Enhanced Multispecificity of Arabidopsis Vacuolar Multidrug Resistance-associated Protein-type ATP-binding Cassette Transporter, AtMRP2*

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Recent investigations have established that Arabidopsis thaliana contains a family of genes encoding ATP-binding cassette transporters belonging to the multidrug resistance-associated protein (MRP) family. So named because of the phenotypes conferred by their animal prototypes, many MRPs are MgATP-energized pumps active in the transport of glutathione (GS) conjugates and other bulky amphipathic anions across membranes. Here we show that Arabidopsis MRP2 (AtMRP2) localizes to the vacuolar membrane fraction from seedlings and is not only competent in the transport of GS conjugates but also glucuronate conjugates after heterologous expression in yeast. Based on the stimulatory action of the model GS conjugate 2,4-dinitrophenyl-GS (DNP-GS) on uptake of the model glucuronide 17β-estradiol-17-β-d-glucuronide (E\textsubscript{217}G) and vice versa, double-label experiments demonstrating that the two substrates are subject to simultaneous transport by AtMRP2 and preloading experiments suggesting that the effects seen result from cis, not trans, interactions, it is inferred that some GS conjugates and some glucuronides reciprocally activate each other's transport via distinct but coupled binding sites. The results of parallel experiments on AtMRP1 and representative yeast and mammalian MRPs indicate that these properties are specific to AtMRP2. The effects exerted by DNP-GS on AtMRP2 are not, however, common to all GS conjugates and not simulated by oxidized glutathione or reduced glutathione. Decyl-GS, metolachlor-GS, and oxidized glutathione, although competitive with DNP-GS, do not promote E\textsubscript{217}G uptake, neither competes with DNP-GS for uptake nor is subject to E\textsubscript{217}G-promoted uptake. A multisite model comprising three or four semi-autonomous transport pathways plus distinct but tightly coupled binding sites is invoked for AtMRP2.

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ATP-binding cassette (ABC)\textsuperscript{1} transporters are starting to assume prominence in considerations of energy-dependent transport in plants. Constituted of one or two copies each of two core structural elements: a hydrophobic, membrane spanning domain (MSD) containing multiple (usually four or six) transmembrane spans and a cytosolically oriented ATP-binding domain (nucleotide binding fold, NBF) containing Walker A, Walker B, and ABC signature sequence motifs, ABC transporters are MgATP-energized pumps that as a superfamily are active in the transport of a broad range of substances including alkaloids, amino acids, sugars and sugar conjugates, peptides and peptide conjugates, heavy metal chelates, and lipids across membranes (1).

Two classes of findings were instrumental in prompting studies of ABC transporters in plants. The first was the molecular cloning of a multidrug resistance (MDR)-like gene from Arabidopsis thaliana (2) and the subsequent independent isolation of other MDR homologs from the same and other plant species (3, 4). Because all of these genes encode ABC transporters bearing strong sequence similarities to one another and to the animal MDR gene products, it seemed likely that ATP-dependent, primary active transport functions, analogous to those catalyzed by the MDRs from other organisms, were also operative in plants. The second class of finding was that intact vacuoles and vacuolar membrane vesicles isolated from plants mediate the MgATP-dependent, H\textsuperscript{+} gradient-independent accumulation of glutathione (GS) conjugates (5, 6).

Of these two classes of finding, investigations of vacuolar GS conjugate transport have provided the most definitive elucidation of the molecular identity and biochemical function of a plant ABC transporter. Analyses of vacuolar GS conjugate uptake, a process implicated in herbicide detoxification, cell pigmentation, the alleviation of oxidative stress, and the storage of antimicrobial compounds, have established that the transporters responsible belong to the multidrug resistance-associated (MRP) family of ABC transporters (7, 8). Although several plant MDRs and their close relatives the pleiotropic drug resistance proteins have been cloned in their entirety (2–4, 9), the transport capabilities of the proteins encoded by these genes has eluded definition. AtPGP1, for instance, is the most thoroughly characterized MDR from a plant source, and

\textsuperscript{1} The abbreviations used are: ABC, ATP-binding cassette; \textit{Bn}-NCC-1, \textit{Brassica napus} nonfluorescent chlorophyll catabolite 1; C3G, cyanidin-3-glucoside; DNP-GS, 2,4-dinitrophenyl-glutathione; E\textsubscript{217}G, 17β-estradiol-17-β-d-glucuronide; GSH, reduced glutathione; GSSG, oxidized glutathione; MRP, multidrug resistance-associated protein; GS, glutathionyl; MSD, membrane spanning domain; NBF, nucleotide binding fold; MDR, multidrug resistance; PAGE, polyacrylamide gel electrophoresis; Mes, 4-morpholineethanesulfonic acid; AVP, \textit{Arabidopsis} vacuolar H\textsuperscript{+} pyrophosphatase.
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Elegant investigations of transgenic plants have shown this ABC transporter to be a plasma membrane protein involved in light-dependent hypocotyl elongation (10, 11), but its mode of action remains obscure.

To date, 15 MRP coding sequences have been identified in Arabidopsis of which five have been isolated as full-length cDNAs and shown to encode functional GS conjugate pumps. Transformation of Saccharomyces cerevisiae ycf1Δ strains from which more than 95% of the coding sequence of the yeast MRP gene YCF1 has been deleted (12), and high affinity MgATP-energized vacuolar GS conjugate transport is grossly impaired (13-15) with expression vectors containing the entire open reading frame of AtMRP1, AtMRP2, AtMRP3, AtMRP4, or AtMRP5 restores GS conjugate transport (16-18).

Despite this basic conformity of core function there are nevertheless strong indications of functional differences among the AtMRPs. Specifically, AtMRP1 and AtMRP2, the only two AtMRPs for which quantitative data are available, exhibit marked differences in transport capacity and substrate preference (17). AtMRP2 but not AtMRP1 is not only able to transport GS conjugates but also other bulky amphipathic anions, such as the linearized tetrapyrrolopyrrole Brassica napus nonfluorescent chlorophyll catabolite 1 (Bn-NCC-1), a product of chlorophyll breakdown (17).

The facility of AtMRP2 for the transport of Bn-NCC-1 is of particular interest in three respects. First, Bn-NCC-1, although not glutathionated, is the most efficacious known substrate for a plant GS conjugate pump (V_{max}/K_m = 4.2 nmol/mg/10 min/μm versus 1.8 nmol/mg/10 min/μm for the next most efficacious AtMRP2 transport substrate, metolachlor-GS; Ref. 17). Second, AtMRP2-dependent uptake of Bn-NCC-1 is nearly 3-fold less sensitive to inhibition by DNP-GS than would be predicted if Bn-NCC-1 and DNP-GS competed for a common binding site. Reciprocally, AtMRP2-dependent DNP-GS uptake is not inhibited appreciably by the inclusion of Bn-NCC-1 in the uptake medium (17). Third, double-label experiments demonstrate simultaneous parallel transport of [14C]Bn-NCC-1 and [3H]DNP-GS by AtMRP2 (17). Although it was initially surprising to find that AtMRP2 can transport both Bn-NCC-1 and GS conjugates, because the functionality, and by implication the pump, responsible for MgATP-energized Bn-NCC-1 uptake by barley vacuoles had earlier been concluded to be different from that responsible for GS conjugate uptake in that GS conjugates did not compete with Bn-NCC-1 for uptake (19), it is now clear, at least in the case of some AtMRPs, that lack of competition between candidate substrates does not automatically preclude their transport by the same transporter.

The general applicability of these findings is not known but a phenomenon that may be related is the capacity of DNP-GS for stimulating the uptake of flavone glucuronides and the model glucuronide 17β-estradiol 17-(β-d-glucuronide) (E217βG) into barley and rye vacuoles via an MRP-like transporter or transporters (20, 21). DNP-GS consistently promotes the uptake of glucuronides by both membrane preparations, whereas other GS derivatives, such as GSGS and decyl-GS, act as activators or inhibitors depending on the membrane source or glucuronide under investigation. A key question with regard to these findings and the known multispecificity of MRPs, such as AtMRP2, is whether the substrate interactions seen in isolated plant vacuolar membranes are a reflection of the properties of individual MRP species or instead reflect interactions between different MRPs or between MRPs and other vacuolar transporters.

In this paper we address this question in two steps. First, by determining whether AtMRP2 localizes to the vacuolar membrane fraction from seedlings of Arabidopsis. Second, by defining the transport characteristics of vacuolar membrane-enriched vesicles purified from AtMRP2-transformed yeast YCF1 disruptants. In so doing, we demonstrate that AtMRP2 is a vacuolar transporter that has a number of unusual properties that distinguish it from other MRPs and confer on it the capacity to account for the complex transport characteristics of native plant vacuolar membranes. It is shown that the multispecificity of heterologously expressed AtMRP2 is not limited to GS conjugates and linearized tetrapyrroles but also extends to glucuronides and that DNP-GS and E217βG mutually promote each other's uptake by interacting with distinct but coupled binding sites. Moreover, it is determined that although AtMRP2 is competent in the MgATP-energized transport of GSH and GSH promotes E217βG transport, the enhancements are not attributable to GSH-E217βG cotransport but instead to nonreciprocal GSH-mediated cis-activation of glucuronide uptake.

**EXPERIMENTAL PROCEDURES**

Heterologous Expression of AtMRP2 or AtMRP1 in S. cerevisiae and Spodoptera frugiperda Cells—For the preparation of transport-competent vacuolar membrane-enriched vesicles containing heterologously expressed AtMRP2 or AtMRP1, plasmids pYES3-AMRP2 and pYES3-AMRP1 were constructed as described (16, 17) and S. cerevisiae ycf1Δ strain DTY168 (MATa his6 leu2-3,112 ura3-52 ycf1::hisG) (12) was transformed with these or empty vector lacking AMRP2 or AMRP1 insert (pYES3) by the LiOAc/polyethylene glycol method (22). Transforms were selected for uracil prototrophy as described previously (23).

For the preparation of membranes containing high levels of heterologously expressed AtMRP2 or AtMRP1 for tests of the efficacy and specificity of antibodies raised against synthetic peptides, insect cell line Sf9, derived from S. frugiperda, was transfected with baculoviruses pVL1392-AMRP2 or pVL1392-AMRP1, containing the coding sequences of AtMRP2 or AtMRP1, respectively. The transfections were performed using a Baculogold Transfection Kit (PharMingen Co., San Diego, CA) according to the manufacturer's recommendations, and amplified recombinant virus was prepared by harvesting and pooling the culture supernatants from Sf9 cells 3 days post-infection.

Preparation of Membrane Vesicles—For the Western analyses shown in Fig. 1, the vacuolar membrane-enriched fraction from Arabidopsis (Col-0) seedlings after growth on Murashige-Skoog medium for 18 days under standard conditions was prepared by a modification of the procedure of Rea et al. (24). For the transport measurements on AtMRP2 and AtMRP1, pYES3-AMRP2/DTY168 or pYES3-AMRP1/DTY168 cells were grown and vacuolar membrane-enriched vesicles were prepared as described (16, 17). For the transport measurements on membranes from S. cerevisiae ycf1Δ strain DTY168 and the isogenic wild type strain DTY7 (12), vacuoles were isolated and vesiculated as described (13). For the transport measurements on heterologously expressed HmMRP3, membrane vesicles were purified from HmMRP3-transfected human embryonic kidney cell line 293 (HER293/MRP3-5 cells) and from empty vector control transfectants (HER293 cells) as described (25). For the routine preparation of insect cell membranes, Sf9 cells infected with amplified pVL1392-AMRP2 or pVL1392-AMRP1 were harvested 4 days post-infection, rinsed in phosphate-buffered saline, and subjected to homogenization, differential centrifugation, and density gradient centrifugation as described (26).

Preparation of Peptide-specific Antibody for Immunodetection of AtMRP2—The AtMRP2-specific rabbit polyclonal antibody used in these investigations (PAB_{AtMRP2}) was raised against synthetic peptide with

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2 R. Sánchez-Fernández, T. G. E. Davies, J. O. D. Coleman, and P. A. Rea, unpublished data.
3 R. Sánchez-Fernández and P. A. Rea, unpublished data.
4 N. Gaedeke, M. Klein, B. Müller-Rober, and E. Martinoia, unpublished data.
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the sequence AESLEEHNISR, corresponding to positions 1569–1579 of the deduced amino acid sequence of AtMRP2 (17). The peptide was synthesized to contain a C-terminal cysteine residue and was coupled to keyhole limpet hemocyanin as described (27). To maximize monospecificity, the antibody was affinity purified against the M, 176,000 band of the membrane vesicles from pVL1392-AtMRP2-infected yeast vacuolar membranes from pVL1392-AtMRP2-infected Sf9 cells (1.8 mg of protein) were subjected to preparative SDS-PAGE on a single-well, 1.5 mm, 7% (w/v) acrylamide slab gel and electrotransferred to nitrocellulose, and two narrow strips were cut from each of the filters and subjected to immunoreaction with crude PABAtMRP2 serum as described below. After visualization of the immunoreactive M, 176,000 band the nitrocellulose membranes were subjected to preparative SDS-PAGE on a single-well, 1.5 mm, 7% (w/v) acrylamide slab gel in the Bio-Rad mini-gel apparatus (28). In the case of the vacuolar membrane-enriched fraction from pYES3-AtMRP2/DTY168 cells, the samples were delipidated in a 1:1 (v/v) mixture of acetone-ethanol at −20 °C as described (29) before denaturation and SDS-PAGE to improve resolution in the high molecular weight range. For immunodetection of AtMRP2, the separated samples were electrotransferred to nitrocellulose membranes using a wet transfer system for 16 h at 4 °C at a current density of 3 mA/cm² in Towbin buffer (20% (v/v) methanol, 25 mM Tris, 192 mM glycine, 0.025% (w/v) SDS) (30). The nitrocellulose blots were probed with purified PABAtMRP2 (1:150) and with secondary horseradish peroxidase-conjugated anti-rabbit antibody (1:50,000) by standard procedures. To assess the enrichment of the membrane fractions from Arabidopsis seedlings, total microsomes and the vacuolar membrane fraction were probed with rabbit polyclonal antibody PAB TPK specific for the Arabidopsis vacuolar H+-pyrophosphatase (AVP), as described (31). Immunoreactive bands were visualized by ECL using the SuperSignaltm system (Pierce).

Measurement of Transport—Uptake of [3H]E217G, [3H]DNP-GS, [3H]GSSG, [14C]Metolachlor-GS, and [3H]GSH from pVL1392-AtMRP2/DTY168 cells and by vacuolar membrane vesicles purified from untransformed DTY168 or DTY7 cells was estimated as described above. For the preloading experiments, 100 µM of the model glucuronide was preincubated at 25 °C for 30 min in standard uptake medium containing 200 µM unlabeled DNP-GS before the addition of 200 µM [3H]E217G. After incubation for a further 10 min, uptake was terminated, and the radioactivity retained after filtration was estimated as described above.

Measurement of Protein—Protein was estimated by a modification of the method of Peterson (32).

Computations—Lines of best fit and kinetic parameters were estimated by nonlinear least squares analysis (30) using the Ultrafit nonlinear least squares analysis program (31). For the determination of the Michaelis–Menten parameters the kinetic parameters estimated by the nonlinear least squares analysis (30) were entered into the Lineweaver–Burk equation, and the reciprocal of initial rates were plotted as a function of the reciprocal of the substrate concentration.

Results

Vascular Membrane Localization of AtMRP2—AtMRP2 was recolocalized with the vascular membrane fraction from Arabidopsis. Regardless of whether Western blots of membranes from recombinant baculovirus pVL1392-AtMRP2-infected Sf9 cells or vascular membrane-enriched vesicles from yeast pYES3-AtMRP2/DTY168 cells (Fig. 1A) or from 18 day-old Arabidopsis seedlings were probed (Fig. 1B), polyclonal antibody PABAtMRP2 raised against the AtMRP2-specific peptide AESLEEHNISR (Fig. 1C), reacted with an M, 176,000 polypeptide species. In all cases the M, 176,000 polypeptide was the only species that reacted with PABAtMRP2, and in the case of membranes from Sf9 cells and yeast, this species was absent from uninfected and vector control cells, respectively (Fig. 1A). On this basis and because it did not react with membranes from Sf9 cells expressing the one other AtMRP, AtMRP1, known to contain the 2C domain encompassing the AtMRP2 sequence AESLEEHNISR (8) (data not shown), PABAtMRP2 was inferred to be monospecific. As would be expected if AtMRP2 is preferentially associated with the vascular membrane fraction from Arabidopsis, parallel Western analyses demonstrated a proportionate enrichment of AtMRP2 and the vacuolar H+-pyrophosphatase, AVP (28), in the vacuolar membrane fraction versus the total microsome fraction from seedlings (Fig. 1B). Moreover, in view of the proximity of the sequence recognized by PABAtMRP2 to the C terminus of AtMRP2 (Fig. 1C), the equivalence of the mobility of the AtMRP2 translation product in all three of the systems examined and, the greater than 97% correspondence between the measured M, of AtMRP2 (176,400 ± 5,400) and the calculated mass of the polypeptide encoded by the open reading frame of AtMRP2 (181 kDa) (17), AtMRP2 appeared to undergo incorporation into the vacuolar membrane fraction with little or no proteolytic processing.

AtMRP2-mediated Glucuronide Transport—AtMRP2 catalyzed glucuronide transport. The capacity of heterologously expressed AtMRP2 for glucuronide transport was assayed as described previously for GS conjugates and Bn-NCC-1 (17) except that the rapid filtration assays were performed using DuraPore (hydrophilic polysilvinydine fluoride) membrane filters instead of cellulose nitrate membrane filters to minimize background binding of this class of compound. S. cerevisiae yeast strain DTY168 was transformed with empty vector (pYES3) or vector containing the entire open reading frame of AtMRP2 (pYES3-AtMRP2) under the control of the constitutive yeast phosphoglycerate kinase gene (PGK) promoter (17). After growth on selective media, vascular membrane-enriched vesicles (23) were prepared from these and untransformed DTY168 cells and assayed for transport of the model glucuronide E176G.

From these experiments it was determined that AtMRP2 catalyzed the low affinity, high capacity MgATP-energized transport of [3H]E217G. When assayed at a concentration of 100 µM, MgATP-dependent uptake by vesicles purified from pYES3/DTY168 cells was approximately linear for the first 10 min.
Mutual Activation of AtMRP2 by Transport Substrates

The results of a screen of the effects of a broad range of GS conjugates demonstrated that at a sub-$K_m$ concentration of [$^{3}H$]E$_2$bG (100 $\mu$m) DNP-GS was the only GS conjugate that activated glucuronide uptake (Table I). Whereas 100 $\mu$m concentrations of GSSG and C3G-GS exerted little or no stimulatory effect on [$^{3}H$]E$_2$bG uptake while metolachlor-GS and decyl-GS inhibited, DNP-GS increased uptake by 3.8-fold versus controls assayed in media lacking GS conjugates (Table I). As confirmed by the finding that 100 $\mu$m concentrations of DNP-GS, GSSG, C3G-GS, and metolachlor-GS were transported at rates of 10.5, 21.3 S. cerevisiae ycf1 and 115.2 ± 18.1 nmol/mg/10 min in media containing DNP-GS, respectively. Individual data points are the means ± S.E. (n = 3–6).

![Fig. 2. Time dependence of MgATP-dependent uptake of [$^{3}H$]E$_2$bG by vacuolar membrane-enriched vesicles purified from pYES3-AtMRP2-transformed (●) and pYES3-transformed (○) S. cerevisiae ycf1A strain DTY168. MgATP-dependent uptake was measured in standard uptake medium containing 100 $\mu$m [$^{3}H$]E$_2$bG and was enumerated as the increase in uptake consequent on the provision of 3 $\mu$M ATP. Values shown are the means ± S.E. (n = 3–6).](image1.png)

![Fig. 3. Effect of DNP-GS on concentration dependence of MgATP-dependent [$^{3}H$]E$_2$bG uptake by vacuolar membrane-enriched vesicles purified from pYES3-AtMRP2/DTY168 cells. Uptake of the indicated concentrations of [$^{3}H$]E$_2$bG in the presence (●) or absence of 100 $\mu$m unlabeled DNP-GS (○) was measured as described in Fig. 1. The data for MgATP-dependent [$^{3}H$]E$_2$bG uptake were fitted to the Michaelis-Menten equation by nonlinear least squares analysis to yield $K_m$ and $V_{max}$ values of 752.3 ± 219.8 $\mu$m and 88.1 ± 7.2 nmol/mg/10 min in media lacking DNP-GS and values of 238.2 ± 79.2 $\mu$m and 115.2 ± 18.1 nmol/mg/10 min in media containing DNP-GS, respectively. Individual data points are the means ± S.E. (n = 3–6).](image2.png)
medium decreased the \( K_m \) for \(^{3}H\)E\(_{217}\)\(_{b}G\) uptake by 3.2-fold, from 752.3 ± 219.8 to 238.2 ± 79.2 \( \mu M \), while increasing \( V_{\text{max}} \) by only 1.3-fold, from 88.1 ± 17.2 to 115.2 ± 18.1 nmol/mg/10 min (Fig. 3).

\( E_{217}\)\(_{b}G\)-promoted \( DNP-GS \) Uptake—The interactions of \( DNP-GS \) and \( E_{217}\)\(_{b}G\) with AtMRP2 were mutual. Not only did \( DNP-GS \) promote the uptake of \(^{3}H\)E\(_{217}\)\(_{b}G\), but \( E_{217}\)\(_{b}G\) promoted the uptake of \(^{3}H\)DNP-GS. Inclusion of 100 \( \mu M \) \( E_{217}\)\(_{b}G\) in the uptake medium increased the \( K_m \) for \(^{3}H\)DNP-GS uptake by vacuolar membrane-enriched vesicles purified from pYES3-AtMRP2/DTY168 cells by 5.3-fold from 126.0 ± 52.6 \( \mu M \) to 20.1 ± 6.7 \( \mu M \) while decreasing \( V_{\text{max}} \) by only 1.3-fold from 23.6 ± 3.5 nmol/mg/10 min to 18.0 ± 1.1 nmol/mg/10 min (Fig. 4).

Some of the GS derivatives, as exemplified by decyl-GS, that did not promote AtMRP2-mediated \(^{3}H\)E\(_{217}\)\(_{b}G\) uptake instead inhibited it. By comparison with GSSG, for instance, which was a weak inhibitor and exerted only 20% inhibition at a concentration of 200 \( \mu M \), decyl-GS inhibited \(^{3}H\)E\(_{217}\)\(_{b}G\) uptake with an \( I_{50} \) of 3.8 ± 1.5 \( \mu M \) (Fig. 5).

In close agreement with what had been determined previously for \(^{3}H\)DNP-GS transport by AtMRP2 (17), Bn-NCC-1 only marginally inhibited \(^{3}H\)E\(_{217}\)\(_{b}G\) uptake (Fig. 5). At concentrations of less than 50 \( \mu M \), Bn-NCC-1 was a 4-fold less inhibitory than would be expected from its \( K_m \) for transport by AtMRP2 (15.2 ± 2.3 \( \mu M \)) (17). At concentrations of 50 \( \mu M \) or more, the inhibitions exerted by Bn-NCC-1 did not exceed 35% (Fig. 5). Because Bn-NCC-1 did not appreciably modulate the transport of either \( E_{217}\)\(_{b}G\) or \( DNP-GS \), it was not investigated further here.

Simultaneous Parallel Transport of \( E_{217}\)\(_{b}G\) and \( DNP-GS\)—\( E_{217}\)\(_{b}G\) and \( DNP-GS \) not only stimulated each other’s uptake but underwent simultaneous parallel AtMRP2-dependent transport. Sub-\( K_m \) (50 \( \mu M \)) concentrations of \(^{3}H\)E\(_{217}\)\(_{b}G\) and \(^{3}H\)DNP-GS were transported into vacuolar membrane-enriched vesicles purified from pYES3-AtMRP2/DTY168 cells at rates of 4.5 ± 1.1 and 8.9 ± 0.5 nmol/mg/10 min, respectively, when added singly to the uptake medium, at rates of 16.2 ± 1.4 nmol/mg/min and 13.6 ± 1.1 nmol/mg/10 min when 50 \( \mu M \) unlabeled DNP-GS was added to the \(^{3}H\)E\(_{217}\)\(_{b}G\) uptake medium and 50 \( \mu M \) unlabeled \( E_{217}\)\(_{b}G\) was added to the \(^{3}H\)DNP-GS uptake medium, respectively, and at an aggregate rate of 32.3 ± 1.8 nmol/mg/10 min when the uptake medium contained both 50 \( \mu M \) \(^{3}H\)E\(_{217}\)\(_{b}G\) and 50 \( \mu M \)^{3}H\)DNP-GS (Fig. 6). Thus, the rate of uptake when 50 \( \mu M \) concentrations of \(^{3}H\)E\(_{217}\)\(_{b}G\) and \(^{3}H\)DNP-GS were provided simultaneously was the sum of the activated rates of uptake (i.e. 32.3 = 16.2 + 13.6 = 29.8 nmol/mg/10 min), not the sum of the individual nonactivated rates of uptake (4.5 + 8.9 = 13.4 nmol/mg/10 min) (Fig. 6).

The simple additivity of the activated rates of uptake of \(^{3}H\)E\(_{217}\)\(_{b}G\) and \(^{3}H\)DNP-GS was instructive in two respects. First, it implied that \( E_{217}\)\(_{b}G\) and \( DNP-GS \) are the means ± S.E. (n = 3–6).

TABLE I

| GS conjugate (100 \( \mu M \)) | Uptake rate | \( E_{217}\)\(_{b}G \) |
|--------------------------|-------------|-----------------|
| None                     | 7.3 ± 0.3   | \( \text{nmol/mg/10 min} \) |
| DNP-GS                   | 27.5 ± 1.1  | \( \text{nmol/mg/10 min} \) |
| GSSG                     | 20.1 ± 4.6  | \( \text{nmol/mg/10 min} \) |
| C3G-GS                   | 8.9 ± 1.1   | \( \text{nmol/mg/10 min} \) |
| Metolachlor-GS           | 4.8 ± 0.8   | \( \text{nmol/mg/10 min} \) |
| Decyl-GS                 | 1.2 ± 0.1   | \( \text{nmol/mg/10 min} \) |

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For the measurements of AtMRP2-dependent GS conjugate uptake, vacuolar membrane-enriched vesicles purified from pYES3-AtMRP2/DTY168 cells were incubated in standard uptake medium containing 100 \( \mu M \) concentrations of \(^{3}H\)DNP-GS, \(^{3}H\)GSSG, \(^{3}C\)HGGS-G, or \(^{3}C\)metolachlor-GS. For the measurements of \(^{3}H\)E\(_{217}\)\(_{b}G\) uptake, pYES3-AtMRP2/DTY168 membranes were incubated in uptake medium containing 100 \( \mu M \) \(^{3}H\)E\(_{217}\)\(_{b}G\) plus or minus 100 \( \mu M \) concentrations of unlabeled DNP-GS, C3G-GS, GSSG, metolachlor-GS, or decyl-GS. MgATP-dependent uptake was measured as described in the legend to Fig. 1. Uptake rates for \( E_{217}\)\(_{b}G\) are the means ± S.E. (n = 3). Rates for AtMRP2-dependent uptake of GS-conjugates are the mean values taken from Lu et al. (17).

For the measurements of AtMRP2-dependent GS conjugate uptake, vacuolar membrane-enriched vesicles purified from pYES3-AtMRP2/DTY168 cells were incubated in standard uptake medium containing 100 \( \mu M \) concentrations of \(^{3}H\)DNP-GS, \(^{3}H\)GSSG, \(^{3}C\)HGGS-G, or \(^{3}C\)metolachlor-GS. For the measurements of \(^{3}H\)E\(_{217}\)\(_{b}G\) uptake, pYES3-AtMRP2/DTY168 membranes were incubated in uptake medium containing 100 \( \mu M \) \(^{3}H\)E\(_{217}\)\(_{b}G\) plus or minus 100 \( \mu M \) concentrations of unlabeled DNP-GS, C3G-GS, GSSG, metolachlor-GS, or decyl-GS. MgATP-dependent uptake was measured as described in the legend to Fig. 1. Uptake rates for \( E_{217}\)\(_{b}G\) are the means ± S.E. (n = 3). Rates for AtMRP2-dependent uptake of GS-conjugates are the mean values taken from Lu et al. (17).
The capacity of GSH to substitute for DNP-GS was examined at four levels: (i) by determining whether [3H]GSH was subject to MgATP-dependent transport by AtMRP2 and able to promote [3H]E217β uptake; (ii) by determining whether GSH promoted [3H]E217β uptake; (iii) by determining whether E217β promoted [3H]GSH uptake; and (iv) by determining whether GSH competed with [3H]DNP-GS for uptake. If the effects of DNP-GS were explicable in terms of its capacity to substitute for GSH in cotransport processes analogous to those inferred for HmMRP1 and cMOAT, criteria i–iv would be expected to apply.

**TABLE II**

| Preloading medium | Rate of [3H]E217β/glip uptake | nmol/mg/10 min |
|-------------------|-------------------------------|----------------|
| −DNP-GS           | 45.4 ± 2.7                    | 44.8 ± 1.4     |
| + DNP-GS          |                               |                |

Fig. 6. Simultaneous MgATP-dependent uptake of [3H]E217βG and [3H]DNP-GS into vacuolar membrane-enriched vesicles purified from pYES3-AtMRP2/DTY168 cells. Uptake of the indicated concentrations of [3H]E217βG and/or [3H]DNP-GS in media lacking or containing unlabeled E217βG (E217βG) or unlabeled DNP-GS ([3H]DNP-GS) was measured as described in the legend to Fig. 1. [3H]E217βG and [3H]DNP-GS were added to the uptake medium at equivalent radiospecific activities. Values shown (uptake rates, nmol/mg/10 min) are the means ± S.E. (n = 3).

**Fig. 7.** Time dependence of MgATP-dependent uptake of [3H]GSH by vacuolar membrane-enriched vesicles purified from pYES3-AtMRP2/DTY168 (○ and □) or pYES3/DTY168 cells (●) measured in the presence (●) or absence of E217βG (100 μM) (○ and □). Uptake of [3H]GSH (1 mM) from standard uptake medium was measured as described in the legend to Fig. 1. Values shown are the means ± S.E. (n = 3).

Direct tests of the applicability of these criteria showed that GSH was indeed amenable to MgATP-dependent transport by AtMRP2 and able to promote [3H]E217βG uptake. Vacuolar membrane-enriched vesicles purified from pYES3-AtMRP2/DTY168 cells catalyzed the MgATP-dependent uptake of 1 mM [3H]GSH at a rate of 2.2 nmol/mg/min, whereas uptake by the equivalent membrane fraction from pYES3/DTY168 cells was negligible (Fig. 7). Addition of 0.5–10 mM concentrations of GSH to the standard uptake medium promoted the MgATP-dependent uptake of 100 μM [3H]E217βG by pYES3-AtMRP2/DTY168 membranes with a Michaelian concentration dependence consistent with Keq and Vmax values of 4.7 ± 1.3 mM and 32.8 ± 10.1 nmol/mg/10 min, respectively (Fig. 8A). Moreover, none of these effects were either attributable to the redox action of GSH or could be simulated by other precursors of the conjugate concerned. The redox-inactive GSH derivative S-methylglutathione (S-methyl-GS) more than 50% substituted for GSH in promoting [3H]E217βG uptake, but the redox-active thiol dithiothreitol did not (Fig. 8A). None of the other precursors of DNP-GS and E217βG, 1-chloro-2,4-dinitrobenzene, glucuronate, or β-estradiol, promoted [3H]E217βG or [3H]DNP-GS uptake (Table III), implying that the effects seen with GSH were specific to this compound. However, in direct contradiction to what would be predicted if GSH and DNP-GS were interexchangeable and GSH and E217βG were subject to cotransport, the uptake of [3H]GSH was not promoted by E217βG but instead inhibited (Fig. 7), and GSH did not compete with [3H]DNP-GS for uptake. Inclusion of 100 μM E217βG in the uptake medium almost totally abolished the MgATP-dependent uptake of 1 mM [3H]GSH (Fig. 7), and neither GSH nor S-methyl-GS competed with 100 μM [3H]DNP-GS for uptake (Fig. 8B).

The finding that the concentrations of DNP-GS required to promote [3H]E217βG uptake were 1–2 orders of magnitude lower than those required for the transport of DNP-GS itself further confirmed that the promotion of [3H]E217βG uptake by GSH or S-methyl-GS did not have the properties expected of symport. The concentrations of DNP-GS sufficient for half-maximal stimulation of the uptake of 50, 100, and 200 μM [3H]E217βG were only 7.2, and 1 μM, respectively (Fig. 9); the corresponding values for half-maximal MgATP-dependent [3H]DNP-GS uptake by pYES3-AtMRP2/DTY168 membranes in the presence or absence of 100 μM E217βG were 20 and 125 μM or greater, respectively (Fig. 4).

**Other MRPs**—The capacity of DNP-GS and E217βG to promote each other’s uptake was a feature peculiar to AtMRP2. None of the other MRPs examined, Arabidopsis AtMRP1, yeast...
YCF1, and human MRP3 (HmMRP3), exhibited the same behavior despite their capacities for the transport of DNP-GS and E17βG individually. MgATP-dependent uptake of a $K_m$ (386 μM) concentration of $[^3H]$E17βG by wild type DTY168 yeast vacuolar membrane vesicles was 4.3-fold greater than that mediated by membranes purified from ycf1Δ strain DTY168 (14.9 ± 2.6 versus 3.5 ± 0.7 nmol/mg/10 min) but insignificantly affected by the addition of a $K_m$ (5.9 μM) concentration of DNP-GS to the uptake medium (Fig. 10A), and addition of a $K_m$ concentration of E17βG simply diminished $[^3H]$DNP-GS uptake by wild type membranes from 16.5 ± 1.2 to 9.2 ± 0.5 nmol/mg/10 min (Fig. 10A). MgATP-dependent uptake of 100 μM $[^3H]$E17βG by vacuolar membrane-enriched vesicles purified from pYES3-AMRP1/DTY168 cells was appreciable by comparison with the near-zero values measured for the corresponding membrane fraction from pYES3/DTY168 cells (0.7 ± 0.1 versus 0.0 ± 0.1 nmol/mg/10 min), yet the addition of 100 μM DNP-GS to the uptake medium largely abolished $[^3H]$E17βG uptake (Fig. 10B). MgATP-dependent uptake of $[^3H]$DNP-GS by the same membranes was unaffected or only weakly inhibited by the addition of E17βG. Although the MgATP-dependent uptake of a $K_m$ (25 μM) concentration of $[^3H]$E17βG (25) by membrane vesicles purified from HmMRP3-transfected HEK293 cells was almost exclusively attributable to HmMRP3 in that the same membrane fraction from empty vector-transfected cells catalyzed little or no uptake (Fig. 10C), addition of a $K_m$ concentrations of DNP-GS to the

**Table III**

| Precursor   | Rate of MgATP-dependent uptake $[^3H]$E17βG | $[^3H]$DNP-GS |
|-------------|-------------------------------------------|---------------|
| Control     | 11.7 ± 0.8                                | 12.8 ± 0.8    |
| CDNB        | 7.5 ± 1.0                                  | 10.8 ± 0.7    |
| 100 μM      | 6.2 ± 0.5                                  | 11.4 ± 1.9    |
| β-Estradiol | 10.0 ± 0.8                                 | 10.6 ± 1.1    |
| Glucurionate| 10.8 ± 2.1                                 | 10.9 ± 1.0    |
| 5 mM        | 11.7 ± 1.0                                 | 10.4 ± 0.6    |
uptake medium did not promote MgATP-dependent uptake (Fig. 10C).

**DISCUSSION**

The results of the experiments described here show that AtMRP2 has the properties of a vacuolar ABC transporter. AtMRP2 localizes to the vacuolar membrane-enriched fraction from *Arabidopsis* seedlings. Heterologously expressed AtMRP2, like native plant vacuolar membranes (20, 21), is not only competent in the transport of GS conjugates and linearized tetrapyrroles (17) but also glucuronides as exemplified by \( E_{17} \beta G \). \( E_{17} \beta G \) and DNP-GS promote each other’s uptake into both yeast vacuolar membrane-enriched vesicles containing heterologously expressed AtMRP2 and into isolated plant vacuoles.

It is now established that many GS conjugates and other amphipathic anions are accumulated in the vacuolar compartment of protoplasts and intact plant cells. In *situ* cytosolic glutathionation of monochlorobimane to its fluorescent derivative, bimane-GS, and accumulation of the latter in the vacuoles of protoplasts and cell suspension cultures has been demonstrated (37) as has a greater than 50-fold accumulation of alachlor-GS in the vacuoles of intact barley leaves (38). Likewise, it is evident that an MRP-like functionality is responsible for the vacuolar uptake of both glutathionated and nonglutathionated dyes by barley aleurone cells (39). However, except for one isolated report of \( M_r \ 170,000 \) band that reacts with antibody raised against a synthetic peptide corresponding to a wheat MRP homolog that is enriched in the vacuolar membrane fraction from *Arabidopsis* is indeed AtMRP2. AtMRP2 is the only MRP, except for AtMRP1, in the *Arabidopsis* genome data base that contains a C2 domain, and although AtMRP1 contains a C2 domain motif (EDSLQNSDISR) resembling the motif against which PAB\(_{AMRP2}\) was raised, this antibody does not react with Sf9 cell-expressed AtMRP1.

Although the capacity of AtMRP2 for the transport of GS conjugates, linearized tetrapyrroles, and glucuronides establishes a basis for the molecular manipulation of three previously identified ABC transporter-like activities in plant vacuoles, it refutes our earlier proposal that AtMRP2 is constituted of two functionally distinguishable semi-autonomous modules residing in different half-molecules (17). A simple two-module model can neither explain how some transport-active GS conjugate for it to stimulate \( E_{17} \beta G \) uptake; although DNP-GS and \( E_{17} \beta G \) promote \( E_{17} \beta G \) uptake; although DNP-GS and \( E_{17} \beta G \) are considered to be transported via semi-autonomous pathways. Other GS conjugates such as metolachlor-GS and C3G-GS compete with DNP-GS for transport. \( E_{17} \beta G \) promotes DNP-GS transport and DNP-GS promotes \( E_{17} \beta G \) transport by interacting with sites distinct from but coupled to the DNP-GS and \( E_{17} \beta G \) transport pathways, respectively. GSH and S-methyl-GS promote \( E_{17} \beta G \) transport by interacting with different sites. Decyl-GS and GSSG compete with DNP-GS for binding to the site responsible for promoting \( E_{17} \beta G \) transport. \( E_{17} \beta G \) blocks the transport of GSH.

**Fig. 11.** Schematic diagram depicting the interactions of \( E_{17} \beta G \) and different GS derivatives with AtMRP2. DNP-GS, GSH, \( Bn-NCC-1 \), and \( E_{17} \beta G \) are considered to be transported via semi-autonomous pathways. Other GS conjugates such as metolachlor-GS and C3G-GS compete with DNP-GS for transport. \( E_{17} \beta G \) promotes DNP-GS transport and DNP-GS promotes \( E_{17} \beta G \) transport by interacting with sites distinct from but coupled to the DNP-GS and \( E_{17} \beta G \) transport pathways, respectively. GSH and S-methyl-GS promote \( E_{17} \beta G \) transport by interacting with different sites. Decyl-GS and GSSG compete with DNP-GS for binding to the site responsible for promoting \( E_{17} \beta G \) transport. \( E_{17} \beta G \) blocks the transport of GSH.

Analogies may therefore be drawn with Lmr(A), a half-molecule ABC transporter from *Lactococcus lactis* that transports drugs with kinetics consistent with cooperative interactions between two or more nonidentical sites (41) and with mammalian P-glycoprotein (MDR) whose kinetic properties implicate a multisite drug-binding model (42).

Of the various models that might be proposed for how \( E_{17} \beta G \) and DNP-GS interact, those involving counter-transport and/or trans-activation are the least plausible inasmuch as stimulation of \( E_{17} \beta G \) uptake into vacuolar membrane-enriched vesicles containing AMRP2 by the addition of DNP-GS to the uptake medium is instantaneous and not enhanced or simulated by preloading the vesicles with DNP-GS. Similarly, a model based exclusively on the cotransport of \( E_{17} \beta G \) and DNP-GS is not capable of explaining all of the characteristics of AtMRP2-mediated transport. Transportability is not the sole criterion to be satisfied by a GS conjugate for it to stimulate \( E_{17} \beta G \) uptake; GS conjugates, other than DNP-GS, are subject to high rates of transport but do not promote \( E_{17} \beta G \) transport. Transport of DNP-GS per se does not appear to promote \( E_{17} \beta G \) uptake; although DNP-GS and \( E_{17} \beta G \) undergo parallel simultaneous transport by AtMRP2, the concentrations of DNP-GS required for the half-maximal promotion of \( E_{17} \beta G \) transport are 1–2 orders of magnitude lower than those required for half-maximal net transport of DNP-GS, itself. Likewise, an extension of the \( E_{17} \beta G \)-DNP-GS cotransport model, namely that DNP-GS promotes \( E_{17} \beta G \) transport by simulating GSH as a cotransported species is also excluded by the nonreciprocal nature of the interactions between GSH and \( E_{17} \beta G \). GSH and its S-methylated, redox-inactive derivative, S-methyl-GS, promote AtMRP2-mediated \( E_{17} \beta G \) transport but \( E_{17} \beta G \), rather than promoting GSH transport, inhibits it. Evidently, GSH, like DNP-GS, promotes AtMRP2-mediated \( E_{17} \beta G \) transport by interacting with a site different from that involved in its own transport. As implied by the inability of both GSH and S-methyl-GS to compete with DNP-GS for uptake, GSH, in turn, appears to be transported via a pathway distinct from that for DNP-GS.

A tentative scheme capable of accounting for the properties of AtMRP2 is depicted in Fig. 11. According to this scheme: (i) \( E_{17} \beta G \), DNP-GS, GSH, and by implication \( Bn-NCC-1 \) undergo transport via different AtMRP2-dependent pathways; (ii)
E_{17}\beta G and DNP-GS promote each other’s transport by binding sites distinct from but tightly coupled to the other’s transport pathway; (iii) GSH and S-methyl-GS, although able to promote E_{17}\beta G transport by DNP-GS and (iv) decyl-GS, and to a lesser extent GSSG, although able to compete with DNP-GS for the site responsible for promoting E_{17}\beta G transport are themselves not able to promote E_{17}\beta G transport.

One of the most striking findings to come from these investigations is an appreciation of how unusual AtMRP2 is in its susceptibility to mutual activation by E_{17}\beta G and DNP-GS. All of the other MRPs examined, regardless of source, catalyze both E_{17}\beta G and DNP-GS transport, but none catalyzes E_{17}\beta G transport in a DNP-GS-stimulated manner. On the contrary, E_{17}\beta G transport by AtMRP1, YCF1, and HmMRP3 is either insensitive to or only slightly inhibited by DNP-GS. Even by comparison with its 94% sequence similar homolog AMRP1 (17), AtMRP2 is unique among the MRPs in terms of its facility for the transport of some substrates cooperatively and others semi-autonomously.

The association of AtMRP2 with multiple semi-autonomous transport pathways and the facility of some substrates to promote the transport of others extends considerably our appreciation of the processes that may converge on this transporter. In the case of GS conjugate and tetrapyrrole transport, the processes that may depend on AtMRP2 and its equivalents not only have the potential of providing a molecular basis for the vacuolar uptake of GS conjugates and glucuronides but also for cross-talk between the GSH-dependent and glucuronate-dependent detoxification pathways in plants.

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