$. The concentration of D-glucose was 20 μM. Arrows indicate the addition of 10 nM nigericin.

mulation of D-glucose is observed, which is not the case when ascorbate is omitted (Fig. 2A). D-Glucose accumulation can be abolished by CCCP. L-Glucose is not accumulated on energization (data not shown).

Plasma membrane vesicles prepared from membranes of the non-transformed mutant strain of *S. pombe* do not show any preferential D-glucose transport whether energized or not (Fig. 2C). The fact that under non-energized conditions the uptake of D- and L-glucose is the same in these vesicles proves that the mutant strain of *S. pombe* YGS-5 lacks the D-glucose facilitator which is present in the original wild type-strain [28].

3.3. Accumulation ratio of D-glucose

Assuming that the internal volume of plasma membrane vesicles is about 1 μl/mg phospholipid (see Material and methods) it can be calculated that at pH 6.3 the accumulation ratio of D-glucose in the best preparations does not exceed the value of 30. The overall protonmotive force in individual vesicle preparations amounts to 130–155 mV, which could drive glucose accumulation more than 100-fold. It must be considered, however, that probably only a part of the vesicles contain an active D-glucose carrier whereas all the vesicles most likely contain cytochrome-*c* oxidase; the degree of D-glucose accumulation, therefore, is a minimal estimate (see discussion of this problem in Refs. [6,29]). In addition the discrepancy in relation to the thermodynamic equilibrium is less pronounced when it is taken into account that the overall protonmotive force is slightly overestimated [25] (see above). Finally, the following results suggest that the equilibrium achieved is mainly a kinetic and not a thermodynamic one.

The accumulation ratio as well as the initial uptake rate depend on the external pH (Fig. 3). The accumulation ratio at external pH 8.2 was ten times lower than that one reached at pH 5.3 while the overall protonmotive force under these conditions did not differ significantly (Table 1). Apparently the pH dependence of the *Chlorella* hexose/H^+ symporter activity which shows an optimum at pH 5.8 [22] leads to a decreased rate of D-glucose uptake with the increasing pH. If passive D-glucose efflux is not affected by external pH, as it holds for neutral amino acids [30], a kinetic equilibrium different from the thermodynamic one will be the consequence. In addition the internal pH of the vesicles, which due to proton pumping is more than half a pH unit higher than the external one, may limit the symporter activity even further. This is supported by the observation that the addition of nigericin, equili-

Table 1
Dependence of pmf and D-glucose accumulation on external pH

External pH	$\Delta\psi$ (mV)	$Z\Delta$pH (mV)	pmf (mV)	D-Glucose accumulation ratio
5.3	−105	34	139	15.5
6.3	−103	37	140	10
7.3	−103	38	141	3.5
8.2	−103	43	146	1.5

Conditions for pmf determination and D-glucose accumulation as in Fig. 1 and Fig. 3, respectively; *Z* is a factor converting pH units into mV and is equal to 59 mV at 25°C.

Table 2
Dependence of D-glucose accumulation on $\Delta\psi$ and ΔpH at external pH of 6.3

Additions	$\Delta\psi$ (mV)	ΔpH	pH_{in}	pmf (mV)	Accumulation ratio
None	−121	0.57	6.87	−155	19.5
Nigericin (10 nM)	−115	0.00	6.30	−115	30
Valinomycin (100 nM)	−30	1.27	7.57	−106	7

Conditions for $\Delta\psi$ and ΔpH determination and D-glucose accumulation were as in Fig. 1 and Fig. 4, respectively.

brating internal and external pH, causes an increase of D-glucose accumulation at external pH values of 6.3 and above (Fig. 3). When valinomycine was added to the vesicles ΔpH was the predominant component of protonmotive force and amounted to 76 mV. The resulting internal pH of 7.6 caused a marked inhibition of D-glucose accumulation while the protonmotive force (> 100 mV) was still quite high. At the valinomycine concentration used, a residual membrane potential of −30 mV was formed (see Table 2), which could be abolished by 10 μM CCCP.

The accumulation ratio of D-glucose in the vesicles, therefore, is not simply defined by the magnitude of the protonmotive force, but also by carrier activity and by substrate leakage.

3.4. The effect of individual components of protonmotive force on accumulation of D-glucose

In the Fig. 4 the uptake of D-glucose into the vesicle is shown at pH 6.3. In this preparation the proton pumping activity of cytochrome-*c* oxidase resulted in an overall pmf of −155 mV (consisting of a $\Delta\psi$ of

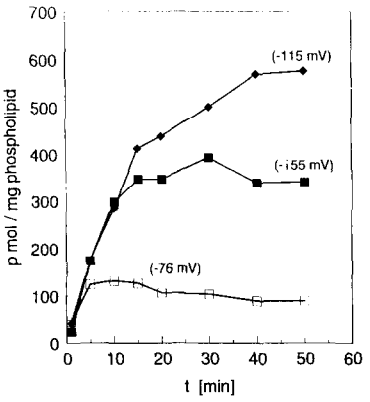

Fig. 4. Effect of ionophores on D-glucose uptake into vesicles. The vesicles were incubated in 50 mM phosphate buffer, pH 6.3, 1 mM $MgSO_4$ and energized at time zero with ascorbate, TMPD, cytochrome *c* (■), in the presence of 10 nM nigericin (♦) or in the presence of 100 nM valinomycin (□). The final concentration of D-glucose was 18.5 μM. The values in brackets indicate the overall protonmotive force in the preparation.

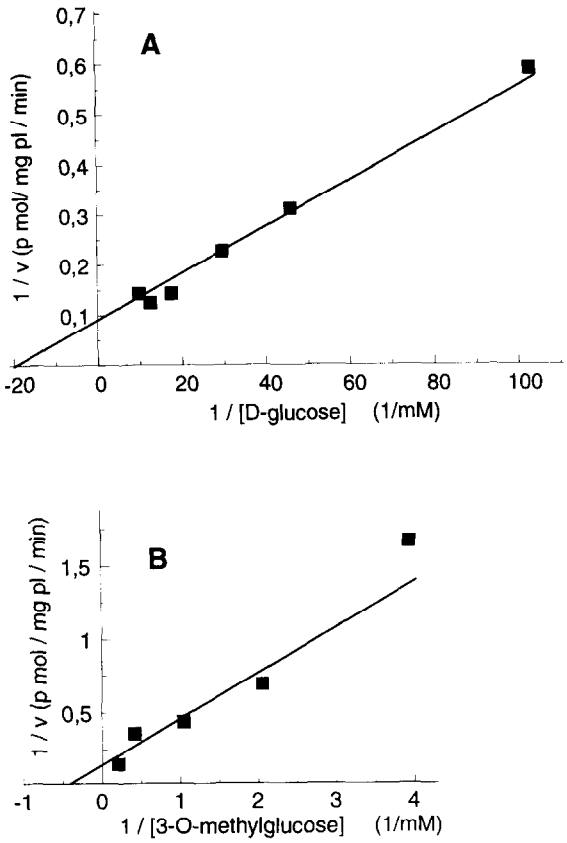

Fig. 5. Lineweaver-Burk plot of D-glucose and 3-*O*-methylglucose transport into vesicles prepared from *S. pombe* TCY 96. The concentration range of D-glucose and 3-*O*-methylglucose was 9.6–105 μM and 0.36–6.3 mM, respectively. (A) D-glucose; (B) 3-*O*-methylglucose; pl, phospholipid.

−121 mV and a ΔpH of 0.57 = −34 mV). D-glucose was accumulated about 20-fold in about 20–30 min; sometimes the amount of internal D-glucose decreased slightly thereafter. The calculated internal pH corresponds to 6.9 (Table 2). When, however, the conditions were set that in the presence of nigericin only $\Delta\psi$ was the driving force, the glucose was accumulated up to 30-fold while the overall pmf was 40 mV lower than in the previous case. The main difference in the two situations is the internal pH, which amounts to pH 6.3 in the latter case and thus is more than half a pH unit less alkaline. It may very well be therefore, that the internal pH of 6.9, suboptimal for carrier activity is responsible for the lower degree of substrate accumulation, although the pmf is 40 mV higher. This is also in accordance with the data of Fig. 3 as discussed above.

3.5. K_m-values for D-glucose of the Chlorella symporter

Fig. 5 shows a Lineweaver-Burk plot for glucose uptake into the vesicles. The K_m value of 53 μM is approx. 3-fold higher than the in vivo value determined

for the *Chlorella* HUP1 gene product [20,22]. A similar *in vivo / in vitro* difference has been previously observed for other substrates and transporters [5,6] and it has been suggested that it may indicate a suboptimal lipid environment for the carrier within the vesicles [5,7]. The K_m value for 3-*O*-methylglucose amounted to 3 mM, which also corresponds to in vivo data [20].

Since the plasma membranes of the mutant *Schizosaccharomyces pombe* YGS-5 cells used here do not show endogenous glucose transport activity and since these cells functionally express the HUP1 gene of *Chlorella* [21], the vesicles described here represent a well defined in vitro system for plant transporters. In the future it will be useful for analysis of plant transporters with specific mutations. The vesicles will also allow to test the effects of different lipid composition on transporter characteristics, a feature that will be advantageous as well.

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