The Arabidopsis ATP-binding cassette protein AtMRP5/AtABCC5 is a high affinity inositol hexakisphosphate transporter involved in guard cell signaling and phytate storage

Nagy, R; Grob, H; Weder, B; Green, P; Klein, M; Frelet-Barrand, A; Schjoerring, J K; Brearley, C; Martinoia, E

Nagy, R; Grob, H; Weder, B; Green, P; Klein, M; Frelet-Barrand, A; Schjoerring, J K; Brearley, C; Martinoia, E (2009). The Arabidopsis ATP-binding cassette protein AtMRP5/AtABCC5 is a high affinity inositol hexakisphosphate transporter involved in guard cell signaling and phytate storage. Journal of Biological Chemistry, 284(48):33614-33622.

Postprint available at: http://www.zora.uzh.ch

Posted at the Zurich Open Repository and Archive, University of Zurich. http://www.zora.uzh.ch

Originally published at: Journal of Biological Chemistry 2009, 284(48):33614-33622.
The Arabidopsis ATP-binding cassette protein AtMRP5/AtABCC5 is a high affinity inositol hexakisphosphate transporter involved in guard cell signaling and phytate storage

Abstract

Arabidopsis possesses a superfamily of ATP-binding cassette (ABC) transporters. Among these, the multidrug resistance protein AtMRP5/AtABCC5 regulates stomatal aperture and controls plasma membrane anion channels of guard cells. Remarkably, despite the prominent role of AtMRP5 in conferring partial drought insensitivity upon Arabidopsis, we know little of the biochemical function of AtMRP5. Our phylogenetic analysis showed that AtMRP5 is closely related to maize MRP4, mutation of which confers a low inositol hexakisphosphate kernel phenotype. We now show that insertion mutants of AtMRP5 display a low inositol hexakisphosphate phenotype in seed tissue and that this phenotype is associated with alterations of mineral cation and phosphate status. By heterologous expression in yeast, we demonstrate that AtMRP5 encodes a specific and high affinity ATP-dependent inositol hexakisphosphate transporter that is sensitive to inhibitors of ABC transporters. Moreover, complementation of the mrp5-1 insertion mutants of Arabidopsis with a MRP5 construct driven from a guard cell-specific promoter restores the sensitivity of the mutant to ABA mediated inhibition of stomatal opening. Additionally, we show that mutation of residues of the Walker B motif prevents from restoring the multiple phenotypes associated with mrp5-1. Our findings highlight a novel function of plant ABC transporters that may be relevant to other kingdoms. They also extend the signalling repertoire of this ubiquitous inositol polyphosphate signalling molecule.
THE ARABIDOPSIS ATP-BINDING CASSETTE PROTEIN ATMRP5/ATABCC5 IS A HIGH-AFFINITY INOSITOL HEXAKISPHOSPHATE TRANSPORTER INVOLVED IN GUARD CELL SIGNALING AND PHYTATE STORAGE

Réka Nagy*, Hanne Grob*, Barbara Weder*, Pornthip Green†, Markus Klein§, Annie Frelet*, Jan K Schjoerring¶, Charles Brearley†, Enrico Martinoia*

From *University of Zurich, Institute of Plant Biology, Zollikerstrasse 107, CH-8008 Zürich, Switzerland
†POSTECH-UZH Global Research Laboratory, Division of Molecular Life Sciences, Pohang University of Science and Technology, Pohang, 790-784, Korea
§ Tobacco Technologies, Tobacco Applied Biology, Philip Morris International, R&D innovation cube, Quai Jeanrenaud 5, CH - 2000 Neuchatel
¶ University of Copenhagen, Faculty of Life Sciences, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark
†University of East Anglia, School of Biological Sciences, Norfolk NR4 7TJ, Norwich, United Kingdom

Running title: ABCC5-mediated inositol hexakisphosphate transport

Correspondence to: Charles Brearley, University of East Anglia, School of Biological Sciences, Norfolk NR4 7TJ, Norwich, United Kingdom
E-mail: C.Brearley@uea.ac.uk; Phone: 0049 (0)1603592197; Fax: 0049 (0)1603592250
Arabidopsis possesses a superfamily of ATP-binding cassette (ABC) transporters. Among these, the multi-drug resistance protein AtMRP5/AtABCC5 regulates stomatal aperture and controls plasma membrane anion channels of guard cells. Remarkably, despite the prominent role of AtMRP5 in conferring partial drought insensitivity upon Arabidopsis, we know little of the biochemical function of AtMRP5. Our phylogenetic analysis showed that AtMRP5 is closely related to maize MRP4, mutation of which confers a low inositol hexakisphosphate kernel phenotype. We now show that insertion mutants of AtMRP5 display a low inositol hexakisphosphate phenotype in seed tissue and that this phenotype is associated with alterations of mineral cation and phosphate status. By heterologous expression in yeast, we demonstrate that AtMRP5 encodes a specific and high affinity ATP-dependent inositol hexakisphosphate transporter that is sensitive to inhibitors of ABC transporters. Moreover, complementation of the mrp5-1 insertion mutants of Arabidopsis with a MRP5 construct driven from a guard cell-specific promoter restores the sensitivity of the mutant to ABA – mediated inhibition of stomatal opening. Additionally, we show that mutation of residues of the Walker B motif prevents from restoring the multiple phenotypes associated with mrp5-1. Our findings highlight a novel function of plant ABC transporters that may be relevant to other kingdoms. They also extend the signalling repertoire of this ubiquitous inositol polyphosphate signalling molecule.

INTRODUCTION

Guard cells form pairs of cells, which are conjoined at their ends, in the epidermis of aerial tissues of plants. The cells surround a central pore, the stoma, through which gas exchange occurs. The principal gases exchanged are CO$_2$ and water vapour, and the function of the stomatal complex may be considered as the maximisation of CO$_2$ assimilation by photosynthesis for the minimization of water loss.

Guard cells, and hence the aperture of the central pore, are sensitive to environmental factors including light, temperature, CO$_2$ and ozone (1). Stomatal closure is initiated by the drought stress hormone abscisic acid (ABA). The closure of stomata is a result of loss of turgor of the delimiting guard cells as a consequence of ion efflux, predominantly Cl$^-$ and K$^+$, and metabolic conversion of organic acids into starch (2).

While the molecular identity of genes encoding the outward and inward K$^+$ conductances are known for Arabidopsis thaliana (3), it remains to be demonstrated whether the recently identified SLAC protein (4,5) encodes the S-type anion channel or is a subunit thereof.

The ATP-binding cassette family of membrane proteins is among the most ubiquitous and variable group of membrane proteins, and is most commonly associated with membrane transport phenomena. The substrates transported are especially diverse, and consequently a major obstacle to the interpretation of ABC transporter function, particularly pertinent in the context of guard cell function, is the identification of the substrate transported.

A recent study identified the ABC transporter AtABCB14 as an apoplast to symplast malate importer capable of modulating stomatal response to CO$_2$ (6), while plants bearing mutation in the MRP type ABC transporter AtMRP5 show partial inhibition of ABA-induced stomatal closure (7); impairment of activation of S-type anion channels by both ABA and cytosolic Ca$^{2+}$ (8); and, additionally, impairment of the activation of plasma membrane Ca$^{2+}$-permeable channel activity by ABA (8). A number of hypotheses have been proposed to explain the phenotype of atmrp5 mutants: these include that AtMRP5 encodes an anion channel, or that AtMRP5 directly regulates a guard cell anion channel (8). Both these possibilities have precedent in the cystic fibrosis transmembrane conductance regulator (CFTR), a mammalian ABC protein that has ion (Cl$^-$) channel activity, and that also modulates the activity of associated ion channels (9).

The activity of other Cl$^-$ conductances, but not CFTR, have been shown to be influenced by inositol polyphosphate signalling molecules.
Most recently, inositol 3,4,5,6-tetraakisphosphate was identified as a physiological regulator of a specific chloride conductance, that of the CIC-3 channel of mammalian secretory epithelia (12).

Inositol 3,4,5,6-tetraakisphosphate and inositol 1,4,5-trisphosphate are not the only inositol phosphates that directly regulate ion channels. In guard cells, inositol hexakisphosphate mobilizes calcium from endomembrane stores (13) and inhibits the inward rectifying K⁺ conductance (14).

We were fascinated by a recent report that disruption of a gene encoding an ATP-binding cassette protein, ZmMRP4, reduced inositol hexakisphosphate accumulation in maize kernels (15). One interpretation of this report is that ZmMRP4 is responsible for transport of inositol hexakisphosphate or a precursor across the vacuole membrane of inositol hexakisphosphate storage tissues of maize kernels.

Our experiments have addressed the function of AtMRP5, an ortholog of ZmMRP4, in planta. By a combination of biochemical, physiological and genetic analysis we identify a hitherto undescribed inositol polyphosphate transport function in Arabidopsis for this example of the most ubiquitous class of membrane transport proteins. Our work links inositol hexakisphosphate transport to the regulation of stomatal response to abscisic acid (ABA).

**EXPERIMENTAL PROCEDURES**

**Chemical analysis of AtMRP5 loss-of-function mutants-** Fifty milligrams of dried seed material was incinerated for 8 h at 550°C and subsequently the ash was solubilized in 2 ml 6 N HCl, briefly heated up to 100°C, purified through Whatman No. 40 ashless filter paper and transferred to double-distilled water to a total volume of 50 ml. Element contents were measured by ICP emission-spectroscopy analysis (Varian Liberty 220; Varian Inc., Palo Alto, CA, USA, equipped with an ultrasonic nebulizer (CETAC U-5000 AT)) according to standard procedures. Inositol hexakisphosphate and inorganic phosphate were determined by suppressed ion conductivity HPLC of acid extracts of Arabidopsis seeds (16). Seeds, 2-4 mg, were boiled in 0.8 ml of 0.6 M HCl for 30 minutes, and the supernatant was diluted 10 x in water. Samples, 50 µL, were analyzed on a Dionex ICS2000 chromatography system fitted with a 25 cm x 2 mm internal diameter Dionex AS11 anion exchange column, with associated AG11 guard column. The column was eluted with a gradient of KOH: time (min), concentration KOH (mM); 0, 0; 20, 100; at a flow rate of 0.25 mL/min. The inositol hexakisphosphate and Pi content of samples was determined from a calibration curve of peak area vs. amount injected in the range 0.2-4 nmol and 0.2-2 nmol respectively. In our hands, leaf inositol hexakisphosphate levels were below the limit of detection of this method.

**Functional analysis in yeast-** The ycf1 yeast mutant was transformed with the expression vector pNEV harbouring no insert (pNEV) or AtMRP5 cDNA (pNEV-MRP5). Microsomal vesicles were isolated as described previously (17). For the transport of inositol hexakisphosphate, Ins(1,3,4,5,6)P₆, prepared according to (18) was added to the reaction mix consisting of transport buffer, 1mM DTT, 5 mM ATP, 10 mM MgCl₂, 10 mM creatine-phosphate and 100µg/mL creatine kinase. Yeast microsomes were added to start the transport assay. Assays were performed at room temperature. At intervals, inositol hexakisphosphate uptake was terminated by the transfer of three aliquots, equivalent to 18.9 nCi (700 Bq) of starting material, onto 0.45µm diameter pore size nitrocellulose filters. Under these conditions, the rate of net transport was linear with time during the first 5 and ½ minutes. Values are corrected for corresponding controls with vesicles isolated from ycf1 yeasts transformed with the empty pNEV vector. ATP independent inositol hexakisphosphate uptake was assessed in the absence of ATP. Transport assays for all treatments were performed with the same vesicle preparation. Experiments were repeated three times, with independent vesicle preparations. Labelled material recovered on the filter was analysed by Partisphere SAX HPLC. For the experiments with the non-hydrolysable ATP-analogue adenyl-5’-yl imidodiphosphate (AMPPNP), the 4mM ATP from the reaction mix was replaced with the equivalent concentration of AMPPNP. For the experiments on ice, tubes containing the reaction mix and the labelled InsP₆ were placed on ice immediately after the addition of the yeast microsomes.

**Inhibition studies-** Yeast microsomes were incubated at room temperature in the reaction mix that contained the indicated potential inhibitors (Table 1). After 8 min,
Ins(1,3,5,6-P)6 was added to the reaction mix and inositol hexakisphosphate uptake was determined as above. The different inhibitors were always tested with the same vesicle preparation as the Ins(1,3,5,6-P)6 uptake control. Experiments were repeated three times. For experiments with different inositol polyphosphates (Table 2), the putative inhibitors/competitors were added to the reaction mix at the concentration indicated in Table 2. The transport assay was started by the addition of microsomes. Assays were performed at room temperature. After 5 and ½ minutes, uptake was terminated by the transfer of three replicates onto 0.45µm diameter pore size nitrocellulose filters. The number of repetitions of the experiment is indicated in Table 2. ATP-dependent AtMRP5-mediated transport was considered as 100%. The reaction mix contained labelled InsP6 at 1.85 KBq / nitrocellulose filter. Partisphere SAX HPLC-Extracts of radioabelled Arabidopsis seedlings, products of transport assays, and preparations of Ins(1,3,5,6-P)6 were all analyzed by anion exchange HPLC on a 23.5 cm x 4.6 mm internal diameter Partisphere SAX WXS cartridge, with guard cartridge (18). The column was eluted at a flow rate of 1 mL/min with a gradient derived by mixing of A, water; B, 1.25 M-(NH4)2HPO4 adjusted to pH 3.8 with H3PO4 according to the following program: time (min), % B; 0, 0; 5, 0; 65, 100; 75, 100. Radioactivity (3H or 33P) in column eluates was estimated by admixture of Optima Flo™ AP (Canberra Packard, Pangbourne, Berks, UK) scintillation fluid at 2 mL.min in a Canberra Packard A515 flow detector fitted with a 0.5 mL flow cell. The integration interval was 0.2 min.

Analysis of stomatal apertures- Arabidopsis (Ws-2) leaves were transformed with the GUS reporter gene under control of the MYB60 promoter (19) by particle bombardment. For detection of GUS activity, leaves were incubated for 8h, at 37°C, in 1 mM X-glucuronic acid, 0.5 % Triton X-100, 50 mM NaHPO4 pH 7.2, and subsequently cleared in 70% ethanol. The AtMRP5 cDNA under the control of the MYB60 promoter was cloned into Gateway™ compatible plant transformation vectors (20). The resulting constructs were used to transform mpr5-1 mutants by the floral dipping method using Agrobacterium (21). Arabidopsis plants (Ws-2, mpr5-1, and MYB60::MRP5, independent homozygous transformed lines from the T3 generation) were grown in soil in a phytotron (8 h light / 16 h dark cycle; 21°C, 70% relative humidity). For stomatal aperture measurements, leaves of 6 weeks old plants were harvested and incubated for 3h in the light in a solution, 30 mM KCl, 1mM CaCl2, 5mM MES-KOH pH 5.8, with or without 5 µM ABA. Epidermal strips, prepared from the abaxial surface, were analysed by light microscopy.

Radiolabelling of Arabidopsis thaliana - Seeds of Ws-2 and mpr5-1 were germinated and grown on a lab prepared ½ strength Murashige and Skoog (MS) medium containing 2% agar. The medium was prepared to match the Duchefa (Haarlem, The Netherlands) M0221 product. The inorganic phosphate (KH2PO4) content of the medium was 0.625 mM. Seedlings were grown for 12 d at 22°C under long day conditions (16 h light / 8h dark cycle) in a Sanyo (Biomedical Europe BV) MLR-351 light cabinet at a fluence rate of 120 µmol/m²/s. Seedlings were subsequently transplanted to agar media containing either 0.625 mM or 5 µM phosphate and grown for a further 3 d, before transfer to liquid media with the same phosphate concentration. The seedlings were labelled in 0.5 mL of liquid media containing 1.125 MBq myo-[3H]inositol (PerkinElmer, Boston, MA), specific activity, 752 GBq/mmol) in the wells of a multiwell plate for the 5 d duration of growth on liquid media. The media was supplemented with 0.2 mL of media lacking radiolabel after 2d.

RESULTS

Ablation of AtMRP5 reduces inositol hexakisphosphate level.- In a recent paper it was shown for maize that the absence of an ATP-binding cassette protein, ZmMRP4, reduced inositol hexakisphosphate accumulation in kernels (15). Phylogenetic studies of ZmMRP4 revealed that AtMRP5 is a very close homologue. Since AtMRP5 is strongly expressed in seeds, we were interested whether knock-out mutants of this ABC transporter exhibit the same low inositol hexakisphosphate phenotype. To this end, we used two independent T-DNA insertion mutants of AtMRP5 in their different genetic backgrounds to determine their corresponding inositol hexakisphosphate contents. Inositol hexakisphosphate accumulates as mixed salts of mineral cations in the globoids of specialized vacuoles in Arabidopsis (22) as in other seeds (23). Seed inositol hexakisphosphate was measured by suppressed ion conductivity (16,24). Example profiles are shown in Fig. 1A,
It has already been extensively reported that \textit{mrp5} mutants have pronounced phenotypes, some of which manifest in guard-cells (7,8,26). Determinations of cations (Fig. 2) which form chelates with inositol hexakisphosphate (phytin) revealed concurrent reductions in mineral composition of seeds of \textit{mrp5-1} and \textit{mrp5-2}. Those minerals that differed significantly between wild-type parent and mutant included Na, which was present only in very low amounts, and which was reduced by 41 and 32 \%. Mg was reduced by 34 and 31 \%, while Ca was reduced by 23 and 31 \%. A small reduction was observed for potassium (17\%), while no significant reduction was determined for Fe, Mn, Zn, Ni and Cu. It is known that several divalent cations strongly bind to phytate, but the species can differ from plant to plant. Our data suggests that iron is not associated with phytate in \textit{Arabidopsis}. Additional data for other minerals that were not found to differ significantly between mutant and wild type genotypes is provided in supplementary Fig. S1.

\textit{AtMRP5 encodes a high affinity inositol hexakisphosphate transporter}. The absence of lower inositol phosphates in the conductivity profiles (Fig. 1A,B) contrasts with the biosynthetic mutant \textit{ipk1-1} and with biosynthetic mutants, for example, of barley (27,28) and maize (29,30). In order to test whether \textit{AtMRP5} is an inositol hexakisphosphate transporter or a membrane protein participating, but not directly catalyzing inositol hexakisphosphate transport, we expressed \textit{AtMRP5} in yeast deficient in the \textit{YCF1} ABC transporter. Preliminary transport experiments with different mutants of MRP-type ABC transporters revealed that \textit{ycf1} exhibited only 10 to 20\% of the MgATP-dependent transport activity compared to the corresponding wild-type strain (W303). This residual activity might be due to one of the MRP-like genes still present in \textit{ycf1}. We were unsuccessful in our attempts to transform \textit{AtMRP5} into double and triple MRP knock-out mutants, so we used \textit{AtMRP5}-transformed \textit{ycf1} for further analysis. Microsomes were prepared and used to perform studies of \textit{[32P]}inositol hexakisphosphate uptake. Inositol hexakisphosphate transport was dependent on \textit{AtMRP5} and ATP and was linear with respect to time up to 5 min 30 sec (Fig. 3A). In the absence of \textit{AtMRP5}, a low level of ATP-dependent inositol hexakisphosphate uptake was observed. Expression of \textit{AtMRP5}

(note that the identity of the inositol hexakisphosphate peak was confirmed for all genotypes by spiking extracts from these genotypes with an inositol hexakisphosphate standard). Figure 1A includes a profile of the biosynthetic mutant \textit{ipk1-1}. On the Dionex AS11 column used, InsP$_3$ and InsP$_4$, which both accumulate in \textit{ipk1-1} elute after inositol hexakisphosphate (24). Our analysis showed that \textit{mrp5-1} and \textit{mrp5-2} mutants do not accumulate inositol hexakisphosphate in the seed; nor do they accumulate lower inositol phosphates, in contrast to \textit{ipk1-1} which accumulates inositol 1,3,4,5,6-pentakisphosphate and inositol 3,4,5,6-tetrafakisphosphate predominantly among tetrafakisphosphate species (25). Our measurements of seed inositol hexakisphosphate in the Col0 genetic background of \textit{mrp5-2}, 24.8 ± 4.1 nmol/mg dry weight; agrees favourably with the data, 22.3 ± 0.4 nmol/mg, of (25). Seed inositol hexakisphosphate was reduced to 0.3 ± 0.6 nmol/mg in the \textit{mrp5-2} mutant (Fig.1C). This value, and that, 1.1 ± 1.1 nmol/mg, of the \textit{mrp5-1} mutant, as compared to its Ws-2 parent, 14 ± 3.1 nmol/mg; are significantly greater depletions than that reported for the biosynthesis mutant \textit{ipk1-1}, 3.9 ± 0.4 nmol/mg (25) for which we obtained a value of 4.1 ± 2.9 nmol/mg (data not shown). These data provide genetic evidence that \textit{AtMRP5} is required to accumulate inositol hexakisphosphate in seeds. Measurement of inorganic phosphate levels in \textit{mrp5} mutants and their parents (Fig.1A,B,D) revealed that depletions of inositol phosphates in the mutants are accompanied by accumulation of inorganic phosphate. The values obtained for seeds of Col0, 35.9 ± 5.4 nmol/mg dry weight; \textit{mrp5-2}, 103.9 ± 9.4 nmol/mg; Ws-2, 18.5 ± 1.0 nmol/mg; and \textit{mrp5-1}, 65.0 ± 4.3nmol/mg, when compared to \textit{atipk1-1}, 30.2 ± 1.2 nmol/mg; indicate that inositol hexakisphosphate transporter mutants show a seed inorganic phosphate accumulation phenotype that is more severe than that of the biosynthetic mutant \textit{atipk1}. Measurements of total phosphorus (Fig. 1E) revealed a reduction, relative to wild type, respectively of 37\% in the \textit{mrp5-1} mutant, and of 34\% in \textit{mrp5-2}. We also attempted to measure inositol hexakisphosphate levels in leaf tissue of \textit{Arabidopsis}. In our hands, the levels were below the level of detection by suppressed ion conductivity, a consequence in part of a number of unidentified anions eluting in the region of inositol hexakisphosphate.
induced a slight increase in inositol hexakisphosphate transport in the absence of ATP, while addition of ATP, to microsomes isolated from AtMRP5-transformed ycf1 cells, resulted in a sixfold increase of the ATP-dependent transport activity.

Replacement of ATP by the non-hydrolysable ATP analogue AMPPNP abolished transport (Table 1). This result further confirms that transport of inositol hexakisphosphate is strictly ATP-dependent.

HPLC analysis of the ^3P recovered from filtered and washed microsomes revealed that, within the timeframe of our uptake experiments, there was no discernible metabolism of Ins(1,3,4,5,6)P_6 to lower inositol phosphate species, nor to inorganic phosphate (Fig. 3B). Thus, the 2-position of inositol hexakisphosphate, and the whole molecule, was metabolically stable. We did not observe phosphorylation of inositol hexakisphosphate, as catalyzed by the KCS1 (31) or VIP1 (32) proteins of S. cerevisiae. Inclusion of a range of ABC transporter inhibitors revealed that the sulfonureas glibenclamide and probenicid had a very minor inhibitory effect, confirming that the ApH plays no or only a minor role in ABC transporter-mediated processes.

Transport experiments performed on ice showed that transport was abolished under these conditions, discounting the possibility that the transport activity reflected binding of inositol hexakisphosphate only, or that transport was a facet of non-specific permeability of yeast microsomes. A kinetic analysis of inositol hexakisphosphate uptake was undertaken at room temperature. Uptake followed saturation kinetics. A double reciprocal plot of uptake against inositol hexakisphosphate concentration yielded a linear relationship and estimations of K_m of 310 nM in one experiment and 263 nM in another (Fig. 3C, S2). V_max values reflecting the expression level of AtMRP5 were estimated at 1.6 to 2.5 µmol min^{-1} mg^{-1} microsome protein (Fig. 3C). The ATP-dependent inositol hexakisphosphate transport activity from non-transformed yeast microsomes corresponded only to 10-20% of that observed with ATMRP5 expressing yeast. Therefore, the obtained K_m values should reflect closely the characteristics of AtMRP5-mediated transport.

In order to characterize the substrate specificity of AtMRP5, we performed competition experiments with a range of inositol polyphosphates identified in plants (Table 2). At a concentration corresponding to a twofold K_m of inositol hexakisphosphate (600 nM), no significant inhibition was observed for Ins(1,4,5)P_3, Ins(1,3,4,6)P_4 and Ins(1,4,5,6)P_4. With Ins(1,3,4,5,6)P_6, transport was reduced by 20%. Scylo-inositol hexakisphosphate did not inhibit transport at 600 nM suggesting that substrate binding exhibits stereospecificity and is not exclusively a consequence of high density of negative-charge on the substrate. Estradiol glucuronide, a compound shown to be transported by AtMRP5 (26) had a slight stimulatory effect. Increasing the concentration of the inhibitors to 1200 nM resulted in an approximately 40% inhibition of myo-inositol hexakisphosphate uptake by both Ins(1,3,4,5,6)P_5 and scylo-inositol hexakisphosphate. Interestingly, at this concentration Ins(1,4,5)P_3 exhibited a stimulatory effect of about 50%.

From this result it is tempting to speculate that Ins(1,4,5)P_3 at higher concentration can accelerate the depletion of the cytosolic InsP_6 and thus the termination of the InsP_6 signalling pathway. In summary, these data ascribe novel function to AtMRP5, that of a specific high affinity myo-inositol hexakisphosphate transporter.

*The inositol phosphate metabolism of atmrp5 mutants is sensitive to external phosphate*. In the context of reduced vacuolar deposition of inositol hexakisphosphate, and accumulation of inorganic phosphate, the atmrp5 transporter mutants potentially afford the opportunity to assess the interplay, previously untested, of intracellular inositol phosphate transport and inositol phosphate metabolism. Inositol hexakisphosphate synthesis is responsive to phosphate supply in suspension culture of Arabidopsis (33). We labelled Ws-2 and mrp5-1 seedlings with inositol and analysed the responsiveness of inositol hexakisphosphate synthesis to phosphate concentration in the growth and labelling media. Our analysis (Fig. 4) revealed that the profile of ^3H-labelled peaks of increasing phosphorylation, from inositol up to inositol hexakisphosphate, were similar for Ws-2 and mrp5-1 mutants treated and labelled.
in low phosphate. The absence of major peaks of label in the InsP3 to InsP5 region is typical of plant tissues. Material eluting before the InsP peak includes inositol and, likely, cell wall sugars derived from inositol. In a typical experiment, at 5 mM phosphate, the label recovered in inositol pentakisphosphate and inositol hexakisphosphate peaks were, respectively, 0.25% and 0.96% for Ws-2, and 0.07% and 0.60% for mrp5-1. These results and the seed inositol phosphate data (Fig. 1) suggest that the control exerted by AtMRP5 on inositol hexakisphosphate accumulation in seeds or whole seedlings is not manifest as an accumulation of metabolic precursors. Moreover, seedlings of Ws-2 and mrp5-1 grown and labelled on high phosphate accumulated approximately 2-fold more label in inositol hexakisphosphate than those transferred to and labelled in low phosphate media. Similar conclusions have been drawn from measurements of inositol hexakisphosphate in suspension cultures of Catharanthus roseus and Arabidopsis grown in low and high phosphate, with Arabidopsis showing weaker responses to phosphate than C. roseus (33). In our experiments, the proportion of label recovered in inositol pentakisphosphate and inositol hexakisphosphate were 0.15% and 1.89% for Ws-2, and 0.25% and 1.46% for mrp5-1 grown and labelled on high phosphate. These data show that, in respect of metabolic flux from inositol to inositol hexakisphosphate, the mrp5-1 mutant is responsive to environmental phosphate status like its wild type Ws-2 parent. We conclude that, unlike the ipk1-1 mutant which is deficient in recognition of external phosphate (25), mrp5 mutants are not defective in sensing environmental phosphate. The data presented above indicate that AtMRP5 makes a dominant contribution to accumulation of inositol hexakisphosphate in seeds of Arabidopsis. Although we are not aware of gene expression studies of inositol hexakisphosphate accumulation in Arabidopsis, of the biosynthetic gene products, IPK1 has a dominant contribution to inositol hexakisphosphate accumulation (25). We undertook expression analysis of the effect of mutation of AtMRP5 on transcript levels of AtIPK1 and of four members of the inositol tris/tetrasphosphate kinase family whose contribution to inositol hexakisphosphate synthesis, while undefined in Arabidopsis (34), is anticipated from studies of maize (30). The data presented in the supplementary information (supplementary figure 3) indicate that mutation of AtMRP5 was without effect on IPK1 or ITPK1 (AtSG16760) in either background, while a doubling of the other transcripts was evident in mrp5-1 only.

Atmrp5 mutants show defective stomatal responses that are partially relieved by guard cell-targeted expression of AtMRP5. The foregoing data point, not only to a novel inositol hexakisphosphate transport function for AtMRP5, but also to a dominant contribution to inositol hexakisphosphate accumulation. ABC transport proteins have not been extensively characterized in plants, but among them AtMRP5 and AtABCB14 have been shown to exert control over a number of cellular targets, particularly ion channels whose regulation underlies the control of stomatal function. Because we have shown that inositol hexakisphosphate is a physiological regulator of vacuolar (Ca2+-permeable) and plasma membrane (inward rectifying K+) conductances (13,14), we sought to determine a role for AtMRP5 in guard cell function. We tested the effectiveness of ABA as an inhibitor of stomatal opening in response to light, because this response is a well documented physiological indicator of MRP5 function, one to which, anion channel activity contributes markedly. The data of Fig. 5C were obtained from epidermal peels of wild type (Ws-2), the mrp5-1 mutant, and a series of lines of mrp5-1 which had been independently transformed with an AtMRP5 construct driven from a promoter, MYB60, that is strongly expressed in guard cells, but not in epidermal cells (Fig. 5A,B). Stomatal opening was strongly inhibited by ABA in Ws-2 but not in the mrp5-1 mutant (Fig. 5C). Significantly, transgenic expression of AtMRP5 restored ABA responsiveness to the mrp5-1 mutant; a result that was confirmed for several independent transgenic lines. Detailed physiological analyses of the above mentioned transgenic lines revealed that guard cell-targeted expression of AtMRP5 did not restore wild-type seed InsP3 content (Fig 5D) and did not restore the root phenotype (S4).

It remains a matter of debate whether the observed stomatal phenotype of mrp5-1 is the result of an interaction between AtMRP5 and unknown proteins, and/or the AtMRP5-mediated transport of a specific molecule. Therefore, to get further insights whether a functional transporter is required, site-directed mutagenesis
of AtMRP5 was undertaken and the mutated cDNAs driven by the CaMV35S promoter were transformed into mrp5-1 (Fig S5A). The mrp5-1 related phenotypes (seed InsP$_6$ content, root length, drought resistance and stomatal aperture) were analyzed for all constructs in the T3 generation. Most of mutants showed an intermediate phenotype as compared to Ws-2 and mrp5-1 for all traits. Only those plants with either a D771A or a D1429A exchange in the Walker B domain of NBD1 or NBD2, behaved like mrp5-1 (S5 B,C,D,E). Because the Walker B domain is known to be essential for the transport activity of most ABC transporters (35), this result suggests that AtMRP5–mediated InsP$_6$ uptake and the stomatal phenotype are both dependent on a functional AtMRP5.

**DISCUSSION**

Inositol polyphosphates and inositol pyrophosphates contribute to diverse cell biological and developmental phenomena including activated exocytosis (36), mRNA export (37,38), cell-cycle activities (39) and establishment of left-right asymmetry of organ development in zebra fish (40). In plants, inositol hexakisphosphate mobilizes calcium and inhibits the inward rectifying K$^+$ conductance of guard cells (13,14).

Despite all these elaborations of higher inositol polyphosphate function, remarkably little is known of the compartmentation of inositol polyphosphate synthesis or of the contribution of compartmentation to the roles identified above. Interestingly, inositol hexakisphosphate is a component of basal resistance to fungal, bacterial and viral pathogens in Arabidopsis, with an indication of a discrete subcellular pool of inositol hexakisphosphate contributing to these phenomena (41). In higher plants, inositol hexakisphosphate accumulates as mixed salts of alkalai and other metal cations in the globooids of specialized vacuoles of storage tissues (23). By corollary, inositol hexakisphosphate is a major component of the laminal layer of hydatid cysts, the reproductive stage of the animal parasite *Echinococcus granulosus* (24). Both of these examples imply vectorial transport of inositol hexakisphosphate or a precursor across cellular membranes; non-cytosolic synthesis of inositol hexakisphosphate; and/or trafficking of membrane-bound inositol hexakisphosphate. These possibilities are all highlighted by the apparent enigma that human multiple inositol polyphosphate phosphatase localizes within the lumen of the endoplasmatic reticulum, while its inositol phosphate substrates are considered to be exclusively cytosolic (42).

Our elaboration of AtMRP5 as an inositol hexakisphosphate transporter, taken with the analysis of ZmMRP4 mutants (15), provides a mechanistic explanation of the accumulation of inositol hexakisphosphate in storage tissues of plants that differs from established conventions. Previous explanations of inositol hexakisphosphate accumulation in membrane-bound protein bodies, including the aleurome bodies of cereals, are tightly linked to morphological examination of vesicle trafficking phenomena as ontogenetic explanations of the origins of protein bodies themselves (43,44). We are reminded of analysis of natural variation of inositol hexakisphosphate accumulation in *Arabidopsis* which mapped a trait in inositol hexakisphosphate accumulation to a 99 kb region that harboured a tonoplast membrane ATPase G-subunit (16). While the exact locus of the trait was not identified, one possible explanation of the variation in inositol hexakisphosphate is that accumulation of inositol hexakisphosphate is dependent on vacuolar transport processes.

We now show that two distinct insertion mutants of *AtMRP5* in different, Col0 and Ws-2, genetic backgrounds both display a low phytic acid phenotype in seed tissue. We show that the low inositol hexakisphosphate seed phenotype is associated with alterations of mineral cation and phosphate status in both genetic backgrounds. The low seed phytate phenotype is a direct consequence of the missing transport activity. Our data demonstrate that AtMRP5 is a specific, high affinity inositol hexakisphosphate transporter.

Beyond our identification of the molecular identity of an inositol phosphate transporter, our analysis reveals, in context of an existing literature, that disruption of a gene encoding an inositol hexakisphosphate transporter contributes to stomatal biology. Because we show that MRP5 expression, exclusively, in guard cells of the *mrp5-1* mutant restores sensitivity of these cells to ABA, we hypothesize that AtMRP5 is involved in stomatal regulation due to its capacity to transport inositol hexakisphosphate. The physiology of guard cells is dominated by the multitude of ion channels and transporters whose regulation is integrated in this specialized epithelial cell, with tonoplast...
transport playing a central role (2). It may be significant that the fast and slow Ca\(^{2+}\) - permeable conductances of the tonoplast are both targets of inositol hexakisphosphate (13). For the meantime, in the absence of direct studies of electrophysiologically targeted effects of inositol hexakisphosphate in our transgenics of \textit{mrp5-1}, it remains plausible that perturbation of cytosolic and/or vacuolar inositol hexakisphosphate concentration due to the absence of its transporter will necessarily deregulate the transport processes which underlie guard cell physiology (Fig 6).

In consideration of tonoplast function, we suggested in a first report (8) that 35S promoter-driven AtMRP5-GFP was localized to the plasma membrane of root cells. It is possible that plasma membrane localization is an ectopic consequence of MRP5 overexpression. Indeed, proteomic analysis predicts AtMRP5 to reside in the vacuolar membrane (45). The latter interpretation is consistent with the low seed inositol hexakisphosphate phenotype of \textit{mrp5-1} and \textit{mrp5-2} mutants: InsP\(_6\) is recognized to accumulate in membrane bound protein bodies of vacuolar nature. In order to further address the physiological location of AtMRP5, we have examined the location of AtMRP5 expressed under the control of its native promoter. No GFP signal was detected when plants were cultivated under the control of its native promoter. No GFP signal was detected when plants were cultivated under the control of its native promoter to 14 drou

These localization studies raise the intriguing question how a vacuolar inositol hexakisphosphate transporter could affect guard cell signalling and the plasma membrane anion channel activity. A hypothetical model linking the phenomena mentioned above is presented in Fig. 6. The impaired AtMRP5-dependent transport of inositol hexakisphosphate into the vacuole could lead to an increase in cytosolic InsP\(_6\) which could act either by complexing divalent cations such as Mg\(^{2+}\) and Ca\(^{2+}\) or triggering a continuous efflux of Ca\(^{2+}\) into the cytosol by an InsP\(_6\)-regulated channel. This could lead in both cases to a disturbance of Ca\(^{2+}\)-dependent signalling pathways. This hypothesis could explain the impaired ABA and Ca\(^{2+}\)signalling observed in \textit{mrp5} mutants. However, it cannot be excluded that the impaired stomatal aperture observed in the \textit{mrp5} mutants during the light phase is not additionally the result of increased InsP\(_6\) binding to unidentified targets or Ca\(^{2+}\)-dependent inhibition of the K\(^+\) inward channel (14).

In conclusion, our identification of a high affinity inositol hexakisphosphate transport associated with the tonoplast membrane adds to the diversity of functions assigned to the ABC transporter class of membrane proteins, and also answers a long standing question of how inositol phosphates traverse membranes. Given the ubiquity of this class of proteins, we can anticipate that inositol phosphate transport will contribute, not only to the regulation of diverse cellular signalling phenomena, perhaps to human disease, but also potentially to the acquisition of environmental inositol hexakisphosphate which comprises one of the most abundant sources of organic phosphate in the environment (47).

REFERENCES

1. Hetherington, A. M. (2001) \textit{Cell} \textbf{107}, 711-714
2. MacRobbie, E. A. C. (1998) \textit{Philos Trans R Soc London} \textbf{B353}, 1475-1488
3. Lebudy, A., Vavasseur, A., Hosy, E., Dreyer, I., Leonhardt, N., Thibaud, J. B., Very, A. A., Simonne, T., and Sentenac, H. (2008) \textit{Proc Natl Acad Sci USA} \textbf{105}, 5271-5276
4. Vahisalu, T., Kollist, H., Wang, Y. F., Nishimura, N., Chan, W. C., Valerio, G., Lamminmäki, A., Broscé, M., Moldau, H., Desikan, R., Schroeder, J. I., and Kangasjärvi, J. (2008) \textit{Nature} \textbf{452}, 487-491
5. Negi, J., Matsuda, O., Nagasawa, T., Oba, Y., Takahashi, H., Kawai-Yamada, M., Uchimiya, H., and Iba, K. (2008) \textit{Nature} \textbf{452}, 483-486
6. Lee, M., Choi, Y., Burla, B., Kim, Y. Y., Jeon, B., Maeshima, M., Yoo, J. Y., Martinoa, E., and Lee, Y. (2008) \textit{Nat Cell Biol} \textbf{10}, 1217-1223
7. Klein, M., Perfus-Barbeoch, L., Frelet, A., Gaedeke, N., Reinhardt D., Mueller-Roever, B., Martinoa, E., and Forestier, C. (2003) \textit{Plant J} \textbf{33}, 119-129
8. Suh, S.J., Wang, Y-F., Frelet, A., Leonhardt, N., Klein, M., Forestier, C., Mueller-Roeber, B., Cho, M. H., Martinoia, E., and Schroeder, J. (2007) J Biol Chem 282, 1916-1924
9. Frizzell, R.A. (1999) Physiol Rev 79: 1-2
10. Yang, L., Reece, J., Gabriel, S. E., and Shears, S. B. (2006). J Cell Sci 119, 1320-1328
11. Vajanapanichan, M., Schultz, C., Rudolf, M. T., Wasserman, M., Enyedi, P., Craxton, A., Shears, S. B., Tsien, R. Y., Barrett, K. E., and Traynorkaplan, A. (1994) Nature 371, 711-714
12. Mitchell, J., Wang, X. Q., Zhang, G. P., Gentsch, M., Nelson, D. J., and Shears, S. B. (2008) Curr Biol 18, 1600-1605
13. Lemptiri-Chlieh, F., MacRobbie, E. A. C., Webb, A. A. R, Manison, N. F., Brownlee, C., Skeper, J. N., Chen, J., Prestwich, G. D., and Brearley, C. A. (2003) Proc Natl Acad Sci USA 100, 10091-10095
14. Lemptiri-Chlieh, F., MacRobbie, E.A.C., and Brearley, C.A. (2000) Proc Natl Acad Sci USA 97, 8687-8692
15. Shi, J., Wang, H., Schellin, K., Li, B., Faller, M., Stoop, J. M., Meeley, R. B., Ertl, D. S., Ranch, J. P., and Glassman, K. (2007) Nat Biotechnol 25, 930-937
16. Bentsink, L., Yuan, K., Koornneef, M., and Vreugdenhil, D. (2003) Theor Appl Genet 106, 1234-1243
17. Tommasini, R., Evers, R., Vogt, E., Mornet, C., Zaman, G. J., Schinkel, A. H., Borst, P., and Martinoia, E. (1996) Proc Natl Acad Sci USA 93, 6743-6748
18. Sweetman, D., Johnson, S., Caddick, S.E., Hanke, D.E., and Brearley, C.A. (2006). Biochem J 394, 95-103
19. Cominelli, E., Galbiati, M., Vavasseur, A., Conti, L., Sala, T., Vuylsteke, M., Leonhardt, N., Dellaporta, S. L., and Tonelli, C. (2005) Curr Biol 15, 1196-1200
20. Curtis, M., and Grossniklaus, U. (2003) Plant Physiol 133, 462-469
21. Clough, S.J., and Bent, A.F. (1998) Plant J 16, 735-43
22. Otegui, M.S., Capp, R., and Staehelin, L. A. (2002) Plant Cell 14, 1311-1327
23. Lott, J.N.A. (1984) In Seed Physiology (Murray DR ed), Academic Press New York vol 1: 139-166
24. Casaravilla, C., Brearley, C. A., Soule, S., Fontana, C., Veiga, N., Bessio, M. I., Ferreira, F., Kremer, C., and Diaz, A. (2006) FEBS J 273, 3192-3203
25. Stevenson-Paulik, J., Bastidas, R.J., Chiou, S.T., Frye, R.A., and York, J.D. (2005) Proc Natl Acad Sci USA 102, 12612-12617
26. Gaedeke, N., Klein, M., Kolukisaoglu,U., Forestier, C., Mueller, A., Ansorge, M., Becker, D., Mannun, Y., Kuchler, K., Schulz, B., Mueller-Roeber, B., and Martinoia,E. (2001) The EMBO J 20, 1875-1887
27. Hatzack, F., Zhang, W., Hansen, P.E., and Rasmussen, S.K. (2001) Biochem J 354, 473-80
28. Dorsch, J.A., Cook, A., Young, K. A., Anderson, J. M., Bauman, A. T., Volkmann, C. J., Murthy, P. P. N., and Raboy, V. (2003) Phytochemistry 62, 691-706
29. Raboy, V., Gerbasi, P. F., Young, K. A., Stoneberg, S. D., Pickett, S. G., Bauman, A. T., Murthy, P. P. N., Sheridan, W. F., and Ertl, D. S. (2000) Plant Physiol 124, 355-368
30. Shi, J., Wang, H. Y., Wu, Y. S., Hazeboek, J., Meeley, R. B., and Ertl, D. S.(2003) Plant Physiol 131, 507-515
31. Saiardi, A., Erdjument-Bromage, H., Snowman, A.M., Tempst, P., and Snyder, S.H. (1999). Curr Biol 9, 1323-6.
32. Mulugu, S., Bai, W., Fridy, P. C., Bastidas, R. J., Otto, J. C., Dollins, D. E., Haystead, T. A., Ribeiro, A. A., and York, J. D. (2007) Science 316, 106-109
33. Mitsuhashi, N., Ohnishi, M., Sekiguchi, Y., Kwon, Y. U., Chang, Y. T., Chung, S. K., Inoue, Y., Reid, R. J., Yagisawa, T., and Mimura, T. (2005) Plant Physiol 138, 1607-1614
34. Sweetman, D., Stavridou, I., Johnson, S., Green, P., Caddick, S. E. K., and Brearley, C. A. (2007) FEBS Lett 581, 4165-4171
35. Frelet, A., and Klein, M. (2006) FEBS Lett 580, 1064-1084
36. Illies, C., Gromada, J., Fiume, R., Leibiger, B., Yu, L., Juhl, K., Yang, S-N., Barma, D. K., Falck, J. R., Saiardi, A., Barker, C. J., and Berggren, P-O. (2007) Science 318, 1299-1302
37. York, J.D., Odom, A.R., Murphy, R., Ives, E.B., and Wente, S.R. (1999) Science 285, 96-100
38. Odom, A.R., Stahlberg, A., Wente, S.R., and York, J.D. (2000) Science 287, 2026-2029
and the parallel samples with an InsP retention time of inositol phosphates can vary, InsP
Fig. 4. double reciprocal plot was used to determine the hexa
and washed microsomes dependent uptake was 80nM. AtMRP5 preparat
(pNEV) or with vector harbouring
Fig. 3. AtMRP5 is a high affinity inositol hexa
uptake by yeast ycf1 mutant cells transformed with an empty vector (pNEV) or with vector harbouring AtMRP5 cDNA (pNEV-MRP5). For non-ATP dependent uptake, the reaction mix lacked ATP. Transport under all conditions was performed with the same vesicle preparation. Legend: open square: pNEV-ATP, filled square: pNEV+ATP, open circle: pNEV-AtMRP5-ATP, filled circles: pNEV-AtMRP5+ATP. The InsP6 concentration used for the time-dependent uptake was 80nM. B. Partisphere SAX HPLC analysis of the 33P recovered from filtered and washed microsomes showing the integrity of inositol hexakisphosphate after transport. C. Inositol hexakisphosphate uptake by microsomes isolated from yeast cells harbouring AtMRP5. Uptake velocities were measured at different inositol hexakisphosphate concentrations, as indicated. D. A double reciprocal plot was used to determine the Km value of AtMRP5.

Fig. 4. Profiles of inositol phosphates from [3H]inositol-labeled seedlings. A. Traces for Ws-2 labelled in: low phosphate, black line, high phosphate, red line. B. Traces for mpr5-1 labelled in: low phosphate, black line; high phosphate, red line; ipk1-1 labelled in high phosphate, blue line. Note, the retention time of inositol phosphates can vary, InsP6, was confirmed for all genotypes by spiking parallel samples with an InsP6 standard. The traces for atmpr5-1 and Ws-2 labelled in high phosphate, and the ipk1-1 trace have been offset on the y-axis to aid visualization.

FOOTNOTES

Acknowledgments
We are grateful to Thomas Flura (ETH, Zürich) for the ICP measurements and to Maik Hadorn for drawing the model. This work was funded by the Swiss National Foundation (grant no 3100AO-117790 to E.M), by the Forschungskredit der Universität Zürich (R.N.), by the EU project PHIME (contract nr. FOOD-CT-2006-0016253 to J.K.S), and by the Biotechnology and Biological Sciences Research Council (grant BB/C514090/1 to C.B.).

FIGURE LEGENDS

Fig. 1. Ablation of AtMRP5 disturbs inositol hexakisphosphate, phosphate and phosphorus levels in seeds. Conductivity profiles of seed extracts. A. Traces for Col0, red line; mpr5-2, black line; ipk1-1, blue line. B. Traces for Ws-2, red line; mpr5-1, black line. Individual traces are offset on the y-axis to aid visualization. C. Seed inositol-hexakisphosphate (IP6), D. inorganic phosphate (Pi) and E. total phosphorus (P) content of two independent AtMRP5 mutants and their corresponding wild types. The data represents the means and standard deviations of four to eight independent measurements.

Fig. 2. Analysis of cation reserves in seeds of Arabidopsis. Seed content of cations that differ significantly in the two independent AtMRP5 mutants (mpr5-1, mpr5-2) as compared to their corresponding wild types. The data represents the means and standard deviations of four independent measurements.

Fig. 3. AtMRP5 is a high affinity inositol hexakisphosphate transporter. A. Inositol hexakisphosphate uptake into microsomes isolated from yeast ycf1 mutant cells transformed with an empty vector (pNEV) or with vector harbouring AtMRP5 cDNA (pNEV-MRP5). For non-ATP dependent uptake, the reaction mix lacked ATP. Transport under all conditions was performed with the same vesicle preparation. Legend: open square: pNEV-ATP, filled square: pNEV+ATP, open circle: pNEV-AtMRP5-ATP, filled circles: pNEV-AtMRP5+ATP. The InsP6 concentration used for the time-dependent uptake was 80nM. B. Partisphere SAX HPLC analysis of the 33P recovered from filtered and washed microsomes showing the integrity of inositol hexakisphosphate after transport. C. Inositol hexakisphosphate uptake by microsomes isolated from yeast cells harbouring AtMRP5. Uptake velocities were measured at different inositol hexakisphosphate concentrations, as indicated. D. A double reciprocal plot was used to determine the Km value of AtMRP5.
Fig. 5. Guard cell-targeted expression of AtMRP5 restores stomatal phenotype in AtMRP5 mutant. (A,B) The MYB60 promoter targets GUS exclusively to guard cells. C. The mrp5-1 mutant is insensitive to ABA (ABA-inhibits opening of stomata in response to light). The MYB60::AtMRP5 transgene restores ABA sensitivity to mrp5-1. Results for Ws-2, mrp5-1 and three independent transgenic T3 lines are shown. At least 200 stomates of abaxial epidermal strips were measured for each genotype. Two independent experiments were performed, and three additional T3 lines of the transgenic gave the same results (data not shown). Error bars represent the SEM. D. The MYB60::AtMRP5 transgene does not complement the seed IP₆ content of mrp5-1. The data represents the means and standard deviations of three independent measurements. For C) The “p-value” indicates the confidence of significance between Light- and Light+ABA treatments within the same genotype. For D) The “p-value” indicates the confidence of significance between wild type and the four other genotypes.

Fig. 6. Hypothetical model that links AtMRP5, InsP₆ signalling and guard cell movements. In wild-type plants, inositol hexakisphosphate stimulates the release of Ca²⁺ by specific channels and inhibits the K⁺ inward channel. To avoid continuous InsP₆ signalling, inositol hexakisphosphate has to be transported into the vacuole by AtMRP5. AtMRP5 mutant plants are impaired in the export of InsP₆ into the vacuole. Increased concentrations of cytosolic InsP₆ might complex divalent cations, or induce continuous Ca²⁺ release thus disturbing Ca²⁺-depending signalling pathways. Furthermore, during the light period, increased InsP₆ levels may reduce K⁺ uptake into guard cells by inhibiting K⁺ inward rectifying channels.
Table 1

The effect of ABC transport inhibitors on AtMRP5-mediated inositol hexakisphosphate transport

| Condition                        | %     | Repetitions |
|----------------------------------|-------|-------------|
| + ATP                            | 100   | 6           |
| + AMPPNP                         | 0     | 2           |
| + 5mM NH₄Cl                      | 77.6±15.5 | 3          |
| + 150 µM glibenclamide           | 28.8±17.8 | 3          |
| + 1mM vanadate                   | 62 ±11.2 | 3          |
| + 1mM probenicide                | -12.5±22.9 | 3          |
| ice                              | 7.3±7.9 | 2           |

Yeast microsomes were incubated in the reaction mix with the potential inhibitors at room temperature. After 8 minutes inositol hexakisphosphate was added to the reaction mix. For the experiments with the non-hydrolysable ATP-analogue (AMPPNP), the 4mM ATP from the reaction mix was replaced with the equivalent concentration of AMPPNP. For the experiments on ice, tubes containing the reaction mix and the labelled InsP₆ were placed on ice immediately after the addition of the yeast microsomes. The InsP₆ uptake was terminated by transfer of three aliquots onto 0.45 µM diameter pore size nitrocellulose filters after 5 and ½ minutes. Values are corrected for corresponding controls with vesicles isolated from YCF1 yeasts transformed with the empty pNEV vector. The different inhibitors and competitors were always tested using the same vesicle preparation. The number of repetitions is indicated above. The reaction mix contained labelled InsP₆ at 1.85 kBq / nitrocellulose filter.
Table 2

The effect of inositol polyphosphates on AtMRP5-mediated inositol hexakisphosphate transport

| Condition                          | %        | Repetitions |
|------------------------------------|----------|-------------|
| +ATP                               | 100      | 6           |
| + 600nM                            |          |             |
| Ins(1,4,5)P₃                        | 111.7±17.6 | 3           |
| Ins(1,3,4,6)P₄                      | 104.1±18.4 | 3           |
| Ins(1,4,5,6)P₄                      | 100.6±16.8 | 3           |
| Ins(1,3,4,5,6)P₅                    | 80.5± 6.6 | 2           |
| Scyllo IP₆                          | 119.8±12.5 | 3           |
| Ins(1,2,3,4,5,6)P₆                  | 52.7± 1.8 | 2           |
| Estradiol glucuronide               | 122.6±16.2 | 3           |
| +1200nM                            |          |             |
| Ins(3,4,6)P₃                        | 157.3± 3.8 | 3           |
| Ins(1,3,4,6)P₄                      | 100.8±30.3 | 2           |
| Ins(1,4,5,6)P₄                      | 80.2±20.2 | 3           |
| Ins(1,3,4,5,6)P₅                    | 61.0± 7.1 | 2           |
| Scyllo IP₆                          | 57.7±13.6 | 2           |
| Ins(1,2,3,4,5,6)P₆                  | 0 ± 0     | 2           |
| Estradiol glucuronide               | 92.4 ± 4.9 | 3           |

The putative inhibitors and/or competitors were added to the reaction mix consisting of transport buffer, 1 mM DTT, 5 mM ATP, 10 mM MgCl₂, 10 mM creatine-phosphate and 100 µg/mL creatine kinase at the concentration indicated above. Yeast microsomes were added to start the transport assay. Assays were performed at room temperature. After 5 and 1/2 minutes, inositol hexakisphosphate uptake was terminated by the transfer of three aliquots/repetition onto 0.45 µm diameter pore size nitrocellulose filters. The putative inhibitors and/or competitors were always tested using the same vesicle preparation. The number of repetitions is indicated above. ATP-dependent AtMRP5-mediated transport was considered as 100%. The reaction mix contained labelled inositol hexakisphosphate at 1.85 kBq / nitrocellulose filter.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6