Directly Energized Uptake of $\beta$-Estradiol 17-($\beta$-D-Glucuronide) in Plant Vacuoles Is Strongly Stimulated by Glutathione Conjugates*

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A directly energized vacuolar pump for glutathione (GS) conjugates has been described for several plant species. Since glucuronate conjugates also occur in plants, we addressed the question whether plant vacuoles take up the abiotic glucuronate conjugate estradiol 17-($\beta$-glucuronide) (E217G) via a GS conjugate pump, which in some cases has been reported to accept various organic anions as substrates, or via a distinct glucuronate transporter. Uptake studies into vacuoles from rye and barley were performed with E217G and metolachlor-GS (MOC-GS), a substrate of the GS conjugate ATPase, to compare glucuronate conjugate transport into vacuoles containing endogenous flavone glucuronides with those lacking specific glucuronate conjugates, respectively. Our results indicate that E217G and MOC-GS are taken up into vacuoles of both plants via a directly energized mechanism since transport was (i) strictly ATP-dependent; (ii) inhibited by vanadate but not by bafilomycin A1, azide, verapamil, nor by dissipation of the vacuolar ΔpH or ΔΨ; (iii) E217G uptake into rye vacuoles was partially driven by other nucleotides in the following order of efficiency: ATP > GTP > UTP ≡ CTP, whereas the non-hydrolyzable ATP analogue 5′-adenylyl-$\beta$-$\gamma$-imidodiphosphate, ADP, or PPi did not energize uptake. E217G transport into rye vacuoles was saturable ($K_m$ ~0.2 mM). The rye-specific luteolin glucuronides decreased uptake rates of E217G and MOC-GS into rye and barley vacuoles to comparable degrees with the mono- and diglucuronidated derivatives (40–60% inhibition) being more effective than the triglucuronide. Inhibition of E217G uptake by luteolin 7-O-diglucuronide was competitive ($K_i$ = 120 μM). Taurocholate had no effect on E217G transport, and uptake of MOC-GS was not inhibited by E217G. Although GS conjugates and oxidized GS decreased MOC-GS transport, E217G uptake into rye and barley vacuoles was stimulated up to 7-fold in a concentration-dependent manner by these substances, with dinitrobenzene-GS being most effective. The stimulation of the GS conjugates was not due to detergent or redox effects and was specific for the E217G pump. GS conjugate stimulation of glucuronate uptake was unique for plants as E217G uptake into yeast micromosomal vesicles was not affected. By comparison with a ΔYCF1 yeast mutant, defective in vacuolar transport of GS conjugates mediated by YCF1, it was shown that E217G was taken up into yeast vesicles via a YCF1-independent directly energized pump. These results indicate that E217G as a glucuronate conjugate is transported across the vacuolar membranes of plants and yeast by a carrier distinct from the GS conjugate ATPase.

In plants, many secondary compounds such as phenolic acids, coumarins, tannins, and flavonoids are detoxified and stored in the large central vacuole, a compartment with low metabolic activity (1). Enzymatic conjugation of these substances with one or more hydrophilic ligands, primarily sugars, catalyzed by respective transferases is an integral part of the detoxification process and leads to higher water solubility. However, in several cases conjugation with glucuronate is also observed. In addition, conjugation of an anthocyanin with glutathione is apparently necessary for its vacuolar deposition in maize (2). Another type of reaction is the esterification of phenolic compounds with polar substituents (3).

Glucuronate conjugates are mainly produced in animals, where they play an important role in the degradation of heme resulting in bilirubin diglucuronide which is secreted into bile. Although a series of phenolic glucuronates have been identified in different plant taxa (4), glucuronotransferases and the subsequent vacuolar deposition are poorly understood. In rye, two glucuronidated flavones, luteolin 7-O-diglucuronyl-4′-O-glucuronide (R1) and luteolin 7-O-diglucuronide (R2), are specifically localized in the mesophyll tissue of primary leaves, whereas the epidermal layers contain glycosylated flavonoids (5). The metabolism of these flavone glucuronates starting from luteolin is sequentially catalyzed by three anabolic cytosolic UDP-glucuronate:flavone-glucuronosyltransferases (6) followed by vacuolar storage via an unknown mechanism and may also involve subsequent degradation of the luteolin triglucuronide initiated by a specific β-glucuronidase located in the cell wall (7). Accordingly, vacuolar steady-state concentrations of the flavone glucuronides show a maximum in the young rye leaf followed by a rapid decline during later development (8). In animals, glucuronate transport across the liver canalicular membrane has been investigated in detail and was shown to be mediated by a directly energized transporter belonging to the
ABC family recognizing several anionic substances called MOAT (multiple organic anion transporter; see Ref. 9).

In plants, final deposition of glutathione conjugates within the plant vacuole is mediated by a directly energized transport process also involving an ABC transporter (10, 11). It has recently been shown that a human (MRP1; Ref. 12) and a yeast (YCFC; Ref. 13) transporter belonging to the ABC family are able to catalyze glutathione (GS) conjugate transport (Refs. 14 and 15 and references therein). Complementation of the cadmium hypersensitive ycf1 deletion mutant with MRP1 restores cadmium tolerance as well as glutathione conjugate transport activity (15). Cadmium tolerance is due to the transport of bis(glutathionato)cadmium complexes in yeast (16). The human and rat liver canalicular MOAT represents a liver-specific isofrom of the human multidrug resistance associated protein MRP1 (therefore denoted MRP2), and the transport function of the heterologously expressed MRP1 (therefore denoted MRP2), and the transport function of the heterologously expressed MRP1 (therefore denoted MRP2), and the transport function of

As outlined above, rye mesophyll cells accumulate specific flavonoid glucuronides. Therefore we started 14C and 3H labeling experiments with these substances to study the mechanism of their vacuolar deposition. However, so far different procedures have failed to specifically label the rye flavone glucuronides. To find evidence for a possible general mechanism of glucuronate transport into plant vacuoles, we investigated the transport of the steroid conjugate E217G, presumably not occurring in plants, and compared the uptake characteristics into rye vacuoles (containing endogenous glucuronates) and barley vacuoles (obviously lacking these compounds). Furthermore, we were interested in comparing the transport of metolachlor conjugated to glutathione (MOC-GS) with E217G, to elucidate whether the vacuolar uptake system for GS conjugates corresponds to a transporter with broad substrate specificity for anionic conjugates as shown for the canicular MOAT/MRP2 (9) or the human multidrug resistance associated protein MRP1 (20).

**EXPERIMENTAL PROCEDURES**

**Chemicals and Isolation of Flavonoid Glucuronides from Rye—** Estradiol 17- (β-d-glucuronide) [estradiol-6,7-3H] (1.8 × 10^10 Bq/mole) and [14C]glucose (23.6 × 10^10 Bq/mole) were obtained from NEN Life Science Products (Bad Homburg, Germany) and Amersham-Buchler (Braun- schweg, Germany), respectively. (phenyl- U-13C)Metolol-glutathione (1.7 × 10^12 Bq/mole, gift of Dr. K. Kreuz, Novartis, Basel, Switzerland) was synthesized as described (23). Percoll and Ficoll 400 were synthesized as described (23). Percoll and Ficoll 400 were supplied by Pharmacia (Freiburg, Germany). Luteolin was from Roth (Karlsruhe, Germany). The flavonoid glucuronides luteolin 7-O-digluconide (R2) and luteolin 7-O-diglucuronyl-4-O-glucuronide (R1; see Ref. 5) were isolated from Secale cereale L. cv. Kustro primary leaves grown in the field for 10 days as described (24) with the following modification: the TLC step was omitted, and separation and final purification were done with a Sephadex LH-20 column (Pharmacia, Freiburg, Germany) using water as eluant. Luteolin-7-O-glucuronide was isolated from *Antirrhinum majus* L. petals (25). Purity of flavonoid glucuronides was higher than 95% as checked by HPLC. Dinitrobenzene-glucuronides (DNB-GS) was prepared chemically by stirring 5 mM 1-chloro-2,4-dinitrobenzene and 10 mM reduced glutathione in water adjusted to pH 8.5 for 24 h at room temperature. Excess of 1-chloro-2,4-dinitrobenzene was removed by 3-fold shaking the mixture out with tert-butyl methyl ether. The aqueous phase was evaporated to dryness, and the concentration of the redissolved DNB-GS was determined photometrically (ε_{250 nm} = 10 × 10^3 cm^-1 mol^-1). All other chemicals and solvents were of highest purity available and were mainly supplied by Sigma (Deisenhofen, Germany), Fluka (Buchs, Switzerland), and Lancaster (Freiburg, Germany). 

**Plant Material and Growth Conditions—**Barley (*Hordeum vulgare* L. cv. Bakara) and rye (*Secale cereale* L. cv. Kustro) were grown on vermiculite for 8 days in a growth cabinet with 13 h of fluorescent light (approximately 100 μmol × m^-2 × s^-1) at 20 °C and 70% relative humidity and were watered daily with Hoagland solution (22).

**Yeasts—** The isogenic Saccharomyces cerevisiae strains DTY7 (Mata, ura3-52, leu2-3,121, his6) and DTY168 (*MataycfΔ::hisG, ura3-52, leu2-3,121, his6) were used for transport experiments into isolated microsomes.

**Preparation of Mesophyll Vacuoles from Primary Leaves of Barley and *Rye—** Protoplasts of both plants and barley vacuoles were isolated as described earlier (26). Contamination of barley vacuoles with other cell constituents was less than 3% as measured by marker-enzyme activities (26). As mechanical shearing forces using different needles and lysis conditions did not result in an acceptable yield of rye vacuoles, a different strategy was established. After digestion, rye protoplasts were washed twice with 0.5 M sorbitol and 1 mM CaCl2, 10 mM MES-KOH, pH 7.2, on a layer of Percoll containing 0.5 M sorbitol and 1 mM EGTA and a layer of MES, pH 6, underneath and sedimented on this layer for 5 min at 200 × g (Rotixa K, Hettich, Tuttlingen, Germany). Either this crude preparation or protoplasts purified on a step gradient as described (26) were used to lyse rye protoplasts by a combination of pH and osmotic shock. One part of concentrated protoplasts was mixed gently with four parts of lysis medium (10% (w/v) Ficoll 400, 20 mM MES-BTP, pH 5.5, 5 mM EGTA, 1 mg/ ml BSA, 5 mM DTT and sorbitol, total osmolarity of 0.22 × osmol/kg^-1) at 30 °C. After 15–20 min of gentle shaking a gradient was prepared consisting of 20 ml of the lysis mixture (bottom), an equal volume of medium A (0.4 M sorbitol, 5.5% (w/v) Percoll, 30 mM KCl, 1 mM DTT, 1 mg/ml BSA, 20 mM MES-BTP, pH 7.2) and 5 ml of medium B (0.4 M glycine betaine, 30 mM KCl, 1 mM DTT, 1 mg/ml BSA, 20 mM MES-BTP, pH 7.2; top) in glass centrifugation tubes. Vacuoles were separated from unlysed protoplasts by centrifugation (5 min at 1000 × g, Rotixa K) and were collected at the interphase of medium A and B. To concentrate the vacuoles, Percoll was added to a final concentration of 20% (w/v), and the gradient and centrifugation steps were repeated using 10-ml glass centrifugation tubes. All steps except for the lysis step were performed on ice and surveyed microscopically. Rye vacuoles were concentrated with less than 1% of nonvacuolar marker enzymes and retained almost 100% of soluble vacuolar hydrolases (data not shown). 

**Preparation of Yeast Microsomes—** Yeast microsomal fractions were isolated from DTY7 and DTY168 strains grown overnight in YPD medium (1% (w/v) Bacto-yeast extract (Difco), 2% (w/v) Bacto-peptone (Difco), 2% (w/v) glucose) at 30 °C to an A°_550 of approximately 10 by disruption of enzymatically prepared spheroplasts in a glass/Teflon pot and pelleting membranes at 100,000 × g for 45 min as described in detail (15). Microsomal pellets were resuspended at an A°_550 of 4 in 1.1 ml glycerol, 50 mM Tris-MES, pH 7.4, 1 mM EDTA, 1 mM DTT, 2 mg/ml BSA, 1 mM phenylmethlysulfonyl fluoride, 1 μg/ml leupeptin. For transport experiments, yeast membranes were either used immediately after isolation or stored in liquid nitrogen.

**Uptake Experiments with Plant Vacuoles—** Uptake experiments with [3H]estradiol 17- (β-d-glucuronide) (E217G) or [14C]metolachlor-glutathione (MOC-GS) were performed as described earlier (26). Unless indicated otherwise, for each time point and condition polyethylene microcentrifugation tubes (0.4-ml capacity) were prepared as follows: 70 μl of medium C (23% (w/v) Percoll, 0.4 M sorbitol, 30 mM KCl, 20 mM MES-BTP, pH 7.2, 0.12% (w/v) BSA, 1 mM DTT, and 1.5 kBq of [3H]E217G, 0.2 kBq of [14C]MOC-GS, or 1.5 kBq of [14C]glucose and further solutes as indicated in figures and tables) were placed on the bottom of the tube. Uptake was started by adding 30 μl of concentrated vacuole suspension. The samples were rapidly overlayered with 200 μl of silicone oil AR 200 and 60 μl of water. The incubation was terminated by flotation of the vacuoles (10,000 × g for 15 s, Beckman Microfuge 11, München, Germany). 50 μl of the aqueous phase was used to determine the radioactivity. Vacuoles were solubilized by the addition of 3.7 kBq of [3H]H2O which equilibrates rapidly between the medium and the vacuolar lumen. [3H]H2O was added directly to medium C in experiments with [14C]MOC-GS and in separate assays of identical composition for the experiments with [3H]E217G. Experiments in the presence of GSH and its conjugates were performed without DTT which had no effect on the uptake of either substrate. Unless stated otherwise,
uptake rates of [3H]E217G or [14C]MOC-GS into barley and rye vacuoles were calculated by subtracting the radioactivity measured after 2 min of incubation from corresponding 18-min values. \( K_{m} \), \( V_{max} \), and \( K \) data were calculated using a computer program (Enzfitter, Elsevier Biosoft, Cambridge, UK).

Identification of [3H]E217G after Uptake into Rye Vacuoles—After incubation of rye vacuoles in medium C supplied with 7.4 kBq [3H]E217G and further solutes as indicated in Fig. 1, 2 × 50 μl of the aqueous supernatants following silicone oil centrifugation containing about 6 μl of vacuolar volume were pooled and subjected to HPLC (22) under the following conditions: Nucleosil RF-8 column (125 × 4.6 mm; 5-μm grain size; CS Chromatographie Langerwehe, Germany); flow rate 1 ml × min⁻¹; solvent A, \( \text{H}_{2}\text{O} \) containing 1% (v/v) \( \text{H}_{3}\text{PO}_{4} \); solvent B, MeOH; linear gradient from 50% to 70% B in 23 min, sample volume 50 μl. 0.5-ml fractions were collected, and the radioactivity was determined by liquid scintillation counting. The radioactive peak of the vacuolar content was compared with that of the authentic substrate [3H]E217G and with unlabeled E217G and \( \beta \)-estradiol, both detected at 260 nm.

Uptake Experiments with Yeast Microsomal Vesicles—Uptake of [3H]E217G was measured by the rapid filtration technique using nitrocellulose filters of 0.45-μm pore size (Schleicher & Schuell, Dassel, Germany) as described (15). In short, 1 part of microsomes was mixed with 6 parts of 0.4 M glycerol, 0.1 M KCl, 20 mM Tris-MES, pH 7.4, 1 mM DTT, 10 μM [3H]E217G, and additional compounds as indicated in figure legends. Incubation was at 25 °C. At times indicated, samples of 100 μl were removed from the incubation medium and filtered through nitrocellulose filters premoistened with medium D (0.4 M glycerol, 0.1 M KCl, 20 mM Tris-MES, pH 7.4). Filters were washed with 5 ml of ice-cold medium D. Radioactivity was determined by liquid scintillation counting after addition of 5 ml ReadySafe (Beckman) and shaking until filters were dissolved. Unless stated otherwise, uptake rates were calculated by subtracting the radioactivity measured after 0.5 min of incubation from corresponding 10-min values. Conditions were repeated with at least three independent microsome preparations, each performed in triplicate.

RESULTS

It has been shown previously that plants have the ability to deposit xenobiotics conjugated to glutathione in their vacuoles by a directly energized, ATP-driven glutathione (GS) conjugate pump (10, 11, 27). In animals, a directly energized transport system named MOAT or MRP2 has been described which is responsible for the removal of conjugates of lipophilic substances with glutathione but also with glucuronate or sulfate across the canalicular membrane of liver cells into bile (for review, see Ref. 28). The present work was performed to address the following questions: (i) do plant vacuolar membranes possess an active transport system that is able to transfer other negatively charged conjugates like \( \beta \)-estradiol 17-(\( \beta \)-d-glucuronide) (E217G) apart from GS conjugates into the vacuole, and (ii) are these conjugate substrates of the transporter described for GS conjugates, thus corresponding to a “plant MOAT-like” pump?

Intact rye vacuoles incubated in the presence of 36 nm [3H]E217G contained a single radioactive compound after HPLC separation that coeluted with authentic labeled or unlabeled E217G at identical retention times (Fig. 1). Up to 60 min of incubation, no further radioactive peak was detected in vacuolar supernatants, excluding a possible modification of the transported compound, especially deglucuronidation to \( \beta \)-estradiol (Fig. 1). Rye as well as barley mesophyll vacuoles took up the abiotic glucuronate conjugate, [3H]E217G, in an ATP-dependent manner (Figs. 1C and 2A and Table I). ATP-stimulated uptake of the glucuronate into barley and rye vacuoles is linear for at least 22 min (Fig. 2A). As shown earlier for barley (10, 29), the vacuolar uptake of \([^{14}\text{C}]\text{MOC-GS} \) into rye vacuoles was ATP-dependent and linear for at least 20 min (Fig. 2B). In the presence of ATP and E217G at a concentration of 8.2 nm, vacuolar transport rates for E217G were comparable for both plant species (8.70 ± 1.69 and 8.48 ± 1.35 pmol E217G × (liter vacuolar volume × s⁻¹) for rye and barley vacuoles, respectively) suggesting the existence of an E217G carrier located in plant vacuolar membranes irrespective of the capacity to synthesize and store endogenous glucuronides. After 18 min of incubation in the presence of 8.2 nm E217G and ATP, vascular concentrations of the glucuronate ranged between 6.61 and 11.75 nm in rye and and between 6.24 and 10.16 nm in vacuoles isolated from rye and barley leaves, respectively. Thus, these results do not clearly demonstrate accumulation against the concentration gradient. Extending the incubation time up to 45 min resulted in only a weak further increase in vascular concentration (data not shown).

In contrast, MOC-GS clearly accumulated in rye and barley vacuoles against the concentration gradient in the presence of

![Fig. 1. \( \beta \)-Estradiol 17-(\( \beta \)-d-glucuronide) ([3H]E217G) is present in rye vacuoles after uptake without any modification. A, unlabeled E217G and \( \beta \)-estradiol could be separated by HPLC as detailed under “Experimental Procedures.” Commercially available E217G contained an additional unknown contaminant marked with an asterisk. Authentic [3H]E217G (B, •) coeluted with the corresponding unlabeled E217G (A) and with the radioactive compound detected in aqueous supernatants of rye vacuoles incubated with [3H]E217G and obtained after silicone oil centrifugation (C, peak elution after 8 min). A, E217G and estradiol were detected photometrically at 260 nm. B and C, 0.5-ml fractions were collected after HPLC, and the radioactivity was determined by liquid scintillation counting. C, vacuoles were incubated in the presence of 36 nm [3H]E217G for 60 min with (•) or without (○) 3 mM MgATP or with 3 mM MgATP and 0.2 mM DNB-GS (■) and with 3 mM MgATP for 2 min (●). For a better resolution, the inset in C shows a magnification of radioactive peaks between 6 and 9 min.](image)
ATP (Fig. 2B and Table I). After 18 min vacuoles of rye contained between 97 and 182 μM (4.8–9.1-fold accumulation) of the MOC-GS (20 μM), and in barley vacuoles concentrations ranged between 89 and 127 μM (4.4–6.3-fold accumulation). The corresponding uptake rates of 131.5 ± 36.5 and 108.6 ± 19.1 nmol of MOC-GS × (liter vacuolar volume × s)⁻¹ for rye and barley vacuoles, respectively, suggest again that both plant species possess comparable transport activities for the deposition of xenobiotic compounds conjugated to glutathione. Uptake rates are in accordance with data published for barley (e.g. Ref. 29).

In the presence of ATP, uptake of E₁₇G into rye vacuoles is a saturable process with an apparent Kₘ of 208 ± 58 μM and a Vₘₐₙₐₜ of 188 ± 53 nmol of E₁₇G × (s × liter⁻¹) (means of three independent experiments; Fig. 3). For barley, vacuolar uptake of MOC- and N-ethylmaleimide-glutathione was shown to be saturable with apparent Kₘ values of 40–60 and 500 μM, respectively, in a previous publication (10).

Inhibitors of different transport ATPases and reagents affecting the vacuolar proton gradient or membrane potential showed comparable effects on the uptake of E₁₇G into vacuoles of both plants (Table I). Transport of the E₁₇G into vacuoles of barley and rye was not affected by bafilomycin A₁ (30). The proton pumping activity of the rye vacuolar ATPase into intact vacuoles was completely abolished in the presence of 20 mM inhibitor (data not shown). In contrast, vanadate, a calcium-channel blocker known to (i) reverse multidrug resistance (31), (ii) to be a potent inhibitor of P-glycoprotein-mediated drug transport (32, 33), and (iii) to stimulate ATP hydrolysis activity of P-glycoprotein (34) did not affect vacuolar uptake of the E₁₇G. Neither dissipation of the pH gradient across the vacuolar membrane by the addition of NH₄Cl nor dissipation of the membrane potential (inside positive) by valinomycin in the presence of K⁺ ions had an effect on E₁₇G uptake into vacuoles of either plant species (Table I). In the absence of ATP, the addition of NH₄Cl did not further reduce E₁₇G uptake compared with uptake rates without ATP. Thus, a ΔpH-dependent, secondary energized glucuronate uptake activity distinct from the vanadate-sensitive system could not be detected, as it has been suggested for abiotic glucosides (22).

**TABLE I**

| Treatment | Scale | Hordeum |
|-----------|-------|---------|
| | Transport of E₁₇G | Transport of MOC-GS | Transport of E₁₇G | Transport of MOC-GS |
| No ATP | 4.7 ± 2.9 | 1.4 ± 0.6 | 7.9 ± 4.9 | 3⁺ |
| + MgATP (control) | 100 | 100 | 110.2 ± 12.5 | 101⁺ |
| + MgATP + bafilomycin A₁ (0.1 μM) | 100.1 ± 20.8 | 105.3 ± 2.5 | 19.0 ± 10.6 | 11⁺ |
| + MgATP + vanadate (1 mM) | 28.7 ± 5.8 | 9.2 ± 0.7 | 98.2 ± 1.6 | 102⁺ |
| + MgATP + NaN₃ (1 mM) | 92.1 ± 6.7 | 106.2 ± 0.8 | 85.1 ± 6.9 | 96⁺ |
| + MgATP + NH₄Cl (5 mM) | 98.1 ± 3.2 | 92.3 ± 8.6 | 89.7 ± 10.0 | ND |
| + MgATP + verapamil (0.1 mM) | 93.7 ± 5.2 | 100.1 ± 1.2 | 91.2 ± 18.1 | ND |
| + MgATP + verapamil (0.1 mM) | 96.2 ± 22.5 | 104.5 ± 1.8 | 3.7 ± 5.6 | ND |

* Data published previously (29).
Essentially, uptake of MOC-GS into barley and rye vacuoles showed an identical sensitivity toward the different effectors compared with \( E_217G \) as the substrate (Table I). Transport of the GS conjugate was insensitive toward bafilomycin A1, azide, verapamil, and dissipation of \( \Delta \psi \) whereas vanadate affected. Estradiol conjugated with glucuronate and sulfate residues (estradiol 3-sulfate-17-(-D-glucuronide)) as anionic substrates were much less inhibitory than the flavone glucuronides (Table III). The unconjugated steroid estradiol inhibited transport of \( E_217G \) about 60%, whereas uptake of metolachlor-GS was not affected. Estradiol conjugated with glucuronate and sulfate residues (estradiol 3-sulfate-17-(-D-glucuronide)) as anionic substrates strongly reduced glucuronate uptake into rye vacuoles. Interestingly, \( E_217G \) added to MOC-GS uptake assays did not affect transport of the GS conjugate into rye vacuoles (Table III).

The bile acid taurocholate which is a substrate of a directly energized vacuolar bile acid transporter recently characterized in barley (29) had no effect on vacuolar uptake of \( E_217G \) into rye vacuoles (Table III).

As shown earlier for barley, various substances conjugated to glutathione inhibited vacuolar uptake of glutathione S-conjugates competitively (10, 35). Here we show that decyl-GS and dinitrobenzene-GS (DNB-GS) present in a 10-fold excess reduced transport of MOC-GS into rye vacuoles by about 85 and 63%, respectively (Table IV). Oxidized glutathione (GSSG) inhibited transport of MOC-GS about 45%. Reduced glutathione (GSH) had only a very low effect on MOC-GS uptake into rye vacuoles.

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**Table II**

Effect of different nucleotides and pyrophosphate on the uptake of \( \beta \)-estradiol 17-(-\( \beta \)-glucuronide) (\( ^{3} \)H\( E_217G \)) into isolated vacuoles of rye.

| Treatment | Transport of \( ^{3} \)H\( E_217G \) | \% MgATP-stimulated uptake rate |
|-----------|----------------------------------------|-------------------------------|
| 3 mm ATP (control), 4 mm \( Mg^{2+} \) | 100 | |
| 3 mm CTP, 4 mm \( Mg^{2+} \) | 46.7 ± 6.2 | |
| 3 mm UTP, 4 mm \( Mg^{2+} \) | 48.4 ± 2.3 | |
| 3 mm GTP, 4 mm \( Mg^{2+} \) | 78.9 ± 0.5 | |
| 3 mm AMP-PNP, 4 mm \( Mg^{2+} \) | 4.1 ± 0.7 | |
| 3 mm ADP, 4 mm \( Mg^{2+} \) | 5.4 ± 1.0 | |
| 0.2 mm PPi, 4 mm \( Mg^{2+} \) | 6.5 ± 4.3 | |

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**Fig. 3.** Concentration dependence of \( \beta \)-estradiol 17-(-\( \beta \)-glucuronide) (\( ^{3} \)H\( E_217G \)) transport into isolated rye vacuoles. A, rye vacuoles were incubated for 18 min in the presence of 3 mm \( Mg^{2+} \) and the concentrations of \( E_217G \) as indicated. Uptake rates were calculated by subtracting the radioactivity determined after 2 min from corresponding 18-min values.

**Table III**

Effect of different nucleotides and pyrophosphate on the uptake of \( \beta \)-estradiol 17-(-\( \beta \)-glucuronide) (\( ^{3} \)H\( E_217G \)) into isolated vacuoles of rye.

| Treatment | Transport of \( ^{3} \)H\( E_217G \) | \% MgATP-stimulated uptake rate |
|-----------|----------------------------------------|-------------------------------|
| 3 mm ATP (control), 4 mm \( Mg^{2+} \) | 100 | |
| 3 mm CTP, 4 mm \( Mg^{2+} \) | 46.7 ± 6.2 | |
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| 3 mm GTP, 4 mm \( Mg^{2+} \) | 78.9 ± 0.5 | |
| 3 mm AMP-PNP, 4 mm \( Mg^{2+} \) | 4.1 ± 0.7 | |
| 3 mm ADP, 4 mm \( Mg^{2+} \) | 5.4 ± 1.0 | |
| 0.2 mm PPi, 4 mm \( Mg^{2+} \) | 6.5 ± 4.3 | |

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**Table IV**

Effect of different nucleotides and pyrophosphate on the uptake of \( \beta \)-estradiol 17-(-\( \beta \)-glucuronide) (\( ^{3} \)H\( E_217G \)) into isolated vacuoles of rye.

| Treatment | Transport of \( ^{3} \)H\( E_217G \) | \% MgATP-stimulated uptake rate |
|-----------|----------------------------------------|-------------------------------|
| 3 mm ATP (control), 4 mm \( Mg^{2+} \) | 100 | |
| 3 mm CTP, 4 mm \( Mg^{2+} \) | 46.7 ± 6.2 | |
| 3 mm UTP, 4 mm \( Mg^{2+} \) | 48.4 ± 2.3 | |
| 3 mm GTP, 4 mm \( Mg^{2+} \) | 78.9 ± 0.5 | |
| 3 mm AMP-PNP, 4 mm \( Mg^{2+} \) | 4.1 ± 0.7 | |
| 3 mm ADP, 4 mm \( Mg^{2+} \) | 5.4 ± 1.0 | |
| 0.2 mm PPi, 4 mm \( Mg^{2+} \) | 6.5 ± 4.3 | |

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 GS-X Stimulates Plant Vacuolar Glucuronate Conjugate Uptake

Table III

Effect of different flavone glucuronides (1), abiotic glucuronides, estradiol, and estradiol conjugates, taurocholate, and luteolin on the ATP-dependent uptake of β-estradiol 17-β-D-glucuronide ([H]E217G) and metabolochlor-glutathione ([14C]MOC-GS) into vacuoles of rye and barley

| Treatment                      | Transport of [H]E217G | Transport of [14C]MOC-GS | % MgATP-stimulated uptake rate | % MgATP-stimulated uptake rate |
|--------------------------------|-----------------------|--------------------------|-------------------------------|-------------------------------|
| Control                        | 100                   | 100                      | 100                           | 100                           |
| R1 (0.2 mM)                    | 70.1 ± 4.2            | 65.6 ± 4.6               | 69.1 ± 8.3                    | 69.3 ± 13.1                   |
| R2 (0.2 mM)                    | 34.0 ± 2.1            | 46.9 ± 4.6               | 33.0 ± 5.9                    | 44.3 ± 1.0                    |
| N (0.2 mM)                     | 43.0 ± 15.1           | 66.4 ± 2.5               | 32.2 ± 1.8                    | 42.9 ± 3.8                    |
| d NP-glur (0.2 mM)             | 93.6 ± 4.2            | 106.0 ± 2.5              | 89.2 ± 5.6                    | 98.1 ± 6.4                    |
| d MUglur (0.2 mM)              | 77.2 ± 7.3            | 1025.3 ± 3.4             | 88.3 ± 17.2                   | 85.6 ± 10.8                   |
| E217G (0.2 mM)                 | ND                    | 98.5 ± 1.6               | ND                            | ND                            |
| Estradiol (0.2 mM)             | 39.4 ± 2.3            | 98.1 ± 4.9               | ND                            | ND                            |
| E2-SO417G (0.2 mM)             | 25.9 ± 2.5            | ND                       | ND                            | ND                            |
| Taurocholate (0.2 mM)          | 88.9 ± 6.7            | ND                       | ND                            | ND                            |
| Luteolin (0.2 mM)              | 45.7 ± 5.0            | ND                       | ND                            | ND                            |

Table IV

Effect of different glutathione conjugates, glutathione, caprinacid acid, and NADP on the ATP-dependent uptake of β-estradiol 17-β-D-glucuronide ([H]E217G), metabolochlor-glutathione ([14C]MOC-GS), and [14C]sucrose into vacuoles of rye and barley

| Treatment                      | Transport of [H]E217G | Transport of [14C]MOC-GS | % MgATP-stimulated uptake rate | % MgATP-stimulated uptake rate |
|--------------------------------|-----------------------|--------------------------|-------------------------------|-------------------------------|
| Control                        | 100                   | 100                      | 100                           | 100                           |
| d ATP Control                  | 4.7 ± 2.9             | 1.0 ± 0.7                | 105.0 ± 6.8                   | 7.9 ± 4.9                     |
| d ATP + decyl-GS (0.2 mM)      | 53.7 ± 73.2           | 14.4 ± 4.9               | 102.3 ± 5.2                   | 6483.3 ± 149.2               |
| d ATP + decyl-BS + Baf. A1 + NH4Cl | 510.3 ± 98.0     | ND                       | 102.3 ± 5.2                   | ND                            |
| d ATP + DBN-BS (0.2 mM)        | 674.9 ± 263           | 37.2 ± 17.1              | 105.4 ± 10.5                  | 6893.3 ± 113.5               |
| d ATP + GSSG (3 mM)            | 248.3 ± 187           | 56.6 ± 2.9               | 103.4 ± 9.2                   | 540.8 ± 134.9                |
| d ATP + GSH (3 mM)             | 98.5 ± 9.3            | 78.5 ± 4.8               | 102.2 ± 4.9                   | 997.7 ± 10.3                 |
| d ATP + caprinacid acid (0.2 mM) | 105.8 ± 26.5           | ND                       | 112.6 ± 23.8                  | ND                            |
| d ATP + NADP (3 mM)            | 50.6 ± 4.4            | ND                       | 58.6 ± 7.3                    | ND                            |

Surprisingly, GS conjugates did not inhibit but strongly stimulated vacuolar uptake of E217G into rye and barley vacuoles (Table IV). The glucuronate conjugate transport rates increased 5-7-fold in the presence of decyl-BS or DBN-BS and 2.5-5.4-fold in the presence of GSSG, whereas GSH did not affect the transport rate. The HPLC analysis of vacuolar contents after uptake of [H]E217G for 60 min in the presence of DBN-BS and ATP confirmed that the stimulation by DBN-BS was excluded due to a rise of the [H]E217G peak (Fig. 1C). Thus, the addition of DBN-BS led to an increase in the transport rate of [H]E217G, and no modification of the transported glucuronate occurred. It can be excluded that the stimulation of glucuronate conjugate transport by oxidized glutathione was due to its redox potential, as NADP added at the same concentration did not enhance but rather reduced E217G uptake in barley and rye vacuoles (Table IV). The unconjugated fatty acid caprinacid acid had no effect on glucuronate uptake. The 5-fold increase of the E217G uptake rate into rye vacuoles induced by decyl-BS was even retained in the presence of bafilomycin A1 and NH4Cl. The stimulation of E217G transport was dependent on the presence of ATP; decyl-BS (0.2 mM) added in the absence of MgATP resulted in E217G uptake rates of about 2% the values in the presence of MgATP which means that decyl-BS did not increase the uptake observed in the absence of ATP (Table I). Finally, the stimulatory effect of GS conjugates was not unspecific; vacuolar uptake of [14C]sucrose into vacuoles of barley and rye showed the typical behavior of an ATP-independent sucrose permease described for green mesophyll tissues (36) without any influence of GS conjugates, oxidized or reduced glutathione, caprinacid acid, or NADP (Table IV).

The stimulation of E217G uptake into rye vacuoles was dependent on the concentration of GS conjugates or GSSG (Fig. 5). Half-maximal increase in E217G uptake rate required 9 ± 2 and 138 ± 19 μM decyl-BS and GSSG, respectively.

It has recently been shown that the gene product encoded by the yeast gene contributes to cadmium tolerance in yeast (13) and that YCF1 is a GS conjugate transporter located in the vacuolar membrane (14-16). To compare vacuolar uptake characteristics of glucuronate conjugates in plants with yeast, transport of E217G into microsomal vesicles isolated from a...
the presence of 3 mM MgATP at various concentrations (50, 100, 200, or 400 μM) of [3H]E217G and in the absence or presence of 0.1, 0.2, or 0.4 mM luteolin 7-O-diglucuronide (R2). A representative experiment is shown. Each data point represents the mean of six replicates. The K, calculated from all experiments was 121 μM. Uptake rates were calculated by subtracting the radioactivity determined after 2 min of incubation from values measured after 18 min.

wild type strain (DTY7) and a ycf1 deletion mutant (DTY168) was studied. As in plant vacuoles, transport of the glucuronate conjugate was dependent on the presence of ATP and strongly inhibited by vanadate (Table V and Fig. 6). Up to 10 min, ATP-dependent uptake into vesicles isolated from DTY7 and DTY168 was linear with time (Fig. 6). Microsomal uptake of E217G into DTY168 vesicles was not significantly reduced when compared with vesicles isolated from the wild type strain DTY7 (Table V), indicating that YCF1 may not be the major transport protein for glucuronate conjugates or it does not accept these conjugates as substrates. In contrast to plant vacuoles, uptake of E217G (10 μM) into DTY7 microsomes was not stimulated in the presence of decyl-GS or DNB-GS, both at 20 μM (Table V) or 200 μM decyl-GS (data not shown).

DISCUSSION

Apart from different glutathione (GS) conjugates tested so far (10, 11), the substrate specificity of the plant vacuolar GS conjugate pump has not been investigated in detail. Two possibilities exist: (i) the GS conjugate pump accepts a variety of different organic anions different from GS conjugates and functions in analogy to the animal liver-specific MOAT/MRP2, or (ii) glutathione and glucuronate conjugates are transported by distinct uptake mechanisms. Here we compared the vacuolar transport of the steroid conjugate β-estradiol 17-β-D-glucuronide (E217G) with that of metolachlor-glutathione (MOC-GS) to be able to distinguish between both possibilities.

Principally, uptake of MOC-GS into rye vacuoles showed the typical basic characteristics of a directly energized transport system, as already shown for barley mesophyll vacuoles (10), vacuolar membrane vesicles from Vigna radiata hypocotyl, and other plant sources (11). Vacuolar uptake was dependent on the presence of MgATP, was insensitive toward inhibitors of the vacuolar proton pumping ATPase, and was strongly inhibited by vanadate (Table I). Dissipation of either the proton gradient (inside acidic) or the membrane potential (inside positive) across the vacuolar membrane had no effect on MOC-GS uptake, ruling out the possibility of a secondary energized mechanism, e.g. via H+ antiport (Table I). The transport rate for MOC-GS entry into rye vacuoles is comparable to that of barley vacuoles (Ref. 29 and this paper). Furthermore, vacuolar uptake, ruling out the possibility of a secondary energized

FIG. 4. Dixon plot of the competitive inhibition of β-estradiol 17-(β-D-glucuronide) ([3H]E217G) uptake into rye vacuoles by luteolin 7-O-diglucuronide. Isolated rye vacuoles were incubated in the presence of 3 mM MgATP at various concentrations (50 (●), 100 (▲), 200 (▲), or 400 (▼) μM) of [3H]E217G and in the absence or presence of 0.1, 0.2, or 0.4 mM luteolin 7-O-diglucuronide (R2). A representative experiment is shown. Each data point represents the mean of six replicates. The K, calculated from all experiments was 121 μM. Uptake rates were calculated by subtracting the radioactivity determined after 2 min of incubation from values measured after 18 min.

FIG. 5. Decyl-glutathione and oxidized glutathione stimulate vacuolar uptake of β-estradiol 17-(β-D-glucuronide) ([3H]E217G) into rye vacuoles. Isolated rye vacuoles were incubated with 3 mM MgATP, 8.2 nM E217G in the presence or absence of increasing concentrations of decyl glutathione (decolyl-GS, A), or oxidized glutathione (GSSG, B). A representative experiment is shown with six replicates per data point. Concentrations achieving half-maximal stimulation of E217G transport were 9 and 138 μM for decyl-GS and GSSG, respectively (two independent experiments). Uptake rates were calculated by subtracting the radioactivity determined after 2 min of incubation from values measured after 18 min.

TABLE V

| Treatment | DTY7 | DTY168 |
|-----------|------|--------|
| -ATP      | 1.8 ± 4.1 | 4.8 ± 8.3 |
| +ATP control | 100 | 71.7 ± 20.7 |
| +ATP + decyl-GS (20 μM) | 97.7 ± 33.1 | ND |
| +ATP + DNB-GS (20 μM) | 117.7 ± 28.9 | ND |
| +ATP + vanadate (1 mM) | 0 ± 19.0 | ND |

Table of values represents three to six independent experiments ± S.D. each performed in triplicate. Uptake rates were calculated by subtracting the radioactivity determined after 0.5 min from corresponding 10-min values. ND, not determined.
accumulation against the concentration gradient could be clearly demonstrated for rye and barley vacuoles, suggesting that xenobiotic glutathione conjugates are deposited in the plant vacuole by a ubiquitous uptake system directly using MgATP as an energy source which is thermodynamically more effective than a secondary active mechanism (27). In contrast to results obtained with V. radiata hypocotyl vesicles (11), verapamil, an agent known to revert multidrug resistance caused by P-glycoprotein, had no inhibitory effect on MOC-GS uptake into rye vacuoles added at 0.1 mM (Table I). It has been shown that vacuolar uptake of GS conjugates and oxidized glutathione (GSSG) is inhibited competitively by other GS conjugates (11, 35, this report, and Table IV) but not by corresponding molecules lacking the glutathione residue (11), suggesting that this residue serves as a tag for vacuum deposition of potentially toxic substances.

Plant vacuolar membranes possess an uptake system for E217G representing a glucuronate conjugate. The transport characteristics of E217G into rye and barley vacuoles were similar to those described for glutathione conjugates (10, 11), chlorophyll catabolites (37), bile acids (29), and an abiotic glucoside (22). It is therefore likely that E217G is recognized by an ABC transporter. (i) Vacuolar uptake of E217G was strongly MgATP-dependent, and in the absence of ATP the glucuronide transport amounted to only about 5% (rye) or 8% (barley) of the transport rates obtained in the presence of MgATP (Table I and Fig. 2). (ii) Transport into barley and rye vacuoles was sensitive toward vanadate, whereas bafilomycin A1 and azide did not affect E217G uptake. Dissipation of ΔpH or ΔΨ did not decrease the glucuronate transport rates (Table I). In addition, inorganic pyrophosphate, which is the substrate of the proton pumping pyrophosphatase present in plant vacuolar membranes (38), was not able to stimulate E217G uptake. Taken together these data suggest that E217G transport does not depend on a secondary active energization mechanism utilizing ΔpH or ΔΨ as driving forces but is energized by a vanadate-sensitive ATPase. (iii) As in other cases where directly energized transport has been observed in plants (10, 22, 29, 37), other nucleoside triphosphates but not ADP and AMP-PNP could partially substitute for ATP (Table II). The latter fact indicates that ATP hydrolysis is necessary for the transport activity.

Compared with vacuolar transport of GS conjugates, bile acids, or chlorophyll catabolites we could not clearly obtain an accumulation of E217G against a concentration gradient within 45 min. However, in the presence of DNB-GS, a 7.7-fold vacuolar accumulation could be observed within 18 min. There are two lines of evidence that vacuolar transport depended on the presence of the glucuronate residue in E217G. (i) The uptake rate decreased about 70% after treatment of E217G with a commercial β-glucuronidase (data not shown). (ii) More significantly, after incubation of rye vacuoles with [3H]E217G the analysis of the vacuolar contents confirmed that the substrate was taken up as such without any structural changes (Fig. 1). Accordingly, uptake of free β-estradiol eventually liberated from E217G by an unspecific β-glucuronidase could be excluded. A comparison of the transport rate of the GS conjugate (see above) to that of E217G showed that vacuoles of both species exhibited about 2-fold higher potential to transport the GS compared with the glucuronate conjugate, both at 20 μM. Compared with rat canalicular membrane vesicles (39), the apparent Km value for E217G uptake into rye vacuoles indicates that the vacuolar transporter may have a lower affinity for the glucuronate than the animal MOAT (200 μM versus 75 μM).

Based on the inhibition studies performed (Table III), we propose that E217G is not transported by the GS conjugate pump but by a separate, novel glucuronate conjugate transporter. Although MOC-GS and E217G uptake was inhibited by the rye-specific flavone glucuronides to comparable degrees and was largely unaffected by the monoglucuronated derivatives of methylumbelliferone and p-nitrophenol, E217G did not inhibit MOC-GS uptake into rye vacuoles when the competitor E217G was present in a 10-fold excess. Vacular uptake of E217G also was not decreased in the presence of GS conjugates but strongly stimulated (Table IV). Methylumbelliferyl- and p-nitrophenol β-d-glucuronide, which did not inhibit E217G uptake into barley and rye vacuoles, were in contrast taken up by isolated canalicular plasma membrane vesicles in an ATP-dependent manner (9). Therefore, the plant vacuolar ATPase for E217G obviously does not transport glucuronates in general as proposed for the animal MOAT. However, our results do not allow us to draw clear-cut conclusions on the chemical structure of the residual part of the molecule bound to glucuronate to serve as a substrate for the E217G transporter. Furthermore, our results indicate that the principal vacuolar GS conjugate transporter does not correspond to a MOAT/MRP2-like pump accepting different anionic substrates. In addition, the transport rate of E217G into yeast microsomal vesicles isolated from a ycf1 deletion mutant is comparable to vesicles isolated from the corresponding wild type (Table V and Fig. 6). Thus, glucuronate and GS conjugates may be transported by distinct membrane proteins in yeast with YCF1 being responsible for yeast GS transport (14–16). Strict ATP dependence and the strong inhibition by vanadate suggest that the yeast E217G transporter may be a further directly energized pump. For plant vacuoles, it can also be excluded that E217G is transported via the bile acid carrier described recently (29), as taurocholate did not inhibit the uptake of E217G into rye vacuoles significantly, although taurocholate is a steroidal derivative. E217G uptake into rye vacuoles was decreased in the presence of the flavone aglycone luteolin and β-estradiol (Table III). Plant phenolics including flavonoid aglycones were shown to inhibit the efflux of dinitrophenol-GS from human colon adenocarcinoma cells (40). Furthermore, unspecific interactions with enzymes and proteins are well known for unconjugated phenolic compounds in plant biochemistry. Thus, we cannot judge from these data...
whether the inhibition by luteolin and estradiol was specific or caused by unspecific hydrophobic interactions. One may speculate that estradiol and luteolin bind to the E$_{17}$G transporter due to similarities in the core structure of E$_{17}$G but are not transported due to the absence of a negative charge.

The striking fact that vacuolar uptake rates and inhibition characteristics of E$_{17}$G transport were comparable in barley and rye and thus independent of the presence of endogenous glucurionate conjugates argues against a specific role of the E$_{17}$G transporter in vacuolar deposition of the rye-specific flavone glucuronides. These flavone glucuronides inhibited uptake of E$_{17}$G to comparable degrees into vacuoles of both plant species (Table III) with the mono- (luteolin 7-O-glucuronide) and diglucuronated (R2) luteolin derivatives being more effective than the triglucuronated one (R1), although R1 is the major component in rye mesophyll vacuoles in young primary leaves (6). The inhibition of E$_{17}$G uptake into rye vacuoles by R2 was competitive (Fig. 4), although with the relatively high $K_i$ of about 120 $\mu$M. Therefore, we cannot exclude the existence of a further high affinity (secondary activated?) carrier specific for flavonol glucuronides in rye in addition to the glucurone conjugate pump described for the abiotic E$_{17}$G as shown for glucoside conjugates in barley (22).

Uptake of E$_{17}$G into rye and barley vacuoles was strongly stimulated by GS conjugates and GSSG (Table IV and Fig. 5), and the resulting transport activity for E$_{17}$G in the presence of decyl-GS was comparable to the rates observed for glutathione conjugates as substrates. The stimulation increased in the following order: GSSG $<$ decyl-GS $<$ DNB-GS and the concentrations required for half-maximal stimulation indicated that substrates with a high affinity toward the GS conjugate pump (10, 35) also have a high "affinity" for stimulation. First, we assumed the stimulation by decyl-GS or DNB-GS to be due to detergent effects of the lipophilic residues of these molecules. However, stimulation was also seen with GSSG which is lacking an aliphatic chain or an aromatic residue, and the amphiphilic C$_{12}$-fatty acid caprinic acid corresponding to the hydrophobic part of decyl-GS had no effect on E$_{17}$G uptake. In addition, GSH did not reduce vacuolar E$_{17}$G transport. Uptake of [14C]glucose into rye and barley vacuoles was not affected by any of the GS compounds tested showing the typical characteristics of a sucrose permease (36) suggesting that GS stimulation was specific for the glucuronate pump. The fact that stimulation of E$_{17}$G uptake by decyl-GS was not decreased in the presence of agents blocking the vacuolar H$^+$-ATPase or dissipating the $\Delta$pH suggests that no secondary active mechanism was involved in the activation but rather the stimulation of a directly energized pump. On the basis of the results shown here, two explanations for the stimulatory effect of glutathione conjugates on vacuolar E$_{17}$G uptake are possible. (i) Different binding sites are present at the same transporter for GS conjugates as a modulator and E$_{17}$G as the substrate. In that case, the activating action of GS conjugates on a glucuronate conjugate pump may be a unique feature of plants as (a) E$_{17}$G uptake into yeast microsomal vesicles was not stimulated by GS conjugates (Table V) and (b) MRPl-dependent E$_{17}$G transport across plasma membrane vesicles isolated from MRPl-transfected HeLa cells is not stimulated but strongly inhibited by leukotriene C$_4$ (21). (ii) The vacuolar glucuronate transporter described here interacts with another protein activated by bound GS conjugates (the GS conjugate pump?). Again, there is no evidence for this possibility in yeast. On the other hand, the interaction between K$^+$ channels and an ABC transporter has been reported (41).

Although detoxification reactions of xenobiotics via conjugation to glucuronate are well known in animal systems (42), to our knowledge no abiotic glucuronolates have been described in plants. Due to the occurrence of endogenous glucuronolates in rye together with corresponding UDP-glucuronolipitransferases and the evidence presented here on the existence of a directly energized glucuronate conjugate transporter for E$_{17}$G, this species may be a suitable model system to study herbicide detoxification via possible glucuronidation.

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