Plant Vacuolar ATP-binding Cassette Transporters That Translocate Folates and Antifolates in Vitro and Contribute to Antifolate Tolerance in Vivo*

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The vacuoles of pea (Pisum sativum) leaves and red beet (Beta vulgaris) storage root are major sites for the intracellular compartmentation of folates. In the light of these findings and preliminary experiments indicating that some plant multidrug resistance-associated protein (MRP) subfamily ATP-binding cassette transporters are able to transport compounds of this type, the Arabidopsis thaliana vacuolar MRP, AtMRP1 (AtABCC1), and its functional equivalent(s) in vacuolar membrane vesicles purified from red beet storage root were studied. In so doing, it has been determined that heterologously expressed AtMRP1 and its equivalents in red beet vacuolar membranes are not only competent in the transport of glutathione conjugates but also folate monoglutamates and antifolates as exemplified by pteroyl-L-glutamic acid and methotrexate (MTX), respectively. In agreement with the results of these in vitro transport measurements, analyses of atmrp1 T-DNA insertion mutants of Arabidopsis ecotypes Wassilewskija and Columbia disclose an MTX-hypersensitive phenotype. atmrp1 knock-out mutants are more sensitive than wild-type plants to growth retardation by nanomolar concentrations of MTX, and this is associated with impaired vacuolar antifolate sequestration. The vacuoles of protoplasts isolated from the leaves of Wassilewskija atmrp1 mutants accumulate 50% less [3H]MTX than the vacuoles of protoplasts from wild-type plants when incubated in media containing nanomolar concentrations of this antifolate, and vacuolar membrane-enriched vesicles purified from the mutant catalyze MgATP-dependent [3H]MTX uptake at only 40% of the capacity of the equivalent membrane fraction from wild-type plants. AtMRP1 and its counterparts in other plant species therefore have the potential for participating in the vacuolar accumulation of folates and related compounds.

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The abbreviations used are: THF, tetrahydrofolate; ABA, abscisic acid; ABC, ATP-binding cassette; AtMRP, A. thaliana MRP; Bn-NCC-1, Brassica napus nonfluorescent chlorophyll catabolite 1; Bistris propane, 1,3-bis[2-hydroxyethyl]amino]propane; CDNB, 1-chloro-2,4-dinitrobenzene; DNP-GS, S-(2,4-dinitrophenyl)-GS; G, 17β-estradiol 17-(β-o-glucuronide); FBP, folate-binding protein; FPGS, polyglutamate synthetase; GGH, γ-glutamyl hydrolase; GST, glutathione S-transferase; HPLC, high performance liquid chromatography; H2O2, hydrogen peroxide; IAA, indole-3-acetic acid; kcat, catalytic constant; kcat/Km, Michaelis constant; M, 2-(N-morpholino)ethanesulfonic acid; MRP, multidrug resistance-associated protein; MTX, methotrexate; NEM-GS, N-ethylmaleimide-GS; p-ABA, p-aminobenzoate; Pte, pteroyl; PteGlu, pteroyl monoglutamate; PteGlu, pteroyl polyglutamate; RT, reverse transcription; WS, Wassilewskija; Col-0, Columbia.

Tetrahydrofolate (THF) and its derivatives, “folates,” are essential cofactors for one-carbon transfer reactions, for instance those crucial for nucleotide biosynthesis and amino acid metabolism. In humans and other animals who cannot synthesize folates de novo, these cofactors must be obtained from dietary sources, principally plant materials. The repercussions of dietary folate deficiencies range from an increased predisposition to megaloblastic anemia and birth defects to cardiovascular disease and certain cancers (1).

Folates are tripartite molecules, consisting of pteridine and p-aminobenzoate, which together constitute the pteroyl moiety, and one or more glutamate residues (Fig. 1A). In plants and most other organisms, the parent folate molecule, pteroyl monoglutamate (PteGlu1), is poly-γ-glutamylated to yield pteroyl polyglutamates (PteGlu6) containing 1–7 additional glutamate residues (2).

The experiments described here were directed at determining whether a multidrug resistance-associated protein (MRP)-type ATP-binding cassette (ABC) transporter might participate in vacuolar folate uptake. The reasons for conducting these studies were 2-fold. The first was recognition that a significant fraction of total cellular folate localizes to the vacuolar compartment of plant cells. Vacuoles purified from pea (Pisum sativum) leaves contain an average of 20% of the total cellular folate, compared with ~50 and 10%, respectively, in mitochondria and chloroplasts (3). Approximately 50% of the principal vacuolar folate in this system, 5-methyl-THF, is polyglutamylated, whereas the principal mitochondrial and plastidial forms are polyglutamylated derivatives of 5-formyl-THF and 5–10-methyl-THF, respectively (3). This is probably a general phenomenon in that between 20 and 60% of total tissue folate, mainly in the form of 5-methyl-THF, of which about 80% is polyglutamylated, can be recovered in the vacuolar fraction of
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Of the 15 unique MRPs encoded by the genome of *Arabidopsis thaliana*, five (AtMRPs 1–5) have been cloned and shown to encode functional transporters after heterologous expression in *Saccharomyces cerevisiae* ycf1Δ strains from which the gene encoding the endogenous vacuolar MRP, yeast cadmium factor 1 (ycf 1), has been disrupted (reviewed in Ref. 13). All five are competent in the transport of GS-conjugates. In addition, several are able to transport other amphipathic anions, including glucuronate conjugates and linearized chlorophyll (tetrapyrrole) catabolites (13).

If plant MRPs are to contribute to the vacuolar sequestration of folates, the membrane localization of these transporters and the form in which the folates are transported and stored must be addressed. Regarding membrane localization, two members of the *Arabidopsis* MRP subfamily, AtMRP1 and AtMRP2 (AtABCC1 and AtABCC2, respectively, according to the new nomenclature described in Ref. 14), have been clearly localized to the vacuolar membrane *in planta* (15, 16). However, on the basis of pilot experiments, only AtMRP1 catalyzes appreciable folate and MTX transport *in vitro*. Although AtMRP4 (ABCC4) is also able to transport these compounds at high capacity (17), its exact membrane localization is unclear. On the one hand, it localizes to the plasma membrane rather than the vacuolar membrane when its partial translation product is fused with green fluorescent protein (18). On the other hand, the results of recent *Arabidopsis* organellar proteomic analyses and studies of constructs in which green fluorescent protein is C-terminally fused to the full-length translation product are consistent with localization to the vacuolar membrane (19). Pending further insight into the membrane localization of AtMRP4, the investigations described here are specifically directed at elucidating the role AtMRP1 might play in vacuolar folate uptake.

Two factors are crucial when considering the form in which folates are transported. The first is that although it has been established that a subset of human and rodent MRPs confer resistance to MTX and have the capacity to transport this and physiological folates, they only do so when these compounds are monoglutamylated; polyglutamylation essentially abolishes transport (7, 8). The second factor is that in plants, as in other organisms, folates are usually polyglutamylated with up to seven γ-linked glutamate residues (2) that serve to enhance cofactor activity and stability. More than 50% of the extractable vacuolar pool of folates is polyglutamylated (3). When account is taken of the likelihood that the vacuole lacks the machinery for folate polyglutamylation, the enzyme folypolyglutamate synthetase (FPGS) and the energy source ATP (3, 20, 21), the implication is clear. If plant MRPs do participate in the vacuolar localization of folates, they are responsible for delivery of only a subfraction of this pool, the monoglutamylated component, into this compartment or, unlike their mammalian counterparts, are able to transport polyglutamates as well as monoglutamates. A primary objective of the investigations reported here was to identify the transport form of vacuolar folates, namely whether they are transported as monoglutamates or polyglutamates, and to determine whether a vacuolar membrane-associated MRP-type functionality, as exemplified by AtMRP1, might be responsible.

To assess the general applicability of the properties of heterologously expressed AtMRP1 and *Arabidopsis* T-DNA knock-
out mutants for this transporter to native membranes from other plant sources, parallel experiments were performed on vacuolar membrane vesicles purified from red beet storage root. This system was considered to be particularly appropriate for these investigations for three reasons. First, red beet storage root is a rich source of high purity transport-competent vesicles derived from the membrane bounding the vacuole (22). Second, previous investigations have established that vacuolar membrane vesicles purified from this source contain an MRP-like functionality or functionalities capable of catalyzing the transport of GS-conjugates (10, 23, 24). Third, red beet storage root is listed as one of the richest natural sources of folates (25), a sizeable fraction of which localize to the vacuole (3). The folate content of red beet storage root is as high as those of green leaves, for instance those of spinach which is considered to be one of the richest sources of this class of vitamins (25).

**MATERIALS AND METHODS**

**Chemicals**—ATP, creatine kinase (type I from rabbit muscle), creatine phosphate, and N-ethylmaleimide (NEM) were purchased from Sigma. Mixed cellulose ester membrane filters (HAWP filters, 0.45-μm pore size) and hydrophilic polyvinylidine difluoride (Durapore) membrane filters (GVWP filters, 0.22-μm pore diameter) were purchased from Millipore Corp. [glycine-2,5-3H]Glutathione ([3H]GSH) (41.5 Ci/mmol) was purchased from PerkinElmer Life Sciences. Mixed cellulose ester membrane filters (Durapore) membrane filters (GVWP filters, 0.22-μm pore diameter) were purchased from Millipore Corp. [glycine-2,5-3H]Glutathione ([3H]GSH) (41.5 Ci/mmol) was purchased from PerkinElmer Life Sciences. [3H]Glu3, 55% [3H]PteGlu4, and 5% [3H]PteGlu5. [3H]NEM-GS was synthesized as described (26). Briefly, 2 nmol of [3H]PteGlu1 were incubated with 25 μg of Escherichia coli FPGS for 24 h at 37 °C in 50 mM Tris-HCl buffer (pH 8.6) containing 10 mM MgCl2,5 0mM KCl, 5 mM ATP, 25 μg of bovine serum albumin, and 20 mM L-glutamate. The reaction was terminated by boiling the mixture for 5 min and subsequent centrifugation at 20,000 g for 10 min. The products of the reaction were purified by BCA-photocromatography as described below for the purification of [3H]PteGlu1, and quantitated and identified by HPLC with chromatography as described below for the purification of [3H]PteGlu1.

**Plant Materials**—For the majority of the investigations of A. thaliana, ecotype Wassilewskia (WS) was employed except for the analyses of atmrp1-2 knock-out mutants, which were performed on ecotype Columbia (Col-0). Fresh red beet (Beta vulgaris) storage roots were purchased locally, stored at 4 °C, and used within 2 days of purchase.

**Isolation of AtMRP1 T-DNA Insertion Mutants**—For the experiments described here, two AtMRP1 insertion mutants, atmrp1-1 and atmrp1-2, respectively, from Arabidopsis eco-

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**RT-PCR**—To assess the steady state levels of AtMRP1 transcripts and therefore the severity of the knock-outs or knock-downs for the atmrp1 mutants, RNA was extracted from 14-day-old atmrp1-1 and atmrp1-2 mutant and wild-type WS and Col-0 plants using TRIzol reagent (Invitrogen) according to the manufacturer’s recommendations. For RT-PCR of AtMRP1, 1-μg aliquots of the RNA samples were reverse-transcribed using Superscript II RNase H- RT (Invitrogen), and the first-strand cDNA products were PCR-amplified using the AtMRP1-specific primer pair AtMRP1-RT-F/AtMRP1-RT-R (5'-CGGAGAAATCCTCTTCCTGCTGTA-3' and 5'-CCGTT-AGCTTCTCTGGTGAGCAGTTT-3’, respectively). PCR amplification was for 3 min at 94 °C followed by 27 cycles of 30 s at 94 °C, 30 s at 62 °C, and 1 min at 72 °C. To assess the efficacy of RNA extraction, aliquots of the same RNA samples were subjected to RT-PCR using the Arabidopsis Actin-8 gene primer pair Actin-F/Actin-R (5'-CTGTGATGATGCTATT-3’ and 5'-CTGTGATGATGCTATT-3’, respectively) using the same thermal profile except that the annealing step was done at 58 °C.

**Phenotypic Characterization of atmrp1-1 and atmrp1-2 Mutants**—For the preliminary phenotypic screens, seeds of atmrp1-1 mutant and wild-type WS plants and of atmrp1-2 mutant and wild-type Col-0 plants were surface-sterilized with 0.05% (w/v) sodium hypochlorite/0.1% (w/v) Tween 20, washed exhaustively with sterile water, and germinated for 48 h at 4 °C on solid MS medium (pH 5.7) containing 1% (w/v) sucrose. Xenobiotics, elicitors of oxidative stress, and phytohormones were incorporated into the medium at the concentrations indicated. Thereafter, the plates were grown vertically under controlled environmental conditions (24 ± 2 °C; continuous cool fluorescent illumination; 70% relative humidity) for 12 days before measuring primary root length. For subsequent detailed screens of the sensitivity of growth to MTX at the whole plant level, surface-sterilized mutant and wild-type seeds were germinated for 48 h at 4 °C on solid MS medium containing 0.5 g/liter Mes, 0.9% (w/v) Difco-Bacto agar, 1% (w/v) sucrose, and the indicated concentrations of MTX in plant tissue culture vessels before transfer to a plant growth room for growth at 22 ± 2 °C and 70% relative humidity under a 16/8-h photoperiod for a further 14 days. In all of the phenotypic screens, precautions were taken to ensure that several independent mutant and wild-type seed batches were treated and screened in parallel under identical conditions.

**Affinity Purification of [3H]PteGlu1**—To remove breakdown products and/or contaminants that were suspected to interfere with transport, the radiolabeled stocks of [3H]PteGlu1 received from the suppliers were further purified by affinity chromatog-
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raphy before use. For this purpose, 20-μl aliquots of the [3H]PteGlu1 stocks (40–45 Ci/mmol) were added to 300 μl of 25 mM potassium phosphate buffer (pH 7.4) and applied to a column packed with folate-binding protein (FBP)-agarose (bed volume 0.5 ml) that had been equilibrated with the same buffer (26). After three successive washes with 2.5 ml of potassium phosphate buffer (pH 7.4) containing 1 mM NaCl, 2.5 ml of potassium phosphate buffer alone, and 300 μl of 0.1 M HCl, the column was eluted with 1 ml of 0.1 M HCl. The final eluate containing purified [3H]PteGlu1 was combined with 0.1 ml of 10 mM 2-mercaptoethanol dissolved in 1 M Tris-base to yield a pH 7.4 solution that was used immediately or stored at −20 °C.

**Heterologous Expression of AtMRP1 in Yeast and Purification of Vacuolar Membrane-enriched Vesicles**—For studies of the transport capabilities of heterologously expressed AtMRP1, S. cerevisiae ycf1Δ strain DTY168 (MATα his6 leu2-3-112 ura3-52 ycf1::hisG) (29) was transformed with pYES3-AtMRP1 or empty pYES3 vector by the LiOAc/polyethylene glycol method (30) and selected for uracil prototrophy by plating on AHC medium containing tryptophan (31, 32). Vacuolar membrane-enriched vesicles purified from pYES3-AtMRP1-transformed S. cerevisiae ycf1Δ strain DTY168 were greenhouse-grown (24 ± 2 °C; 10/14 h photoperiod; 80% relative humidity) for 4–5 weeks, and protoplasts were prepared from rosette leaves as described (33). MTX uptake was estimated by incubating equal amounts of the wild-type and atmrp1-1 protoplast suspensions in uptake medium (10 mM CaCl2, 1 mM MgCl2, 0.5 M sorbitol and 10 mM Tris/Mes (pH 5.5)) containing 19 nM [3H]MTX at 25 °C for 2.5 h with gentle agitation on an orbital shaker. For the estimation of total protoplast (“cellular”) [3H]MTX content, the protoplasts were pelleted by centrifugation at 60 × g for 5 min and washed three times with fresh uptake medium before the removal of aliquots for liquid scintillation counting. For the estimation of vacuolar [3H]MTX content, intact vacuoles were isolated from the protoplasts by differential osmolysis and Ficoll flotation centrifugation through 10% (w/v) and 4% (w/v) Ficoll solutions (34). Bovine serum albumin (5 mg/ml) was added to the 10% Ficoll solution to diminish adherence of membranes from other sources to the surfaces of the vacuoles during fractionation (35). Intact vacuoles were collected from the 0/4% Ficoll interface for liquid scintillation counting.

**Protein Estimations**—Protein was estimated by a modification of the method of Bradford (36)

**RESULTS**

*MgATP-dependent Transport of Folate Monoglutamate (Pteroyl-L-glutamate, PteGlu1)*—Both heterologously expressed AtMRP1 and vacuolar membrane vesicles purified from red beet storage root are competent in the transport of PteGlu1. The concentration dependence of uptake of affinity-purified [3H]PteGlu1 by vacuolar membrane-enriched vesicles purified from pYES3-AtMRP1-transformed S. cerevisiae ycf1Δ strain DTY168 approximates Michaelis-Menten kinetics to yield $K_m$ and $V_{max}$ values of 188 ± 68 μM and 10.1 ± 1.8 nmol/mg/20 min, respectively (Fig. 2A). The corresponding values for native vacuolar membrane vesicles purified from red beet storage root are 195 ± 14 μM and 6.4 ± 0.2 nmol/mg/20 min, respectively (Fig. 2B). Crucial, however, is transport to be measured reliably, is the purity of the [3H]PteGlu1, employed as transport substrate. For the experiments shown in Fig. 2 and all of the other experiments employing [3H]PteGlu1, the radiolabeled...
FBP-agarose-purified [³H]PteGlu₁ exceeded those of the unpurified compound by severalfold such that purification of the [³H]PteGlu₁ stock was accompanied by a decrease in the $K_m$ from 403 ± 112 to 195 ± 15 μM concomitant with an increase in the $V_{max}$ from 2.3 ± 0.4 to 6.4 ± 0.2 nmol/mg/20 min (data not shown). The reason for this difference is not known, but it is suspected to result from contamination of the stock with breakdown products that interfere with uptake of the parent compound, [³H]PteGlu₁, but which lack radiolabel and/or do not undergo appreciable MgATP-dependent uptake themselves. HPLC analysis of the [³H]PteGlu₁ stock before and after affinity purification discloses additional species in the former that are absent from the latter.

MgATP-dependent transport of the antifolate MTX by yeast vacuolar membrane-enriched vesicles containing heterologously expressed AtMRP1 or purified red beet vacuolar membrane vesicles are fundamentally equivalent to those of [³H]PteGlu₁. In both membrane preparations, MgATP-dependent [³H]MTX uptake approximates Michaelis-Menten kinetics to yield $K_m$ and $V_{max}$ values of 243 ± 55 μM and 8.6 ± 0.9 nmol/mg/20 min, respectively, for heterologously expressed AtMRP1 (Fig. 3A) and 223 ± 37 μM and 3.3 ± 0.2 nmol/mg/20 min, respectively, for red beet vacuolar membrane vesicles (Fig. 3B).

Vanadate inhibitory of [³H]PteGlu₁ and [³H]MTX transport—All four of the transport processes examined, MgATP-dependent uptake of [³H]PteGlu₁ or [³H]MTX into yeast vacuolar membrane-enriched vesicles containing heterologously expressed AtMRP1 or into red beet vacuolar membrane vesicles, are susceptible to inhibition by vanadate (Table 1). On this basis and the insensitivity of these processes to the V-ATPase inhibitor bafilomycin A₁ or the protonophore gramicidin-D (data not shown), the transport measured appears to be largely attributable to the primary energization of ABC transporters by MgATP rather than secondary $H^+$-coupled transport. The only qualitative difference between the kinetics of [³H]PteGlu₁ and [³H]MTX transport by heterologously expressed AtMRP1 and the red beet membrane preparations is that ~20% of the transport measured in the latter is insensitive to inhibition by vanadate. This necessitates subtraction of the uninhibitable component from the total uptake measured when enumerating the concentration of vanadate required to inhibit the inhibitable component by 50%. When this correction is applied, the $I_{50}$ values for the inhibition of [³H]PteGlu₁ and [³H]MTX uptake by vacuolar membrane-enriched vesicles containing heterologously expressed AtMRP1 and red beet vacuolar membrane vesicles fall in the same range: 3.3 ± 0.5 and 6.7 ± 1.9 μM for [³H]PteGlu₁ and [³H]MTX uptake by AtMRP1; 3.2 ± 1.0 and 9.6 ± 4.6 μM for [³H]PteGlu₁ and [³H]MTX uptake by red beet (Table 1).

Transport of PteGlu₁, and GS-conjugates by a common MRP-type functionality—AtMRP1 was first characterized in terms of its capacity to catalyze the vanadate-inhibitable, MgATP-dependent transport of GS-conjugates (32), a property it shares with many other MRP-type ABC transporters (13). Accord-

5 V. Naponelli, A. D. Hanson, and J. Gregory, unpublished data.
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FIGURE 3. Concentration dependence of MgATP-dependent $[^3H]$MTX uptake by vacuolar membrane-enriched vesicles purified from DTY168/pYES3-AtMRP1 cells (A) and by vacuolar membrane vesicles purified from red beet storage root (B). The data were fitted to a Michaelis-Menten function to yield $K_m$ and $V_{\text{max}}$ values of 243 ± 55 µM and 8.6 ± 0.9 nmol/mg/20 min, respectively, for heterologously expressed AtMRP1 and 223 ± 37 µM and 3.3 ± 0.2 nmol/mg/20 min, respectively, for red beet vacuolar membrane vesicles. Values shown are means ± S.E. ($n = 3$).

TABLE 1
Sensitivity of MgATP-dependent $[^3H]$PteGlu$_{15}$, $[^3H]$MTX, or $[^3H]$NEM-GS uptake by yeast vacuolar membrane-enriched vesicles containing heterologously expressed AtMRP1 and vacuolar membrane vesicles purified from red beet storage root to inhibition by vanadate

| Transport substrate | Heterologously expressed AtMRP1 | Red beet vacuolar membrane vesicles |
|---------------------|---------------------------------|-------------------------------------|
| PteGlu$_{15}$       | 3.3 ± 0.5                       | 3.2 ± 1.0                           |
| MTX                 | 6.7 ± 1.9                       | 9.6 ± 4.6                           |
| NEM-GS              | 2.3 ± 0.5                       | 4.4 ± 0.5                           |

The rates of MgATP-dependent uptake of 100 µM concentrations of $[^3H]$PteGlu$_{15}$, $[^3H]$MTX, or $[^3H]$NEM-GS from assay media containing 0–80 µM vanadate were estimated as described in Figs. 2 and 4. Each data set was fitted to a single negative exponential function by nonlinear least squares analysis to yield estimates of the concentrations of vanadate required for 50% inhibition of net uptake ($I_{50}$ values). Note that the $I_{50}$ values calculated for red beet vacuolar membrane vesicles are those for the inhibitable component obtained by subtracting the uninhibitible component, which accounted for approximately 20% of total uptake, from total uptake. Values shown are means ± S.E. ($n = 3$).

An intriguing finding, however, is that although NEM-GS interacts with the same MRP-type functionality as PteGlu$_{15}$, it does so noncompetitively. Although inclusion of 100 µM NEM-GS in the uptake medium decreases the $V_{\text{max}}$ value for $[^3H]$PteGlu$_{15}$ uptake by vacuolar membrane-enriched vesicles purified from yeast heterologously expressing AtMRP1 from 10.1 ± 1.8 nmol/mg/20 min to 5.7 ± 0.8 nmol/mg/20 min, it has little or no effect on the $K_m$ value, which has values of 181 ± 52 and 188 ± 68 µM, respectively, in the presence and absence of NEM-GS (Fig. 5A). Qualitatively similar results are obtained when the same experiment is performed on red beet vacuolar membrane vesicles; the $V_{\text{max}}$ value for $[^3H]$PteGlu$_{15}$ uptake is decreased from 6.4 ± 0.2 nmol/mg/20 min to 2.3 ± 0.1 nmol/mg/20 min while leaving $K_m$ relatively unaffected at values of 195 ± 15 and 181 ± 12 µM (Fig. 5B) when NEM-GS is included in the uptake medium. Because under all four conditions $[^3H]$PteGlu$_{15}$ uptake closely approximates Michaelis-Menten kinetics to yield strictly linear Hanes-Woolf plots, the implication is that PteGlu$_{15}$ and NEM-GS interact with a common functionality in both membrane preparations but at different sites. The interaction of heterologously expressed AtMRP1 or its equivalents in red beet with NEM-GS interferes with $[^3H]$PteGlu$_{15}$ transport without interfering with the binding of the latter to the transporter.

Polyglutamylated Folate as a Poor Transport Substrate—By comparison with the folyl monoglutamates, PteGlu$_{15}$ and MTX, folyl polyglutamates (PteGlu$_n$) undergo only very low rates of MgATP-dependent uptake. Net uptake of 50 µM $[^3H]$PteGlu$_n$ into yeast vacuolar membrane-enriched vesicles containing heterologously expressed AtMRP1 or red beet vacuolar membrane vesicles is 7–10- and 3-fold lower than the net uptake of 100 µM $[^3H]$PteGlu$_{15}$ measured after 10 or 20 min under the same conditions (Fig. 6 A and B). Moreover, whereas the inclusion of 50 µM PteGlu$_n$ in the $[^3H]$PteGlu$_{15}$ uptake medium weakly but consistently inhibits uptake mediated by heterologously expressed AtMRP1, suggesting that PteGlu$_1$ and PteGlu$_n$ compete for common binding sites (Fig. 6A), the converse
is seen in the red beet system. Addition of 50 μM PteGlu₃ increases the net uptake of 100 μM [³H]PteGlu₃ by red beet vacuolar membrane vesicles by factors of 1.3 and 2.2 after 10 and 20 min, respectively (Fig. 6B). The significance of this effect is not known, but the increase in [³H]PteGlu₃ uptake seen when PteGlu₃ is added to the assay medium is at least partially inhibited by vanadate (Fig. 6B), which is consistent with the participation of an ABC transporter.

Isolation and Genomic Characterization of AtMRP1 Knockout Mutants—With the aim of gaining insight into the function of AtMRP1 in the intact plant, or at least processes for which its loss of function has discernible phenotypic consequences, two T-DNA insertion mutant alleles of AtMRP1, atmrp1-1 and atmrp1-2 from ecotypes Wassilewskia (WS) and Columbia (Col-0), respectively, were obtained. The former was isolated in in-house screens; the latter was obtained from the Arabidopsis Biological Resource Center Salk collection.

As confirmed by the results shown in Fig. 7, both T-DNA insertions, atmrp1-1 that maps to exon 23 of the genomic sequence of AtMRP1, and atmrp1-2 that maps to exon 25 (Fig. 7A), are associated with a knock-out or severe knockdown of expression (Fig. 7B). RT-PCR of total RNA extracted from atmrp1-1 or atmrp1-2 mutants and the corresponding wild types using the AtMRP1-specific primer pair AtMRP1-RT-F/AtMRP1-RT (see “Materials and Methods”) yields a strong signal that matches the 512-bp amplification product expected
from the mature transcript from the wild-type RNA extracts but no signal from the mutant extracts (Fig. 7B).

**MTX Hypersensitivity of atmrp1-1 and atmrp1-2 Knockout Mutants**—Having established that both atmrp1-1 and atmrp1-2 are null mutants and that heterologously expressed AtMRP1 is competent in the MgATP-dependent transport of toxic amphipathic anions, such as MTX, as well as some GS-conjugatable xenobiotics, screens were initiated to assess the sensitivity of these mutants by comparison with wild-type plants to the toxic effects of these agents.

In the first instance, seeds from atmrp1-1 and atmrp1-2 plants and their corresponding WS and Col-0 wild-type equivalents were germinated and grown in the light on sterile plates containing a broad range of concentrations of the antifolate MTX, CDNB (a cytotoxic generic glutathione S-transferase (GST) substrate), the GS-conjugable herbicides atrazine (a triazine derivative) or metolachlor (a chloroacetanilide), menadione (an elicitor of oxidative stress), or abscisic acid (ABA, a stress hormone). In this way it was determined that although neither of these mutants is more susceptible than their corresponding wild types to any of the GS-conjugatable xenobiotics tested or menadione or ABA, both are more sensitive to MTX in the growth medium. When germinated and grown vertically on MS medium supplemented with 1–10 nM atrazine or metolachlor, 5–60 μM CDNB, 5–100 μM menadione or 1–5 μM ABA, no difference between the mutant and wild-type seedlings was discernable; all showed similar degrees of root growth inhibition (data not shown). However, when the same experiments were conducted with MTX, differences between the atmrp1-1 and atmrp1-2 mutants and their corresponding wild types were evident in the nanomolar range.

Expansion of the screens to the effects of MTX at the level of the intact seedling reinforced these findings. When grown on solid MS medium containing 0–40 nM MTX in plant tissue culture vessels under a 16/8-h photoperiod for a further 14 days after germination, significant, albeit small to moderate, differences between the atmrp1 mutants and their corresponding wild types are evident at both the root and shoot levels (Fig. 8).

At the highest MTX concentrations examined, 30 and 40 nM, the mutants are more prone than the wild types to chlorosis, anthocyanin accumulation, and eventual necrosis (Fig. 8).

**Protoplasts from atmrp1-1 Mutants Are Defective in Vacuolar *[3H]MTX Accumulation**—In view of the capacity of heterologously expressed AtMRP1 for MgATP-dependent MTX transport in vitro and localization of this transport protein to the vacuolar membrane in planta, the most straightforward explanation for the increased susceptibility of atmrp1-1 and atmrp1-2 mutants to the toxic action of this antifolate is that by comparison with their wild-type counterparts they are impaired in the vacuolar sequestration of this compound. To test this proposal, two approaches were adopted. In the first, total cellular *[3H]MTX uptake and the vacuolar levels achieved by protoplasts isolated from the leaves of atmrp1-1 mutant and wild-type WS plants were compared. In the second, the capacities of vacuolar membrane-enriched vesicles purified from atmrp1-1 mutant and wild-type WS liquid root cultures for MgATP-dependent *[3H]MTX uptake in vitro were compared.

The results obtained from both approaches were consistent with a deficiency in vacuolar MTX sequestration in atmrp1-1 mutants. When incubated in culture medium containing 19 nM *[3H]MTX for 2.5 h, protoplasts isolated from atmrp1-1 mutants and wild-type WS plants achieve similar total cellular levels of the antifolate (61.7 ± 3.1 and 63.8 ± 2.6 fmol/mg protein, respectively) (Fig. 9A). However, if aliquots of the same samples of protoplasts are gently disrupted by differential osmolysis to release intact vacuoles and the *[3H]MTX contents of these are estimated, there is a marked difference depending on whether the vacuoles are fractionated from atmrp1-1 mutant or wild-type WS protoplasts. Vacuoles prepared from wild-type WS protoplasts achieve *[3H]MTX contents of 4.4 ±
0.1 pmol/mg protein, whereas the [3H]MTX content of the corresponding fraction from atmrp1-1 mutant protoplasts is only 2.1 ± 0.2 pmol/mg protein (Fig. 9B). An ~50% diminution of the vacuolar [3H]MTX content of atmrp1-1 mutant versus wild-type WS protoplasts despite comparable levels of total protoplasm uptake indicates that the atmrp1-1 mutants are not impaired in total cellular MTX uptake or extrusion but are instead impaired in delivery of this compound into the vacuole. In agreement with this interpretation, the rates and extents of MgATP-dependent [3H]MTX uptake by vacuolar membrane-enriched vesicles purified from atmrp1-1 mutant root cultures when measured at an initial concentration of 50 nM are diminished by ~60 and 40%, respectively, by comparison with the corresponding membrane fraction from wild-type WS root cultures (Fig. 10). Moreover, regardless of the source of the membrane vesicles, MgATP-dependent [3H]MTX uptake is more than 85% inhibited by the inclusion of 100 μM vanadate in the uptake medium (data not shown), which implies that not only the AtMRP1-dependent component but also the AtMRP1-independent component of uptake is largely attributable to ABC transporters and not to secondary H^+-coupled, V-ATPase-energized uptake.

**DISCUSSION**

The findings presented establish that heterologously expressed AtMRP1 is not only competent in the MgATP-dependent transport of GS-conjugates but also monoglutamylated folates, as exemplified by PteGlu1 and MTX, and that this is a property shared by vesicles derived from the vacuolar membrane of red beet storage root. Moreover, it is shown that whereas both of the knock-out mutant alleles of AtMRP1, atmrp1-1 and atmrp1-2 from Arabidopsis ecotypes WS and Col-0, respectively, are indistinguishable from their wild-type...
counterparts when grown under standard conditions or on solid media containing GS-conjugable xenobiotics, they are more sensitive to nanomolar concentrations of MTX in the growth medium. In the case of the mutant allele that was characterized further, atmrp1-1, MTX hypersensitivity is associated with impaired vacuolar transport and sequestration of this antifolate. Evidently, AtMRP1 and its functional equivalent in the vacuolar membrane of red beet translocate folate monoglutamate and antifolates in vitro and the former contributes to antifolate tolerance in vivo.

Estimates of the internal volume of red beet vacuolar membrane vesicles prepared in the same manner as in this study yield a value of 10 µm/mg membrane protein (10, 37). Assuming a 1:1 mixture of right-side-out and inside-out vesicles, uptake of ~5 nmol/mg protein after 60 min from a medium containing 100 µM [3H]PteGlu₄ amounts to an accumulation ratio of 10. The corresponding value for the uptake of [3H]NEM-GS by the same preparation is 16. Given that the transport capabilities of yeast vacuolar membrane-enriched vesicles containing heterologously expressed AtMRP1 are similar to and often greater than those of red beet vacuolar membrane vesicles, it is apparent that transport is against a moderately steep concentration gradient in both preparations.

The substrate selectivity of AtMRP1 strictly parallels those of the MRPs from mammalian sources that have been implicated in the transport of folates. Human MRPs 1 and 3, for instance, are high capacity, low affinity MTX and folate transporters exhibiting little or no affinity toward polyglutamates (7). MRP3 and several other human MRPs confer resistance to MTX in transfected cultured cells (8). Equivalently, AtMRP1 is a high capacity, intermediate affinity folate and MTX transporter exhibiting little or no activity toward polyglutamates; it mediates very low rates of [3H]PteGlu₄ transport, and the inclusion of PteGlu₄ in the uptake medium only weakly inhibits [3H]PteGlu₄ uptake. Knock-out mutants for AtMRP1 are hypersensitive to MTX in the growth medium and deficient in its vacuolar sequestration.

AtMRP1 and its functional equivalents in other plants have the characteristics of MgATP-dependent vacuolar pumps capable of contributing to the detoxification of non-glutathionylated amphipathic anions. That this should be the case, however, raises the following question: why does AtMRP1 appear not to confer tolerance toward CDNB or metolachlor, a generic cytotoxin and herbicide, respectively, whose GS-conjugates are also in vitro transport substrates (12, 32)?

The answer to this question is not known, but functional redundancy or a lack thereof may be an important consideration. Because only a subset of plant MRPs have appreciable folate and antifolate transport activity, the problems of functional redundancy associated with screens involving GS-conjugable xenobiotics, namely the ability of most MRPs to transport GS-conjugates, apply less to MTX. An alternate or supplementary explanation is that, unlike the screens deploying GS-conjugable xenobiotics, the efficacy of MTX as a screening agent is probably not rate-limited by upstream enzymes, such as GSTs, whose activity in the case of GS-conjugable xenobiotics would set an upper limit on the rate of formation of transport-active derivatives. In lieu of findings to the contrary, one other possibility that cannot be ruled out is that the importance of vacuolar sequestration has simply been overstated in that conjugation of GS-conjugable xenobiotics, alone, might be sufficient for the detoxification of many xenobiotics regardless of whether the conjugates remain in the cytosol or are transported into the vacuole (38–40).

The MTX hypersensitivity profiles of atmrp1 mutants are reminiscent of the properties of atmrp2 knock-out mutants. Despite the capacity of heterologously expressed AtMRP2 for high rates of GS-conjugate transport, the highest reported to date for any MRP regardless of source (16, 41), the most striking phenotype of atmrp2 mutants is not associated with GS-conjugate transport but instead with the vacuolar sequestration of chlorophyll catabolites (40), amphipathic anions whose transport, as exemplified by the linearized tetrapyrrole Brassica
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napus nonfluorescent chlorophyll catabolite 1 (Bn-NCC-1), does not depend on upstream GST action (41).

Another striking similarity between AtMRP1 and AtMRP2 is that both interact with both GS-conjugates and non-glutathionylated amphipathic anions but not in a simple competitive manner. Heterologously expressed AtMRP2 catalyzes the transport of GS-conjugates, Bn-NCC-1 and glucurononides such as 17β-estradiol 17-(β-d-glucuronide) (E217βG), but not in a manner consistent with the interaction of these three classes of substrate with a common binding or transport site (41). For instance, DNP-GS and Bn-NCC-1 exert little or no effect on each other’s transport via AtMRP2; both are transported at similar rates regardless of whether the other transport substrate is in the uptake medium (41). Rather than competing with each other for uptake by AtMRP2, DNP-GS stimulates the uptake of E217βG and vice versa (16). The kinetics of AtMRP2-mediated transport are consistent with a scheme in which DNP-GS, E217βG, and Bn-NCC-1 undergo transport through different AtMRP2-dependent pathways and that DNP-GS and E217βG promote each other’s transport by binding sites distinct from but tightly coupled to the other’s transport pathway (13, 16). The behavior of AtMRP1 is not as pronounced as that of AtMRP2 in this regard. Nonetheless, the binding and/or transport sites for NEM-GS and PteGlu1 behave as if distinct, but coupled. NEM-GS noncompetitively inhibits PteGlu1 transport and vice versa, implying that the two substrates interact with nonidentical binding sites on the same functionality. This is not a property peculiar to AtMRP1 because the equivalent transport processes in red beet vacuolar membrane vesicles behave similarly.

If MRP-type transporters contribute to the accumulation of vacuolar folates in planta, they likely do so by catalyzing uptake of the monoglutamylated rather than the polyglutamylated component. Because polyglutamylated folates represent such a sizeable fraction of the total vacuolar folate pool, a pressing question that remains to be answered is how polyglutamylated folates make their appearance in this compartment? Must alteration of the monoglutamylated rather than the polyglutamylated component result in the formation of new efflux transporters or increase the activity of the existing transporters? The answer to this question requires a more detailed understanding of the role of MRP-type transporters in folate transport.

A subsidiary consideration that has a bearing on whether AtMRP1 and its equivalents in other plant vacuolar membranes actually transport folate monoglutamates in vivo are their relatively low affinities for these compounds in vitro. The $K_m$ values for PteGlu1 transport by heterologously expressed AtMRP1 and red beet vacuolar membrane vesicles (188 and 195 μM, respectively) are far higher than the probable steady state cytosolic concentrations of these compounds in plant cells. Assuming a typical folate content of 2 nmol/g fresh weight (42) of which 5% or less is monoglutamylated (3), it is likely that the steady state cytosolic concentration of PteGlu1 seldom exceeds 1 μM if the cytosol amounts to ~34 μl/g fresh weight (43, 44), and about 20% of the total pool is allocated to this compartment (3). In short, if AtMRP1 and its equivalents are to participate in vacuolar folate sequestration, the conditions for their assay in vitro are far from optimal and/or the transporters in question operate far from their maximal capacity in vivo.

Notwithstanding these uncertainties, there can be little doubt of the relevance of AtMRP1 for the detoxification of antifolates despite its correspondingly low in vitro $K_m$ value for MTX (243 μM) in that atmrp1 knock-out mutants are hypersensitive to nanomolar concentrations of this compound. Moreover, even when exposed to only nanomolar concentrations of MTX, there is a marked difference between the capacity of wild-type and atmrp1 knock-out mutant protoplasts for vacuolar sequestration of this antifolate, which might imply that even when operating suboptimally MRP-type transporters can make a significant contribution to the intracellular distribution of these and related compounds.

That said, there is still appreciable MTX uptake into the vacuoles of atmrp1-1 mutant protoplasts, and in vitro measurements of the uptake of [3H]MTX by vacuolar membrane-enriched vesicles purified from atmrp1-1 root cultures reveal considerable residual vanadate-inhibitable MgATP-dependent uptake. Knowing that heterologously expressed AtMRP4 is capable of high rates of PteGlu1 and MTX transport (17) and that at least a fraction of total cellular AtMRP4 may localize to the vacuolar membrane (19), there is a possibility that this transporter also contributes to the vacuolar uptake of folyl monoglutamates.

It is instructive to note that because physiological folates compete with antifolates for binding to their target enzymes, dihydrofolate reductase and FPGS, any factor that influences physiological folate pool size could affect antifolate efficacy. It is therefore conceivable that a diminution of vacuolar folate uptake, as might be the case for atmrp1 knock-out mutants, would confer MTX hypersensitivity by comparison with wild types not only because of a decrease in the capacity for vacuolar MTX sequestration but also because of a decrease in cellular folate pool size.

Of the many facets of vacuolar folate uptake that have yet to be resolved, one that is especially perplexing is the seeming localization of folate polyglutamates and γ-glutamyl hydrolase (GGH) to the same compartment (3). If this is correct and the activity of GGH in vivo approximates its activity in vitro, the vacuolar folate polyglutamate pools of Arabidopsis and red beet would be predicted to have half-lives of only ~7 s and 5 min, respectively, under steady state conditions (3). This paradox has yet to be reconciled, but a possibility that cannot be ignored is that it is not monoglutamates but instead polyglutamates that are transported into the vacuole in vivo by a non-MRP-type functionality, and that the former are derived from the latter intravacuolarly through the action of a vacuolar GGH, i.e. there may be parallels between what happens in plants and what happens in mammals, where lysosomes do not store folates but instead import folate polyglutamates, hydrolyze them, and export monoglutamates (45, 46). Alternatively, vacuolar folate polyglutamates are protected from intravacuolar hydrolysis through their interaction with FBPs or by the presence of a potent GGH inhibitor in this compartment. Although there is no experimental evidence for or against either possibility, the latter is the less likely in that the inhibition of vacuolar GGH would not only diminish the deglutamylation of folate polyglu-
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tamates but also the deglutamylation of p-amino benzoate polyglutamates, which would disrupt folate recycling (3). Another possibility, perhaps the most straightforward one, is that folate polyglutamates, or at least a sizeable fraction of this pool, and GGH do no reside in precisely the same vacuolar compartment but instead localize to different intravacuolar compartments or different subpopulations of vacuoles in the same cells or different cells within the same or different tissues.

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