FEBS Letters 420 (1997) 86–92 FEBS 19640

Transport of lucifer yellow CH into plant vacuoles - evidence for direct energization of a sulphonated substance and implications for the design of new molecular probes

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Received 25 September 1997

Abstract Contrasting observations exist which indicate that in plants the fluorescent dye lucifer yellow CH (LYCH) either can be used as a tracer for endocytosis or as a substrate for an anion transporter located at the vacuolar membrane. In addition, LYCH as a disulphonated substance may represent an analogue of sulphonated or sulfated natural compounds like some flavonoids. We performed uptake experiments with LYCH into isolated rye vacuoles and observed saturable (K_m = 0.3–0.6 mM) vacuolar transport and accumulation of the dye against the concentration gradient only when MgATP was present. GTP and, to a low extent, UTP could substitute for ATP, while the non-hydrolysable ATP analogue AMP-PNP did not drive LYCH uptake. Vanadate and probenecid, the latter substance is known to inhibit organic anion transport at the liver canalicular membrane, both strongly decreased the vacuolar uptake of LYCH, while bafilomycin A1, a specific inhibitor of the vacuolar H^+-ATPase, had no effect. Together with the fact that abolishment of the ΔpH via CCCP had only a weak influence on LYCH accumulation, our results indicate that this compound is taken up into rye vacuoles by a directly energized process. Uptake of LYCH was strongly inhibited by other sulfated compounds including sulfobromophthalein and the flavones apigenin 7,4′-disulfate and luteolin 7,4′-disulfate arguing for the presence of a vacuolar transporter for structurally different sulphonated or sulfated compounds. Glucuronates like the rye-specific flavone luteolin 7-O-diglucuronide also strongly decreased uptake of the dye, whereas only a weak effect was observed in the presence of glutathione and a glutathione conjugate, suggesting that LYCH uptake is not mediated via the vacuolar glutathione conjugate pump.

Key words: ABC (ATP-binding cassette) transporter; Fluid-phase endocytosis; Fluorescent dye; Lucifer yellow, organic anion; Sulphonated compound; Vacuole; (*Secale cereale* L.)

1. Introduction

A number of fluorescent probes such as the pH-sensitive 2′,7′-bis(2-carboxyethyl)-5-(and -6)-carboxyfluorescein (BCECF) [1], the Ca^{2+}-indicator dyes fura-2 [2] and Quin-2 [3], or fluorescein isothiocyanate have been reported to accumulate in the vacuole when they are supplied to certain plant cells or protoplasts. Especially, the vacuolar accumulation of the fluorescent dye lucifer yellow CH (LYCH), a disulphonated 4-aminonaphtalimide (MW 457), has been investigated during the last years [4,5]. LYCH has several advantages as a tracer: it is assumed to be nontoxic, the high quantum yield does not vary between pH 1–10, it is membrane impermeable and highly dissociated at physiological pH values (pK < 0.7 of the disulphonate groups) [6,7].

The observation that LYCH when exposed to different plant tissues, cells or protoplasts did not remain confined to the extracellular space but readily entered the vacuole e.g. in differentiating root cells ([8], reviewed in [4]) led to the suggestion that the dye accumulates in the vacuole via vesicle-mediated fluid-phase endocytosis derived from the plasma membrane [4,9]. In addition, the absence of a vacuolar transport system for the dye was postulated due to the apparent impermeability of the vacuolar membrane towards LYCH investigated either via injection into the cytosol using microinjection or patch clamp techniques ([4], and references cited) or via in vitro incubation of vacuoles isolated from suspension cultured carrot cells [10]. On the other hand, it has been reported that the dye is able to cross the vacuolar membrane after microinjection into the cytosol [11,12].

LYCH has also been used to demonstrate fluid-phase endocytosis in cultured animal cells (e.g. [13]) and yeast [14,15]. Steinberg et al. [16–18] have shown that LYCH is sequestered from the cytosol into the lysosomal system after ATP^{4-} permeabilization of the plasma membrane of mammalian cells. The drug probenecid (*p*-[dipropylsulphamoyl]-benzoic acid), a competitor of organic anion transport in animal cells, inhibited sequestration of LYCH into the lysosomes [17,18] indicating that a transport system localized at the lysosomal membrane recognizes LYCH. Subsequently, probenecid inhibition of plant vacuolar LYCH accumulation has been reported for suspension-cultured cells of carrot [19], *Morinda citrifolia* [20], and for onion epidermal cells [21] using fluorescence microscopic techniques. The existence of an organic anion transporter located at the vacuolar membrane of plants was therefore proposed. However, direct evidence for vacuolar and lysosomal uptake of LYCH has not yet been reported.

In animals, sulfated substances have been shown to be excreted across the liver canalicular membrane by a directly energized transporter belonging to the family of ABC transporters and recognizing several anionic substances called therefore MOAT (multiple organic anion transporter)

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Abbreviations: ABC, ATP-binding cassette; AMP-PNP, 5′-adenylyl β,γ-imidodiphosphate; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; $E_2$17G, β-estradiol 17-(β-D-glucuronide); GS conjugate, glutathione conjugate; GSSG, oxidized glutathione; LYCH, lucifer yellow carbohydrazide; MOAT, multiple organic anion transporter; MRP, multidrug resistance-associated protein; PPi, inorganic pyrophosphate; YCF1, yeast cadmium factor 1

PII S0014-5793(97)01492-0

[22,23]. As for MOAT, the human multidrug-associated protein MRP1 is capable of transporting glutathione (GS) conjugates and other negatively charged conjugates [24,25].

On the one hand, a number of sulfated secondary products, especially flavonoids [26] are known to occur in plants and on the other hand, several directly energized transport systems belonging to the ABC transporter family located at the vacuolar membrane of plants have been described for the deposition of biotic and abiotic compounds like GS conjugates [27,28], including the fluorescent bimane-GS conjugate [29], chlorophyll catabolites [30], bile acids [31], estradiol 17-(β-D-glucuronide) ($E_2$17G) [32], and a herbicide glucoside [33]. We investigated therefore whether (i) LYCH is taken up by isolated rye vacuoles, and in that case (ii) to analyze the energization mechanism of its uptake, and (iii) whether LYCH could be used as a model substance for biotic and abiotic sulphonated and sulfated compounds in plants. Knowledge of the uptake mechanism of this model substrate could have furthermore important basic implications for the design of molecular probes destinated to be targeted to the plant vacuole in order to analyze the vacuolar metabolism in planta.

2. Materials and methods

2.1. Chemicals

Lucifer yellow CH (lithium salt) was obtained from Molecular Probes Europe (Leiden, The Netherlands). The flavonoid luteolin 7-O-diglucuronide (R2) was isolated from *Secale cereale* L. cv. Kustro primary leaves according to [34]. Sulfated flavonoids were a kind gift of Dr. R.K. Ibrahim (Concordia University, Montreal, Canada). Concentrations of stock solutions of the flavonoids used were prepared in buffer adjusted photometrically using the following extinction coefficients: $\varepsilon_{340\ nm}$=17 783 and 20 890 $(M \times cm)^{-1}$ for luteolin and apigenin derivatives [35], respectively. All other chemicals were purchased as described [32].

2.2. Plant material and preparation of mesophyll protoplasts and vacuoles

Rye (*Secale cereale* L. cv. Kustro; Lochow-Petkus, Bergen, Germany) was grown under standardized conditions in a phytotron [32]. Mesophyll protoplasts [36] and vacuoles [32] were prepared from primary leaves according to a procedure described previously with a minor modification: The adaxial epidermis of 8 day-old primary leaves was abraded carefully using emery paper (P 400, Carborundum Abrasives, Germany).

2.3. Uptake experiments with rye mesophyll protoplasts

Uptake experiments of lucifer yellow CH (LYCH, 0.3 mM) into protoplasts were performed as described [37] with the following modifications: Instead of silicone oil AR 200, a mixture of AR 20 with AP 100 (1:2, v/v) was used for silicone oil centrifugation. Protoplasts recovered after the uptake experiment were mixed with 0.1% (v/v) Triton X-100 to a final volume of 2.1 ml. Protoplast volume was determined by addition of 3.7 kBq of ^3H_2O [37]. Accordingly, 0.1 ml were removed for liquid scintillation counting (determination of volume) and the remainder was used for fluorescence spectroscopy. For permeabilization, protoplasts were incubated with 5 mM ATP for 15 min on ice prior to uptake experiments. Permeabilization was terminated by the addition of 5 mM $MgSO_4$.

2.4. Uptake experiments with rye vacuoles

Vacuolar uptake experiments with LYCH were designed as described [38]. If not stated otherwise, 30 μl of densely packed vacuoles were added to 70 μl of uptake medium (23% (v/v) Percoll/0.4 M sorbitol/30 mM KCl/20 mM 2-(*N*-morpholino)-ethane sulfonic acid (Mes)-bis[tris(hydroxymethyl)-methylamin]-propane (Btp), pH 7.2/0.12% (w/v) BSA/1 mM DTT) in the presence of 50 μM of LYCH, and further solutes as indicated in figures and tables. The vacuolar volume was determined by the addition of 3.7 kBq of ^3H_2O. After silicone oil centrifugation [25], the aqueous supernatants of two samples (50 μl each) were pooled. Ten μl were removed for liquid scintillation counting (determination of vacuolar volume), while 1910 μl of 0.1% aqueous Triton X-100 were added to the remaining 90 μl. Experiments in the presence of glutathione and its conjugates were performed without DTT which had no effect on the uptake of lucifer yellow.

2.5. Quantitation of lucifer yellow and calculation of uptake rates

Concentrations of LYCH within protoplasts and vacuoles were determined by measuring the fluorescence of the lysates using a Jobin Yvon (Longjumeau, France) spectrofluorometer, model JY 3D, excitation at 430 nm (bandwidth 10 nm), emission at 530 nm (bandwidth 10 nm). Standard curves of LYCH dissolved in 0.1% (v/v) Triton X-100, were found to be linear in the concentration range used. As low as 0.5 pmol LYCH could be detected in a volume of 2 ml. None of the inhibitors or competitors added in uptake experiments had an effect on the LYCH emission spectrum, as measured in control experiments without organelles. Uptake rates were calculated as pmol LYCH after spectrofluorimetry per μl volume of protoplasts or vacuoles determined by liquid scintillation counting. Unless stated otherwise, vacuolar uptake rates were calculated by subtracting the LYCH concentration measured after 2 min of incubation from corresponding 30 min values. For each condition and time point, at least three independent experiments with three (vacuoles) or six (protoplasts) replicates were performed. K_m and V_{max} values were calculated using a computer program (Enzfitter, Elsevier Biosoft, Cambridge, UK).

2.6. Microscopy

For fluorescence microscopy of vacuoles loaded with LYCH, the vacuoles were incubated in uptake medium supplied with 0.33 mM LYCH and further solutes as indicated, but without ^3H_2O. After 1 h, a modified silicone oil centrifugation was performed: Instead of water, 60 μl of 0.4 M glycinebetaine/30 mM KCl/20 mM Mes-Btp, pH 7.2/1 mg ml^{-1} BSA/1 mM DTT was added being the upper phase. After centrifugation, the upper phase containing the vacuoles was removed and observed with a Leitz DM RB microscope (Leitz, Wetzlar, Germany) with fluorescence attachment using a blue filter (L4, Leitz; dichroitic mirror, 510 nm; excitation filter, 450–490 nm; barrier filter, 515–560 nm). Photographs were taken on Kodak (UK) Ektachrome EPL 135 film, 400 ASA. Fig. 1 (black and white) was generated from color slides by computer aided image processing using Adobe Photoshop (Edinburgh, UK).

3. Results and discussion

Mesophyll protoplasts isolated from rye primary leaves were incubated in the presence of 0.3 mM LYCH. No uptake of the dye into the vacuole or cytosol could be detected using either fluorescence microscopy (up to 18 h of incubation) or spectrofluorimetry (up to 4 h of incubation; data not shown).

Table 1
Vacuolar accumulation of lucifer yellow CH (LYCH) in the presence of MgATP after 30 min of incubation and effect of inhibitors and the protonophore CCCP

Treatment	LYCH concentration (μM)		(*n*)
	medium	internal (vacuole) ± S.D.	
(control)	50	105.3 ± 10.9	(4)
+bafilomycin A1 (0.1 μM)	50	93.1 ± 19.1	(3)
+CCCP (5 μM)	50	89.0 ± 5.9	(3)
+probenecid (1 mM)	50	18.4 ± 6.1	(3)

Isolated rye mesophyll vacuoles were incubated for 30 min in the presence of 50 μM LYCH, 3 mM MgATP and further compounds as indicated. Vacuolar concentrations of LYCH were determined as explained in Section 2. Values are means of three to four independent experiments ± S.D. Each single experiment consisted of three replicates per condition. Uptake rates were calculated by subtracting the LYCH concentration determined after 2 min from corresponding 30 min values.

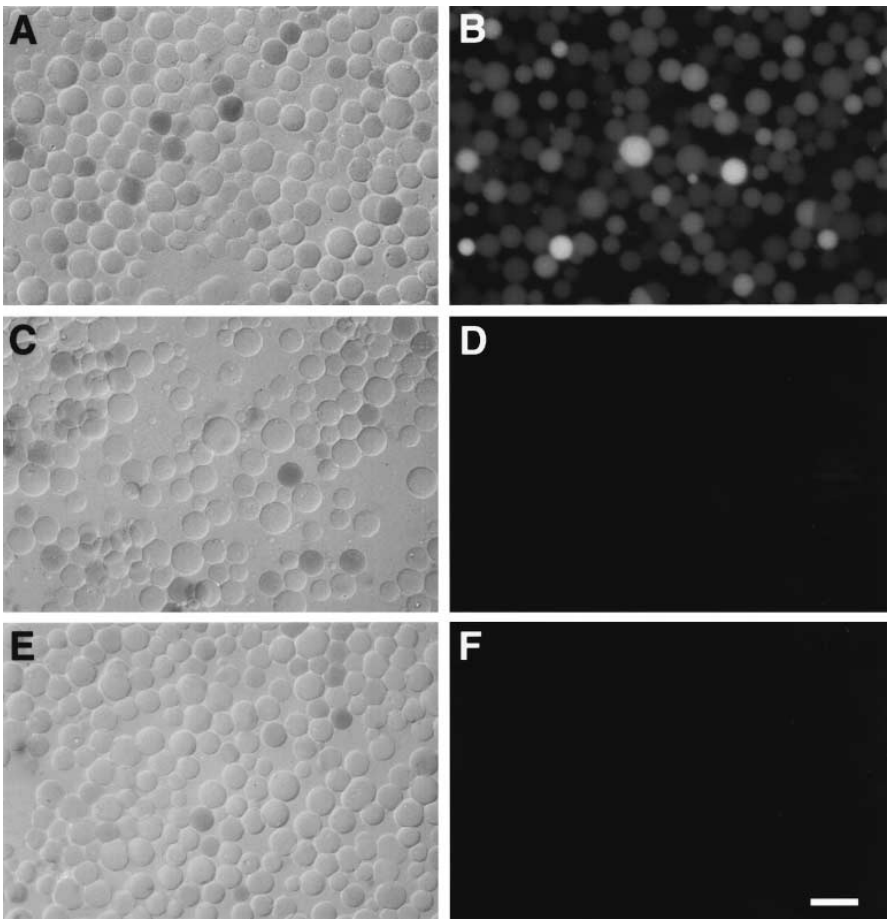

Fig. 1. MgATP-dependent uptake of lucifer yellow CH (LYCH) in rye mesophyll vacuoles is inhibited by probenecid. Vacuoles were incubated for 60 min as described in Section 2 in the presence of 0.33 mM LYCH and with 3 mM MgATP (A, B), without MgATP (C, D), and with 3 mM ATP and 1 mM of probenecid (E, F). After incubation, vacuoles were centrifuged through a silicone oil layer into fresh glycinebetaine medium and were investigated with DIC optics (A, C, E) or fluorescence emission using a blue filter for excitation (pairs A/B, C/D, and E/F show identical vacuolar samples each). Only in the presence of MgATP, the fluorescent dye LYCH accumulates in the vacuoles and leads to a substantial staining (B). In the absence of ATP (D) and with ATP and the organic anion transport inhibitor probenecid (F), vacuoles do not accumulate any fluorescence. Bar = 45 μm.

The addition of $(NH_4)_2SO_4$ or CCCP, both dissipating pH gradients across membranes, did not have stimulating effects on LYCH uptake into protoplasts. In addition, permeabilization treatment of protoplasts with ATP^{4-} for 15 min on ice did not lead to an internal LYCH fluorescence. The lack of LYCH uptake into rye mesophyll protoplasts is in contrast to results published by Wright and Oparka [39] reporting dye uptake into mesophyll protoplasts of various dicotyledonous species, especially from the genus *Nicotiana*. In addition, vacuolar accumulation of LYCH was detected in different plant species after addition of the dye to protoplasts or cell suspensions or after apoplastic loading [4]. Although LYCH sequestration into the vacuole has been reported for intact roots [8] and aleurone protoplasts of barley [4], no vacuolar accumulation of the dye could be detected using barley mesophyll protoplasts (E. Martinoia, unpublished). Thus, demonstration of vacuolar LYCH accumulation in protoplasts or intact cells may depend on the plant species, tissue and experimental conditions chosen.

In contrast, isolated rye vacuoles took up the fluorescent LYCH in the presence of 3 mM MgATP as shown by fluorescence microscopy (Fig. 1A, B) and spectrofluorometry (Fig. 2). In the absence of ATP, no vacuolar fluorescence could be visualized after 1 h of incubation with 0.33 mM LYCH (Fig. 1C, D). Vacuolar uptake of the dye in the presence of MgATP

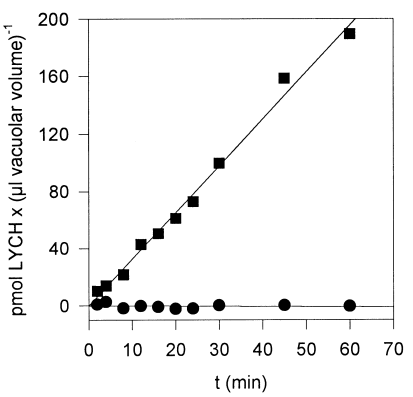

Fig. 2. Time-dependent uptake of LYCH into rye vacuoles. Vacuoles were incubated with 50 μM LYCH in presence (■) or absence (●) of 3 mM MgATP. Mean values of representative experiments consisting of three replicates per condition. Vacuolar contents of the LYCH were corrected for the values extrapolated at time zero reflecting the contamination of the vacuoles with LYCH after their separation from the surrounding medium.

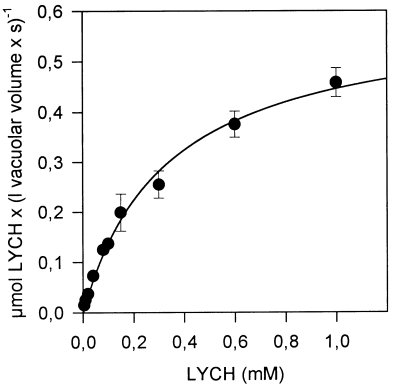

Fig. 3. Concentration dependence of LYCH transport into isolated rye vacuoles. Rye vacuoles were incubated for 24 min in the presence of 3 mM MgATP and the concentrations of LYCH as indicated. Uptake rates were calculated by subtracting the vacuolar LYCH concentration after 2 min of incubation from values measured after 24 min. A representative (mean of three replicates) of three independent experiments is shown.

was linear with time for at least 45 min (Fig. 2). In the presence of MgATP and 50 µM of LYCH, the vacuolar transport rate was 63.1 ± 15.4 nmol LYCH × (l vacuolar volume × s)$^{-1}$. This value is comparable to the uptake rate determined for the abiotic $E_2$17G at 50 µM into rye vacuoles (36.4 nmol $E_2$17G × (l vacuolar volume × s)$^{-1}$) but much lower than rates observed with GS conjugates [27,32]. A two-fold accumulation of the dye against the concentration gradient was observed already after 30 min uptake in the presence of MgATP (Table 1) suggesting that LYCH was very efficiently deposited into the vacuole. In the presence of MgATP, uptake of LYCH into rye vacuoles was a saturable process (Fig. 3). K_M values measured in three independent experiments ranged between 0.33 and 0.64 mM, and V_{max} values were between 0.59 and 0.87 µmol × (l vacuolar volume × s)$^{-1}$. For other abiotic conjugates transported into the plant vacuole by ATP-driven pumps K_M values of 40–60 µM and 210 µM were reported for the GS conjugate of the herbicide metolachlor (barley vacuoles) [27] and for $E_2$17G (rye vacuoles) [32], respectively.

The following results indicate that the fluorescent dye LYCH was taken up into rye vacuoles via a directly energized transport system possibly involving an ABC transporter:

(i) In the absence of MgATP, only 2.5% of the vacuolar uptake rate observed in the presence of MgATP (100%) was obtained (Table 2). Conventional fluorescence microscopy clearly confirmed this result: rye vacuoles incubated with LYCH in the absence of ATP did not possess any LYCH fluorescence (Fig. 1D) when compared to vacuoles incubated with MgATP and the dye (Fig. 1B). ADP or the non-hydrolyzable ATP analogue AMP-PNP could not drive LYCH uptake (Table 2) suggesting that ATP hydrolysis was a prerequisite for transport of the dye. Thus, the lack of dye accumulation in vacuoles isolated from *Daucus carota* cell suspensions reported by Hillmer et al. [10] may be due to the lack of MgATP in these experiments.

(ii) MgGTP could partially substitute for MgATP (45% of the rate observed with MgATP, Table 2) while MgUTP had a much weaker stimulating effect (11%). The addition of inorganic pyrophosphate which is the substrate of the proton pumping pyrophosphatase [40] instead of ATP drove only about 10% of the rate observed with ATP.

(iii) The LYCH uptake was decreased to 13.5% of the control rate in the presence of vanadate. Vanadate acts as a phosphate analogue and is known to inhibit directly energized ABC transporters. In contrast, azide, inhibiting F-type ATPases, has almost no effect on LYCH transport (Table 2). Inhibition of the vacuolar H^+-ATPase by bafilomycin A1 [41] and addition of CCCP did not consistently reduce LYCH accumulation (Tables 1 and 2). However, $(NH_4)_2SO_4$, which like CCCP dissipates the ΔpH, reduced the LYCH uptake rate to about 65% of the control (Table 2). This effect may partially be due to inorganic SO_4^{2-} anions inhibiting the transport of the disulphonated dye.

(iv) As already reported for mammalian cells [17], plant cells and protoplasts [9], 1 mM probenecid inhibited the

Table 2
Effect of different nucleoside-triphosphates, pyrophosphate, and inhibitors on the uptake of LYCH into mesophyll vacuoles of rye

Treatment		LYCH transport activity
		% of control ± S.D.
−ATP		2.5 ± 0.1
+3 mM MgATP	(control)	100.0
+3 mM MgGTP		44.5 ± 2.9
+3 mM MgUTP		10.7 ± 0.5
+0.2 mM PPi		10.6 ± 5.3
+3 mM MgADP		1.6 ± 1.1
+3 mM AMP-PNP		4.6 ± 0.1
+3 mM MgATP	+bafilomycin A1 (0.1 µM)	88.4 ± 18.1
+3 mM MgATP	+azide (1 mM)	90.8 ± 2.0
+3 mM MgATP	+vanadate (1 mM)	13.5 ± 7.8
+3 mM MgATP	+CCCP (5 µM)	84.5 ± 5.6
+3 mM MgATP	+$(NH_4)_2SO_4$ (5 mM)	64.6 ± 11.1
+3 mM MgATP	+valinomycin (5 µM)	64.5 ± 3.2
+3 mM MgATP	+verapamil (1 mM)	84.0 ± 13.6
+3 mM MgATP	+vinblastine (1 mM)	56.1 ± 5.2
+3 mM MgATP	+probenecid (0.1 mM)	47.8 ± 8.5
+3 mM MgATP	+probenecid (1 mM)	20.9 ± 8.7

Vacuoles were incubated in the presence of 50 µM LYCH, the nucleoside-triphosphates and further compounds as listed in the table. Mg^{2+} was supplied as $MgSO_4$, all nucleotides were added as the respective sodium salts from a 0.2 M stock solution in 0.2 M Btp (final pH 7.5). Pyrophosphate (PPi) was added as KPPi. 100% corresponds to an ATP-stimulated uptake rate of 63.1 ± 15.4 nmol LYCH × (l vacuolar volume × s)$^{-1}$. Values are means of at least three independent experiments ± S.D. Each experiment consisted of three replicates per condition. The vacuolar uptake rate was calculated by subtracting the vacuolar LYCH concentration determined after 2 min from corresponding 24 min values.

ATP-dependent uptake of LYCH into rye mesophyll vacuoles, as shown by spectrofluorimetric analysis (Tables 1 and 2) and fluorescence microscopy (Fig. 1F). Probenecid is largely accepted as an inhibitor of organic anion transport in animal cells, and it was shown to inhibit the transport of carboxyfluorescein diacetate and bimane-GS from the renal proximal tubule cell to the lumen [42]. In view of the finding that the central vacuole of onion epidermal cells vesiculates in the presence of probenecid, it is important to note that isolated rye mesophyll vacuoles appeared stable and intact for at least 1 h when incubated with this inhibitor (Fig. 1E). In contrast, the Ca^{2+}-channel blocker verapamil, a substrate for the mammalian multidrug resistance transporter MDR/P-glycoprotein, only weakly inhibited vacuolar LYCH uptake (Table 2). An insensitivity against verapamil was also shown for the uptake of $E_2$17G and metolachlor-GS into rye vacuoles [32]. Vinblastine sulfate, another substrate of human MDR, reduced LYCH uptake to about 56% of the control value (Table 2). However, the inhibition may be due to the inorganic sulfate anion instead of the drug cation. In addition, different effects of vinblastine on the transport of organic anions into membrane vesicles isolated from the liver canalicular membrane were reported: while transport of the cysteinyl leukotriene LTC_4 and $E_2$17G was inhibited by vinblastine [43,44], (2,4-dinitrophenyl)GS transport is not affected by the drug [45].

Taken together, LYCH uptake met the criteria of a directly energized organic anion transporter being responsible for LYCH accumulation in plant vacuoles. Strict ATP dependence, the partial substitution of ATP by GTP, insensitivity towards inhibitors of the vacuolar H^+-ATPase but strong inhibition by vanadate have already been described for the vacuolar transport of GS conjugates [27,28], glucuronates [32], a herbicide glucoside [33], and chlorophyll catabolites [30]. Furthermore, these data are in accordance with the characteristics described for organic anion transport via the animal MOAT/MRP2 and MRP1 [23]. Both isoforms have been shown to catalyze the efflux of negatively charged conjugates of catabolites or abiotic substances. However, due to the facts that, for rye vacuoles, (i) bafilomycin A1 reduced the LYCH uptake rate to 90% of the control rate, (ii) CCCP had a comparable effect, (iii) inorganic pyrophosphate slightly stimulated, and (iv) LYCH transport was inhibited to about 64% by valinomycin, a K^+-ionophore dissipating the trans-tonoplast membrane potential (inside positive) [46], it cannot be excluded that secondary energized transporters driven by the ΔpH or by $\Delta\Psi$ may be associated with rye vacuolar membranes and contribute to the ATP-driven uptake of the dye. However, it may be possible that the inhibition of LYCH uptake by valinomycin is due to direct effects on the ABC-like transporter described here, either through a modulation of its activity by the membrane potential or by direct inhibition of the transporter, as shown for the competitive inhibition of rhodamine 123 transport by valinomycin in yeast ($K_I \approx 15$ μM) [47].

The multiple organic anion transporter MOAT/MRP2 present on the canalicular membrane of animal liver cells, had been shown to be responsible for the efflux of different substances conjugated to negatively charged groups like glutathione, glucuronate, and sulfate as demonstrated by transport experiments with canalicular membrane vesicles and by studies using mutant mouse strains which are defective in the biliary secretion of nonbile organic anions [23]. Transport of $E_2$17G catalyzed by MRP1 was strongly inhibited by the cholestatic secondary bile salt glycolithocholate-3-sulfate, but not by non-derivatized bile acids for which a separate transporter exists [48]. Using isolated plant vacuoles, competition experiments argue for distinct directly energized vacuolar transporters responsible for the accumulation of GS conjugates [27,28], bile acids [31], abiotic glucosides and glucuronates [32,33], and chlorophyll catabolites [30]. However, some data suggested that there may be interactions between different substrates

Table 3
Effect of various conjugates, glutathione and bile acids on the ATP-stimulated uptake of LYCH into rye vacuoles

Treatment	(mM)	LYCH transport activity
		% of control ± S.D.
control		100.0
α-naphthyl β-glucuronide	0.5	90.2 ± 20.6
luteolin 7-O-diglucuronide	0.5	19.8 ± 1.7
β-estradiol 17-(β-glucuronide)	0.5	0.9 ± 16.3
β-estradiol 3-sulfate-17-(β-glucuronide)	0.5	0* ± 18.9
β-estradiol 3-sulfate	0.5	9.7 ± 9.9
sulfobromophtalein	0.5	0* ± 13.8
α-naphthyl-sulfate	0.5	22.1 ± 0.5
β-naphthyl-sulfate	0.5	23.6 ± 3.5
apigenin 7,4′-disulfate	0.5	0* ± 13.2
luteolin 7,4′-disulfate	0.5	0* ± 10.5
taurolithocholic acid 3-sulfate	0.5	0* ± 12.9
taurocholic acid	0.5	16.8 ± 11.9
glycocholic acid	0.5	42.9 ± 23.5
decyl-glutathione	0.5	69.4 ± 20.8
oxidized glutathione	3	46.3 ± 9.8
reduced glutathione	3	97.4 ± 17.2
apigenin 6-C-glucoside	0.5	42.0 ± 2.6

Vacuoles isolated from primary leaves of rye were incubated in the presence of 50 μM LYCH and further compounds as indicated for each condition. 100% corresponds to ATP-stimulated uptake rates as given in the legend of Table 2. All experiments were performed in the presence of 3 mM ATP and 4 mM $MgSO_4$. Values are means of three independent experiments ± S.D. Each single experiment consisted of three replicates per condition. Uptake rates of both substrates were calculated by subtracting the vacuolar LYCH concentration determined after 2 min from corresponding 24 min values.
0% values marked with an asteriks (*) correspond to 'negative' uptake rates between 0 and −20%, that is the LYCH concentration after 2 min was higher than after 24 min of uptake under these conditions.

or the corresponding transporters: vacuolar transport of $E_2$17G was reported to be strongly stimulated in a concentration-dependent manner by GS conjugates and GSSG [32], and chlorophyll catabolites were also accepted as substrates for a plant MRP homologue AtMRP expressed in yeast (Tommasini et al., submitted). We performed competition experiments with a set of sulfate conjugates, glucuronates, GS conjugates and bile acids in order to investigate the specificities of the LYCH uptake system (Table 3). LYCH uptake was strongly inhibited by sulfated conjugates (Table 3). Besides estradiol 3-sulfate, the model organic anion sulfobromophtalein, the secondary bile salt taurolithocholic acid 3-sulfate, naphthyl-sulfates (irrespective of the position of the sulfate group), and the flavone disulfates apigenin 7,4′-disulfate and luteolin 7,4′-disulfate were inhibitory. These results suggest that other biotic or abiotic *sulfated* substances may be equally accepted as substrates for the directly energized transporter described here apart from the *disulphonated* dye LYCH. The abiotic naphthyl-glucuronide did not reduce LYCH transport into rye vacuoles (Table 3), while dye uptake was strongly inhibited by $E_2$17G and the rye-specific vacuolar flavone glucuronide luteolin 7-O-diglucuronide [49]. Besides the glucuronate derivative of the steroid also β-estradiol 3-sulfate-17-(β-glucuronide) strongly reduced LYCH uptake. Comparable results were obtained when $E_2$17G transport into rye and barley mesophyll vacuoles was studied, and estradiol glucuronide transport into rye vacuoles was shown to be competitively inhibited by the luteolin diglucuronide [32], suggesting that LYCH and $E_2$17G are taken up by one or comparable transport systems. In contrast to $E_2$17G transport, uptake of LYCH into rye vacuoles was not stimulated but rather inhibited by decyl-GS and GSSG (about 70 and 46% of the control rate, respectively; Table 3) while reduced glutathione did not affect LYCH uptake. In addition, the fact that the bile acids glycocholate and taurocholate also acted inhibitory on dye transport (43 and 17% of the control rate) is in conflict with results obtained when vacuolar uptake of GS conjugates and $E_2$17G was studied [31,32]. However, although distinct directly energized transporters for bile acids and organic anions were identified also in mammals [23], competitive inhibition of organic anion transport by bile acids was reported in some cases [45], and taurocholate inhibition of LYCH uptake could have been partly due to the presence of a sulfate group.

The different effects of glucuronates, GS conjugates, and bile acids on LYCH transport and also the inhibition in the presence of apigenin 6-C-glucoside (42% of control; Table 3), a flavone shown to be transported by a barley-specific flavonoid glucoside antiporter [33], suggest that a more detailed functional analysis is necessary in order to obtain information on the existence of one or several transporters for different organic anions. The latter point may be addressed in a more promising way via the identification of plant MRP-like genes and their expression in heterologous systems (Tommasini et al., submitted).

A number of abiotic fluorescent probes like rhodamine 123 [50–52] and the fluorescent properties of some cytotoxic drugs such as daunorubicin and doxorubicin [51] have been successfully established in animal systems in order to study MDR function as a drug efflux pump and have the potential to be used as quick screening methods in clinical research. Besides for MDR, fluorescent dyes may also be applied to study the function of other ABC transporters: (i) the influx of the fluorescent dihydrorhodamine 6G was shown to be increased in cells expressing the human cystic fibrosis transmembrane conductance regulator [53], (ii) calcein and calcein derivatives were used to investigate MDR and MRP function in human tumor cells [54], and (iii) the GS conjugate of monochlorbimane formed intracellularly was applied to study deposition of GS conjugates in plant cells [29] and YCF1 function in yeast [55]. Here we demonstrated that LYCH may be a candidate to study directly energized transport of sulphonated and possibly also sulfated organic anions across the vacuolar membrane in plant cells and the animal lysosomal membrane. Apart from LYCH, other established fluorescent dyes like fluorescein isothiocyanate, carboxyfluorescein [20,21], the Ca^{2+}-indicator Quin-2 [3] may be substrates of organic anion transporters. Furthermore, substrates containing an electrophilic function and therefore readily being used for conjugation reactions to GS, pH- or ion-sensitive probes or other vacuolar reporter dyes carrying sulfate or sulphonate groups may be additional classes of interesting substances efficiently targeted to the vacuole in intact plant cells via a directly energized transporter.

4. Conclusion

Using isolated rye vacuoles, a directly energized vacuolar transporter for the fluorescent dye lucifer yellow CH was characterized for the first time. Results obtained argue for a MOAT-like vacuolar carrier for sulphonated and sulfated compounds belonging to the family of ABC transporters. In view of these findings it must be concluded that LYCH cannot unequivocally be used as a marker for plant fluid-phase endocytosis.

Acknowledgements: Financial support by the Deutsche Forschungsgemeinschaft Bonn (Az. We 630/12-2) and of the Freunde und Förderer der Universität zu Köln is gratefully acknowledged. We thank Dr. R.K. Ibrahim, Concordia University, Montreal, Canada, for the kind gift of sulfated flavones, P. Burchard, G. Sachs, and R. Schmitz for experimental help, Prof. H. Bothe and Prof. K. Schmitz, both University of Cologne, for the possibility to use the spectrofluorometer and the fluorescence microscope, and K. Fischer for help with Fig. 1.

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