Arabidopsis Immunophilin-like TWD1 Functionally Interacts with Vacuolar ABC Transporters

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Previously, the immunophilin-like protein TWD1 from *Arabidopsis* has been demonstrated to interact with the ABC transporters AtPGP1 and its closest homologue, AtPGP19. Physiological and biochemical investigation of *pgp1/pgp19* and of *twd1* plants suggested a regulatory role of TWD1 on AtPGP1/AtPGP19 transport activities. To further understand the dramatic pleiotropic phenotype that is caused by loss-of-function mutation of the *TWD1* gene, we were interested in other TWD1 interacting proteins. AtMRP1, a multidrug resistance-associated (MRP/ABCC)-like ABC transporter, has been isolated in a yeast two-hybrid screen. We demonstrate molecular interaction between TWD1 and ABC transporters AtMRP1 and its closest homologue, AtMRP2. Unlike AtPGP1, AtMRP1 binds to the C-terminal tetratricopeptide repeat domain of TWD1, which is well known to mediate protein-protein interactions. Domain mapping proved that TWD1 binds to a motif of AtMRP1 that resembles calmodulin-binding motifs; and calmodulin binding to the C-terminus of MRP1 was verified. By membrane fractionation and GFP-tagging, we localized AtMRP1 to the central vacuolar membrane and the TWD1-AtMRP1 complex was verified *in vivo* by coimmunoprecipitation. We were able to demonstrate that TWD1 binds to isolated vacuoles and has a significant impact on the uptake of metolachlor-GS and estradiol- β -glucuronide, well-known substrates of vacuolar transporters AtMRP1 and AtMRP2.

INTRODUCTION

FK506 binding proteins (FKBPs) form, together with the cyclosporin A (CsA) binding cyclophilins and parvulins, three structurally unrelated classes of proteins known to function as *cis-trans*-peptidylprolyl isomerases (PPIases; Schiene and Fischer, 2000). Small FKBPs, such as FKBP12, are thought to modulate signal transduction pathways (Luan, 1998). Cyclophilin-CsA and FKBP12-FK506 complexes have been shown to bind to calcineurin (PP2B), a

Ca²⁺, calmodulin-regulated Ser/Thr-specific protein phosphatase, thereby blocking Ca²⁺-dependent signaling (Harrar *et al.*, 2001) and leading to inhibition of T-cell activation. CsA and FK506 are therefore widely used to treat and prevent graft rejection in organ transplantation patients. Additionally, these products of soil-borne microorganisms have recently been shown to play a role in reversing multidrug resistance (MDR) in several types of cancer by inhibiting the efflux of anticancer drugs (Mealey *et al.*, 1999). FKBP12 has been demonstrated to function as a physiological regulator of the cell cycle. Cells from FKBP-deficient (FKBP12^{−/−}) mice are arrested in the G1 phase of the cell cycle (Agdhasi *et al.*, 2001). Disruption of this gene also affects the proper function of calcium release channels in the heart muscle, leading to cardiovascular disorders in mutant mice (Shou *et al.*, 1998).

High-molecular-weight FKBPs are composed of one or more FKBP12-like (also referred as PPIase) domains and differ from their small counterparts by containing a tetratricopeptide repeat (TPR) domain (Das *et al.*, 1998; Pratt *et al.*, 2001) and a C-terminus that in most cases contains a putative calmodulin-binding domain (Harrar *et al.*, 2001). Mammalian FKBP52, the best investigated example, is associated with HSP90 via its TPR domain in the native steroid hormone receptor complex (Silverstein *et al.*, 1999). Plant high-molecular-weight FKBPs seem to bind plant HSP90 by means of the same TPR interaction as the mammalian homologues (Pratt *et al.*, 2001). FKBP73 and FKBP77 from wheat have been identified as part of a HSP90 heterocomplex *in vitro* (Reddy *et al.*, 1998); and, very recently, TWD1

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Abbreviations used: FKBP(s), FK506 binding protein(s); PPIases, *cis-trans*-peptidylprolyl isomerases; MDR, multidrug resistance; TPR, tetratricopeptide repeat; *twd1*, twisted dwarf1; ABC, ATP-binding cassette; β -gal, β -galactosidase; PGP, P-glycoprotein; CaM, calmodulin; CaMbd, CaM-binding domain; EGFP, enhanced green fluorescent protein; aa, amino acid(s); PAGE, polyacrylamide gel electrophoresis.

from *Arabidopsis* has been shown to bind both HSP90 and calmodulin (Kamphausen *et al.*, 2002).

Although yeast seems to be viable without immunophilins, as demonstrated by a yeast mutant strain in which the entire set of immunophilin genes is disrupted (Dolinski *et al.*, 1997), drastic phenotypes have been associated with mutations in individual plant immunophilins. Loss-of-function mutation in the cyclophilin40 homologue of *Arabidopsis* lead to reductions in the number and size of juvenile rosette leaves (Berardini *et al.*, 2001). The chloroplast localized AtFKBP13 is responsible for import and accumulation of the Rieske protein subunit of the cytochrome *bf* complex in the thylakoid lumen. DsRNAi silenced plants show a substantial increase in the accumulation of Rieske protein in the chloroplast (Gupta *et al.*, 2002). The *Arabidopsis* T-DNA mutant *pasticcino1* (*pas1*), which lacks a 72-kDa FKBP, is characterized by ectopic cell division, abnormally developed cotyledons and leaves, fusion of tissues, and impaired root development, as well as hypersensitivity toward cytokinin (Faure *et al.*, 1998; Vittorioso *et al.*, 1998). The *Arabidopsis* FKBP42 mutant *twisted dwarf 1* (*twd1*) results in a drastic reduction of cell elongation combined with a disoriented growth behavior (Geisler *et al.*, 2003). The *twd1* and *ultracurvata2* (*ucu2*) mutants are both defective in AtFKBP42 and phenotypically indistinguishable (Pérez-Pérez *et al.*, 2004). TWD1 has been shown to be membrane-anchored and immunolocalized on both the central vacuole and the plasma membrane (Kamphausen *et al.*, 2002; Geisler *et al.*, 2003). TWD1 forms a protein-protein complex via the C-terminal domains of the ABC transporters AtPGP1 and AtPGP19 (Noh *et al.*, 2001; Murphy *et al.*, 2002; Geisler *et al.*, 2003), which belong to the MDR subfamily of ABC transporters (recently renamed as ABCB in humans; Dean *et al.*, 2001). The single gene mutation *twd1* and double *atpgp1/atpgp19* (*atmdr1*) mutants exhibit similar phenotypes including epinastic growth, reduced inflorescence size, and reduced polar auxin transport in hypocotyls (Noh *et al.*, 2001; Geisler *et al.*, 2003), suggesting a regulatory role of TWD1 on AtPGP1/AtPGP19 transport activities (Geisler *et al.*, 2003).

FKBPs have been suggested to function as regulators of MDR-like ABC transporters (Hemenway and Heitman, 1996; Geisler *et al.*, 2003). Moreover, MDR resistance can be partially overcome by immunosuppressant treatment (Cardenas *et al.*, 1994; Mealey *et al.*, 1999), but any attempts to link a direct association with a change in transport activity have failed so far. Here we show that TWD1 interacts with the C-termini of multidrug resistance-associated (MRP/ABCB)-like ABC transporters AtMRP1 and AtMRP2. Interacting domains in AtMRP1 and TWD1 were mapped. We demonstrate TWD1-AtMRP1 interaction on the membrane of central vacuoles and show that TWD1 can modulate ABC transporter uptake activities on isolated vacuoles.

MATERIALS AND METHODS

Yeast Two-hybrid Analysis

A stretch of TWD1 containing a TPR-like domain (E₁₆₃–L₃₁₄), which omits a calmodulin-binding domain (Q₃₁₀–G₃₂₈; Kamphausen *et al.*, 2002) as well as the C-termini of *Arabidopsis* ABC transporters AtMRP1, AtMRP2, AtMRP4, AtMRP5, and AtMRP7 were amplified by PCR (for MIPS codes and primer sequences see Table 1) and cloned as GAL4-binding domain (BD) and activation domain (AD) fusions into the two-hybrid vectors pACT2 and pAS2 (Clontech, Palo Alto, CA), respectively.

The C-terminus of AtMRP13 as well as the TPR domains of PAS1 and ROF1 were cloned by two-step RT-PCR as described in Geisler *et al.* (2003) using a gene-specific primer located in the 3' untranslated region of the correspond-

ing genes (see Table 1 for primer sequences). The coding regions were amplified by PCR using Vent DNA Polymerase (New England Biolabs, Frankfurt, Germany) and inserted into the indicated sites of two-hybrid vectors pACT2 and pAS2 (see Table 1). All constructs were sequenced to verify the absence of PCR errors.

For interaction analysis, three to five independent transformants of two independent transformations for each construct were analyzed for activation of histidine growth reporter (HIS auxotrophy) and LacZ (β -galactosidase) reporter activity. Therefore, single colonies were resuspended in 1 ml of sterile water and 5 μ l of each suspension were spotted on SD plates containing 25 mM 3-amino-1,2,4-triazole lacking leucine, tryptophan, and histidine (–HIS). Growth was judged after 3 days. β -galactosidase activity was quantified by liquid culture assay using standard protocols.

Recombinant Expression of the AtMRP1 C-terminus

The insert of clone pACT2-AtMRP1 (BE11) encoding the C-terminus of AtMRP1 was cut out from the two-hybrid vector using *Bgl*III sites flanking the insert and ligated in-frame into the *Bam*HI site of pQE32 (Qiagen, Hilden, Germany). This way, the resulting peptide was expressed as an N-terminal 6 $\times$ His-tagged fusion protein in *Escherichia coli* strain BL21D3 pLysC (Stratagene, La Jolla, CA). Due to an internal *Bgl*III site (base pairs 4172), the C-terminus was split into two halves, but only the N-terminal part of the interacting protein (E₁₂₁₇–S₁₃₉₂) was successfully expressed in *E. coli*.

In Vitro Binding Assays

A TWD1-affinity matrix was synthesized and incubated with cleared *E. coli* supernatants from cells overproducing the C-terminus of AtMRP1 or vector control lysates as described in Geisler *et al.* (2003). Some reactions were carried out in the presence of 500 μ M spinach calmodulin (Sigma, Buchs, Switzerland) with (addition of 100 μ M CaCl₂) or without calcium (addition of 5 mM EGTA). Eluted proteins were detected by Western blot analysis using monoclonal anti-pentaHIS and anti-RGSHis (Qiagen).

Calmodulin Overlays

Total lysates of *E. coli* containing the expressed AtMRP1 C-terminus were transferred to filters and probed with 100 ng/ml biotinylated CaM (Calbiochem, Darmstadt, Germany) in TBS plus 2% BSA. Blots were washed with TNT (TBS plus 0.05% Tween 20) in the presence (500 μ M CaCl₂) or absence of calcium (5 mM EGTA). Bound CaM was detected with alkaline phosphatase coupled to streptavidine (Promega, Madison, WI).

Cloning of AtMRP1

The cDNA of AtMRP1 was isolated by screening a size selected *Arabidopsis* cDNA library made from plants, which were treated with the herbicide primisulfuron (Tommasini *et al.*, 1997). A 1005-base pair AtMRP1-/AtMRP2-specific PCR fragment amplified from *Arabidopsis* genomic DNA (upper primer 5' tgccaatcactcataccatgtgtcg, lower primer 5' gagagagtgcacgaa(c/t)gtctctgcagg) was used as radiolabeled probe. Positive lambda clones were purified and integrated plasmid vectors were excised via in vivo excision. Clone pBSC-24 contained the longest ORF of AtMRP1. Because pBSC-24 was lacking the first 387 base pairs of the predicted complete ORF, the 5' end of AtMRP1 was cloned by one-step RT-PCR using the Titan RT-PCR system (Roche Diagnostics, Rotkreuz, Switzerland) and 1 μ g of *Arabidopsis* RNA followed by a second PCR using a 10-fold dilution of the one-step RT-PCR reaction as template. Nested PCR primers contained *Not*I and *Sph*I restriction sites (underlined in Table 1) that enabled completion of the ORF in pBSC-24 resulting in vector pBSC-MRP1.

Transgenic Plants

A *Not*I adapter was ligated into a *Xho*I site downstream the stop codon of vector pBSC-MRP1, and the AtMRP1 ORF was subcloned as *Not*I-*Xho*I fragment into vector pRT Ω (Überlacker and Werr, 1996), resulting in pRT Ω -MRP1. The entire gene of an enhanced GFP version (EGFP) was PCR amplified and inserted into the *Hind*III (base pairs 4526) and *Xba*I (base pairs 4729) sites of AtMRP1 (pRT Ω -MRP1), respectively (see Figure 5A). The cassette containing the CaMV 35S promoter, MRP1-EGFP and the polyadenylation signal was excised with *Asc*I and inserted into vectors pGPTV-bar (Überlacker and Werr, 1996) and the resulting binary constructs pGPTV-MRP1-12 and pGPTV-MRP1-13 were used to transform *Arabidopsis* via vacuum infiltration using *Agrobacterium* strain GV3101. Resistant transformants were selected on soil by watering with a BASTA solution (1:20,000).

Genetic nomenclature was used following the community standards for *Arabidopsis* genetics (Meinke and Koornneef, 1997).

Transfection of Onion Epidermis Cells and Confocal Laser Scanning Microscopy

Onion epidermis cell layers were transfected with both constructs pGPTV-MRP1-12 and pGPTV-MRP1-13 after coating 0.6- μ m gold microparticles (Bio-Rad, Hercules, CA) using a particle inflow gun as described in Ibrahim *et al.*

Table 1. Sequences of the oligonucleotide primers used for the PCR amplification reactions and origin of constructs described in this study

Construct (MIPS code)	Upper primer/lower primer	RT primer sequence	GAL4 fusion	Template vector	Reference
pACT2-MRP1 (At1g30400)	— ^a	—	AD	— ^a	This work
pACT2-MRP1-CaMbd (At1g30400)	5' cagggatccctgctgttctcatggagttcg/ 5' ctgctcgagcatctcgacattgtccagtc	—	AD	pRT Ω -MRP1	This work
pACT2-MRP2 (At2g34660)	5' gttgtgactctcgagcagtggtctcgagggg 5' cagggatccctcactgatattccctcagaatcc/	—	AD	pGEM-MRP2	Geisler and Klein, unpublished
pACT2-MRP4 (At2g47800)	5' ctgctcgagtattccggcagatcgagagc 5' cagggatccagtagcagattgtaggagag/	—	AD	pNEV-MRP5	Gaedeke <i>et al.</i> (2001)
pACT2-MRP5 (At1g04120)	5' ctgctcgagataattcagggtattccagtag — ^a	—	AD	— ^a	This work
pACT2-MRP7 (At3g13100)	5' cagggatccctggttctccagtagatggatgtg/ 5' ctgctcgagctgagaagctctgacaaagc	5' taggcaagtcacgaagactc	AD	— ^b	This work
pACT2-MRP13 (At2g07680)	—	—	BD	—	Geisler <i>et al.</i> (2003)
pAS2-BusB (At3g21640)	—	—	BD	—	Geisler <i>et al.</i> (2003)
pAS2-PPIase (At3g21640)	—	—	BD	—	Geisler <i>et al.</i> (2003)
pAS2-TPR (At3g21640)	—	—	BD	—	Geisler <i>et al.</i> (2003)
pAS2-TWD1-CaMbd (At3g21640)	5' tggatcatatggccatggaggg/ 5' acgggatcccaaggcttctctgtctgctgc	—	BD	pAS2-TWD1	Geisler <i>et al.</i> (2003)
pBDGal4-PAS1 (At3g54010)	5' acggaattcgaactccagagcatcatggac/ 5' acggtcgacagcagcggtcgcatcagcttc	5' tctgagtctacagtgcggttg	BD	— ^b	This work
pBDGal4-ROF1 (At3g25230)	5' acggaattcgaactgaacactgaggagaag/ 5' acggtcgactttctgctctagcttcacttc	5' cgttttcaagacctcaggtgc	BD	— ^b	This work
pBSC-MRP1 ^c (At1g30400)	5' ctccgtgagattcgaggattg/ 5' tgatccagagtcacatcaatgg 5' tagcggccgcatggggttgagccgttgattgg/ 5' ggactctccgtacgtttaataaactgtc	5' tgatccagagtcacatcaatgg	—	— ^b	This work

Restriction sites used for subcloning are underlined.

^a Obtained by library screening (see MATERIALS AND METHODS).

^b Cloned by RT-PCR.

^c Two pairs of nested PCR primers were used in a nested RT-PCR (see MATERIALS AND METHODS).

(2000). After bombardment at low-pressure helium flow (26 psi), epidermal layers were incubated at RT for 16–24 h in the dark.

Transgenic *Arabidopsis* plants were grown for 7 days on MS plates including BASTA (1:20,000) under continuous light. Transfected onion epidermis cells and young *Arabidopsis* seedlings were analyzed by confocal laser scanning microscopy (CLSM). FITC fluorescence using the corresponding filter sets was recorded, and stored images were then colored as green or red colors using Adobe Photoshop 5.5 (Adobe Systems, San Jose, CA).

Membrane Fractionation

Arabidopsis microsomes were prepared and separated by continuous sucrose gradient centrifugation as described in Geisler *et al.* (2003). Nitrocellulose membranes were probed with anti-AtMRP1 and the following marker enzyme antibodies: anti-P-type H⁺-ATPase, anti-V-type H⁺-ATPase (2E7), anti-PPase and anti-BIP as described in Geisler *et al.* (2003). The anti-AtMRP1 antibodies were raised in rabbits by injecting 8 mg of synthetic peptide (C-NYIEIPSEAPLVENNR), which was conjugated with keyhole limpet hemocyanin. The peptide sequence corresponds to the internal hydrophilic region of AtMRP1 (N₁₂₁₀–R₁₂₂₆).

For vacuolar pull down assays, *Arabidopsis* vacuolar membranes (50 μ g protein) were incubated for 30 min with 20 μ g TWD1-3 or 15 μ g 14-3-3-GF14- ω protein in sorbitol buffer (400 mM sorbitol, 30 mM KCl, 20 mM HEPES, pH 7.2) at 4°C. Proteins were expressed from plasmids pTWD1-3 (Geisler *et al.*, 2003) and pMP681 (Baunsgaard *et al.*, 1998), purified by Ni-affinity chromatography, and dialyzed against sorbitol buffer. Vesicles were pelleted by ultracentrifugation and washed twice with sorbitol buffer. Equal volumes of pellets and supernatants (20 μ l of 360 μ l final volume) were separated by 15% PAGE, and TWD1-3 and 14-3-3-GF14- ω were detected using monoclonal anti-pentaHIS antibodies.

Coimmunoprecipitation

Immunoprecipitations using protein G-coupled anti-AtMRP1 were carried out as described in Geisler *et al.* (2003) with the following modifications: microsomal membranes prepared from *Arabidopsis* wild-type leaves were enriched for vacuolar membranes by pooling immunopositive TWD1 fractions obtained by sucrose gradient centrifugations (fractions 8–10; see Figure 5C). Immunoprecipitations using anti-HA affinity matrix were carried out according to the manufacturer (Roche Diagnostics, Rotkreuz, Switzerland) with microsomes derived from *Arabidopsis* plants expressing an N-terminal HA-tagged version of TWD1 (TWD1-HA; Geisler *et al.*, 2003). After cross-linking, membrane proteins were solubilized using 0.1% (vol/vol) Brij 35 and 0.05% (wt/vol) CHAPS in 10 mM potassium phosphate, pH 7.8, 250 mM sucrose, and 20% (vol/vol) glycerol. Eluted proteins were separated by PAGE using 7.5%, 12.5% Laemmli gels, or 4–12% Criterion XT Bis-Tris gels (Bio-Rad Laboratories, Reinach, Switzerland) in the presence or absence of 1 mM DTT.

Vacuolar Uptake Experiments

Vacuoles were prepared from *Arabidopsis* cell suspension cultures (Axelos *et al.*, 1992) and transport studies using the silicon oil centrifugation technique was carried out as described in Frangne *et al.* (2002). Some reactions were performed in the presence of 1 μ M bovine brain calmodulin and 1 μ M purified TWD1-3 protein (Geisler *et al.*, 2003). Uptake was started by addition of 30 μ l concentrated vacuole suspension and stopped after 10 min by centrifugation. ¹⁴C-metolachlor-GS and ³H-estradiol- β -glucuronide was determined by scintillation counting of aqueous phases and –ATP assays were considered as time 0. The vacuolar volume was calculated by the addition of 0.05 μ Ci ³H₂O in separate assays. Conjugate-uptake experiments were per-

formed with three independent vacuole preparations with five replicas each for each time point.

RESULTS

TWD1 Interacts Specifically with AtMRP1 and AtMRP2

Previously, we have isolated multidrug resistance (MDR)-like ABC transporter AtPGP1 as TWD1 interacting protein by screening an *Arabidopsis* cDNA library with the entire cytosolic domain of TWD1 as bait (Geisler *et al.*, 2003). One of 48 sequenced prey clones encoded the C-terminal peptide (BE11) of multidrug resistance-associated (MRP)-like ABC transporter AtMRP1 (Lu *et al.*, 1998). This interaction is specific because yeast strains expressing both proteins expressed high levels of β -galactosidase activity and were able to grow on medium lacking histidine, indicating a direct interaction between these two fusion proteins (Figure 1A). Negative controls of GAL4-binding domain (BD) or activation domain (AD) alone were not interacting with TWD1 or AtMRP1, respectively. Interestingly, as in the case of the AtPGP1 TWD1 interaction (Geisler *et al.*, 2003), the AtMRP1 prey also coded for the C-terminus of AtMRP1. This domain comprises the C-terminal nucleotide binding fold covering the Walker A and B boxes and the intermediate ABC signature (Sanchez-Fernandez *et al.*, 2001; Martinoia *et al.*, 2002).

We generated GAL4-BD fusions of homologous stretches of AtMRP2, the closest homologue of AtMRP1 sharing 85% sequence identity, and some selected members of the *Arabidopsis* MRP/ABCC gene family (Kolukisaoglu *et al.*, 2002; Figure 1B). These peptides were tested for their ability to bind TWD1. AtMRP2—but not AtMRP4, AtMRP5, AtMRP7, and AtMRP13—interacted specifically with TWD1 in the yeast two-hybrid system as judged from β -galactosidase and His auxotrophy analysis. β -galactosidase activity (but not the growth assay on $-$ HIS plates) for MRP2 was about 30% lower, possibly indicating a slightly lower magnitude of interaction.

To verify the two-hybrid data *in vitro*, a part of the two-hybrid peptide BE11 of AtMRP1 (E₁₂₁₇–S₁₃₉₂) was expressed in *E. coli*. This peptide was incubated as total lysate with purified TWD1-3 protein (M₁–K₃₃₇) immobilized on Affigel beads, constituting a highly specific TWD1 affinity matrix (Geisler *et al.*, 2003). The TWD1 matrix was able to specifically sediment the AtMRP1 C-terminus of 20 kDa from soluble *E. coli* extracts (Figure 1C, lane 1). No proteins were detected in controls in which the empty Affigel resin or a vector control lysate (Figure 1C, lane 2 and lane 3, respectively) were used. Disproportional staining of TWD1-3 and AtMRP1-1 peptides is due to different affinities of the used anti-pentaHis and anti-RGSHIS recognizing TWD1-3 and AtMRP1-1, respectively.

Interaction with AtMRP1 Is Mediated Mainly by the Putative TPR Domain of TWD1

Homology modeling revealed that the putative TPR domain localized in the C-terminal part of TWD1 has the potential to form a scaffold (our unpublished results) known to act as protein-protein interaction surface (Owens-Grillo *et al.*, 1996; Das *et al.*, 1998; Pratt *et al.*, 2001). To assess whether the TPR domain provided the interaction with AtMRP1, we tested GAL4-BD fusions covering the PPIase-like (M₁–E₁₆₃) and the TPR domains (E₁₆₃–K₃₃₇) of TWD1 in the yeast two-hybrid system. Indeed, AtMRP1 interacted with the C-terminal stretch comprising the TPR domain but not with the N-terminus containing the PPIase-like domain as monitored by β -galactosidase and His auxotrophy analysis (Figure 2). Because the TPR construct (TPR+CaMbd) used contained a

motif with the potential to bind calmodulin downstream of the TPR repeats, we generated GAL4-BD fusions of the TPR motifs alone. This construct revealed similar two-hybrid profiles as the TPR+CaMbd construct proving that the TPR domain is mainly responsible for TWD1 complexation. Compared with the entire TWD1 bait, the TPR constructs displayed a reduced β -galactosidase activity, whereas the ability to grow on $-$ HIS plates was in the same range. However, because the β -galactosidase activities (5.95 ± 0.53 and 5.41 ± 0.56 U/mg) were significantly higher than the vector control levels (0.98 ± 0.22 U/mg), the TPR domain alone seems to be sufficient for interaction with AtMRP1.

The C-terminus of AtMRP1 Contains a Calmodulin-binding Domain

A search for candidate motifs that might be responsible for interaction to TWD1 revealed the existence of a putative calmodulin-binding domain (CaMbd) as well located in the C-terminus of AtMRP1 (W₁₂₃₁–Y₁₂₄₆; Figure 3A). Calmodulin-binding motifs are more conserved in terms of structural features than in terms of primary sequence homology (Geisler *et al.*, 2000a, 2000b): many CaM targets contain hydrophobic residues at positions 5 and 8 that are flanked by aromatic residues at positions 1 and 15. Although this rule is not followed strictly in AtMRP1, in an α -helical wheel presentation the peptide shows a segregation of basic and polar residues to one side and hydrophobic residues to the other side (Geisler *et al.*, 2000b), typical for CaMbd (our unpublished results). A putative CaMbd is also well conserved in AtMRP2 (Figure 3A).

Indeed, by using calmodulin-overlays, we were able to show that the expressed C-terminus was able to bind calmodulin in a calcium-dependent manner. Calmodulin-positive bands matched exactly the size of the known molecular weight of AtMRP1 C-terminus as could be judged from Western analysis of samples run in parallel (Figure 3B). Smaller soluble peptides of the *E. coli* cell lysate also reacted with calmodulin (see asterisks in Figure 3B), but are probably unspecific because CaM binding took place in the absence of calcium and was also found with vector control lysates (our unpublished results).

The C-terminal Calmodulin-binding Domain of AtMRP1 Is Mainly Responsible for TWD1 Interaction

To elucidate whether the identified CaMbd motif in the AtMRPs corresponds to the acceptor for the TPR motif of TWD1, we deleted this peptide (E₁₂₁₇–P₁₂₅₁) from the GAL4-AD-MRP1 fusion construct. Two-hybrid analysis showed that with the entire soluble part of TWD1, β -galactosidase activity as well as growth on $-$ HIS plates was heavily reduced (Figure 3C). Even more obvious was the effect with the C-terminus of TWD1, which contains mainly TPR stretches. In summary, this indicates that the putative CaMbd alone can function as acceptor for the TWD1 TPR domain.

On the basis of these findings, we asked whether calmodulin would affect the binding of the AtMRP1 C-terminus. Preincubations with a high concentration of spinach calmodulin (500 μ M) before addition of the AtMRP1 target seemed to have no effect on AtMRP1 binding to the TWD1-matrix in pull-down assays (Figure 3D).

PAS1 Is Able To Interact with AtMRP1 But Uses a Different Interaction Domain

A FKBP gene family with 17 putative members (Harrar *et al.*, 2001) has been identified in *Arabidopsis*. Members that contain three repetitions of TPR motifs are TWD1, both ROF

Figure 1. TWD1 interacts specifically with the C-termini of AtMRP1 and AtMRP2. (A) Yeast two-hybrid analysis of TWD1 interacting clones. Two-hybrid screening of an *Arabidopsis* cDNA library using TWD1 (BD-BusB) resulted in the identification of AtMRP1 (clone BE11). Homologous stretches of selected AtMRPs were fused to a GAL4 activation domain and tested for interaction with the soluble part of TWD1 (BD-BusB). Negative controls are from top to bottom: BD-BusB/AD vector, BD vector/AD-MRP1, and AD vector/BD vector. Activation of histidine growth reporter (growth on -HIS) is indicated by + and -; LacZ reporter activities are displayed as units per mg; error bars represent standard deviations from three to five independent transformants. (B) *Arabidopsis* MRP-like ABC transporters cluster into two clades. The tree was modified from Kolukisaoglu *et al.* (2002) and identity and accession numbers can be deduced from there. Positive and negative two-hybrid interactions of AtMRPs tested against TWD1 in A are indicated with + and -, respectively. (C) In vitro interaction between TWD1 and AtMRP1. A TWD1 affinity matrix was incubated with cleared *E. coli* lysates containing the expressed C-termini of AtMRP1 (lane 1) or the vector control (lane 3). As negative control, empty Affigel beads were incubated with the C-terminus of AtMRP1-1 lysate (lane 2). Matrix-eluted proteins were separated by PAGE and immunoprobed against penta-His and anti-RGSHis recognizing the TWD1-3 and AtMRP1-1 peptides, respectively. Note that unproportional staining of TWD1-3 and AtMRP1-1 peptides is due to different affinities of the used antisera toward their antigens (see text).

Figure 2. The TPR domain of TWD1 domain is responsible for the interaction with AtMRP1. TWD1 fragments fused to an GAL4 binding domain tested for interaction with AD-MRP1 are represented by boxes as follows: PPlase, *cis-trans*-peptidyl prolyl isomerase domain; TPR, tetratricopeptide repeat; C, calmodulin-binding domain; M, membrane anchor. Amino acid positions are indicated. Negative controls are from top to bottom: BD-BusB/AD vector, BD vector/AD-MRP1, and AD vector/BD vector. Activation of histidine growth reporter (growth on -HIS) is indicated by + and - and LacZ reporter activities are displayed as mean units per mg; error bars represent standard deviations from three to five independent transformants.

isoforms, ROF1 and ROF2 (Vucich and Gasser, 1996; Harrar *et al.*, 2001), and PASTICCINO1 (PAS1; Faure *et al.*, 1998; Vittorioso *et al.*, 1998; Figure 4A). To demonstrate specificity toward AtMRP1, we therefore tested TPR domains of ROF1 and PAS1 for AtMRP1 interaction in the yeast two-hybrid system. PAS1, but not ROF1 is able to interact with AtMRP1 (Figure 4B). Unlike TWD1, PAS1 seems not to interact with the CaMbd of AtMRP1 but with different regions of the C-terminus as two-hybrid analysis with AtMRP1 lacking the CaMbd showed very similar two-hybrid profiles (Figure 4B).

AtMRP1 and TWD1 Form a Complex on the Central Vacuolar Membrane

The intracellular localization of AtMRP1 is unknown; however, the transport specificity of AtMRP1 approximates that of endogenous vacuolar GS-conjugate pumps (Lu *et al.*, 1998, 2001; Sanchez-Fernandez *et al.*, 2001; Martinoia *et al.*, 2002).

To determine precisely the intracellular localization of AtMRP1 we cloned AtMRP1 into a plant binary vector and constructed two independent C-terminal fusions with an enhanced version of the green fluorescent protein (EGFP). Both EGFPs were inserted into the C-terminal extension (CTE) after the second nucleotide binding fold of AtMRP1 (Figure 5A).

Confocal microscope analysis of heterozygous transgenic seedlings showed high levels of AtMRP1-EGFP fluorescence in the root apex and root epidermis (Figure 5Bi), whereas plants transformed with vector control revealed no GFP fluorescence (our unpublished results). Surprisingly, no fluorescence was found in the shoot. In the root hair zone, root hairs revealed fluorescent caps at their tips (arrows in Figure 5Bii). Fluorescence surrounded the cells, however, even from close-ups it was difficult to differentiate between tonoplast and plasma membrane, mainly because in fully turgid cells the cytoplasm is limited to just small stripes.

Therefore, we expressed AtMRP1-EGFP in onion epidermis cells that were transiently transformed by particle bom-

bardment. The vacuolar GFP marker KC01, a component of the slow-vacuolar K⁺ channel (Schönknecht *et al.*, 2002) labeled the tonoplast, that surrounds the central vacuole, sparing the cytoplasm, which is mainly restricted to a small spot (red color and asterisk in Figure 5Biii). Both EGFP constructs (green color in Figure 5B, iv and v) showed very similar expression patterns compared with the vacuolar control, suggesting that AtMRP1 resides on the tonoplast. In some onion cells small vesicles of unknown origin were labeled in addition to the tonoplast (arrows in Figure 5B, v and vi).

To immunologically verify these data, we probed *Arabidopsis* microsomes separated by linear sucrose gradient density centrifugation with a polyclonal antiserum that was raised against a 17-mer peptide of AtMRP1 (N₁₂₁₀-R₁₂₂₆). This antiserum detected a single band of the expected size (~180 kDa) in fractions 8–10 (sucrose concentrations between 35 and 43%) of the sucrose gradient (Figure 5C). AtMRP1 colocalized with the vacuolar V-type H⁺-ATPase (same distribution of peak fraction), whereas markers for the plasma membrane (P-type H⁺-ATPase) or ER (BIP) cross-reacted with other fractions (Figure 5C).

A unique feature of TWD1 is the existence a C-terminal hydrophobic α -helical region, which anchors it apparently both in the plasma membrane and the central vacuolar membrane, the tonoplast. Using electron microscopy, a HA-tagged version of constitutively overexpressed TWD1 (TWD1-HA) has been immunolocalized in the plasma membrane and the vacuolar membrane (Kamphausen *et al.*, 2002). In a parallel study, using membrane fractionation and cellular immuno-localization techniques, a plasma membrane location has been verified (Geisler *et al.*, 2003). However, also in those continuous sucrose gradients TWD1-positive fractions show a clear overlap with vacuolar fractions (identified using the vacuolar V-type H⁺-ATPase marker) of lower sucrose percentage/higher membrane density (fractions 8–10; Geisler *et al.*, 2003).

Figure 3. A calmodulin-binding domain in AtMRP1 mediates interaction to TWD1. (A) Alignment of a putative calmodulin-binding domain (CaMbd) of AtMRP1 (MIPS code At1g30400) and AtMRP2 (At2g34660) with selected CaMBds. CaMBds of different origin were taken from Geisler *et al.* (2000b) and aligned using MegAlign (DNASTar, Madison, WI). Position of conserved residues essential for calmodulin binding are boxed, and conserved residues are printed in bold. (B) Calmodulin overlay the AtMRP1 C-terminus. Soluble *E. coli* extracts of cells expressing the MRP1 C-terminus (AtMRP1-1: residues 1217–1392) were transferred onto nitrocellulose and probed using anti-RGSHis antisera or biotinylated calmodulin (CaM-overlay) in the presence (+Ca) or absence of calcium (–Ca). The asterisk marks binding of *E. coli* proteins that were found also with vector control lysates. (C) The putative CaMbd domain of AtMRP1 is mainly responsible for the interaction with TWD1. AD-MRP1 fragment with (■) and without putative CaMbd (□) are tested against indicated TWD1 fragments fused to a GAL4 binding domain. Activation of histidine growth reporter (growth on –HIS) is indicated by + and – and LacZ reporter activities are displayed as mean units per mg; error bars represent standard deviations from three to five independent transformants. (D) Calmodulin does not affect the interaction between TWD1 and AtMRP1 in vitro. A TWD1 affinity matrix was incubated with cleared *E. coli* lysates containing the expressed C-termini of AtMRP1 (control, lane 1). Some reactions were preincubated with spinach calmodulin in the presence (+Ca, lane 2) or absence of calcium (–Ca, lane 3). Matrix-eluted proteins were separated by PAGE and immunoprobed against anti-pentaHis.

To verify the dual location of TWD1, we reanalyzed selected fractions derived from linear sucrose gradient separation of TWD1-HA microsomes. In contrast to our previous study, we analyzed equal protein amounts of each fraction and used as a vacuolar marker antisera directed against a mung bean vacuolar PPase. Western blotting clearly indicates that TWD1-HA-positive fractions overlap to similar extents with both plasma membrane (40 and 50% sucrose) and vacuolar fractions (30 and 40% sucrose; Figure 6A).

To demonstrate the TWD1-AtMRP1 complex in vivo, AtMRP1 and TWD1 were immunoprecipitated from solubilized wild-type and transgenic TWD1-HA (Geisler *et al.*, 2003) microsomes, respectively, after cross-linking with thiol-cleavable DTBP (Figure 6B). Cross-linking was used to avoid disruption of protein-protein interactions during strong detergent treatments to solubilize both membrane proteins from the tonoplast.

Using immobilized anti-AtMRP1 antiserum for immunoprecipitation, wild-type TWD1 was detectable in a Western analysis (Figure 6B, lane 3), suggesting coprecipitation with AtMRP1 (Figure 6B, lane 1). The same was true for TWD1-HA being slightly bigger than the wild-type protein

(Figure 6B, lane 6). This was the case under reducing conditions (+DTT), which cleave the DTBP cross-linker between AtMRP1 and TWD1. Under nonreducing conditions, a high-molecular-weight complex of more than 250 kDa was detected using anti-TWD1 (Figure 6B, lane 5), suggesting either multi-merization of more than one TWD1 with one AtMRP1 or the involvement of so far unknown components. No AtMRP1 or TWD1 could be detected in control experiments using empty protein G, respectively (lanes 2, 4, and 7).

Vice versa, using an anti-HA affinity matrix we were able to detect AtMRP1 beside TWD1-HA; however, the entire complex could not be analyzed by PAGE under nonreducing conditions (lane 9).

Purified TWD1 Protein Modulates Uptake of AtMRP-like ABC Transporter Model Substrates into Isolated Vacuoles

All cloned AtMRPs have been shown to transport GS conjugates, such as metolachlor-GS (MOC-GS), a glutathionated chloroacetanilide herbicide, to different degrees in vitro (Rea *et al.*, 1998; Sanchez-Fernandez *et al.*, 2001; Martinoia *et al.*, 2002). But only for AtMRP1 and AtMRP2 are quantitative

Figure 4. The PAS1 but not ROF1 TPR domain of TWD1 interacts with AtMRP1 in yeast two-hybrid assays. (A) Domain structures of high-molecular-weight FKBP from *Arabidopsis*. Functional domains of At-FKBP12, TWD1, ROF1, ROF2, and PAS1 are boxed and indicated as follows: *cis-trans* peptidyl-prolyl isomerase domain (FKBP), red; tetratricopeptide repeats (TPR) blue; CaMbd, violet; membrane anchor, green. (B) Specificity of PAS1-AtMRP1 interaction in the yeast two-hybrid system. TPR fragments of PAS1 and ROF1 were fused to a GAL4 binding domain and tested for interaction with the C-terminus of AtMRP1 including the CaMbd (AD-MRP1+CaMbd) or lacking the CaMbd (AD-MRP1-CaMbd). Negative controls are from top to bottom: AD-MRP1/BD vector and AD vector/BD vector. Activation of histidine growth reporter (growth on -HIS) is indicated by + and - and LacZ reporter activities are displayed as mean units per mg; error bars represent standard deviations from three to five independent transformants.

data available (Liu *et al.*, 2001). The overall transport capacity of AtMRP2, the sole AtMRP that has been roughly localized to vacuolar-enriched membranes (Liu *et al.*, 2001), exceeds that of AtMRP1. The substrate specificity of AtMRP1 fits better that of an endogenous vacuolar GS-conjugate pumps than does AtMRP2 (Lu *et al.*, 1998, 2001).

To investigate whether the interaction of TWD1 with vacuolar ABC transporters (AtMRP1 and AtMRP2) has a physiological impact on their transport activities, we measured the uptake of two ABC transporter model substrates into isolated vacuoles in the presence of purified TWD1-3 and calmodulin. Isolated *Arabidopsis* vacuoles offer an ideal test system, because the C-termini of transporters as putative interacting domains are thought to face the cytoplasm (Rea *et al.*, 1998; Sanchez-Fernandez *et al.*, 2001; Martinoia *et al.*, 2002) and are therefore accessible for interfering proteins in this assay. MOC-GS uptake was significantly reduced by both, TWD1-3 (34% inhibition) and calmodulin (26% inhibition) preincubation. On the other hand, TWD1-3 protein stimulated the uptake of 17 β -estradiol 17-(β -D-glucuronide) (E₂17 β G), a glucuronide test substrate, whereas calmodulin had no significant effect.

Vacuolar microsomes offered in excess were able to quantitatively sediment the TWD1-3 protein as demonstrated by Western analysis (Figure 7B, lane 1) when equal volumes of bound (P) and unbound fractions (SN) were used. No TWD1-3 protein was detected in bound fractions when microsomes were omitted from the assay (Figure 7B, lane 3), indicating the specificity of the interaction. Regarding the expected molar ratio of AtMRP1/TWD1, the high amount of sedimented TWD1 indicates that other transporters besides AtMRP1 (i.e., AtMRP2) might contribute to this effect. As a specific control, we tested the *Arabidopsis* 14-3-3 protein GF14- ω known to structurally resemble TPR domains (Das *et al.*, 1998). This isoform of 14-3-3 (Baunsgaard *et al.*, 1998) was also able to show some binding to vacuolar membranes but to a much lesser extent (<50%; Figure 7B, lanes 5 and 6).

DISCUSSION

Previously, TWD1 has been demonstrated to interact with *Arabidopsis* p-glycoprotein ABC-transporter AtPGP1 and its closest homologue, AtPGP19 (Geisler *et al.*, 2003). Physiological and biochemical investigation of *atpgp1 atpgp19* double mutants and *twd1* plants suggest that a functional TWD1-AtPGP1/AtPGP19 complex is required for proper plant development. Therefore, a regulatory role of TWD1 on AtPGP1/AtPGP19 transport activities has been suggested (Geisler *et al.*, 2003).

To further understand the dramatic pleiotropic phenotype that is caused by loss-of-function mutation of the *TWD1* gene, we were interested in other TWD1-interacting proteins. AtMRP1, a full-size ABC transporter of the MRP/ABCC subclass (Kolukisaoglu *et al.*, 2002; Martinoia *et al.*, 2002), has been isolated in a yeast two-hybrid screen as TWD1-interacting protein. In this study, we demonstrate molecular interactions between TWD1 and *Arabidopsis* ABC transporters AtMRP1 and its closest homologue AtMRP2. Using GFP-tagging and membrane separation we localized AtMRP1, like TWD1, to the central vacuolar membrane and show that TWD1 has an impact on ABC transporter uptake activities into isolated vacuoles.

TWD1 Interacts Specifically with AtMRP1 and AtMRP2 via Its TPR Domain

Quantification of histidine reporter growth and the LacZ reporter of retransformed prey clones for isolated AtMRP1 with the TWD1 bait (BD-BusB) confirmed the interaction between the two proteins. In vitro interaction using a highly specific TWD1 affinity matrix (Geisler *et al.*, 2003) verified the two-hybrid data (Figure 1). To further sustain specificity of interaction, we tested other C-termini of homologues of AtMRP1 in the two-hybrid system. Based on the AGI data (Arabidopsis Genome Initiative, 2000), 14 MRP-like transporter genes have been suggested for *Arabidopsis*

Figure 5. AtMRP1 is localized on the central vacuolar membrane. (A) Structural model of AtMRP1. Functional domains of AtMRP1 are boxed. A putative calmodulin-binding domain (CaMbd) and extension of the isolated two-hybrid clone BE11 as well as the sites of insertion of enhanced GFPs (EGFP) into constructs pGPTV-MRP1-12 and pGPTV-MRP1-13 are indicated. Abbreviations are as follows: NTE, N-terminal extension; TMD, transmembrane domain; NBF, nucleotide binding fold; LR, linker region; CTE, C-terminal extension. (B) Laser scanning confocal analysis of plant material expressing a GFP-tagged version of AtMRP1. Images represent internal optical sections generated by CLSM from root material of heterozygous plants expressing ectopically (i and ii) and onion epidermis cells transiently expressing AtMRP1-EGFP (iv and v). The tonoplast surrounding the cytoplasm including the nucleus is indicated by asterisks; small GFP-labeled cytoplasmic structures obtained with construct pGPTV-MRP1-13 are marked by arrows. (i) Root apex. (ii) Close-up of the root hair zone. (iii) Onion epidermis cell transiently expressing the tonoplast marker KCO1-GFP (Schönknecht *et al.*, 2002; red color). (iv) Onion epidermis cell expressing AtMRP1-EGFP (construct pGPTV-MRP1-12, green color). (v) Onion epidermis cell expressing AtMRP1-EGFP (construct pGPTV-MRP1-13, green color). (vi) Same cells in a bright field. (C) *Arabidopsis* microsomal fractions from wild-type plants separated by linear sucrose gradient centrifugation were probed with anti-AtMRP1 antisera. Origin of immunopositive fractions was ascertained by Western blots using antisera against the marker proteins vacuolar V-type H⁺-ATPase, ER localized BIP, and the plasma membrane-bound P-type H⁺-ATPase (Geisler *et al.*, 2000a).

(Kolukisaoglu *et al.*, 2002; Martinoia *et al.*, 2002) that can be assigned—with the exception of MRP13—to two clades (Figure 1B). None of the selected transporters, covering most subbranches of the phylogenetic tree of the AtMRP gene family, interacted with TWD1. The only other tested AtMRP to interact with TWD1 was AtMRP2, the closest homologue

of AtMRP1. The fact that binding to TWD1 has been found only for AtMRP1 and AtMRP2 is an interesting finding and further proves specificity of this interaction.

A similar picture is true for AtPGPs interacting with TWD1 on the plasma membrane. Here, only AtPGP19, the closest homologue of AtPGP1 was shown to be a TWD1-

Figure 6. In vivo interaction between TWD1 and AtMRP1. (A) TWD1-HA overlaps with vacuolar and plasma membrane fractions in a continuous sucrose gradient. *Arabidopsis* microsomal fractions from plants expressing TWD1-HA were separated by linear sucrose gradient centrifugation and equal amounts of protein of selected fractions were probed with anti-HA and anti-MRP1. Origin of immunopositive fractions was ascertained by Western blotting using antisera against the marker proteins vacuolar PPase, ER localized BIP and the plasma membrane-bound P-type H⁺-ATPase (Geisler *et al.*, 2000a). (B) Immunoprecipitation of a TWD1-AtMRP1 complex. Microsomal membranes from *Arabidopsis* wild-type (lanes 1–5) or TWD-HA plants (lanes 6–9) were cross-linked with DTBP, solubilized using 0.1% Brij 35 and 0.05% CHAPS and immunoprecipitated using an anti-AtMRP1 or anti-HA affinity matrix, respectively. Immunoprecipitated proteins were separated by 7.5% (lanes 1, 2, and 5), 12.5% (lanes 3, 4, 6, and 7) or 4–12% PAGE (lanes 8 and 9) in the presence (+) or absence of DTT (–) and probed with anti-AtMRP1 (lanes 1–2, 8–9), anti-TWD1 (lanes 3–5), and anti-HA (lanes 6–9), respectively. As negative controls, unspecific binding of proteins to empty protein G was monitored (lanes 2 and 4). Note that the size difference of the coprecipitated wild-type TWD1 in lane 3 having a slightly smaller weight than the HA-epitope-tagged TWD1 (lane 6) is due to the lack of the HA-epitope. Molecular size markers on the left and right correspond to lanes 1–2, and 5 and lanes 3–4, 6–7, respectively; positions of TWD1 and AtMRP1 are indicated.

interacting protein (Geisler *et al.*, 2003). As with AtPGP1, the interacting AtMRP1 clone BE11 isolated from a two-hybrid screen covers nearly the entire C-terminus of AtMRP1. AtMRP1 and AtMRP2 bear—in contrast to all other AtMRPs and P-glycoproteins/MDRs/ABCs (Liu *et al.*, 2001)—a so-called C-terminal extension domain (CTE; see Figure 5A), which extends their C-terminus far longer (405 compared with 109 residues) than in AtPGP1. Based on in vitro interaction experiments, the TWD1-interacting region was further limited to 175-aa residues (E₁₂₁₇–S₁₃₉₂). However, identification of a putative calmodulin-binding domain just upstream of the Walker A motif of the C-terminal NBF allowed to map the TWD1 docking domain down to 34 aa (E₁₂₁₇–P₁₂₅₁) as deletion of this domain in the yeast two-hybrid construct abolishes interaction (Figure 3). This do-

Figure 7. TWD1 binds to and modulates uptake of ABC transporter model substrates into vacuoles. (A) Uptake of ¹⁴C-metolachlor-GS and ³H-estradiol-β-glucuronide were carried out in the absence (control) or presence of each 1 μM purified TWD1-3 protein (+TWD1-3) and 1 μM calmodulin (+CaM), respectively. Conjugate uptake was measured with five replicates for each time point. Relative activities are displayed as means with standard deviations from three independent vacuole preparations. Import activities being statistically different (Mann-Whitney *U* test, *p* > 0.05) compared with control experiments are indicated by an asterisks. (B) Purified TWD1-3 protein binds specifically to vacuolar membranes. *Arabidopsis* vacuoles (+vac) were incubated with purified TWD1-3 or 14-3-3 protein (14-3-3-GF14-ω; Baunsgaard *et al.*, 1998) and pelleted by ultracentrifugation. Equal volumes of pellets (P) and supernatants (SN) were separated by PAGE and TWD1-3, and 14-3-3-GF14-ω were detected using anti-pentaHIS antibodies.

main shows typical features of CaMbds (see above). Calmodulin binding to the expressed AtMRP1 C-terminus verifies the identity of this domain.

Vice versa, mapping of TWD1 motifs required for AtMRP1 binding demonstrated that AtMRP1 recognizes the C-terminal TPR domain, whereas AtPGP1 was shown to interact with the N-terminal PPIase-like domain (Geisler *et al.*, 2003). This is not surprising because TPRs are often implicated in protein-protein interactions observed with diverse proteins and other FKBP (Das *et al.*, 1998; Harrar *et al.*, 2001). Recently, a region of TWD1 containing the TPR domain has been shown to bind AtHsp90.1 and to prevent

citrate synthase aggregation in analogy to the human PPIase TPR domains (Kamphausen *et al.*, 2002).

However, besides TWD1, only the TPR domain of PAS1 but not of ROF1 was able to interact with AtMRP1, emphasizing the specificity of interaction. Interestingly, TWD1 and PAS1 seem to use different docking sites on the AtMRP1 molecule because deletion of the putative CaMbd did not reduce interaction with PAS1 (Figure 4B). Based on immunological data, PAS1 has a nuclear localization (Carol *et al.*, 2001), making an interaction between PAS1 and vacuolar localized AtMRP1 (or AtMRP2) unlikely. This again highlights the role of TWD1 as the sole membrane-associated FKBP that interacts with integral membrane proteins such as the AtMRPs.

TWD1 Functionally Interacts with Vacuolar ABC Transporters

AtMRP1 has been identified by screening an ordered expression library with polyclonal antibodies raised against *Arabidopsis* plasmalemma-enriched membranes (Galaud *et al.*, 1999). Using AtMRP1-specific antisera, we immunodetected AtMRP1 in microsomal fractions from vacuoles as shown by linear sucrose gradient centrifugation. Cross-reaction of the antibodies to the close homologue vacuolar-localized AtMRP2 (see below) cannot be ruled out because the 17-mer peptide used as antigen is also well conserved in the latter (88% sequence identity). However, because a band was exclusively observed in the tonoplast enriched fractions, our results indicate that even assuming a cross-reaction, both AtMRP1 and AtMRP2 reside on the vacuolar membranes.

GFP-tagging clearly showed that AtMRP1 resides on the tonoplast. In line with these data, transport activities assigned to AtMRP1 are found on vacuoles (Lu *et al.*, 1997, 1998).

This is to our knowledge the first detailed localization of an MRP-like ABC transporter in plants. The closest homologue of AtMRP1, AtMRP2, has been detected in vacuolar-enriched fractions of *Arabidopsis* using polyclonal antisera directed against a short peptide of AtMRP2 (Liu *et al.*, 2001; A₁₅₆₉-R₁₅₇₉). The AtMRP2 antibody has been suggested to be monospecific because it does not cross-react with heterologous produced AtMRP1. However, it seems very likely that both transporters reside on vacuolar membranes.

Considering the vacuolar location of AtMRP1 (and probably also of AtMRP2), we wanted to explore if the shown interaction of both proteins has an impact on vacuolar ABC transporter activities. Purified TWD1-3 protein inhibits metolachlor-GS conjugate uptake but stimulates estradiol- β -glucuronide import into vacuoles (Figure 7A). Both are well-described substrates for AtMRP1 and AtMRP2. Therefore these transporters are good candidates for catalyzing this uptake in vivo. Stronger effects of the TWD1 action might be masked by endogenous TWD1 still partially bound to the respective transporters in isolated vacuoles. In addition, other signaling components (like protein kinases) lacking in the in vitro assay might amplify the modulating effect of TWD1 in the cell. Finally, it could be, that activation of vacuolar AtMRP-mediated transport activities via TWD1 is increased under stress conditions. If this would be the case, modulation by TWD1 could already have taken place during vacuole isolation because treatment of plants with cellulases and pectinases is a typical stress inducing treatment.

Calmodulin, having the potential to bind to the same epitope as TWD1, could partially mimic TWD1 inhibition on metolachlor-GS uptake, but failed in stimulating estradiol- β -glucuronide transport. As the CaMbd, the most probable acceptor for the TPR domain of TWD1, is located in the

direct proximity of the MRP1 C-terminal nucleotide-binding fold (NBF2; see Figure 5A), it is conceivable that interaction of this domain with TWD1 affects ATP binding and/or hydrolysis. Furthermore, up- and downregulation of transport activities via protein interaction is not unusual, especially because the two different substrates might use different substrate binding sites. Interestingly, TWD1 mimics an effect observed with glutathione conjugates: although GS-X transport is competitively inhibited by other GS-X conjugates, several GS-X are able to strongly enhance estradiol glucuronide transport in intact vacuoles (Klein *et al.*, 1998) or by heterologously expressed AtMRP2 (Liu *et al.*, 2001). On the other hand, the possibility that AtMRP1 and AtMRP2 are negatively regulated by TWD1 or CaM cannot be excluded.

In contrast to these results we found no interference of CaM on AtMRP1-TWD1 interaction with in vitro pull-down assays. This might indicate on the one hand, that calmodulin and TWD1 use different binding domains (thus CaM does not bind to the designated motif), which is unlikely because no other putative CaMbd could be identified in the AtMRP1 C-terminus. On the other hand, it might be possible that TWD1 and CaM do not interfere sterically while binding AtMRP1. This, however, is also not likely because both proteins are relatively space filling compared with the small stretch of maximal 34 residues acting as contact surface. Third, and most likely, it might be that the TPR of TWD1 owns a far higher affinity for the interaction domain of the AtMRPs compared with CaM.

Using a vacuolar pull-down assay, specific binding of TWD1-3 protein to vacuolar microsomes was demonstrated (Figure 7B). This is further support that the C-termini of interacting transporters as putative interacting domains are indeed facing the cytoplasm and are therefore accessible for TWD1 proteins in this assay. This fits well with current secondary structure models for AtMRPs (Rea *et al.*, 1998; Sanchez-Fernandez *et al.*, 2001; Martinoia *et al.*, 2002).

In contrast, the *Arabidopsis* 14-3-3 isoform GF14- ω (Baunsgaard *et al.*, 1998), known to structurally resemble TPR domains although overall sequence identity is low (Das *et al.*, 1998), binds to a lesser extent (<50%), emphasizing the specificity of TWD1-3 binding. Indeed, 14-3-3 proteins have been shown to downregulate currents of vacuolar SV channels by using whole-vacuole recordings (van den Wijngaard *et al.*, 2001). Interestingly, docking of 14-3-3 proteins to the plasma membrane H⁺-ATPase is induced by phosphorylation (Baunsgaard *et al.*, 1998), which is so far not known to be essential for binding of proteins to TPR domains.

Finally, our data indicate that TWD1 has a dual regulatory function on both the plasma membrane and vacuolar membrane. TWD1 forms complexes with pairs of ABC transporters of distinct subclasses: ABCB (AtPGP1/AtPGP19; Geisler *et al.*, 2003) and ABCC (AtMRP1/AtMRP2). This unusual distribution of high-molecular-weight FKBP might be a typical feature in higher plants. Recently, using quantitative immunogold electron microscopy wheat high-molecular-weight TaFKBP73 and TaFKBP77 have been shown to be present in the cytoplasm, but being transported into the nucleus upon heat-shock treatments (Dwivedi *et al.*, 2003).

Specificity of interaction between TWD1 and ABC transporters is mediated by protein-protein interactions with distinct domains of the multidomain FKBP TWD1. The PPIase-like domain and the TPR domain seem to be responsible for specificity of interaction with different subclasses of ABC transporters.

In vivo association between TWD1 and AtMRP1 was established by coimmunoprecipitation assays (Figure 6B). Therefore, one might assume that absence of TWD1 (posi-

tively modulating a vacuolar transport complex consisting of AtMRP1 and AtMRP2) is responsible for certain aspects of the *twd1* phenotype. This model is supported by several studies showing that immunophilins play a regulatory role in different multiprotein complexes (Cameron *et al.*, 1995; Timerman *et al.*, 1995; Hemenway and Heitman, 1996; Mealey *et al.*, 1999). However, in contrast to *atpgp1/atpgp19* plants resembling *twd1* mutants, *atmrp1* and *atmrp2* single and *atmrp1/atmrp2* double mutant plants, respectively, show no obvious phenotype (Ü. Kolukisaoglu, M. Klein, and E. Martinoia, unpublished results). Detailed morphological and biochemical analyses revealed that similar phenotypes of *twd1* and *atpgp1/atpgp19* plants are limited to early developmental stages. The *twd1* mutant exhibits a far more drastic reduction of cell elongation that corresponds to further reduced height of the entire plant. Disorientation of growth behavior resulting in pronounced twisting of all organs can be observed only in *twd1* plants. Compared with *atpgp1/atpgp19* plants, polar auxin transport rate in hypocotyls is also further reduced in *twd1* mutants (Geisler *et al.*, 2003). Therefore, it cannot be ruled out that the combination of mutations of TWD1 interacting partners might reveal the entire picture of the phenotype that was observed in *twd1* mutants. It is obvious that the differing aspects of the rather pleiotropic *twd1* phenotype needs to be addressed separately to result in a genetic dissection of the "twd1 syndrome." Generation of quadruple mutants that combine *atmrp1*, *atmrp2*, *atpgp1*, and *atpgp19* mutations would certainly be a next step in recombining the genetic network of TWD1 function. However, the genetic establishment of these lines is no short-term task, as the genes for the ABC transporters AtMRP2 and AtPGP1 are in close genetic proximity on the long arm of chromosome 2.

Loss of a TWD1-AtMRP1/AtMRP2 complex could therefore contribute to the *twd1* phenotype due to a lack of vacuolar import of yet unknown *in vivo* conjugate substrates. Regarding the role of AtPGP1 and AtPGP19 in indole-3-acetic acid (IAA) transport, it is tempting to speculate that AtMRP1 and AtMRP2 could act as transporters for IAA conjugated to amino acids. Removal of IAA-conjugates forming negatively charged organic substrates has been suggested to be catalyzed by ABC transporters such as AtMRP5 (Gaedeke *et al.*, 2001; Luschnig, 2002) and for related HsMRP1 glutamate conjugate transport has been demonstrated (König *et al.*, 1999).

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