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Minireview

The multidrug resistance-associated protein (MRP/ABCC) subfamily of ATP-binding cassette transporters in plants

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Abstract **In many different plant species, genes belonging to the multidrug resistance-associated protein (MRP, ABCC) subfamily of ABC transporters have been identified. Following the discovery of vacuolar transport systems for xenobiotic or plant-produced conjugated organic anions, plant MRPs were originally proposed to be primarily involved in the vacuolar sequestration of potentially toxic metabolites. Indeed, heterologous expression of different *Arabidopsis* MRPs in yeast demonstrates their activity as ATP-driven pumps for structurally diverse substrates. Recent analysis of protein–protein interactions and the characterization of knockout mutants in *Arabidopsis* suggests that apart from transport functions plant MRPs play additional roles including the control of plant transpiration through the stomata. Here, we review and discuss the diverse functions of plant MRP-type ABC transporters and present an organ-related and developmental analysis of the expression of *Arabidopsis MRPs* using the publicly available full-genome chip data.**

Keywords: Transport; Detoxification; Guard cell regulation; Vacuole; Tonoplast; Plasma membrane

1. Introduction

Multidrug resistance-associated proteins (MRPs) were originally identified in drug-resistant human lung cancer cell lines (HsMRP1; ABCC1, [1]). Consequently, ABCC1-mediated ATP-dependent extrusion of organic anions such as leukotriene C4 and other related glutathione conjugates (GS-X) was demonstrated [2]. Structurally, MRPs and multidrug resistance proteins (MDRs) exhibit the same arrangement of domains (two membrane-spanning domains called TMDs, each followed by a cytosolic loop termed nucleotide binding fold (NBF) which contains the Walker A and B motives together with the characteristic ABC signature). They differ in the N-terminal region where an extremely hydrophobic N-terminal extension (NTE or TMD0) of around 220 amino acids is observed for the MRPs but not for MDRs. This TMD0 is connected via a cytosolic, so-called CL3 loop to the remaining protein. For some MRPs it has been shown that TMD0 is required for correct targeting and a recent study on ABCC1 demonstrates that this domain is essential for trafficking or retention of the protein towards/in the plasma membrane [3]. A study of the membrane topology of human MRP employing glycosylation-site mutants indicated that the hydrophobic TMD0 forms a third membrane-spanning domain of five transmembrane α-helices and that the N-terminus is most likely facing the extracellular side of the membrane bilayer [4]. Hydrophathy plots of animal and plant MRPs resemble each other to great extend, suggesting a similar arrangement of the membrane-spanning domains in animals and plants [5].

The first evidence for the presence of MRPs in plants was the observation that glutathionated compounds are transported into the vacuole by direct ATP-dependent energization and independently of the proton motif force [6], suggesting that these transporters are an integral part of the plant detoxification mechanism [7]. Since plants have no efficient system to excrete compounds to the environment, they have to store potentially toxic compounds within the large central vacuole which has only a very limited metabolic activity. Detoxification in plants and animals occurs by functionally similar enzymatic and transporter steps with the difference that the 'extrusion' pumps are located on different membranes. Recent investigations revealed that plant MRPs are not only implicated in detoxification but also in guard cell regulation [8,9]. Since the function of most MRPs has still to be elucidated, additional functions may be discovered in the near future.

2. The *MRP* gene families in *Arabidopsis* and rice

Several papers have analyzed the genome of *Arabidopsis* and rice with respect to MRPs [10–15]. Very similar results were obtained either calculating phylogenetic trees with the complete protein sequences or with parts consisting of the N- or the C-terminal regions containing the NBDs only. Fifteen *MRPs* have been identified in *Arabidopsis*, one possibly being a pseudogene (*AtMRP15*). In order to complete the list of rice *MRP* genes we have performed separate BLAST searches with all annotated *Arabidopsis* MRP protein sequences against the TIGR Rice genome database (annotation version 3.0; BLASTP). All identified rice sequences exhibiting homology to *Arabidopsis* MRPs were again separately compared with the rice genome (BLASTP). Several rice sequences with only low homology to MRPs were after comparison to the *Arabidopsis* genome identified as non-MRP-type ABC transporters resulting in a total of 17 or 18 rice MRPs. In Table 1, we present

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Table 1
Proposed nomenclature for the 17 rice MRP genes

O. sativa short name	TIGR ORF	Number of introns	Lengths		Number of transmembrane helices in TMD			Next ORF	
			gDNA (nt)	*Protein* (Aa)	*TMD0*	*TMD*1	*TDM*2	Upstream	Downstream
OsMRP1	Os04g52900	28	11 746	1704	5	5	6	Hypothetical protein	Hypothetical protein
OsMRP2	Os01g67580	11	6889	1432	2 (3)	4 (5)	6	Similar to F12A21.16	Hypothetical protein
OsMRP3	Os01g07870	9	6624	1555	5	5 (6)	6	Retrotransposon protein, putative, unclassified	bZIP transcriptionsfactor
OsMRP4	Os02g18700	11	8224	1236	–	4 (5)	3	Hypothetical protein	BURP domain, putative
OsMRP5	Os02g18670	11	5802	1224	–	5	5	Hypothetical protein	Neurofilament triplet m protein
OsMRP6	Os04g49890	12	7743	1225	2	2 (3)	4 (5)	Hypothetical protein	OsMRP7
OsMRP7	Os04g49900	10	7419	1360	–	5 (6)	4	OsMRP6	Retrotransposon protein, putative, unclassified
OsMRP8a	Os01g25390	3	8257	764	5	4 (6)	–	Transposon protein, putative, CACTA, En/Spm sub–class	OsMRP8b
OsMRP8b	Os01g25380	7	3958	824	–	–	6	OsMRP8a	Ulp 1 protease family
OsMRP9	Os04gl3210	11	11 907	1620	5	5 (6)	5	OsMRP10	Hypothetical protein
OsMRP10	Os04g13220	10	8512	1562	5	4 (5)	6	Hypothetical protein	OsMRP9
OsMRP11	Os06g36650	11	5722	1233	–	3 (5)	4	Transposon protein, putative, CACTA, En/Spm sub-class	Retrotransposon protein, putative, unclassified
OsMRP12	Os06g08560	33	15 423	1544	5 (6)	3 (4)	6 (7)	Transposon protein, putative, mutator sub-class	Expressed protein
OsMRP13	Os03g04920	10	6875	1555	4	6 (7)	5 (6)	Hypothetical protein	Expressed protein
OsMRP14	Os05g10730	11	6652	1522	5	5 (6)	6	Hypothetical protein	Ndr family
OsMRP15	Os06g06440	10	8411	1522	5	4 (6)	6	U-box domain, putative	Expressed protein
OsMRP16	Os11g05700	10	7767	1522	5	4 (6)	5	Hypothetical protein	amino acid transporter-like protein
OsMRP17	Os12g37580	11	6011	1267	–	4 (5)	5	Protein kinase domain, putative	Prefoldin subunit 3, putative

The nomenclature for OsMRP1 to OsMRP12 is in accordance with [13]. Transcription unit/gene model identifiers used by Garcia et al. have been replaced by locus identifiers released by The Institute of Genomic Research (Rice Genome Annotation (Version 3; http://www.tigr.org/tdb/e2k1/osa1/)) by blasting the TIGR ORF's to the rice database. In order to identify the definitive number of rice MRPs all *Arabidopsis* MRP protein sequences were compared to the rice genome database using BLASTP followed by BLASTP searches of all significant rice hits against the rice genome. Final verification was performed using the Aramemnon web interface (http://aramemnon.botanik.uni-koeln.de; see [72]) with the TIGR ORFs as input and searches for homologues heterospecific genes. Apart from sequence alignements, Aramemnon returns consensus transmembrane topology alignements integrating results from several hydrophobicity predicition programs. The comparison to the known *Arabidopsis* MRPs was used to identify the position of TMDs and the number of transmembrane helices in each TMD. The number of transmembrane helices given in brackets include predicted hydrophobic segments with a hydrophobicity consensus score lower than 0.5.

a nomenclature for the rice *MRP* genes which extends an existing but incomplete nomenclature [13] together with annotations of up- and downstream genes. Notably, two genes *OsMRP8a* and *OsMRP8b* are annotated as separate ORFs oriented in a head-to-tail configuration with 2 kb of intergenic region between these ORFs. As separate units, each ORF codes for a truncated MRP-type protein. Both ORFs exhibit highest homology to *AtMRP4* and *OsMRP8a* aligns to the TMD0 and TMD1 regions of *AtMRP4* while *OsMRP8b* exhibits highest homology to NBD1, TMD2 and NBD2 of *AtMRP4*. Protein sequence signature searches confirmed that only OsMRP8b contains the ABC signature of NBD1. We therefore propose that OsMRP8a and OsMRP8b encode a single MRP protein and that the ORFs are separated by a ~2 kb intron. This suggestion is supported by different alternative gene models including the 'Rice Interim Structural Annotation Update'.

The *Arabidopsis* and rice genomes possess a large clade II. Since no mammalian or fungal MRPs cluster with the clade II this subclade represents a plant-specific lineage. This clade contains nine (10 including *AtMRP15*) and 15 *MRP*-genes in *Arabidopsis* and rice, respectively. In clade I four members, *AtMRP1*, *2*, *11* and *AtMRP12* have been identified in *Arabidopsis* while only a single rice gene belongs to this clade. Interestingly, if plant genomes are compared corresponding orthologues group together with more than one member either from *Arabidopsis* or rice (Table 2), possibly reflecting functional redundancy. However, two exceptions exist: (i) *AtMRP5*/*OsMRP13* are represented by only one member in both genomes. AtMRP5 is known to regulate guard cell movement and it is tempting to speculate that such a central function has to be tightly controlled by one gene product. (ii) In both plants one gene, *AtMRP13* and *OsMRP12* do not group with both clades. These two *MRPs* lack the N-terminal extension typical for other *MRPs* but nevertheless belong to the MRP gene family due to sequence homology. A 'truncated' MRP is not unique for plants: the yeast Yor1, which has been shown to confer resistance to oligomycin [16] also lacks the TMD0. Rice and *Arabidopsis* are distantly related plants. Therefore, it can be postulated that in each plant genome members of individual subgroups exist. This assumption is supported by sequence alignements using tomato, soybean and maize EST sequences [17]. Interestingly, the current rice genome annotation suggests that five *MRP* genes code for proteins lacking the TMD0 structurally resembling *AtMRP13* (Table 1). Deletion of the TMD0 in different MRPs did not result in catalytically inactive transport proteins [3]. However, the gene models vary when different gene prediction algorithms are applied demonstrating that cloning of the corresponding cDNAs is necessary to assure the absence of the TMD0 in all of these cases.

Table 2
Comparison of subgroups of *Arabidopsis* MRP genes with genes identified from rice and maize demonstrates that orthologs exist in each subgroup

Arabidopsis thaliana	*Oryza sativa*/*Zea mays*
Clade I	
AtMRP1; AtMRP2; AtMRP11; AtMRP12	OsMRP1
Clade II	
AtMRP3; AtMRP6; AtMRP7	OsMRP3; OsMRP4; OsMRP5/ZmMRP2
AtMRP4; AtMRP14	OsMRP8; OsMRP9; OsMRP10
AtMRP5	OsMRP13
AtMRP8	OsMRP11; OsMRP14/ZmMRP1
AtMRP9; AtMRP15*	OsMRP2; OsMRP6; OsMRP7
AtMRP10	OsMRP15; OsMRP16; OsMRP17/ZmMRP3
AtMRP13	OsMRP12

AtMRP15 marked with an asterisk putatively is a pseudogene. The AtMRP nomenclature used was proposed by [11].

Notably, the number, positions and phases of introns are highly conserved in plant MRPs. MRPs of clade II from *Arabidopsis* contain no introns in the TMD0 and TMD1. A similar picture is observed in rice, where no or only one to three introns are present in this region. In the region between the first NBD and the C-terminus 8–10 introns in defined positions can be found in rice and *Arabidopsis*. Clade I MRPs contain a 26–27 introns spread over the complete gene.

3. Molecular characterization of plant MRP genes confirms and extends the functional spectrum for plant MRPs

3.1. Analysis of transport characteristics of Arabidopsis MRP transporters

In spite of the availability of genome data and MRP sequences in model plants such as *Arabidopsis*, rice or poplar and in spite of successful cloning of plant *MRP* coding sequences from *Arabidopsis*, maize or wheat [8,10,18–24] only five plant MRPs from *Arabidopsis* (AtMRP1 to 5) have been investigated with respect to their transport characteristics and their action as typical MRP-type ATP-dependent pumps for organic anions has been confirmed [8,18–20,24]. In all of these cases, *Arabidopsis* MRPs were heterologously expressed in *Saccharomyces cerevisiae* lacking either the major vacuolar GS-X pump Yeast cadmium factor1 (Ycf1p) or Ycf1 in combination with the Bile pigment transporter 1 (Bpt1p) and transport experiments were performed either with microsomal vesicles or with vacuolarmembrane enriched vesicles. Ycf1p represents the major vacuolar GS-X pump and at the same time confers cadmium tolerance to yeasts by its action as a vacuolar transporter for bis(glutathionato)cadmium complexes [25–27]. Although *S. cerevisiae* contains six *MRP* subfamily members, Δycf1Δbpt1 yeasts are devoid of any detectable ATP-dependent GS-X transport activity making this strain an ideal system for glutathione conjugate transport experiments with a minimal background activity [28,29].

Research on heavy metals has attracted much interest in the last decade. Besides academic interest this is due to toxic effects of these metals when entering the food chain. At the same time some heavy metals such as iron and zinc are micronutrients which are often present in limiting amounts in soils. The practical goal of plant scientists is therefore to produce plants with improved uptake of essential micronutrients but only a limited amount of toxic heavy metals. Engineering plants which extract large amounts of heavy metals from the soil and accumulate them in their shoot will help to decontaminate polluted areas. With respect to MRPs, heavy metal tolerance and increased accumulation could be engineered in plants by ectopic expression of *Ycf1* in *Arabidopsis* [30]. Transgenic plants exhibited a strongly increased bis(glutathionato)cadmium transport activity, were more tolerant to cadmium and lead and accumulated up to 2.5-fold more cadmium. These results suggest that vacuolar transport is a limiting factor in heavy

metal resistance in plants and that modulation of the heavy metal uptake capacity in vacuoles improves accumulation and tolerance. In plants, the major Cd-complexing agents are phytochelatins which after complexing Cd end up in the vacuole. However, although the structure of phytochelatins as glutathione derivatives and the demonstrated capacity of MRPs as bis(glutathionato)cadmium pumps theoretically suggest that MRPs could also transport Cd-phytochelatin complexes, data from fission yeast demonstrate that the vacuolar half-size ABC transporter Hmt1p is capable of transporting phytochelatins, both as apo-phytochelatin as well as the Cd-phytochelatin complex [31]. In *Arabidopsis*, ABC transporters belonging to the half-molecule 'ATM' family [10] exhibit highest similarity to ScHmt1. However, their function as phytochelatin pumps has not been established.

Expression of AtMRP1-3 in Δycf1 yeasts resulted in MgATP-dependent uptake of GS-X into yeast vesicles [18–20]. Typically, transport of the model glutathione conjugate *S*-(2,4-dinitrophenyl)glutathione (DNP-GS) was not energized by non-hydrolysable MgAMPPNP, inhibited by *ortho*-vanadate which acts as a metastable analog of inorganic phosphate (reported I_{50} values for AtMRP1 and 2 are 8 and 6 μM, respectively; see [18,19]) and insensitive as well to the vacuolar H^+-ATPase inhibitor bafilomycin A1 as proton uncouplers such as gramicidin D or FCCP. The latter finding clearly demonstrates that energization of GS-X transport does not occur via a pre-existing pH gradient established by vacuolar proton pumps. The K_m values for DNB-GS transport in vesicles isolated from Δycf1 yeasts transformed with AtMRP1-3 ranged between 60 and 90 μM (Table 3). When kinetic parameters of the two closely related AtMRPs 1 and 2 were compared, the K_m values for DNB-GS and metolachlor-GS were comparable while the K_m for oxidized glutathione was 3-fold higher for AtMRP1 than for AtMRP2 (220 vs. 73 μM, respectively; see [19]). Since the V_{max} for all substrates analyzed was clearly greater for AtMRP2 than for AtMRP1, AtMRP2 appears to represent a GS-X pump with higher catalytic capacity when compared to AtMRP1. In spite of their close homology reflected by 87% of amino acid identity [12], AtMRP2 expressed in yeast displayed an enhanced multispecificity which could not be found for AtMRP1. First, AtMRP2 but not AtMRP1 was able to transport *Bn*-NCC1, an *O*-malonylated chlorophyll catabolite of *Brassica napus* with high affinity (K_m of 15 μM; see [19]). Interestingly, *Bn*-NCC1 and DNP-GS were not competitively inhibiting each other suggesting spatially separated substrate binding sites. Second, a comprehensive analysis of the transport properties of AtMRP2 expressed in yeast implies that (i) AtMRP2 is able to transport the typical MRP glucuronide substrate 17β-estradiol 17-(β-D-glucuronide) ($E_2$17G) as well as glutathione conjugates, (ii) some (but not all) glutathione conjugates and $E_2$17G reciprocally activate each others transport from *cis* and not from *trans* via distinct but coupled binding sites, (iii) reduced glutathione is a substrate for ATP-dependent transport by AtMRP2, (iv) GSH and *S*-methyl-glutathione but not the thiol dithiothreitol are able to stimulate $E_2$17G-transport but the glucuronide rather inhibits than activates GSH-transport [32]. These authors therefore suggest that AtMRP2 is unique in the sense that it mediates transport via multiple binding sites/pathways in a semi-autonomous manner and that each pathway is subject to individual cross-regulation by other AtMRP2 substrates. In view of earlier results obtained with isolated barley and rye vacuoles where ATP-dependent transport of $E_2$17G was strongly activated by different GS conjugates (but not GSH) while metolachlor-GS uptake was partially inhibited (and not activated) by different glucuronides [33] it is important to note that the cross-activation found for vacuoles with an unknown set of MRP transporters can at least partially be explained solely by the action of a single MRP. However, the importance and physiological relevance of cross-activation/substrate modulation of AtMRP2 will have to be re-examined in *atmrp* knockout mutants since glucuronide uptake into vacuoles isolated from wild-type *Arabidopsis* rosette leaves is not strongly activated by glutathione conjugates (Frelet and Klein, unpublished). Single mutants in *atmrp1* and *2* as well as the *atmrp1 atmrp2* double mutant still possess vacuolar GS-X transport activity suggesting that further AtMRPs are involved in vacuolar GS-X sequestration (Frelet, Kolukisaoglu, Schulz and Klein, unpublished).

As demonstrated for AtMRP2 also AtMRP3, 4 and 5 which represent members of clade II share features of a broader substrate specificity or multiple functions in plants. In attempts to identify *Arabidopsis* MRPs that like HsMRP1 [27] complement the cadmium hypersensitivity of the Δycf1 yeasts, strains transformed with *AtMRP1* to *5* and *7* were tested for enhanced cadmium resistance. AtMRP3, 4 and 7 but not AtMRP1, 2 and 5 partially restore cadmium resistance ([20] and Plaza, Bovet, Klein, Geisler and Martinoia, unpublished) although it has not yet been established whether these AtMRPs act like

Table 3
Substrate specificities of individual AtMRPs heterologously expressed in yeast and potential to restore cadmium resistance in the hypersensitive Δycf1 yeast background

Compound class	Substance	AtMRP				
		1	2	3	4	5
Glutathione conjugate	DNP-GS	74	66	~80	n.d.	Yes
	GSSG	220	74	n.d.	n.d.	n.d.
	Metolachlor-GS	64	75	n.d.	n.d.	n.d.
Glucuronide	$E_2$17G	Low	750	n.d.	n.d.	Yes
Chlorophyll catabolite	*Bn*-NCC1	No	15	Yes	n.d.	n.d.
Folate (analogs)	Methotrexate	n.d.	n.d.	n.d.	Yes	n.d.
	Folic acid	Yes*	n.d.	n.d.	Yes*	n.d.
Restores Cd resistance in Δycf1 yeasts		No	No	Yes	Yes+	No

Summarized is the current status of transport experiments performed with vesicles isolated from yeasts transformed with AtMRPs 1–5. Values represent published K_m values in μM [18–20]. 'Yes' or 'no' means that the transport capacity was analyzed without the determination of kinetic properties [8,24]. Statements marked with * are published in [14]. +, Plaza, Bovet, Klein, Geisler and Martinoia, unpublished; n.d., not determined.

YCF1p as transporters for Cd-GSH complexes. Expression of *AtMRP3* is strongly induced by cadmium stress while transcript levels of *AtMRP4* and 7 are only marginally affected by heavy metals [34].

Present evidence obtained via GFP-tagging, confocal microscopy and sucrose density fractionation suggests that AtMRP1 and 4 localize to the tonoplast and the plasma membrane in plant cells, respectively [24,35]. Using a peptide-specific polyclonal antibody raised against AtMRP2, Liu et al. [32] demonstrated an enrichment of a positively reacting band in a vacuolar membrane-enriched vesicle fraction isolated from *Arabidopsis* while any in situ data is missing. Therefore, plasma-membrane localized AtMRP4 may define a novel pathway for cadmium detoxification in plants. Furthermore, AtMRP3 like AtMRP2 mediates *Bn*-NCC1 chlorophyll catabolite transport suggesting that several isoforms are able to detoxify chlorophyll catabolites during senescence. These properties of selected AtMRPs confirm the earlier finding that isolated vacuoles are able to accumulate chlorophyll catabolites via an ATP-dependent mechanism [36]. Although AtMRP4 and 5 have supposed roles in the regulation of ion channel activities in guard cells thereby controlling plant transpiration and gas exchange through stomata they were also shown to possess transport activities. AtMRP5 is able to catalyze $E_2$17G transport [8] and AtMRP4 like AtMRP1 mediates transport of folate [14,24]. It has been shown previously that different human MRP transporters confer resistance to the anticancer folate biosynthesis inhibitor methotrexate [37–39] and are capable of transporting methotrexate and physiological folates with a preference for non-polyglutamylated forms [40,41]. In plants, folate biosynthesis is split into a minimum three different subcellular compartments represented by plastids, mitochondria and the cytosol [42]. Shuttling of intermediates or products is not well investigated. Folates are involved in important metabolic processes including synthesis of purines, thymidilates, methionin and pantothenate. In C3 plants folates mediate large metabolic fluxes mainly during photorespiration as a cofactor of the mitochondrial glycine decarboxylase/serine hydromethyltransferase complex. The addition of polyglutamyl tails via folylpolyglutamyl synthetase has cytosolic, plastidic and mitochondrial isoforms. Recently, a non-ABC plastidic folic acid transporter from *Arabidopsis* termed AtFOLT1 was described sharing extensive homology with mammalian mitochondrial folate transporters and whose activity was demonstrated by complementation assays [43]. However, *atfolt1* null mutants contained normal levels of folates in chloroplasts suggesting the presence of further folate import routes across the envelope. Moreover, compartmentation studies demonstrated the occurrence of about 20% of total cellular folates in vacuoles of pea leaves and presence of folates in red beet root vacuoles [44]. A large proportion of the total folate carried polyglutamyl tails (about 50% of total folates in the case of pea vacuoles). Interestingly, polyglutamylated folates coexisted in vacuoles together with the γ-glutamyl hydrolyases which are responsible for the efficient removal of polyglutamyl tails raising the possibility of the existence of distinct vacuolar subpools separating the polyglutamylated folates from the hydrolase. These data demonstrate that the vacuole is a fourth essential compartment of folate metabolism and support the existence of transporters mediating transtonoplast flux of folates with AtMRP1 being a potential candidate due to its tonoplast localization. However, it has to be resolved whether AtMRP4 as a plasma membrane transporter exports folates into the apoplastic space and whether the extracellular presence of folates is of any physiological relevance.

At present, substrate specificities of individual plant MRPs must be interpreted with precaution. First, cDNA cloning, yeast expression followed by characterization of transport properties with standard substrates is still missing for nine AtMRPs and none of the MRPs isolated from other plants such as the crops maize or wheat have been rigorously tested in uptake assays although expression analysis of the maize *ZmMRP1* and *2* genes and of the wheat *TaMRP1* revealed increased transcription of *ZmMRP1* and *TaMRP1* in response to xenobiotics [21,22] suggesting their importance for detoxification processes. Secondly, the availability of single or multiple knockout mutants in all *Arabidopsis* MRPs (Kolukisaoglu, Schulz, Martinoia and Klein, M., unpublished) allows to initiate a detailed analysis of vacuolar or plasma membrane transport properties in mutants. Thirdly, the use of substrates which are likely to be absent in plant cells in in vitro assays again raises the question of endogenous substrates for plant MRPs.

3.2. Evidence for MRP-mediated transport of phenolic compounds

The investigation of vacuolar transport mechanisms for glycosylated flavonoids suggested the presence of specific flavonoid/H^+-antiporters for endogenously synthesized compounds as opposed to xenobiotic compounds possessing comparable conjugation patterns which were transported by direct energization via an ABC-type glucoside pump whose molecular identity remains unknown [45,46]. Thus, in contrast to mammalian transport processes for conjugates where, e.g., addition of glutathione- or glucuronosyl-residues serves as an efficient tag to define transport via MRP-type ABC transporters [47], the most prominent 'tag' to produce more hydrophilic compounds in plants, glucose, does not determine whether a compound is transported by ABC transporters or via H^+-antiport.

For the vacuolar uptake of glucosylated flavones from barley different transport mechanisms exist depending on the species chosen: they are subject to transport via a H^+-antiport mechanism or ABC-type directly energized transport, respectively, when transport into the specific barley vacuoles is compared to *Arabidopsis* vacuoles which do not synthesize flavones [45,46]. Other glucosylated compounds, namely *p*-hydroxycinnamic acid, *p*-hydroxybenzoic acid and glucosylated chlorsulfuron, are transported via a H^+-antiport mechanism into *Beta vulgaris* tonoplast vesicles [48] while transport of hydroxyprimisulfuron-glucoside, the detoxification product of the herbicide primisulfuron, into barley vacuoles uses an ABC-type transport mechanism [45]. Interestingly, the 2-O-β-D-glucose conjugate of salicylic acid which is an important signal in systemic acquired resistance and hypersensitive cell death during plant–pathogen interactions is transported in a H^+-dependent manner into tonoplast vesicles of tobacco suspension cultures [49] while an ABC-type energization mechanism has been found when transport into soybean vacuolar vesicles was studied [50]. These data suggest coexistence of ABC transporters and a minimum of one further membrane protein family on plant vacuolar membranes with the latter catalyzing phenylpropanoid/H^+-antiport. However, the molecular identity of a plant glucoside ABC transporter remains to be shown. In contrast, flavone glucuronides occurring in the

rye mesophyll and sulphated or sulfonated compounds require an ABC transporter for accumulation in the vacuole [51]. Since $E_2$17G and flavone glucuronide uptake exhibited competitive inhibition it is likely that vacuolar MRP-type transporters mediate sequestration of naturally occurring anionic conjugates.

Several lines of evidence indicated the importance of MRP-type ABC transporters in vacuolar flavonoid transport. (i) Analysis of mutants led to the identification of genes encoding glutathione *S*-transferases (GSTs) in different species including maize (*Bronze2*), Petunia (*AN9*) or *Arabidopsis* (*TT19*), all involved in late steps of anthocyanin and/or PA biosynthesis [52–54]. Glutathionated compounds are known high-affinity substrates for MRPs in all species investigated so far including plants [55]. (ii) The structurally unidentified in vitro-product of a GST-catalyzed reaction between the anthocyanin cyanidin 3-glucoside (C3G) and reduced glutathione underwent ATP-dependent transport via AtMRP1 and AtMRP2 expressed in yeast [19]. However, the existence of anthocyanin conjugates with glutathione in vivo had been questioned and a model where the Petunia GST AN9 acted as an adaptin for flavonoids guiding them towards the vacuolar membrane for transport thereby controlling the free flavonoid concentration in the cytosol had been proposed instead [56]. Analysis of single knockout mutants in all 14 *AtMRP* genes including mutants in *atmrp2* did not lead to the identification of flavonoid-related phenotypes including transparent testa seed color so far (Klein, Frelet, Schulz, and Kolukisaoglu, unpublished). In contrast, the isoflavonoid medicarpin conjugated to glutathione was transported into tonoplast vesicles isolated from etiolated hypocotyls of mung bean via an ABC-type mechanism while unconjugated medicarpin was taken up at a much lower rate and independent of the presence of MgATP [57]. (iii) Antisense mutants of the vacuolar membrane-localized maize MRP ZmMRP3 which was controlled transcriptionally by regulators of anthocyanin biosynthesis exhibited a marked reduction in anthocyanin production and pigment mislocalization [23].

3.3. Interaction of plant MRPs with other proteins

In animals, two ABCC-type membrane proteins are involved in transmembrane ion fluxes: The cystic fibrosis transmembrane conductance regulator CFTR has a chloride channel activity and controls K^+ channels [58]. The sulfonylurea receptor SUR is tightly associated with an inward-rectifying potassium channel [59] to form the ATP-sensitive K^+ channel. CFTR and SUR are sensitive to sulfonylureas [60] and blocked by glibenclamide in numerous tissues [61]. In plants, AtMRP5 has been shown to regulate guard cell movement and electrophysiological studies indicate that this MRP is not a channel but regulates anion channel currents (see below).

An interaction of ABC transporters with the immunophiline TWD1 (twisted dwarf) has recently been described [35,62]. TWD1 is the only immunophiline of *Arabidopsis* which contains a membrane-spanning domain and it could be localized in the plasma membrane as well as in the vacuolar membrane [35,62,63]. In the cytosol the interaction occurs between the *cis–trans* peptidyl-prolyl isomerase-like domain of TWD1 which lost the enzymatic activity and the C-terminal domain of AtPgp1 and its closest homologue AtPgp19. This interaction is part of the complex regulation of auxin, a phytohormone, transport (for more details see Minireview on plant Pgps in this volume). TWD1 localized in the vacuolar membrane interacts with AtMRP1 and its closest homologue AtMRP2 but not with MRPs belonging to other clades such as AtMRP4, AtMRP5, AtMRP7 and AtMRP13. Unlike AtPgp1 and AtPgp19, AtMRP1 binds to the C-terminal tetratricopeptide repeat domain of TWD1. This domain is well known to mediate protein–protein interactions. Domain mapping proved that TWD1 binds to a motif of AtMRP1 resembling calmodulin-binding motifs. Using the overlay technique, calmodulin binding could indeed be verified, but calmodulin did not affect TWD1 binding. It could be shown that TWD1 binds to vacuolar membranes and that addition of TWD1 into transport assay resulted in a significant decrease of the metolachlor-GS uptake and an increase in vacuolar estradiol-glucuronide uptake. Thus, these results indicate that post-translational events such as protein–protein interaction with calmodulin and immunophilines are an integral part of the regulatory network of ABC transporters.

3.4. In search of plant CFTR/SUR homologs: AtMRP4 and AtMRP5 affect stomatal regulation and AtMRP5 controls guard cell ion channels

Pharmacological evidence using sulfonylureas and potassium channel openers in combination with electrophysiological experiments demonstrated the presence of CFTR- and SUR-type membrane proteins in the plasma membrane of guard cells which as a pair together with the central pore form stomata, a highly differentiated structure of plant epidermal tissues responsible for gas exchange and transpiration [64,65]. Stomatal opening and closure are tightly regulated by different endogenous and environmental signals ultimately controlling ion fluxes across the plasmalemma of guard cells. These results – most importantly glibenclamide-inducible stomatal opening and inhibition of closure and inhibition of the guard cell protoplast slow anion current by glibenclamide – were exciting since they argued for the participation of ABC proteins in guard cell regulation.

Two *AtMRP* genes, *AtMRP4* and *5*, are strongly but not exclusively expressed in guard cells [8,9]. Both membrane proteins expressed as GFP-fusions localize to the plasma membrane ([24]; Frelet, Klein and Martinoia, unpublished). The analysis of knockout mutants in both genes demonstrated that *atmrp4* and *atmrp5* mutants are more, respectively less, susceptible to drought stress. Present evidence supports the hypothesis that AtMRP5 is directly involved in ion channel regulation and represents a plant homologue of CFTR or SUR. The observations that (i) *AtMRP5* expressed in mammalian HEK293 cells binds glibenclamide [66] and (ii) stomata of *atmrp5* mutants are insensitive to glibenclamide [8] provide direct evidence that AtMRP5 mediates glibenclamide-induced stomatal opening. Overexpression of *AtMRP5* in the *Arabidopsis atmrp5* mutant background [24] or in *Nicotiana tabacum* (Suh, Frelet, Grob, Gaedeke, Schmidt, Mueller-Roeber, Klein and Martinoia, submitted) resulted in reversion of drought tolerance and plants with increased water loss. In tobacco guard cell protoplasts ectopically expressing *AtMRP5*, the activity of the slow (S-type) anion channel was consistently lower than in untransformed controls while guard cell K^+ inward and outward rectifying currents were similar or only marginally smaller in overexpression lines, respectively. Stomatal closure is triggered by a Ca^{2+}-dependent activation of S-type anion channels which upon depolarization activate K^+ outward rectifiers [67]. Since the decrease of slow anion channel activity

coincides with larger stomata opening and increased drought susceptibility of overexpression plants, these results demonstrate for the first time that a plant ABC transporter can directly regulate ion channels most probably at the level of the S-type anion channel.

Physiological analysis of stomata movements and gas exchange analysis suggests that AtMRP4 is unlikely to be a simple antagonist of AtMRP5 which raises the issue whether both MRPs are involved in rather distinct processes controlling plant transpiration. While stomatal movements of *atmrp5* mutants are insensitive to phytohormonal control by auxin or the plant water stress hormone abscisic acid (ABA), which normally induces stomatal closure and inhibits opening in the light [9], *atmrp4* mutants still respond to ABA [24]. Stomatal pore sizes were greater in the dark and light in *atmrp4* mutants when compared to the wild type while *atmrp5* mutants displayed slightly smaller apertures only in the light but not in the dark. Kinetic analysis of transpiration rate changes in response to light and darkness revealed that *atmrp4* mutants have a pronounced transpirational response to the 'light on' trigger while the reduction of transpiration after switching off the light source was comparable to the wild type. In addition, CO_2 partial pressures falling below 200 ppm resulted in transpiration rates that 1.5-fold higher in *atmrp4* mutants when compared to the wild type. However, *atmrp4* mutant guard cells do not exhibit changes in ion channel activities under our experimental conditions.

Clearly, the molecular action and targets/interaction partners of both MRPs need to be identified in order to define better the function of AtMRPs in guard cell regulation and a proper electrophysiological characterization of guard cells of both mutants is needed to understand the nature of channel regulation by these AtMRPs. Furthermore, it will be interesting to find out whether more AtMRPs are involved in ion channel regulation. Two points are worth to be mentioned: (i) Under standard growth conditions all mutants as well as tobacco plants ectopically expressing *AtMRP5* appeared identical in growth and development when compared to the corresponding wild types and no drastic phenotypic changes as seen in classical hormone mutants disrupting, e.g., ABA signaling were observed. (ii) In spite of differences in transpiration rates the net photosynthesis was not altered in *atmrp* mutants suggesting that carbon fixation rates are not limited. These findings open a possible pathway to the generation of crop plants exhibiting increased drought susceptibility via subtle and not drastic changes of guard cell regulation by ABC transporters.

4. Expression analysis of the MRP gene family in *Arabidopsis*

Our preliminary analysis of the presence of all *AtMRP* genes in different tissues via RT-PCR demonstrated that all 14 genes are expressed in all tissues investigated without clearly detectable indications of any organ-specificity [12]. The complete sequencing of the *Arabidopsis* genome achieved in the year 2000 [68] together with in situ synthesis of highdensity oligonucleotides on glass slides [69] and the availability of a full-genome array (ATH1; developed by Affymetrix and The Institute for Genomic Research) allows the global evaluation of gene expression in different tissues or during *Arabidopsis* development [70]. In the context of this review we have used the 'Genevestigator' web interface (https://www.genevestigator.ethz.ch/; see [71]) to perform an analysis of the *AtMRP* genes present on the ATH1 gene chip with respect to tissue distribution and *Arabidopsis* development. All data presented here were analyzed by the end of September 2005 using the Gene Atlas and Gene Chronologer tools of the Genevestigator web page.

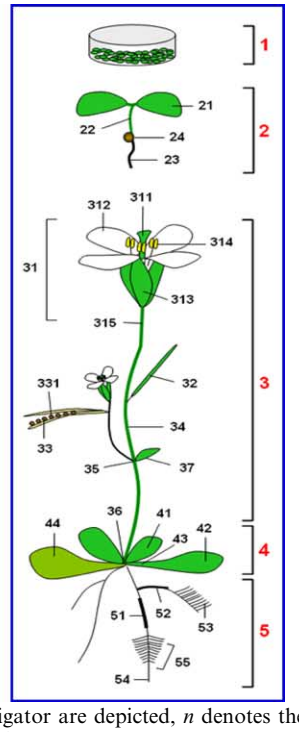
The dataset was restricted to the Col-0 ecotype representing the sequenced genome and all chipsets (AtGenExpress, NASC-Arrays, GEO, ArrayExpress, Gruissem Laboratory and the Functional Genomics Center Zurich) were chosen for analysis. Notably, *AtMRP3* (At3g13080) and *AtMRP11* (At1g30420) are not present on the ATH1 array and *AtMRP9* and *12* represent ambiguous probesets. Thus, signal intensities of these two genes must be interpreted with care since probably two homologous genes are detected. It should be noted that comparison of multiple probesets representing different genes in different tissues or life stages is difficult on the basis of absolute values. Therefore, analysis is restricted to the results for a given gene/probeset across different experiments and no conclusions are drawn cross comparing expression level values of different members of the *AtMRP* gene family under a given condition.

Genes representing members of clade I are expressed in all tissues and organs. In the seedling, highest expression of *AtMRP1* is found in the hypocotyl and the radicle, for *AtMRP2* in the hypocotyl and for *AtMRP12* in cotyledons although expression differences are not pronounced in all cases (Table 4). For the genes belonging to clade II, *AtMRP5* and to a lower extent *AtMRP6* and *7* are most abundant in the primary root, *AtMRP8* exhibits a certain specificity for the hypocotyl while expression of *AtMRP9* and *10* is remarkably lower in the hypocotyl when compared to other seedling parts. All clade I genes (*AtMRP1,2* and *12*) are highly expressed in roots while only *AtMRP5*, *6* and *7* exhibit a certain specificity for expression in root tissue when compared to, e.g., rosette leaves. Preferential expression of *AtMRP7* in roots is in accordance with former RT-PCR analysis [12]. Apart from guard cells, plants transformed with a *Pro AtMRP5*::*uidA* construct revealed high GUS activity in the vascular tissue and *atmrp5* mutants exhibited a nutrient- and salt-dependent defect in root elongation growth [8,66]. Furthermore, the expression atlas demonstrates highest expression levels of *AtMRP5* in seeds – a feature only shared by *AtMRP14*. Again, GUS data support high abundance of *AtMRP5* transcript in seeds (Klein and Frelet, unpublished). It is preliminary but tempting to speculate that AtMRP5 is also instrumental in the regulation of ion channels involved in mineral nutrient supply apart from guard cell ion channel control. Furthermore, the growth stage-specific expression patterns demonstrate high transcript abundance for *AtMRP5* and 14 (the closest *AtMRP4*-homolog) at late (45–50 days) stages while transcript levels drop during germination (1–5 days; Table 5). Thus, both genes could be involved in processes controlling seed integrity and germination efficiency such as the regulation of dormancy.

Differences between juvenile and adult leaves are not very pronounced for all *AtMRP* genes. However, *AtMRP2*, *5*, *7*, *8*, *9*, *10* and *12* exhibit a tendency to lower expression values in adult, expanded rosette leaves. Interestingly, expression of all *AtMRP*s except for *AtMRP12* and *13* is strongly increased in senescent leaves when compared to all other rosette leaf stages with *AtMRP2* and *AtMRP8* exhibiting 5- and 4-fold

Table 4
Tissue- and organ-specific expression of *AtMRP* genes

	AtMRP												
Organ	**n**	**1**	**2**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**12**	**13**	**14**
1 cell suspension	42	3393	3858	2149	1846	447	369	2561	82	1481	976	67	3773
2 seedling	320	2366	1301	1030	1171	279	195	899	301	552	427	279	2987
21 cotyledons	31	1990	921	988	850	130	203	548	550	646	637	451	2661
22 hypocotyl	32	2884	1605	1243	1655	210	363	1497	106	80	356	384	2638
23 radicle	32	2704	1267	1104	2127	414	482	495	227	333	515	404	2897
3 inflorescence	139	1908	1557	1185	2908	132	104	1332	1378	843	345	225	5039
31 flower	58	2247	1899	1291	2077	119	132	1891	2397	1163	424	213	3915
311 carpel	6	838	1165	548	2158	98	78	1467	4584	791	302	160	3295
312 petal	6	4208	1569	652	2241	108	127	2623	646	287	353	184	5826
313 sepal	6	3473	4472	3655	2244	154	368	3734	5936	5574	1339	337	6261
314 stamen	8	2266	2582	1318	1679	122	150	1253	1587	506	133	231	2014
3141 pollen	2	616	257	131	557	92	189	468	227	222	13	368	243
315 pedicel	3	1776	1173	1075	1604	109	123	1564	378	114	266	249	4465
32 silique	11	933	1645	878	2618	100	53	1027	1288	331	184	155	2480
33 seed	32	1279	1090	1287	5563	170	75	397	563	811	119	181	9919
34 stem	7	1730	801	813	1319	201	80	1713	101	49	282	349	2608
35 node	3	2010	796	731	3088	116	131	2071	213	77	433	416	3231
36 shoot apex	17	2183	1418	633	2027	98	79	928	363	450	522	268	3070
37 cauline leaf	3	3865	2932	4187	1239	149	235	2524	1460	1871	833	439	4176
4 rosette	577	2267	1286	1029	931	190	238	881	460	646	481	373	3714
41 juvenile leaf	86	2056	1222	1193	1052	202	398	834	622	850	613	298	2888
42 adult leaf	189	2012	930	1260	845	181	239	608	510	551	528	275	3181
43 petiole	12	1627	494	411	702	165	128	748	91	239	291	320	3011
44 senescent leaf	3	3962	4679	2819	1526	298	917	2622	1808	1353	524	393	3457
5 roots	187	4748	2829	1240	2476	983	771	996	243	324	942	465	2570
52 lateral root	43	3720	1837	1417	1872	437	292	1311	181	319	469	380	2859
55 elongation zone	31	1600	1900	1740	2458	958	485	388	139	176	554	359	3003



The signal intensity values from ATH1/Col-0 chip experiments integrated into the Gene Atlas tool of Genevestigator are depicted, *n* denotes the number of chip experiments available by September 2005. The *Arabidopsis* image is Courtesy of Genevestigator(*r*).

higher expression values when compared to adult leaves, respectively. It is unclear whether this almost family wide effect reflects the need for massive storage of chlorophyll catabolites or whether many different potentially toxic compounds have to be handled by ABC transporters during senescence processes. Another intriguing observation is that the expression levels of *AtMRP1*, *2*, *4* and *14* are higher in cauline leaves when compared to rosette leaves.

Notably, different genes exhibit highest relative expression in tissues belonging to the flower. *AtMRPs 2*, *4* and *8–12* are most abundant in sepals. Especially, expression of *AtMRP10* is 19-fold higher in sepals than in petals. As for the higher expression in cauline than rosette leaves it remains to be shown whether this preference reflects physiological function. It is tempting to speculate that sepals undergo rapid senescence processes once the flower opened and (self-)pollination occurred. In contrast, expression values for *AtMRP1* are higher in petals than in sepals.

Genome-wide analysis of gene expression throughout *Arabidopsis* development reveals three characteristic patterns (Table 5): Most *AtMRP* genes exhibit an expression maximum at 14–17 days after germination (d) (*AtMRP1*, *2*, *6–8*, *12*, *13*).

Table 5
Growth stage-dependent expression patterns of *MRP* transporters of *A. thaliana*

AtMRP	Plant age (days after germination)								
	1–5	6–13	14–17	18–20	21–24	25–28	29–35	36–44	45–50
1	1796	2401	3619	2133	2214	2090	2291	2180	1094
2	935	1350	2265	1052	1312	1062	1256	1849	1288
4	935	1063	1052	1056	703	1250	912	1709	1184
5	384	1234	1736	958	1346	1054	1019	1904	6310
6	203	295	595	167	121	186	181	154	140
7	68	211	547	166	68	247	147	217	78
8	551	891	1102	815	818	792	999	1812	609
9	198	312	362	340	346	743	663	1099	975
10	1083	550	568	415	563	646	691	637	333
12	162	450	737	458	450	535	401	407	94
13	194	282	493	284	333	261	322	300	198
14	5610	3037	3186	2708	4262	3406	3996	3307	8452
n	23	306	360	83	27	188	217	27	28

Depicted are the signal intensity values as given by the Gene Chronologer tool of Genevestigator. *n* indicates the number of chip experiments.

AtMRP5 and *14* are most abundant in the last developmental stage while *AtMRP4* and *9* are most abundant in the preceding stage (36–44 days). *AtMRP10* does not fit to any of these developmental patterns since it depicts highest abundance at germination followed by decreased but constant expression throughout the rest of the life cycle with a second but smaller reduction after 45 days. Thus, in this respect *AtMRP10* expression is antagonistic to the expression of *AtMRP5* and 14. Unfortunately, neither the gene expression atlas (Table 4) nor growth stage-specific expression patterns (Table 5) give clear hints with regard to organ or growth stage specificity of *AtMRP13* lacking the TMD0. The most intriguing observation for this gene is that its expression is lowest in cell suspensions.

A recent study compares relative expression levels of 10 *ZmMRPs* from maize by RNA gel blot analysis [23]. Interestingly, expression of four *ZmMRP* probes cannot be detected in any tissue investigated which is in contrast to results from *Arabidopsis*. Three *ZmMRPs* exhibit specific expression patterns in seedlings (*ZmMRP1*), the adult leaf (*ZmMRP2*) and shoot, leaf, husk and tassel (*ZmMRP3*), respectively. *ZmMRP3* expression needs the presence of the B and PI transcription factors controlling anthocyanin pigmentation in maize. It seems that the expression pattern of different *MRPs* points to an enhanced tissue specificity in maize when compared to ubiquitous expression in *Arabidopsis* and it will be interesting to include more expression data, e.g., from rice or poplar to explore whether tissue-specific expression of plant *MRP* genes occurs in these species.

Expression levels do not necessarily mean higher protein abundance or transport activity. In view of gene redundancy in plant MRP families on the one hand and the famous multifunctionality of ABC transporters on the other hand it is difficult to draw conclusions on MRP-affected physiological processes from expression data. Furthermore, although the lifecycle of *Arabidopsis* has been dissected to many different stages, expression-profiling is still integrating over many cell types within one tissue and associated with the loss of cell specificity. Future work will be needed integrating mutant analysis (subcellular) protein localization or promoter–reporter gene systems to pinpoint the individual relevance of each MRP protein.

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