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The plant multidrug resistance ABC transporter AtMRP5 is involved in guard cell hormonal signalling and water use

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Summary

Carbon dioxide uptake and water release through stomata, controlling the opening and closure of stomatal pore size in the leaf surface, is critical for optimal plant performance. Stomatal movements are regulated by multiple signalling pathways involving guard cell ion channels. Using reverse genetics, we recently isolated a T-DNA insertion mutant for the *Arabidopsis* ABC-transporter AtMRP5 (mrp5-1). Guard cells from mrp5-1 mutant plants were found to be insensitive to the sulfonylurea compound glibenclamide, which in the wild type induces stomatal opening in the dark. Here, we report that the knockout in *AtMRP5* affects several signalling pathways controlling stomatal movements. Stomatal apertures of mrp5-1 and wild-type Ws-2 were identical in the dark. In contrast, opening of stomata of mrp5-1 plants was reduced in the light. In the light, stomatal closure of mrp5-1 was insensitive to external calcium and abscisic acid, a phytohormone responsible for stomatal closure during drought stress. In contrast to Ws-2, the phytohormone auxin could not stimulate stomatal opening in the mutant in darkness. All stomatal phenotypes were complemented in transgenic mrp5-1 plants transformed with a cauliflower mosaic virus (CaMV) 35S-*AtMRP5* construct. Both whole-plant and single-leaf gas exchange measurements demonstrated a reduced transpiration rate of mrp5-1 in the light. Excised leaves of mutant plants exhibited reduced water loss, and water uptake was strongly decreased at the whole-plant level. Finally, if plants were not watered, mrp5-1 plants survived much longer due to reduced water use. Analysis of CO_2 uptake and transpiration showed that mrp5-1 plants have increased water use efficiency. Mutant plants overexpressing *AtMRP5* under the control of the CaMV 35S promoter again exhibited wild-type characteristics. These results demonstrate that multidrug resistance-associated proteins (MRPs) are important components of guard cell functioning.

Keywords: ABC transporter, stomata, regulation, water use, AtMRP5.

Introduction

Guard cells are highly specialised cells located in the epidermis of aerial plant organs. They play a major role in controlling gas exchange (mainly photosynthetic carbon dioxide uptake and water release by transpiration) between the plant and the surrounding atmosphere. Stomatal pore size is regulated by dynamic changes in the intracellular concentrations of inorganic and organic ions (including K^+ and malate^{2-}), and soluble sugars (e.g. sucrose). Precise control of stomatal aperture is critical for optimal plant performance under natural growth conditions, and requires a sophisticated and highly coordinated interplay of ion channels, metabolite conversions and signal transduction pathways (Grabov and Blatt, 1998; Leckie *et al.*, 1998; Schroeder *et al.*, 2001a). Our current understanding of

guard cell signal transduction and the role individual genes play in this process, has undoubtedly benefited from the analysis of mutants impaired in stomatal physiology (e.g. Gaedeke *et al.*, 2001; Kinoshita *et al.*, 2001; Pei *et al.*, 1998; Szyroki *et al.*, 2001; Wang *et al.*, 2001). It has been emphasised that a detailed understanding of stomatal physiology will have a major impact on the genetic engineering of drought tolerance in higher plants, including crops (Schroeder *et al.*, 2001b).

The ATP-binding cassette (ABC) superfamily represents a large family of proteins that mediate the ATP-dependent transport of solutes (Higgins, 1992). Among ABC transporters identified in animal cells, both cystic fibrosis transmembrane conductance regulator (CFTR) and sulfonylurea receptor (SUR) are receptors for the sulfonylurea compound, glibenclamide (Schmid-Antomarchi *et al.*, 1987; Schultz *et al.*, 1996). These transporters exhibit either an ion channel activity (CFTR) or regulate the activity of other channel proteins (SUR; for review, see Higgins, 1995). The clinical importance of mutations affecting these proteins, leading to cystic fibrosis and non-insulin-dependent diabetes, respectively, has contributed to the expansion of the research in this field.

In plants, more than 129 open reading frames encoding ABC transporters have been identified (Martinoia *et al.*, 2002; Sánchez-Fernández *et al.*, 2001). Among them, 14 encode multidrug resistance-associated proteins (MRPs) and are generally thought to play a major role in cellular detoxification, by transporting toxic compounds, including xenobiotics, from the cytosol into intracellular storage organelles, namely the vacuole. Despite recent progress in the molecular and biochemical analysis of plant ABC transporters, a large gap still exists with respect to the physiological function of most of them (Martinoia *et al.*, 2002). Until now, only a single knock-out mutant for an MRP-type ABC transporter, namely AtMRP5 from *Arabidopsis thaliana*, has been reported in higher plants (Gaedeke *et al.*, 2001). The *AtMRP5* gene is expressed in guard cells and other tissues, including roots, as shown by promoter-β-glucuronidase (GUS) fusions. The AtMRP5 protein exhibits glutathion and glucuronide-conjugate transport activity when expressed in the yeast, *Saccharomyces cerevisiae* (Gaedeke *et al.*, 2001). We have previously demonstrated that glibenclamide regulates plant ionic channels and induces stomatal opening in the dark in several plant species, including *Vicia faba, Commelina communis* (a monocotyledonous species) and *Arabidopsis* (Gaedeke *et al.*, 2001; Leonhardt *et al.*, 1997, 1999, 2001), providing strong evidence for a role of ABC transporters in guard cell signal transduction. When the *AtMRP5* gene was disrupted by a T-DNA insertion, stomatal opening was no longer triggered by an application of glibenclamide (Gaedeke *et al.*, 2001), indicating that AtMRP5 may function as a receptor for glibenclamide in guard cells, further strengthening the view that ABC transporters are instrumental for stomatal movements. Here, we demonstrate that AtMRP5 also plays an important role in guard cell physiology in intact plants. *AtMRP5* knockout mutants exhibited a reduced transpiration rate when compared to wild-type control plants, resulting in strongly reduced water uptake and increased drought tolerance on the whole-plant level.

Results

In addition to their well-known role in detoxification, pharmacological studies indicate that, some plant ABC transporters, like their animal counterparts, might catalyse or be involved in regulating ion fluxes (Leonhardt *et al.*, 1997, 1999). Using reverse genetics, we recently isolated a T-DNA insertion mutant for the *Arabidopsis* ABC-type transporter AtMRP5 (mrp5-1). Stomata of mrp5-1 mutants were insensitive towards the sulfonylurea glibenclamide (Gaedeke *et al.*, 2001), an agent known to induce stomatal opening in the dark (Leonhardt *et al.*, 1997, 1999). The observation that glibenclamide-induced stomatal opening was impaired in the mrp5-1 mutant indicated that AtMRP5 participates in the regulation of stomatal movements. However, glibenclamide is a pharmacological modulator but non-physiological agent, and therefore we tested whether stomatal movement was altered in mrp5-1 mutants *in planta*.

Stomatal regulation by light, Ca^{2+}, abscisic acid and auxin is impaired in mrp5-1 plants

In order to evaluate the stomatal response of mrp5-1 plants to different physiological signals, epidermal strip assays were conducted. In a first set of experiments, stomatal apertures were measured after 3-h incubation in the light or in darkness (Table 1). In darkness, no differences

Table 1 Stomatal apertures of Ws-2 and mrp5-1 plants under different light conditions and in the presence or absence of 5 μM fusicoccin

Conditions		Mean stomatal aperture (μm) ± SEM	*n*
Light	Ws-2	2.78 ± 0.2	1980
	mrp5-1	2.27 ± 0.15	2340
Darkness	Ws-2	1.21 ± 0.15	960
	mrp5-1	1.2 ± 0.14	1020
Darkness + 5 μM fusicoccin	Ws-2	3.49 ± 0.17	500
	mrp5-1	3.8 ± 0.15	500

n corresponds to number of stomata measured. About 100 stomata were measured for each independent experiment. Mean stomatal apertures of Ws-2 and mrp5-1 plants in the light are significantly different (*t*-test, 0.05).

between the mutant and wild-type Ws-2 plants were observed. In contrast, under light, the stomatal aperture of mrp5-1 plants was slightly but significantly reduced in epidermal strips as well as in intact plants (Figure 1a). Fusicoccin, a fungal toxin, stimulates the plasma membrane H^+-ATPase, resulting in membrane hyperpolarisation, and concomitant activation of inward rectifying K^+ channels and stomatal opening in the dark. Addition of 5 μM fusicoccin induced pronounced stomatal opening in darkness in both wild-type and mrp5-1 plants.

The knockout in *AtMRP5* drastically altered the response of guard cells towards classical modulators of stomatal movements (Assmann, 1993). (i) Increasing concentrations of external Ca^{2+} known to trigger stomatal closure under white light (Allen *et al.*, 2001; DeSilva *et al.*, 1985; Gilroy *et al.*, 1990) classically induced stomatal closure in Ws-2 plants (Figure 1b, closed circles), but had only a negligible effect on stomata of mrp5-1 plants (Figure 1b, open squares). (ii) Increasing concentrations of abscisic acid (ABA), a phytohormone synthesised in response to drought stress and leading to stomatal closure (Assmann, 1993; MacRobbie, 1981; Schroeder *et al.*, 2001a), led to a progressive closure of wild-type stomata which were allowed to open for 3 h in the light prior to ABA treatment (Figure 1c). Maximal stomatal closure was obtained with 1 μM ABA. Conversely, in mrp5-1 plants, ABA did not affect the stomatal aperture ($n = 6$). (iii) Wild-type stomata incubated in darkness progressively opened in the presence of increasing concentrations of the auxin indole-3-acetic acid (IAA) (Figure 1d; Blatt and Thiel, 1994). In contrast, stomata of mrp5-1 plants remained closed even at concentrations as high as 1 mM ($n = 4$). These experiments demonstrated that

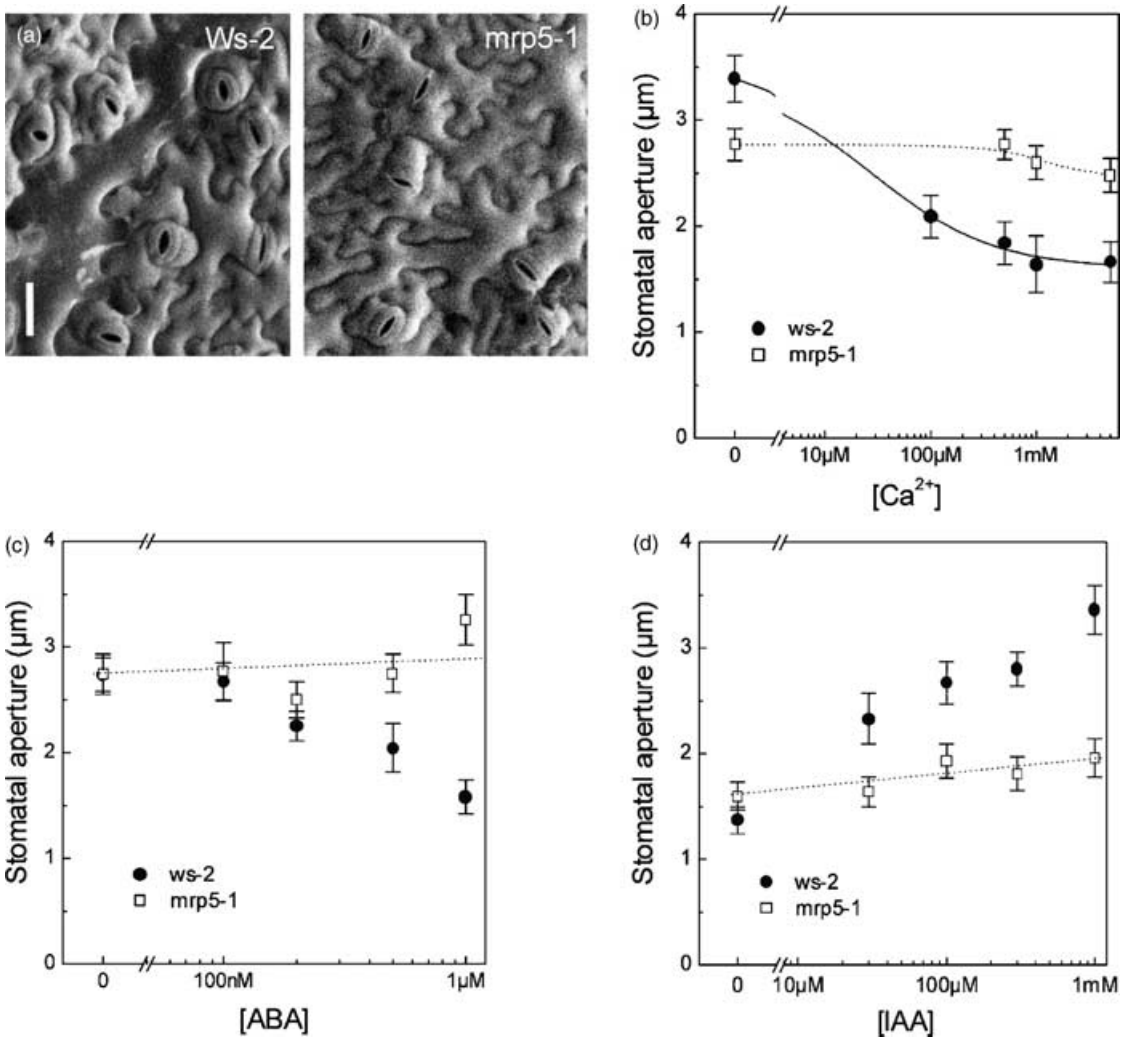

Figure 1. Comparison of stomatal apertures in wild-type (Ws-2) and mrp5-1 mutant.
(a) Scanning electron microscopic image of the abaxial epidermis of rosette leaves. Plants were kept in the light for 3 h before freezing the leaves. Bar = 50 μm.
(b–d) Response of stomatal apertures to externally added Ca^{2+} (b), abscisic acid (c) and indole-3-acetic acid (IAA) (d). Epidermal strips were incubated for 2 h in the light for Ca^{2+} and abscisic acid or in the dark for IAA. Mean of four independent experiments with at least 100 stomata measured per condition.

mrp5-1 plants still possess the potential to open and close stomata. However, several signal transduction pathways responsible for responses to environmental changes are strongly affected.

Based on 2500 stomata observed for each genotype (six different plants per genotype, three different leaves per plant), a frequency of 160 ± 10 and 177 ± 10 stomata mm^{-2} was calculated for mrp5-1 and Ws-2, respectively. These values were not significantly different, demonstrating that the stomatal densities of Ws-2 and mrp5-1 plants were similar.

Overexpression of AtMRP5 *in* mrp5-1 *mutants restores wild-type hormonal regulation of stomata*

In order to verify that the observed de-regulation of stomatal movements is due to the mutation in *AtMRP5*, we generated transgenic plants carrying the *AtMRP5* coding sequence under the control of the cauliflower mosaic virus (CaMV) 35S promoter in the mutant background. We selected several independent transformants and performed experiments in the T_2 generation using homozygous lines. RT-PCR experiments using primers specific for the *AtMRP5* coding region (Gaedeke *et al.*, 2001) confirmed the absence of *AtMRP5* transcript in the mrp5-1 mutant (Figure 2). The combination of an *AtMRP5*-specific sense primer with a primer specific for the left border of the integrated T-DNA (Schulz *et al.*, 1995) detected a fusion transcript containing 1242 bases from the 5′ part of the *AtMRP5* coding region and a segment of the T-DNA. These results reflected the structural organisation of the T-DNA insertion in the *AtMRP5* gene (Gaedeke *et al.*, 2001). Corresponding reactions performed on RNA of Ws-2 plants were negative. Furthermore, RT-PCR experiments performed with RNA isolated from the T_3 families carrying the CaMV 35S-*AtMRP5* construct in the homozygous *mrp5-1* background, using both primer combinations demonstrated that *AtMRP5* transcript is present and overexpressed in these transgenic lines due to the transformation of the cDNA into the mutant background. The analysis of stomatal apertures in mrp5-1/35S-*AtMRP5* plants revealed that the wild-type situation was restored for all treatments or conditions tested. Stomata exposed to the light again closed in the presence of ABA and external calcium. In the dark, glibenclamide and IAA induced stomatal opening in the complemented lines (Table 2).

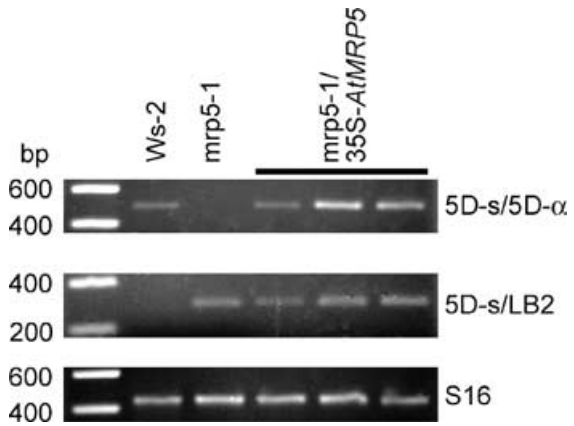

Figure 2. Expression of AtMRP5 in mrp5-1 and independent mrp5-1/35S-AtMRP5 T_3 plants.
Results of RT-PCR analyses using primers specific for *AtMRP5* (upper panel), the *AtMRP5*/T-DNA left border junction (middle panel) and 40S ribosomal protein S16-specific cDNA (lower panel) are shown. Left lane: size markers.

Reduced sensitivity of mrp5-1 seeds towards ABA-mediated germination inhibition

The insensitivity of mrp5-1 stomata towards ABA prompted us to investigate whether the lack of AtMRP5 in the mutant also affects other known physiological responses to this phytohormone such as the inhibition of seed germination by exogenously applied ABA (Leung and Giraudat, 1998; Zeevart and Creelman, 1988). The dose–response curves

Table 2 Complementation of stomatal regulation defects in transgenic mrp5-1/35S-*AtMRP5* plants

	Stomatal apertures (%)		
Conditions	Ws-2	mrp5-1	mrp5-1/35S-*AtMRP5*
Light	100 (3.34 ± 0.24 μm)	100 (2.75 ± 0.13 μm)	100 (4.06 ± 0.17 μm)
+1 μM ABA	46.4 ± 4.5	99.1 ± 5.0	66.8 ± 4.1
+5 mM Ca^{2+}	50.0 ± 4.1	89.8 ± 4.8	70.6 ± 4.7
Darkness	52.7 ± 3.9	60.6 ± 6.1	48.5 ± 6.2
+8 μM glibenclamide	83.9 ± 8.9	62.3 ± 7.6	87.5 ± 8.2
+1 mM IAA	99.1 ± 7.1	70.3 ± 5.2	93.8 ± 3.8

The light condition for each genotype was set to 100%. The stomatal apertures for this condition are given in brackets ± SEM. For each genotype and condition, four independent experiments were performed. At least 200 stomata were counted per condition and experiment. Two independent mrp5-1/35S-*AtMRP5* lines were analysed.

illustrated in Figure 3(a) demonstrated that the germination rates of different independent homozygous mutant F_2 lines of a backcross of mrp5-1 into Ws-2 (Gaedeke *et al.*, 2001) was clearly less inhibited by ABA in agar medium than several independent batches of Ws-2 seeds. A 50% inhibition of germination requires about 1 μM ABA for Ws-2, but at least 3 μM for mrp5-1 seeds. This result argues for a reduced sensitivity of mrp5-1 mutants towards ABA with regard to seed germination. However, compared to the well-described ABA-insensitive dominant negative mutants *abi1* and *abi2* (Gosti *et al.*, 1999; Leung *et al.*, 1994, 1997; Meyer *et al.*, 1994), the insensitivity of mrp5-1 towards ABA-mediated germination inhibition is much less pronounced. In contrast, several transgenic T_2 families of mrp5-1 mutants transformed with the 35S-*AtMRP5* construct exhibited an ABA-induced germination inhibition almost identical to that of the wild type. Thus, the 35S-*AtMRP5* transgene complemented the ABA-insensitive phenotype of mrp5-1 roots. Furthermore, reduced growth of mrp5-1 primary roots previously described by Gaedeke *et al.* (2001) was complemented in the transgenic situation (Figure 3b).

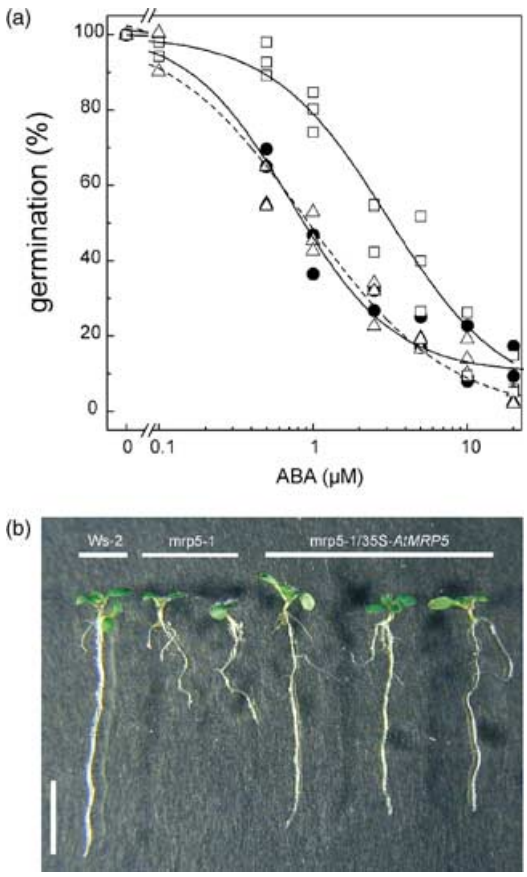

Figure 3. mrp5-1 exhibits reduced sensitivity to germination inhibition by abscisic acid (ABA).
(a) ABA dose–response curve for germination inhibition. Germination rate was determined as described in Experimental procedures. Three independent seed batches of Ws-2 (●), three independent F_3 homozygous mrp5-1 lines (□) and offspring of three independent transgenic mrp5-1/35S-*AtMRP5* T_2 lines (△) were tested.
(b) CaMV35S-*AtMRP5* complements the root phenotype of mrp5-1. Complemented seedlings on the right belong to three independent mrp5-1/35S-*AtMRP5* lines. Plants were grown for 8 days in continous light. Bar = 0.5 cm.

Mrp5-1 plants exhibit a reduced transpiration rate

To examine whether the results obtained with isolated epidermal strips reflect alterations in gas exchange at the leaf or whole-plant level, we investigated the effects of the knockout in *AtMRP5* on water loss, water uptake, transpiration rates and CO_2 fixation. Figure 4(a) illustrates the mean of four experiments of water loss kinetics of excised mrp5-1 or Ws-2 leaf rosettes. Water was retained more efficiently by rosettes of mrp5-1 compared to Ws-2 plants. Considering all experiments ($n = 6$), Ws-2 plants lost 1.56 ± 0.62-fold more water than mrp5-1 plants, 6 h after detaching the rosette. Similar results were obtained with cut single leaves (data not shown). Overexpression of *AtMRP5* in the mutant plants is associated with increased water loss compared to mrp5-1 ($n = 5$). Compared to wild-type plants, water loss was even slightly increased (Figure 4a).

In a complementary series of experiments, we determined the water volume entering intact Ws-2 and mrp5-1 plants using a potometric method. While no difference in water uptake between wild-type and mutant plants was observed in darkness (not shown), the water uptake rate was reduced by a factor of 1.48 ± 0.35 in mrp5-1 plants in the light (Figures 4b; $n = 7$ for each genotype). As observed for water loss, the increased water uptake was restored in mutants overexpressing *AtMRP5* (Figure 4b; $n = 5$). In order to mimic water stress or changes in the soil water potential, water uptake rates were compared before and after the addition of ABA or polyethylene glycol (PEG 3350) to the root compartment. After 24 h of the application of 10 μM ABA, water entry in the light was reduced twofold in Ws-2 plants, but only about 1.3-fold in mrp5-1 plants. A similar difference in water entry was observed after the application of 10% PEG. These results reflect the differential behaviour of Ws-2 and mrp5-1 stomata in the dark and light (Figure 1a) as well as differences in the sensitivities towards ABA (Figure 1c). Taken together, these experiments demonstrated that the water consumption of mrp5-1 mutant plants was lower than that of Ws-2 plants, under standard but also under stress conditions.

We investigated the effect of the absence of AtMRP5 on the assimilation and transpiration rates by measuring gas exchange either in single leaves attached to intact plants (mounted into a gas exchange cuvette), or with whole plants inserted completely into a two-compartment chamber (see Experimental procedures). Transpiration and assimilation rates as a function of the light intensity analysed at 400 ppm CO_2 were reduced in mrp5-1 single, fully

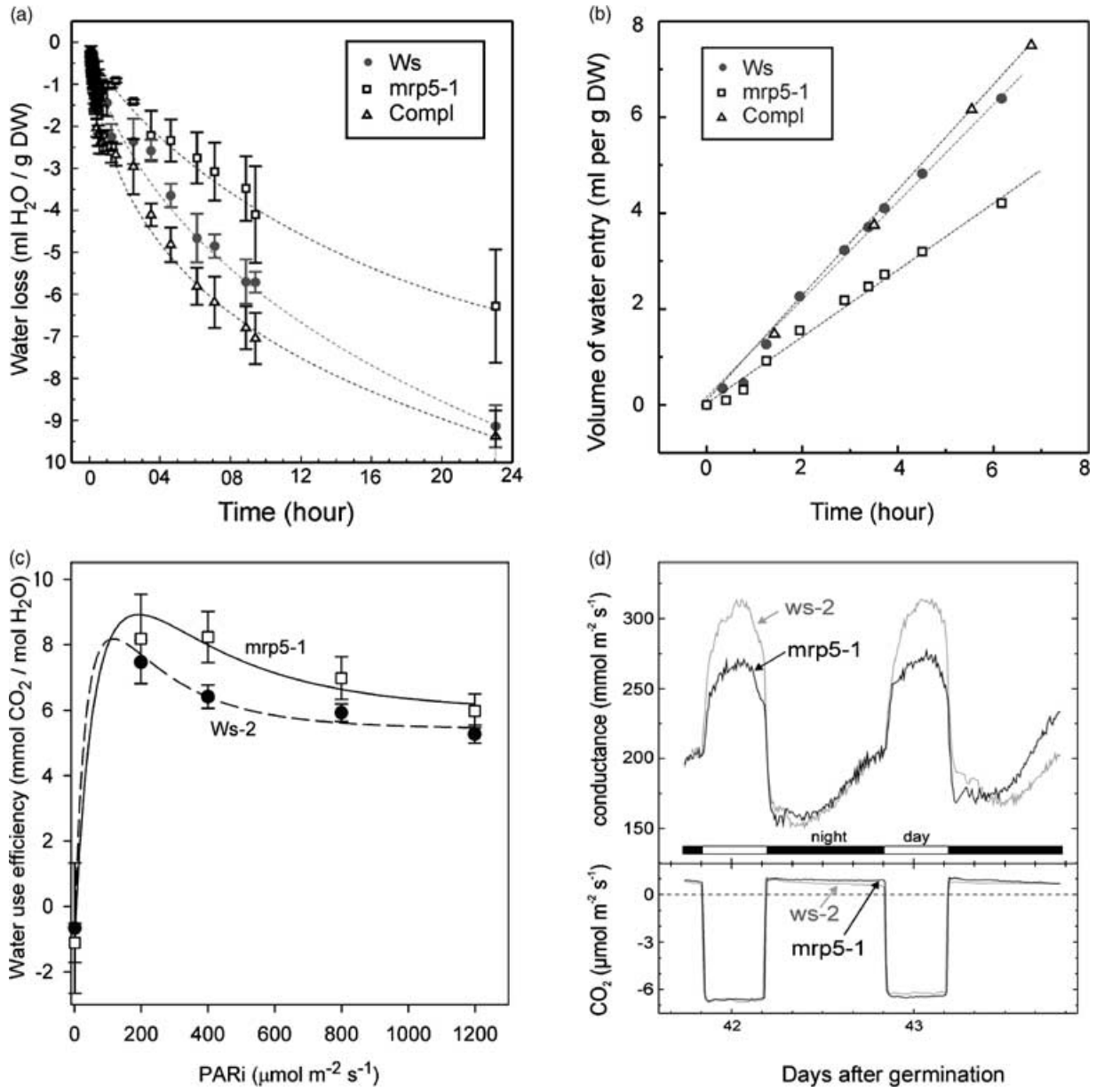

Figure 4. Reduced transpiration and increased water use efficiency (WUE) of mrp5-1.
Water loss (a) and water uptake (b) are reduced in mrp5-1 (squares) compared to Ws-2 plants (Ws, circles) and restored in mrp5-1/35S-*AtMRP5* plants (Compl, triangles). Water loss from excised leaf rosettes was measured using a precision balance.
(c) Light–response curves of single-leaf gas exchange measurements. Plants were shaded during the measurements and PAR (controlled by an in-chamber quantum sensor) was set stepwise from 0 to 1200 and back to 0 μmol m^{-2} sec^{-1}. At each new light dose the leaf was allowed to equilibrate for at least 40 min before measurements were made. The WUE was calculated as the ratio of the photosynthesis rate divided by the transpiration rate.
(d) Mean leaf conductance and CO_2 fluxes measured on intact plants during a day/night cycle.

adult leaves. At saturating light intensities, the lack of AtMRP5 caused a decrease of 22 and 14% in the transpiration and assimilation rates, respectively. These changes account for an improved water use efficiency (WUE) of about 10% in the range between 200 and 600 μmol m^{-2} sec^{-1} PAR in the mutant (Figure 4c).

Continuous recording of the mean leaf conductance and whole-plant CO_2 fluxes in a dedicated chamber (Lascève *et al.*, 1997) showed that both genotypes normally reacted to a day–night cycle by closing the stomata during the dark period, pre-opening in the second part of night period and opening under light (Figure 4d). No significant difference was observed for CO_2 fixation at ambient CO_2 partial pressure, whereas a significant reduction of the mean leaf conductance was observed in mrp5-1 plants, a result consistent with the one observed in stomatal bioassays (see

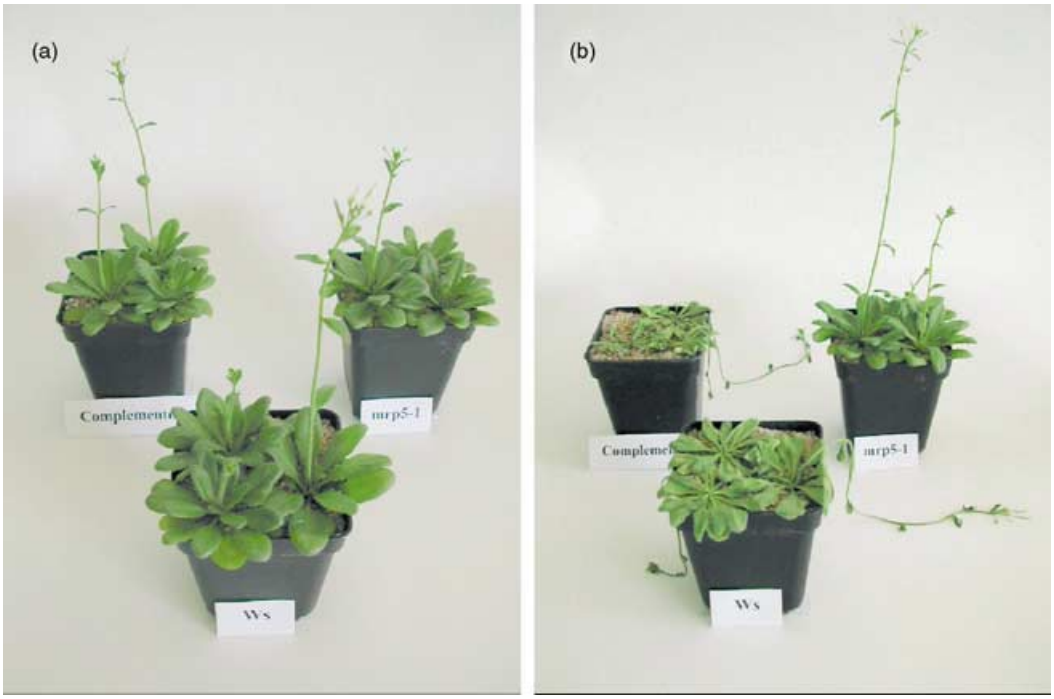

Figure 5. The ABC transporter knockout mutant mrp5-1 is more resistant to drought stress than wild-type plants (Ws) and mutants overexpressing *AtMRP5* (mrp5-1/35S-*AtMRP5*; complemented).
Six-week-old plants grown in a growth chamber on sand with regular irrigation (see Experimental procedures) are illustrated before (a) and after 6 days without water (b).

Figure 1a). This reduction corresponds to a 22% improvement in WUE in the mutant. Ws-2 and mrp5-1 plants opened their stomata when CO_2 was absent and closed them at elevated CO_2 concentrations (data not shown). This result indicates that the stomatal response to CO_2 was not affected in mrp5-1 plants.

Mrp5-1 mutant plants survive longer on non-irrigated soils than Ws-2 and mrp5-1/35S-AtMRP5 plants

When 4- to 5-week-old sand-grown plants were deprived of water in the phytotron, drought-stress symptoms such as turgor loss and wilting were visible in Ws-2 plants already after 48 h. In contrast, mrp5-1 mutant plants were only slightly affected at that time. In all similar experiments performed (plants either grown in sand or soil), the same difference in turgor loss was observed, demonstrating that water loss in the knockout of *AtMRP5* is reduced, allowing this plant to survive longer with a given amount of water (Figure 5). Soil-grown Ws-2 plants in which the drought-stress symptoms appeared later compared to sand-grown plants also developed senescence symptoms such as chlorophyll degradation earlier than mrp5-1 mutants (not shown). Consistently, mrp5-1 mutant plants expressing *AtMRP5* under the control of the CaMV 35S promoter wilted much earlier than the mrp5-1 plants and slightly faster than wild-type plants (Figure 5).

Discussion

Guard cells represent the best characterised plant cell type with respect to ion transport and signal transduction (Schroeder *et al.*, 2001a). Stomatal opening is driven by the plasma membrane H^+-ATPase which is activated by auxin and light (Lohse and Hedrich, 1992; Shimazaki *et al.*, 1986). Activation results in membrane hyperpolarisation, driving the uptake of K^+ via inward rectifying potassium channels (Schroeder *et al.*, 1987; Thiel *et al.*, 1992). Stomatal closure can be triggered by raising the cytosolic Ca^{2+} concentration to approximately 1 μM or by drought stress due to abscisic acid production (Assmann, 1993; Lemtiri-Chlieh and MacRobbie, 1994; MacRobbie, 1981; Schroeder and Hagiwara, 1989; Schroeder *et al.*, 2001a). Multiple substances, second messengers and genes have been identified in the past years which directly or indirectly modulate ion channel activities in guard cells, and regulate the opening or closing of stomata (Schroeder *et al.*, 2001a). These studies indicated that different signal transduction pathways must be responsible for the opening or closing reaction. Several genes involved in the ABA signalling pathway have been identified. ABI1 and ABI2 both code for PP2C phosphatases (Leung *et al.*, 1994, 1997; Meyer *et al.*, 1994). The dominant *abi1-1* and *abi2-1* mutants are ABA insensitive and it has been proposed that ABI1 negatively regulates the ABA transduction pathway (Gosti *et al.*, 1999; Leung

et al., 1997). In contrast, a mutation in the *ERA1* gene, which encodes a farnesyltransferase β-subunit, results in plants which are hypersensitive to ABA (Cutler *et al.*, 1996; Pei *et al.*, 1998). In *abi1*, *abi2* and *era1* mutants, the regulation of guard cell ion channels is impaired (Allen *et al.*, 1999; Armstrong *et al.*, 1995; Pei *et al.*, 1997). Under some experimental conditions, normal CO_2-dependent stomatal closure was reported for *abi1-1* and *abi2-1* (Leymarie *et al.*, 1998), whereas in other cases, CO_2 responsiveness of guard cells was affected (Webb and Hetherington, 1997), arguing for the existence of multiple pathways involved in stomatal closure.

We have recently shown that a knockout mutant in AtMRP5 (*mrp5-1*) has shorter primary roots and more lateral roots when compared to the wild-type Ws-2 plant. Furthermore, the sulfonylurea compound, glibenclamide, no longer triggered stomatal opening in the mutant. Here, we demonstrate that mrp5-1 exhibits an altered stomatal behaviour in response to several physiological conditions. Both closure of stomata by ABA and Ca^{2+} as well as opening by auxin were impaired. In contrast, light still induced the opening of stomata with a small decrease in aperture compared to the wild type. These observations are in line with observations made using other stomatal mutants, suggesting that different signalling pathways are involved in stomatal movement. AtMRP5 is an actor in the pathway which is ABA, Ca^{2+} and auxin dependent. The observation that mutants transformed with a CaMV 35S-*AtMRP5* transgene again exhibited wild-type properties of stomata under all these conditions proves definitively that AtMRP5 is involved in stomatal regulation (Figure 1; Table 2). The slight differences observed between the different experiments reflect small differences in plant growth during the year, an observation also made by other laboratories. Mutant and wild-type plants exhibited no difference in stomatal aperture in the presence of fusicoccin, a classical agent triggering maximal opening (Marre, 1979). These observations allow different interpretations: (i) AtMRP5 itself could act as an ion channel as observed for the mammalian CFTR which exhibits chloride channel activity (Akabas, 2000; Schultz *et al.*, 1996). Interestingly, no functional anion channel has been cloned from plants up to date; (ii) AtMRP5 could regulate an ion channel as suggested for the SUR in animal cells which physically interacts with K_{ir}-type potassium channels (Bryan and Aguilar-Bryan, 1999; Schmid-Antomarchi *et al.*, 1987); (iii) Alternatively, AtMRP5 may also be a transporter for as yet unknown signalling molecules or partially contribute to the regulation of the guard cell metabolism. Taking into account results obtained by Leonhardt *et al.* (1999) and the present study, we cannot, at present, decide whether glibenclamide acts as an activator or inhibitor of MRP5. It is tempting to speculate that the binding of glibenclamide results in a conformational change on the receptor, thereby affecting downstream signalling or metabolic pathways. Thus, there is no discrepancy between the pharmacological effect glibenclamide has, and the effect of a mutation that removes the putative target of the drug.

We directly approached the question whether AtMRP5 is an ion channel, by expressing AtMRP5 in insect cells. Electrophysiological studies did not reveal any current attributable to AtMRP5 (not shown). From our results with epidermal strips, it is very likely that ion fluxes are altered in guard cells of mrp5-1 plants, since mutant stomata are largely insensitive to classical modulators such as ABA, auxin and Ca^{2+}. Strongly reduced transpiration rates, resulting in plants which survive longer if they are not irrigated, may also support the view that *At*MRP5 exerts a strong influence on guard cell ion fluxes. The fact that mutants overexpressing AtMRP5 consistently show a phenotype which has slightly larger values for stomatal aperture, water loss and faster wilting compared to the wild-type plants, indicate that the transcript level of AtMRP5 might be an additional regulatory factor in stomatal control.

On the other hand, mrp5-1 stomata responded normally to fusicoccin and no difference between mrp5-1 and wild-type leaves was observed when CO_2-induced stomatal closing was tested in gas-exchange experiments (data not shown). Thus, mrp5-1 still possesses all guard cell channel activities necessary to open or close their stomata, supporting the hypothesis that AtMRP5 itself is not a major ion channel. The multimeric mammalian complexes composed of SUR and K_{ir} channel isoforms provide evidence that both proteins are needed for the regulation of K^+ fluxes. Therefore, such a situation seems unlikely for AtMRP5. The absence of effects of multiple (ABA, Ca^{2+}, auxin), but not all modulators (light, although not completely; fusicoccin; CO_2) argues for the fact that AtMRP5 is able to integrate several but not all signals regulating stomatal movement.

Our results do not allow to distinguish whether AtMRP5 acts late in the guard cell signal transduction chains or whether it directly (physically) interacts with ion channels. However, in the latter case it must be concluded that the target channel of AtMRP5 is also regulated in an AtMRP5-independent manner. Alternatively, different subsets of guard cell ion channels respond differentially to environmental signals, and only some of them are affected by AtMRP5. In guard cells, an appreciable number of K^+ channels are expressed. Interestingly, in *Arabidopsis*, a T-DNA insertion in the *KAT1* gene, which encodes a guard cell influx K^+ channel, did not affect the function of stomata (Szyroki *et al.*, 2001), although a dominant negative variant of KAT1 overexpressed in *Arabidopsis* significantly reduced light-induced stomatal opening (Kwak *et al.*, 2001). It is therefore tempting to speculate that functionally redundant ion channels may be regulated by diverse regulatory mechanisms. To understand the biological function of AtMRP5, even a detailed electrophysiological analysis will

most likely not allow to distinguish between direct and indirect effects of AtMRP5 on guard cell ion fluxes. Instead, a search for proteins interacting with AtMRP5 may be a better way to locate its exact interception in guard cell signalling chains.

One possible way to improve crop yield in arid zones is to reduce transpirational water loss by engineering guard cell signal transduction pathways (Schroeder *et al.*, 2001b). Reduced irrigation also results in reduced salt deposition, and hence limited salt stress. Under the conditions used in our experiments, a knockout of a plant ABC transporter-generated *Arabidopsis* plants which could survive longer with a given amount of water. These plants are not drought tolerant *in sensu stricto*, since they use less water and cannot survive better on soils with a strongly negative water potential. Furthermore, mutant plants exhibited increased WUE, i.e. less water was required to produce a biomass equivalent to that of the wild type. Since stomata of mrp5-1 plants are less open in the light, but at the same time, insensitive towards ABA, it appears likely that the reduced transpiration during an initial mild water stress is more relevant for the overall water loss than stomatal closure at a later stage when ABA exerts its effect. It appears that engineering AtMRP5 function may pave the way to new crop plants that exhibit an improved tolerance towards drought stress.

Experimental procedures

Plant culture

A. thaliana plants were grown in sand watered with half-strength Hoagland's solution (Epstein, 1972) in a growth chamber (8-h light period, 22°C; 16-h dark period, 21°C; relative humidity 70%) and a light of 250–300 μmol $m^{-2}sec^{-1}$ PAR. Plants were watered daily.

Analysis of stomatal apertures

Stomatal apertures and density of 4–5-week-old plants were measured according to the method of Lascève *et al.* (1997). After 30 min in darkness, initial stomatal apertures were recorded. Ca^{2+}- or ABA-dependent stomatal apertures were measured after 2 h in the light (300 μmol m^{-2} sec^{-1}) at 20°C and after 2 h in the dark for IAA and fusicoccin. All experiments were repeated at least five times. Error bars represent standard error to the mean (SEM) with a confidence interval of 95% and *n* is the number of independent experiments. Plot and curve fitting (non-linear regression by the Levenberg–Marquardt method) were done using Origin 5.0 (MicroCal Software Inc., Northampton, MA, USA). Microscopic analysis of stomatal aperture of intact leaves was carried out with an S3500N variable pressure scanning electron microscope from Hitachi (Tokyo, Japan), equipped with a cool stage. Fresh leaf samples were detached and immediately frozen in liquid nitrogen. Frozen samples were mounted on the cool stage at a cooling temperature of −22°C and immediately analysed at a pressure of 20 Pa and an acceleration voltage of 15 kV. The rapidity of the cooling and the direct analysis allows reliable determination of the aperture of large numbers of stomata.

Production and characterisation of transgenic plants

The full-length *AtMRP5* cDNA (Gaedeke *et al.*, 2001) was cloned into the *Sma*I site of pBinAR between the CaMV 35S promoter and nos terminator, resulting in plasmid p35S-*AtMRP5*. Homozygous mrp5-1 plants were transformed by the floral dip method (Clough and Bent, 1998). Transformed seeds were selected on 0.8% agar plates containing 20 mg l^{-1} hygromycin B. Independent families were re-selected in the T_2 and T_3 generation on hygromycin plates in order to identify homozygous T_2 lines. The presence of *AtMRP5* transcript was verified by RT-PCR analysis of total RNA (RNeasy Plant Kit; Qiagen, Hilden, Germany) isolated from sterile-grown seedlings of Ws-2, *mrp5-1* and *AtMRP5* expressing transformants using a reverse transcription kit (Promega, Mannheim, Germany). AtMRP5 transcript (primers 5D-s, 5D-α), an AtMRP5/T-DNA junction transcript (primers 5D-s, LB2) and 40S ribosomal protein S16-specific cDNAs, respectively, were amplified by PCR for 30 cycles at an annealing temperature of 52°C. For primer sequences, see Gaedeke *et al.* (2001).

Germination tests

For the inhibition of seed germination by exogenous abscisic acid (ABA), several seed batches of surface-sterilised seeds (about 100 seeds per batch and treatment) of *A. thaliana* Ws-2, mrp5-1 and homozygous transgenic mrp5-1/35S-*AtMRP5* T_2 families were grown vertically on 1/2 MS medium (Duchefa, Haarlem, NL, cat. no. M0233) supplemented with 1% (w/v) sucrose, 0.8% (w/v) phytoagar (Duchefa). Plates were kept in the dark at 4°C for 3 days to break seed dormancy and then transferred to 20°C in continuous light. The rate of germination and the number of seeds exhibiting a fully emerged radicle tip were scored 3 days after germination using a binocular.

Whole-plant and single-leaf gas exchange measurements

Four-week-old plants were removed from the sand and inserted into an experimental chamber composed of independent shoot and root compartments (Lascève *et al.*, 1997). The shoot compartment was inserted into an open-flow gas circuit. Water vapour pressures at inlet and outlet of the shoot compartment were continuously measured with dew point hygrometers (Hygro M4, General Eastern, Woburn, MA, USA). Changes in CO_2 concentration were followed with a differential infrared gas analyser (225 MK 3, ADC, Hoddesdon, UK). Leaf temperature was measured with a thermistor probe (409, YSI, Yellow Springs, OH, USA) stuck on the abaxial side of a leaf with ultrathin double-sided adhesive tape. Data were continuously recorded with a personal computer fitted with data acquisition software (Labview, National Instruments, TX, USA). The leaf surface area was measured daily from enlarged photographs. To ensure a rapid uptake via the transpiration stream, ABA was added to the nutrient solution 1 h after illumination of the plant.

Single-leaf gas exchange measurements were performed with a LI-COR LI-6400 photosynthesis system (DMP, Fehraltorf, Switzerland), equipped with LED light source 6400–02 fixed on top of the 6 cm^2 standard cuvette/IRGA. Fully expanded leaves of 4–5-week-old plants and of comparable developmental status were chosen by counting the leaf number from the center of the rosette. Gas flow was adjusted to a constant rate of 0.25 mmol sec^{-1} and the CO_2 partial pressure supplied by a CO_2 cylinder was set to 400 ppm. Relative humidity was 40% and the chamber temperature was set

to 22°C. Six independent Ws-2 and mrp5-1 plants were analysed. The error (SEM) of the WUE was calculated by simulation of the distribution with 2×10^4 values.

Kinetics of water loss from excised rosettes

These experiments were carried out under standard conditions as described by Weyers and Meidner (1990). Water loss is expressed as $(g(t)–g(t_0))$/DW where $g(t)$ is the mass of the rosette measured at time t, $g(t_0)$ is the mass of the rosette measured at the beginning of the experiment and DW is the dry weight of the rosette.

Measurement of water flow into rooted plants

Water uptake of 4- to 5-week-old plant was measured using a homemade potometer, the root and shoot compartments being isolated by the application of an inert synthetic mastic at the crown level. All experiments were conducted in the phytotron. Rates of water flow were measured in a capillary by the movement of an air bubble.

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