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Abstract

Stomatal pores formed by a pair of guard cells in the leaf epidermis control gas exchange and transpirational water loss. Stomatal closure is mediated by the release of potassium and anions from guard cells. Anion efflux from guard cells involves slow (S-type) and rapid (R-type) anion channels. Recently the SLAC1 gene has been shown to encode the slow, voltage-independent anion channel component in guard cells. In contrast, the R-type channel still awaits identification. Here, we show that AtALMT12, a member of the aluminum activated malate transporter family in Arabidopsis, represents a guard cell R-type anion channel. AtALMT12 is highly expressed in guard cells and is targeted to the plasma membrane. Plants lacking AtALMT12 are impaired in dark- and CO□-induced stomatal closure, as well as in response to the drought-stress hormone abscisic acid. Patch-clamp studies on guard cell protoplasts isolated from atalmt12 mutants revealed reduced R-type currents compared with wild-type plants when malate is present in the bath media. Following expression of AtALMT12 in Xenopus oocytes, voltage-dependent anion currents reminiscent to R-type channels could be activated. In line with the features of the R-type channel, the activity of heterologously expressed AtALMT12 depends on extracellular malate. Thereby this key metabolite and osmolite of guard cells shifts the threshold for voltage activation of AtALMT12 towards more hyperpolarized potentials. R-Type channels, like voltage-dependent cation channels in nerve cells, are capable of transiently depolarizing guard cells, and thus could trigger membrane potential oscillations, action potentials and initiate long-term anion and K(+) efflux via SLAC1 and GORK, respectively.

*At*ALMT12 represents an R-type anion channel required for stomatal movement in *Arabidopsis* guard cells

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Stomatal pores formed by a pair of guard cells in the leaf epidermis control gas exchange and transpirational water loss. Stomatal closure is mediated by release of potassium and anions from guard cells. Anion efflux from guard cells involves anion channels of the S- and R-type. Recently the SLAC1 gene has been shown to encode the slow, voltage-independent anion channel component in guard cells. In contrast, the R-type channel still awaits identification. Here we show that *At*ALMT12, a member of the ALuminum activated Malate Transporter family in *Arabidopsis*, represents a guard cell R-type anion channel. *At*ALMT12 is highly expressed in guard cells and targeted to the plasma membrane. Plants lacking *At*ALMT12 are impaired in dark- and CO_2-induced stomatal closure as well as in response to the drought-stress hormone abscisic acid. Patch clamp studies on guard cell protoplasts isolated from *atalmt12* mutants revealed reduced R-type currents compared to wild-type plants when malate is present in the bath media. Following expression of *At*ALMT12 in *Xenopus* oocytes, voltage-dependent anion currents reminiscent to R-type channels could be activated. In line with the features of the R-type channel, activity of heterologously expressed *At*ALMT12 depends on extracellular malate. Thereby this key metabolite and osmolite of guard cells shifts the threshold for voltage activation of *At*ALMT12 towards more hyperpolarized potentials. R-Type channels, like voltage-dependent cation channels in nerve cells, are capable to transiently depolarize guard cells and thus could trigger membrane potential oscillations, action potentials and initiate long-term anion and K^+ efflux via SLAC1 and GORK, respectively.

Introduction

Stomatal closure is initiated by the release of anions which in turn depolarizes the membrane potential and activates the outward-rectifying potassium channels (Roelfsema and Hedrich, 2005). The rapid (R-type) and the slow (S-type) activating anion channel are responsible for anion efflux (Schroeder and Keller, 1992; Raschke *et al.,* 2003). The slow, voltage-independent anion channel component in guard cells was recently shown to require the *SLAC1* gene (Negi *et al.,* 2008; Vahisalu *et al.,* 2008). In this multisensory cell type SLAC1 activation and associated stomatal closure depend on the protein kinase OST1 and distinct CPKs (Geiger *et al.,* 2009; Geiger *et al.,* 2010). SLAC1 activation by the latter kinases is prevented by PP2C protein phosphatases ABI1 and 2 (Leung *et al.,* 1997; Merlot *et al.,* 2001) which are addressed by a cytosolic abscisic acid (ABA) receptor (Ma *et al.,* 2009; Park *et al.,* 2009). The water stress hormone ABA activates both the S-type as well as the voltage-dependent R-type channel (Roelfsema *et al.,* 2004; Levchenko *et al.,* 2005). However, the nature of channels, underlying the rapid component, remains yet unknown. This guard cell anion channel – also named GCAC1 (Guard Cell Anion Channel 1)/QUAC (QUick activating Anion Channel) - exhibits voltage-dependent features of neuronal calcium and sodium channels (Hedrich *et al.,* 1990; Kolb *et al.,* 1995; Hille, 2001). Upon depolarization this channel type activates with fast kinetics, while hyperpolarization causes deactivation. Malate represents both a key metabolite and major organic osmolite in guard cells (Fernie and Martinoia, 2009; Meyer *et al.,* 2010). During stomatal closure malate is partially converted to osmotic inactive starch, but malate is also released from the cell to the apoplast (van Kirk and Raschke, 1978; Roelfsema and Hedrich, 2005). External malate shifts the voltage gate of the R-type channel towards more negative membrane potentials, favoring channel opening at the resting state and in turn depolarization of the guard cell (Hedrich and Marten, 1993; Raschke *et al.,* 2003; Konrad and Hedrich, 2008). Additionally it has also been shown that apoplastic malate is required for an efficient stomatal opening (Lee *et al.,* 2008).

ALMT channels (ALuminum activated Malate Transporter) have been first described as plasma membrane located, Al^{3+}-activated malate channels by patch clamp studies in root cells (Kollmeier *et al.,* 2001; Pineros and Kochian, 2001) and genetically identified in *Triticum aestivum* (*Ta*ALMT1) (Sasaki *et al.,* 2004) and *Arabidopsis thaliana* (*At*ALMT1) (Hoekenga *et al.,* 2006). These channels play a central role in aluminum resistance by releasing malate from the root tip thereby chelating aluminum in the rhizosphere (Sasaki *et al.,* 2004; Hoekenga *et al.,* 2006). In a later study it has been shown that certain *At*ALMTs can also reside on the tonoplast acting as vacuolar malate channels (Kovermann *et al.,* 2007). Interestingly, one member of the ALMT protein family, *Zm*ALMT1, however, activates Al^{3+}-independently and transports inorganic anions such as Cl^-, NO_3^-, and SO_4^{2-} rather than malate (Pineros *et al.,* 2008). Very recently an *Arabidopsis* ALMT protein (*At*ALMT12) has been described to be strongly expressed in guard cells and to be permeable for chloride and nitrate (Sasaki *et al.*, 2010). Loss-of-function mutants were impaired in stomatal closure. However, under the conditions used by the Sasaki laboratory, neither the S-type nor the R-type channel activities appeared altered in guard cells. Since the authors observed the GFP fluorescence predominatly in the endoplasmic reticulum (ER), they predicted *At*ALMT12 to function in release of inorganic anions from the ER. Thus the role of *At*ALMT12 for stomatal closure remains elusive.

Based on independent, parallel studies to those of the Sasaki laboratory (2010) we here provide convincing evidence evidence i) that *At*ALMT12 is expressed in the plasma membrane of guard cells; ii) that guard cells of loss-of-function mutants are impaired in malate dependent R-type channel activity and iii) that *At*ALMT12 is malate permeable and thus well suited for release of malate from guard cells as shown for *Vicia faba* (Keller *et al.,* 1989; Dietrich and Hedrich, 1994).

Results

Tissue specific expression analysis of *At*ALMT12

In order to identify potential candidates for R-type channels we searched for ALMTs expressed in guard cells. Gene expression data from microarray experiments (http://www.bar.utoronto.ca/) indicated strong mRNA accumulation of *AtALMT12* in guard cells. To verify the microarray data we transformed *Arabidopsis* plants with the *ß-Glucuronidase* (GUS) gene under the control of a 2018 base pairs (bp) promoter region (*pAtALMT12*) upstream of the genomic sequence of *AtALMT12*. Strong GUS activity in *pALMT12::GUS* transformants was detected in guard cells of different tissues (Figure 1 a, b; Figure SI1 a, b). Additionally, a signal was also observed in the pollen tissue as well as in the stele of roots (Figure SI1 b, c). These observations were consistent with the microarray gene expression data and data presented by Sasaki *et al.* (2010).

Subcellular localization of an *At*ALMT12-GFP fusion protein

In order to investigate the subcellular localization of *At*ALMT12, the green fluorescence protein (GFP) was fused to the C- as well as the N-terminal end of *At*ALMT12. Transient expression of these constructs in *Arabidopsis* protoplasts under the control of the *35S*-promoter revealed that *At*ALMT12-GFP was targeted to the plasma membrane (Figure 1 c-f). In order to verify plasma membrane localization of *At*ALMT12 in guard cells, where its promoter activity has been detected, we also performed independent confocal laser scanning microscopy analyses of stable transformed *Arabidopsis*. In general most channels are present only at very low protein numbers and can therefore hardly be visualized using their own promoter. We thus generated transgenic Arabidopsis plants expressing the *At*ALMT12-GFP construct under the control of the stomata-specific promoter *AtMYB60* (Cominelli *et al.,* 2005, Nagy *et al.*, 2009). Confocal microscopy analysis showed GFP fluorescence along the guard cell periphery thus confirming plasma membrane localization recognized by transient expression in *Arabidopsis* protoplasts before (Figure 1 g, h). Taken together, our studies

identified *At*ALMT12 as a protein of the *Arabidopsis* guard cell plasma membrane rather than intracellular membrane systems.

Analysis of mutant lines carrying a T-DNA insertion in the *AtALMT12* gene

Guard cells are sensitive to environmental- and endogenous changes including light, CO_2 and ABA. To unravel the physiological role of *At*ALMT12 in guard cells we analyzed stomatal movements in two independent *atalmt12* mutant lines (*atalmt12-1* and *atalmt12-2*) identified in the JIC T-DNA insertion mutant collection (Tissier *et al.,* 1999). The absence of the *AtALMT12* transcripts in the mutants was demonstrated by RT-PCR (Figure SI2). In a first step we investigated whether wild-type plants and *atalmt12* mutants differ in their reaction to the plant hormone abscisic acid (ABA), which is synthesized in response to drought stress. As observed by Sasaki *et al.* (2010) in the presence of ABA stomata of wild-type guard cells closed efficiently. In contrast, both *atalmt12* mutant plants barely responded to ABA and stomata remained largely open even after 2 h of incubation with ABA (Figure 2). In all experiments light stimulated opening of guard cells was similar in wild-type and mutant plants. In order to see whether the difference in ABA response could also be observed in response to other stimuli at the whole plant level, we monitored the stomatal conductance in *atalmt12* mutants and wild-type plants in response to light and CO_2. Compared to wild-type plants *atalmt12* mutants exhibited a much slower decline of stomatal conductance in response to light-dark transitions (Figure 3 a, b). Furthermore, increase of [CO_2] from 365 p.p.m. to 800 p.p.m caused a rapid stomatal closure in wild-type which was less pronounced in mutant plants (Figure 3 c, d).

Electrophysiological studies on *atalmt12* guard cell protoplasts and *At*ALMT12 expressing *Xenopus laevis* oocytes

The fact that *atalmt12* mutant plants are impaired in stomatal closure and members of this family operate as anion channels (Pineros *et al.,* 2008), directed us to examine the electrical properties of this potential anion channel in guard cells. To study the anion channel transport

capacity of *Arabidopsis* guard cells, we performed patch clamp studies with protoplasts isolated from wild-type plants and *atalmt12-1* mutants. The *slac1* mutant exhibits a largely reduced S-type current, while R-type currents remained unaffected by the loss of this anion channel function (Vahisalu *et al.*, 2008; Geiger *et al.*, 2009; Geiger *et al.*, 2010). Consequently, we tested whether guard cells isolated from the *atalmt12* mutants appear altered in plasma membrane R-type anion currents. In contrast to *Vicia faba* (Hedrich *et al.,* 1990; Marten *et al.,* 1991; Raschke *et al.,* 2003), R-type channels in *Arabidopsis* guard cells have not been characterized in detail. Therefore in the first place we analyzed the R-type properties of wild-type guard cells because knowledge about the R-type characteristic represents a basic requirement to understand the phenotype of the *atalmt12* mutants. To resolve R-type-specific anion release currents we performed patch clamp studies using 75 mM sulfate-based pipette solutions (cf. Vahisalu *et al.,* 2008). With 20 mM sulfate in the extracellular medium and the plasma membrane clamped to a holding potential of -180 mV, depolarizing voltage pulses elicited inward currents (Figure SI3 a; cf. Sasaki *et al.,* 2010). Under these conditions anion currents reversed around the Nernst potential of sulfate and displayed fast activation and deactivation kinetics. Thus *Arabidopsis* guard cells appear to express plasma membrane anion channels with voltage dependent properties of the R-type channel GCAC1/QUAC found in *Vicia faba* (Kolb *et al.,* 1995; Schulz-Lessdorf *et al.,* 1996; Raschke *et al.*, 2003). However, no difference in anion channel activity could be detected between wild-type and *atalmt12* mutant plants under these conditions (Figure SI3 b). In *Vicia faba* malate was shown to activate R-type currents (Hedrich and Marten, 1993; Raschke *et al.*, 2003). We thus challenged *Arabidopsis* wild-type and *almt12-1* mutant guard cell anion channels with malate. In the presence of extracellular malate, R-type anion currents of *almt12-1* mutants appeared reduced by 40% when compared to wild-type guard cells (Figure 4 a, b). Subtracting wild type currents from those observed in *atalmt12* guard cells resulted in a bell-shaped current-voltage curve. This electrical behaviour points to strong voltage dependence and channel activation upon depolarization which both are hallmarks of the R-type anion channels. Such differential R-type currents between wild type and *atalmt12-1*

guard cell protoplasts have been not observed by Sasaki et al. (2010) because their experiments were performed solely under external chloride-based conditions meaning in the absence of the R-type channel gating modifier malate. Thus based on the characteristic voltage-dependent features of the malate-dependent currents (Figure 4), the loss-of-function phenotype suggested that *AtALMT12* likely encodes a malate-sensitive component of the R-type anion channel of *Arabidopsis* guard cells.

To study the malate-sensitive component of the R-type current in guard cells, we expressed *At*ALMT12 in *Xenopus* oocytes. Following injection of *AtALMT12* cRNA into oocytes, depolarizing voltages elicited outward currents under chloride-based external solutions (Figure SI4 a). The amplitudes of these anion uptake currents appeared to depend on the external chloride concentration (Figure SI4 b; cf. Sasaki *et al.,* 2010). Upon replacement of external chloride by malate, however, voltage pulses elicited both inward and outward currents of up to 7 µA (Figure 5 a, b). Interestingly, Sasaki et al. (2010) could only observe outward currents (anion uptake) and no inward currents (anion release) in the presence of external malate and sulfate (Figure 5, SI5). This discrepancy to our results may arise from the use of less physiological external pH conditions by Sasaki et al. (2010). For further characterization we examined the voltage-dependent gating of *At*ALMT12 with a double voltage pulse protocol under extracellular ionic conditions similar to those used in patch clamp experiments with guard cell protoplasts. Therein the pulse to -200 mV was followed by a depolarizing pulse to +60 mV, before applying trains of hyperpolarizing pulses (Figure 5 a). After opening of *At*ALMT12 channels at +60 mV they were forced to deactivate as a function of the subsequent negative-going voltage steps. In line with the R-type currents in protoplasts (Figure 4), the voltage dependence of these steady-state currents could be also described by a bell-shaped current-voltage curve (Figure 5 b). In the presence of comparable external malate concentrations (Figure 5 b: 25 mM in oocytes; Figure 4: 20 mM in protoplasts) channel activation already occurred at less depolarized potentials in oocytes than in protoplasts very probably because of the divergent cytosolic composition (Figure 4, 5 b). Channel gating depended on the concentration of malate (Figure 5 b, c). Upon an

increase in the malate concentration the half-maximal activation potential shifted towards more negative membrane potentials (Figure 5 c; Table SI1; Hedrich and Marten, 1993; Raschke *et al.*, 2003). When sulfate was injected into oocytes, *At*ALMT12-mediated inward currents increased (Figure SI5), indicating that also sulfate is a preferred substrate of the anion channel (Roberts, 2006). This is in agreement with the finding that presence of K_2SO_4 reduces the impact of Cl^- in stomatal action (Schnabl and Raschke, 1980). In contrast to *At*ALMT1, *At*ALMT12 currents were not stimulated by extracellular Al^{3+} treatment (Figure 6 a).

Malate is not only a key metabolite and signaling component for guard cells, but was shown to represent a substrate for R-type channels in *Vicia faba* guard cells (Keller *et al.,* 1989; Hedrich and Marten, 1993). Following injection of malate (20 mM final cytosolic concentration) buffered to pH 7.5 inward currents were recorded with moderate negative-going membrane potentials (Figure 6 b). Note, that malate injection activates pronounced R-type like currents with *At*ALMT12 in the absence of malate in the oocyte external medium. Addition of 10 mM malate into the external medium further maximized these inward currents. These experiments show that similarly to the *Vicia faba* R-type channel (Hedrich and Marten, 1993) malate functions as a gating modifier as well as a permeating substrate of *A*tALMT12.

Discussion

In *Arabidopsis* the ALMTs constitute a small gene family of 14 members which can be subdivided into three clades (Kovermann *et al.*, 2007). The best characterized *At*ALMT1 which is located to the root plasma membrane is a member of clade 1 while the vacuolar *At*ALMT9, mainly expressed in the leave mesophyll, is assigned to clade 2 (Hoekenga *et al.,* 2006; Kovermann *et al.*, 2007). Microarray data suggest that *At*ALMT12, a member of clade 3, is strongly expressed in guard cells. Our present study and an independent one recently published by Sasaki *et al.* (2010) could confirm the predicted predominant expression of

*At*ALMT12 in this cell type. Using two different *AtALMT12* loss-of-function mutants we observed an impaired stomatal closure when leaves were exposed to ABA. Similar results were obtained by Sasaki *et al.* (2010) with independent mutant lines. These authors could also show impaired stomatal closure in the presence of Ca^{2+} and in the dark. In our study, furthermore gas exchange measurements revealed a delayed and not complete stomatal closure in plants lacking the *At*ALMT12 protein. These results convincingly show that *At*ALMT12 is required for efficient stomatal closure.

_At_ALMT12 is a strongly voltage-dependent plasma membrane anion channel

Sasaki *et al.* (2010) stated that *At*ALMT12 is an outward rectifying (anion uptake into the cytosol) channel permeable for chloride and nitrate, but not sulfate and malate as demonstrated in our work. From Sasaki`s studies AtALMT12 function in stomatal movement remained elusive since the localization for *At*ALMT12 was observed in both, the endoplasmic reticulum as well as the plasma membrane. This equivocal localization may be due to the transient expression used by these authors, while in our study a clear plasma membrane localization of AtALMT12 was observed in plants stably transformed with a similar construct. Particularly the predicted direction of the anion movements makes it difficult to attribute a role in stomatal closure to *At*ALMT12 located to the plasma membrane. Furthermore working in the absence of malate the authors could not detect differences in R-type channel activities between guard cells of wild-type and *atalmt12* mutant *Arabidopsis* plants. Therefore, Sasaki *et al.* (2010) postulated a predominant role of *At*ALMT12 in release of inorganic anions from the endoplasmic reticulum into the cytosol. However, since the volume of the endoplasmic reticulum is relatively small it is thus rather unlikely that anions released from this compartment have a major impact in stomatal closure. Furthermore, *At*ALMT-mediated currents measured on native membranes showed only inward rectification so far (Kovermann *et al.*, 2007; Zhang *et al.,* 2008). Assuming a slight negative membrane potential between the ER and the cytosol, due to the activity of a V-type ATPase, no anion release to the cytosol

would be detectable taking into account the current-voltage curves presented by Sasaki *et al.* (2010).

In previous studies, ALMTs have been characterized as channels for dicarboxylates such as malate and fumarate, but also for inorganic anions like Cl^-, NO_3^-, and SO_4^{2-} (Pineros *et al.,* 2008). Since the latter substrate specificity was also known for R-type channels (Roberts, 2006) and the fact that *At*ALMT12 is highly expressed in guard cells, we here explored whether *At*ALMT12 could act as a so far non-identified plasma membrane R-type channel. Our studies on guard cell protoplasts and *At*ALMT12-expressing oocytes provide evidence that *At*ALMT12 represents a malate-sensitive component of the R-type anion channel in guard cells of *Arabidopsis*. In both systems, protoplasts and oocytes, R-type anion currents showed similar voltage dependence and kinetics. In line with *Zm*ALMT1 from *Zea mays* but in contrast to TaALMT1 or *At*ALMT1, *At*ALMT12 was not induced by Al^{3+} (Sasaki *et al.*, 2004; Hoekenga *et al.,* 2006; Pineros *et al.,* 2008,). We thus suggest renaming it QUAC1 in accordance with the naming of the S-type anion channel of guard cells, SLAC1 (Raschke *et al.*, 2003; Negi *et al.,* 2008; Vahisalu *et al.,* 2008). Due to the fact that in the *atalmt12/quac1* mutant guard cells only malate-dependent anion currents appeared to be affected, one would predict that QUAC1 is based either on different channels exhibiting similar current-voltage curves, but possibly different substrate specificities and malate dependencies. Alternatively, QUAC1 could be formed by heteromers with *At*ALMT12 as one subunit responsible for malate sensing. Clade 3 of the *At*ALMT protein family is constituted by three additional members, *At*ALMT11, 13 and 14 (Kovermann *et al.,* 2007). *At*ALMT11 is not likely to be a candidate for an additional component of the QUAC channel because the predicted structure is constituted by only two membrane domains and the protein is weakly expressed in guard cells and other tissues (http://www.bar.utoronto.ca/). However, *At*ALMT13 and *At*ALMT14 might be candidates for further members of a heteromeric QUAC complex although no expression data are available so far. Future studies on the guard cell expressed QUACs thus have to focus on the identification of additional components of the R-type channel**.**

In former studies it has been shown that beside Cl^-, NO_3^-, and SO_4^{2-} the R-type channels exhibits also permeability for malate (Keller *et al.,* 1989; Hedrich and Marten, 1993; Roberts, 2006). Malate in the apoplast has been postulated to play an important role in activating anion release possibly by inducing a shift in the voltage dependence of R-type anion channels (Hedrich and Marten, 1993; Raschke *et al.,* 2003; Konrad and Hedrich, 2008). As shown in Figure 6 b, *At*ALMT12 is not only sensitive to malate, but also permeable for this organic ion and it is therefore tempting to speculate that this component of QUAC might play an important role in this feed-forward stimulus for long-term anion release.

***At*ALMT12/R-type/QUAC and SLAC poise guard cells for voltage- and volume control**

SLAC1, addressed by ABA signaling kinase/phosphatase pairs, seems to be required (i) to drive long term efflux of osmotically active anions from guard cells and stomatal closure, and (ii) thus for an effective decrease in transpiration when soil water is limiting (Negi *et al.,* 2008; Vahisalu *et al.,* 2008; Geiger *et al.*, 2009). QUAC-like channels in *Vicia faba* also appear to be addressed by the water stress hormone (Raschke *et al.,* 2003; Roelfsema *et al.,* 2004). In contrast to SLAC1, QUAC1 activation in oocytes did neither require the presence of OST1 nor plant specific CPKs (cf. Geiger *et al,* 2009; Geiger *et al.,* 2010). These observations strongly suggest that SLAC1 and QUAC1 likely represent response elements on different branches of the ABA signaling pathway leading to stomatal closure (Levchenko et al. 2005).

Voltage-dependent properties (activation, de- and inactivation) of the R-type channels are reminiscent of depolarization-activated cation channels in neurons (Hedrich *et al.,* 1990; Kolb *et al.,* 1995; Schulz-Lessdorf *et al.,* 1996; Hille 2001). Due to the inverse anion gradients across the plasma membrane of plant cells relative to that of animal origin, it is tempting to speculate that the malate-sensitive QUAC1 channel is involved in membrane potential oscillations (Konrad and Hedrich, 2008; Raschke *et al.,* 2003). This notion is supported by the fact that malate promotes oscillations in membrane voltage and thus appears to be involved in membrane excitability (Konrad and Hedrich, 2008). Furthermore stimulation by

modifiers of the QUAC-type channels gating (Lohse and Hedrich, 1995) seems to trigger action potentials in guard cells (Blatt and Thiel, 1994).

Experimental procedures

Plant material and growth conditions

Arabidopsis thaliana wild-type plants (Col-0) and mutant plants were grown in controlled environment chambers in potting soil or on agar medium under a 8h/16h light to dark regime (90 µmol m^{-2} s^{-1}) and dark at 21°C (60 % relative humidity). The floral dip method (Clough and Bent, 1998) was used for obtaining transgenic lines.

Selection of *atalmt12* T-DNA mutant lines

T-DNA insertion lines of *AtALMT12* were obtained from the JIC collection (Tissier *et al.,* 1999) and plants homozygous for the T-DNA insertion were isolated by PCR genotyping. Two independent T-DNA insertion lines of *AtALMT12* were identified (*atalmt12-1* and *atalmt12-2*). Wild-type lines were selected as those plants that genotyped as wild-type (WT-like) during homozygous PCR screening of the JIC mutant line seed batch. In all assays of stomatal measurements (Figure 2, 3) *atalmt12-1* and *atalmt12-2* mutant lines were compared to the corresponding WT lines (WT-like -1 and -2). For details see Supporting Information.

Subcellular localization and tissue-specific expression

Tissue-specific expression of *At*ALMT12 in *Arabidopsis* was analysed by amplifying a 2018 bp promoter region fused to the *GUS* reporter gene in the pGPTV-Bar vector (Becker *et al.,* 1992). C- and N-terminal GFP fusion constructs with *At*ALMT12 were generated in the pUC18-GFP5T-sp (Meyer *et al.,* 2006) and a modified pART7 vector (Endler *et al.,* 2006) for transient expression and in a modified pMDC83 vector for stable transformation (Nagy *et al.,* 2009). For details see Supporting Information.

Stomatal aperture and gas exchange measurements

Gas exchange was measured with a portable gas exchange system (LI-6400; LI-COR). For response to ABA detached rosette leaves were treated with 10 µM ABA after pre-incubation in opening buffer. For details see Supporting Information.

Electrophysiological techniques

Plant growth conditions and isolation of guard cell protoplasts for electrophysiological studies were performed as described previously (Geiger *et al.,* 2009). Using the patch clamp technique protoplasts were studied in the whole-cell configuration essentially as described elsewhere (Wolf *et al.,* 2006). The standard bath solution was composed of (in mM) 2 $MgCl_2$, 0.5 $LaCl_3$, 10 MES pH 5.6/Tris and either (20 $CaGluconate_2$ plus 20 Cs_2SO_4) or 20 CaMalate. The pipette solution consisted of (in mM) 75 Cs_2SO_4, 2 $MgCl_2$, 5 Mg-ATP, 10 Hepes pH 7.1/Tris. To obtain a free Ca^{2+} concentration of 1 µM, the pipette solution additionally contained 5 mM EGTA plus 4.2 mM $CaCl_2$. The osmolality of the pipette and bath media was adjusted to 440 and 400 mosmol/kg, respectively, with D-sorbitol. Oocyte measurements were performed using the two-electrode voltage-clamp technique (TEVC). Oocytes were perfused with a standard solution containing 10 mM Mes/Tris pH 5.6, 1 mM $CaGluconate_2$, 1 mM $MgGluconate_2$, 1 mM $LaCl_3$ and variable concentrations of NaH-malate, NaCl and/or NaGluconate. If necessary, osmolality was adjusted to 220 mOsmol/kg using D-sorbitol. Injection of 50 nl of a 200 mM Na^+-malate solution solution resulted in a final malate concentration of 18 mM in the oocyte because a mean volume of 500 nl for the spherical oocytes was calculated from the averaged oocyte diameter of 1 mm. Voltage pulse protocols similar to those used for studies of guard cell R-type channels (Hedrich *et al.*, 1990, Marten *et al.,* 1991) were applied from a holding voltage of -20 mV. Details of solutions and pulse protocols are mentioned in the figure legends. For further details of data acquisition and analysis see Supporting Information.

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Supporting Information (SI)

Supporting Experimental Procedures

Figure legends

Figure 1. *At*ALMT12 is expressed in guard cells und localizes to the plasma membrane**.**
(a, b) *pAtALMT12::GUS* gene expression in leaves of young plants (a), close-up of leaf guard cells (b). (c, d) Fluorescence microscopy images of *Arabidopsis* mesophyll protoplast transiently expressing *At*ALMT12-GFP (c) and GFP-*At*ALMT12 (d) fusion protein. (e, f) Transmission pictures of the same *Arabidopsis* mesophyll protoplasts as in the fluorescent images (c) and (d). (g, h) Fluorescence microscopy image (g) and transmission picture (h) of an *Arabidopsis* guard cell stably expressing *At*ALMT12-GFP fusion protein under control of the *MYB60* promoter. Chloroplasts show red auto fluorescence in (c, d, g). Scale bars: (a) 1mm, (b) 20 µm, (c, d, g) 10 µm.

Figure 2. Impaired stomatal closure in *atalmt12* mutant plants in response to the phytohormone abscisic acid (ABA).

ABA (10 μM) was added to detached whole leaves at time = 0 (100%). (n = 3 at 2 h and at 1 h for *atalmt12-2*; n = 5 at 1 h for *atalmt12-1*; about 60 stomata of three to four different leaves of one plant were measured in each experiment and at each time point). Stomatal apertures at time = 0 corresponded to an average aperture of 3.64 ± 0.26 µm (WT-like -1), 3.57 ± 0.24 µm (*atalmt12-1*), 4.32 ± 0.07 µm (WT-like -2) and 4.19 ± 0.07 µm (*atalm12-2*). Data represent means ± S.E.M.

Figure 3. Mutations in *AtALMT12* affect stomatal closure in response to various stimuli.
(a, b) Time courses of stomatal conductance in *atlmt12-1* mutants and WT-like -1 plants (a) and *atalmt12-2* mutants and WT-like -2 plants (b) in response to change in light intensity. The number of experiments was n = 6 for WT-like -1 and *atlmt12-1*, n = 4 for WT-like -2 and n = 5 for *atalmt12-2*. (c, d) Time courses of stomatal conductance in *atalmt12-1* mutants and WT-like -1 plants (c) and *atalmt12-2* mutants and WT-like -2 plants (d) in response to elevated CO_2 levels The number of experiments was n = 6 for WT-like -1, n = 5 for *atlmt12-1* and n = 4 for *atalmt12-2* and WT-like -2. Data represent means ± S.E.M.

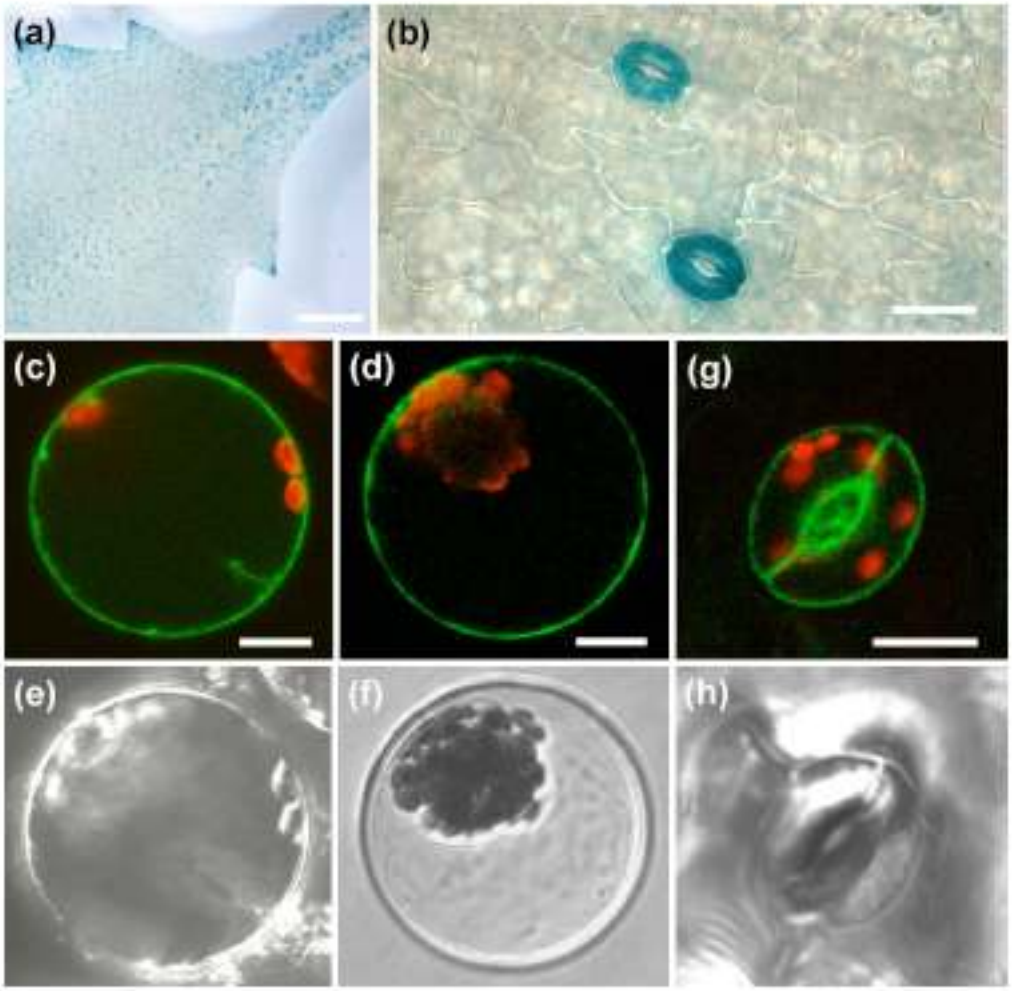
Figure 4. Voltage-dependent activation of R-type currents from wild type and *atalmt12-1* guard cell protoplasts.
(a) Representative current responses elicited upon a voltage ramp from +70 to -180 mV (WT in black and *atalmt12-1* in red). The holding voltage was -180 mV. (b) Steady-state current densities (I_{ss}/C_m) plotted against the clamped voltages. Experiments were performed in the presence of 20 mM external malate. *At*ALMT12 loss-of-function mutants were characterized by a decrease in the current density compared to wild-type protoplasts. Data points represent mean ± S.E.M. The number of experiments was n = 6 for wild type (WT) and *atalmt12-1*. The inset shows the *At*ALMT12-mediated current component derived from the subtraction of the residual currents of *atalmt12-1* protoplasts from currents observed in WT protoplasts.

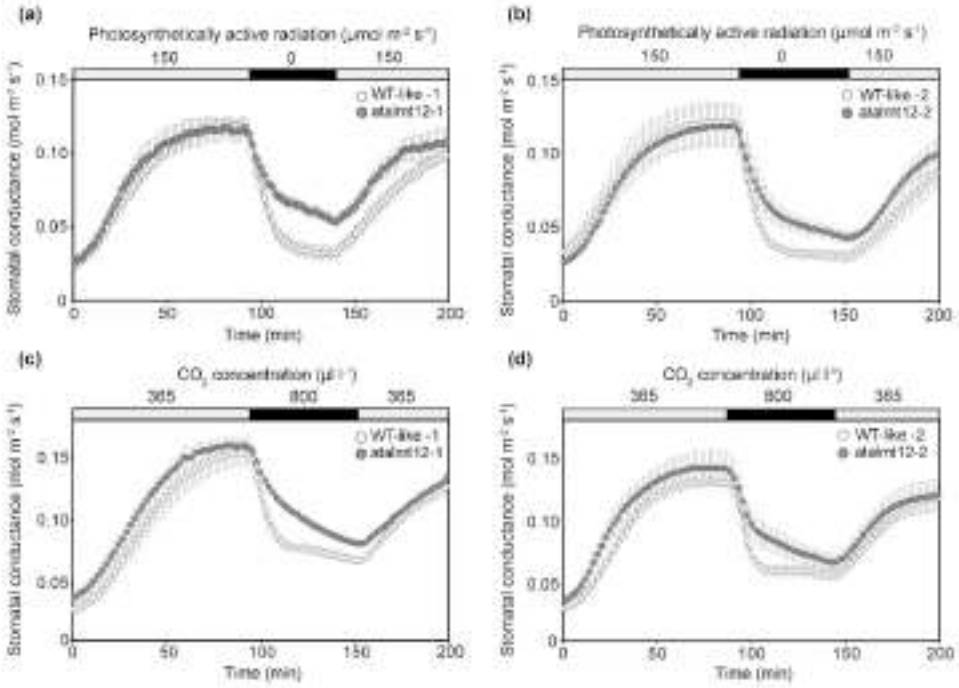
Figure 5. Whole-oocyte current recordings from *At*ALMT12-expressing oocytes measured in malate-based external solutions.

(a) Representative current responses evoked upon a channel-activating voltage pulse (+60 mV) followed by test voltage pulses (as indicated). The standard bath medium contained 5 mM malate. Arrows label the positions at which steady state (I_{ss}) and tail currents (I_{tail}) were determined. The latter was used for calculation of the relative open probability. (b) Steady state currents I_{ss} and (c) relative open probability P_o as a function of voltage are shown at different extracellular malate concentrations (as indicated). Note the shift in P_o and peak inward current towards more negative voltages with increasing extracellular malate concentrations. Data points represent mean ± S.D. with the number of experiments n = 6.

Figure 6. Whole-oocyte current recordings from *At*ALMT12-expressing oocytes in the presence of Al^{3+} and in the presence of cytosolic malate.

(a) Steady state currents of *At*ALMT12-expressing oocytes in 25 mM external NaCl solution in the presence or absence of 1 mM $AlCl_3$ at -100 mV. The final malate concentration of injected oocytes was around 20 mM. In contrast to other known ALMT transporters *At*ALMT12 was not activated by Al^{3+}. Bars represent mean ± S.E.M.; n = 4 for water-injected and n = 5 for malate-injected oocytes. (b) Steady state currents of *At*ALMT12-expressing oocytes at a membrane potential of -150 mV. Oocytes were either injected with water or malate (final concentration around 20 mM) prior to current recordings under chloride- or malate-based external conditions (i.e. 25 mM chloride or 10 mM malate in the bath solution). In the presence of cytosolic and extracellular malate *At*ALMT12-derived anion currents appeared maximized. Bars represent mean ± S.E.M. (n ≥ 4).


(a)
(b)
(c)
(d)
(g)
(e)
(f)
(h)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150
0
150
Stomatal conductance (mol m⁻² s⁻¹)
0.15
0.10
0.05
0
WT-like -1
atalmt12-1
0
50
100
150
200
Time (min)
(b)
Photosynthetically active radiation (μmol m⁻² s⁻¹)
150
0
150
Stomatal conductance (mol m⁻² s⁻¹)
0.15
0.10
0.05
0
WT-like -2
atalmt12-2
0
50
100
150
200
Time (min)
(c)
CO2 concentration (μl l⁻¹)
385
800
385
Stomatal conductance (mol m⁻² s⁻¹)
0.15
0.10
0.05
0
WT-like -1
atalmt12-1
0
50
100
150
200
Time (min)
(d)
CO2 concentration (μl l⁻¹)
385
800
385
Stomatal conductance (mol m⁻² s⁻¹)
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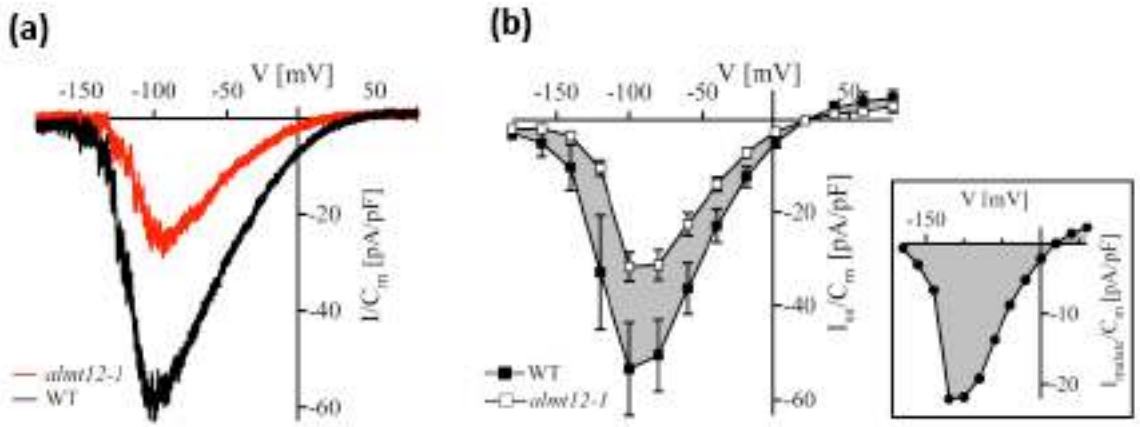
0.10
0.05
0
WT-like -2
atalmt12-2
0
50
100
150
200
Time (min)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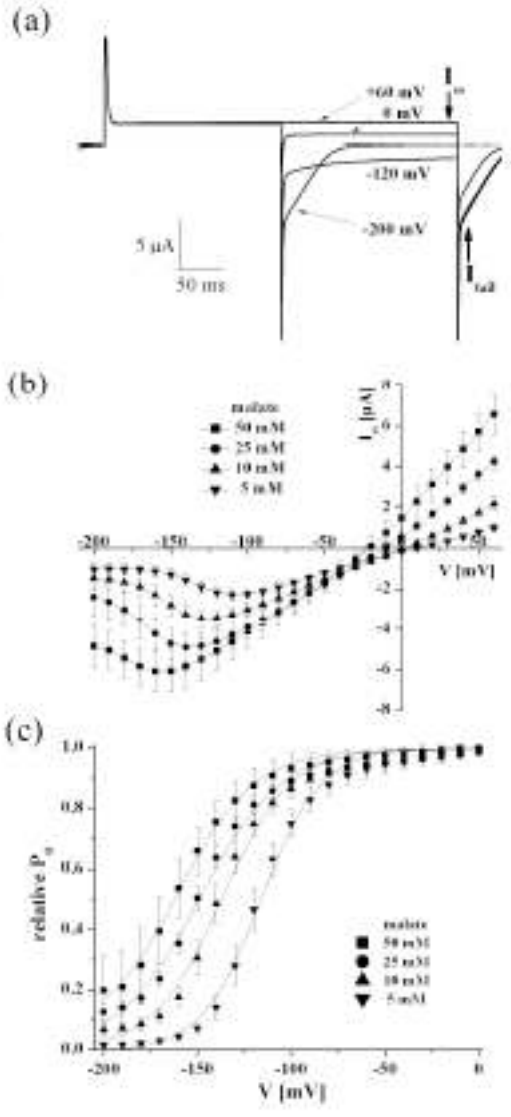
V [mV]
-150
-100
-50
50
-20
-40
-60
I/C_m [pA/pF]
almt12-1
WT
(b)
V [mV]
-150
-100
-50
50
-20
-40
-60
I_ss/C_m [pA/pF]
WT
almt12-1
V [mV]
-150
-10
-20
I_transc/C_m [pA/pF]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+60 mV
0 mV
-120 mV
-200 mV
5 µA
50 ms
(b)
malate
50 mM
25 mM
10 mM
5 mM
-200
-150
-100
-50
V [mV]
(c)
relative P_o
malate
50 mM
25 mM
10 mM
5 mM
-200
-150
-100
-50
V [mV]

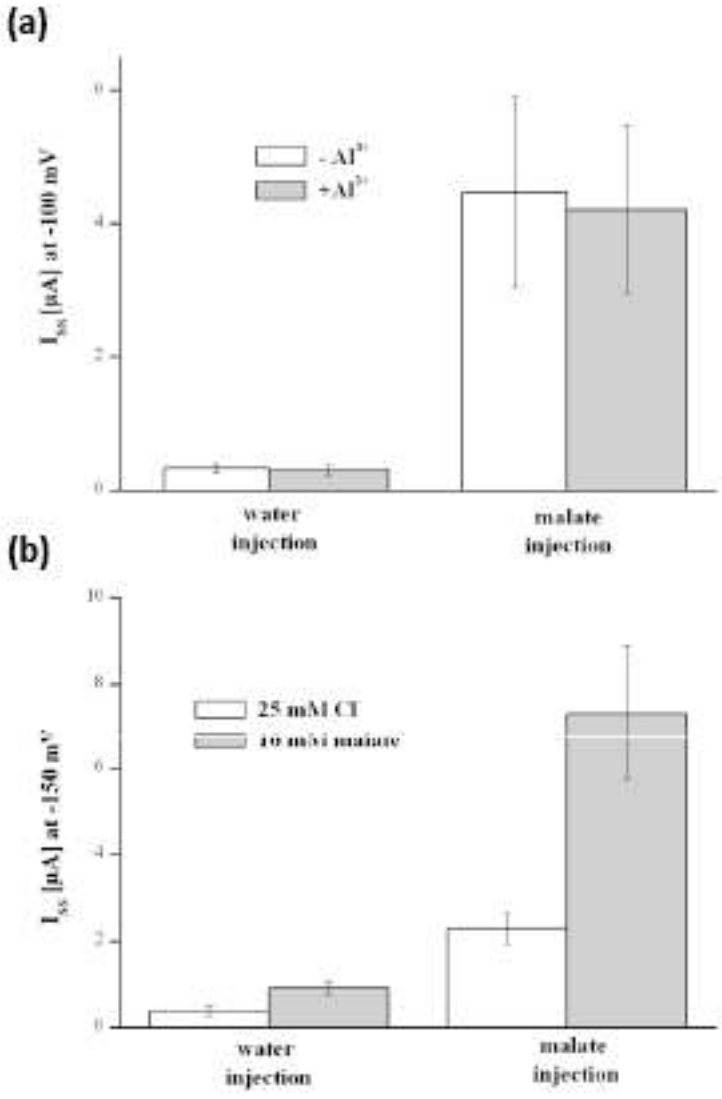

(a)
I_{ss} [µA] at -100 mV
- Al^{3+}
+ Al^{3+}
water injection
malate injection
(b)
I_{ss} [µA] at -150 mV
25 mM Cl
malate
water injection
malate injection

Figure SI1

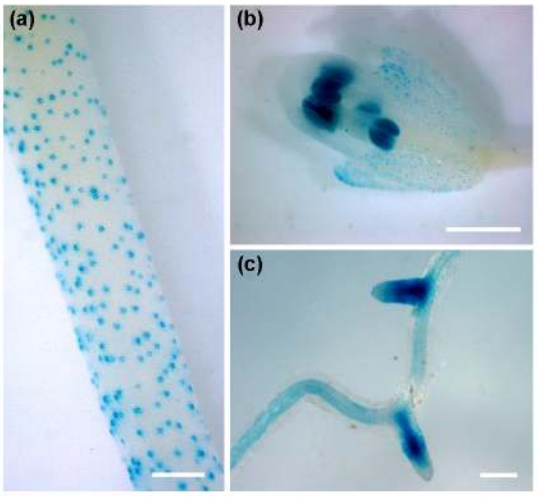
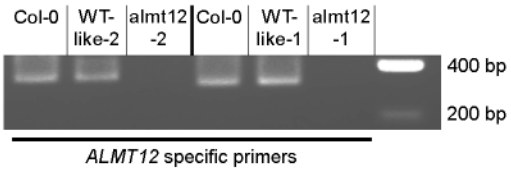


Figure SI2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almt12-2
Col-0
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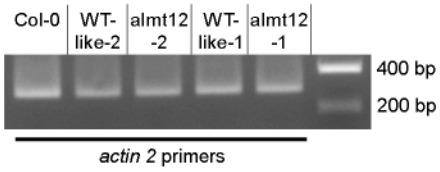
200 bp
ALMT12 specific primers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200 bp
actin 2 primers

Figure SI3

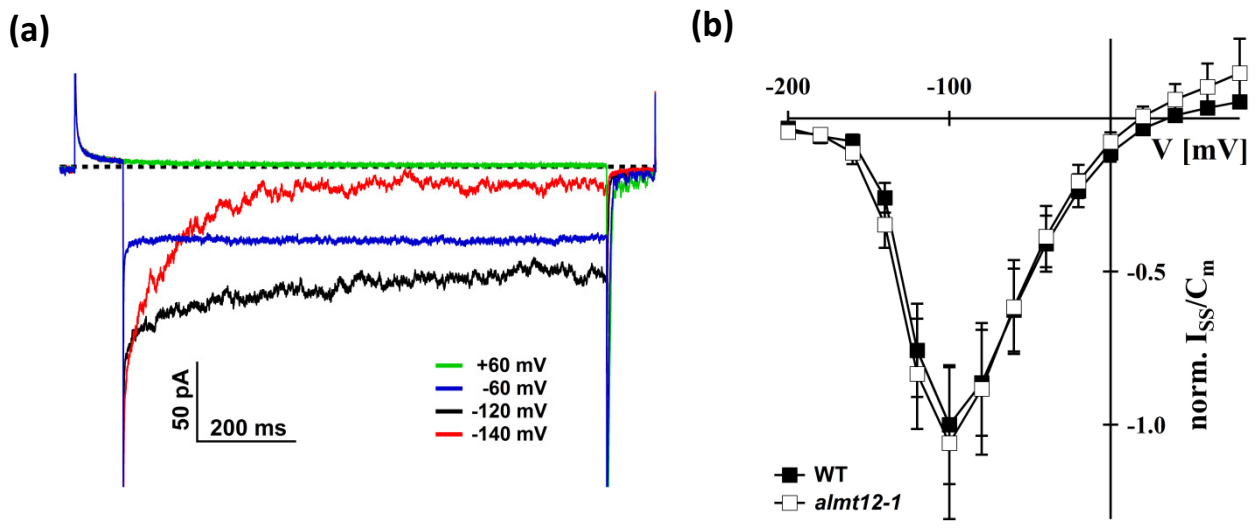

(a)
50 pA
200 ms
+60 mV
-60 mV
-120 mV
-140 mV
(b)
-200
-100
V [mV]
-0.5
-1.0
norm. I_{SS}/C_m
WT
almt12-1

Figure SI4

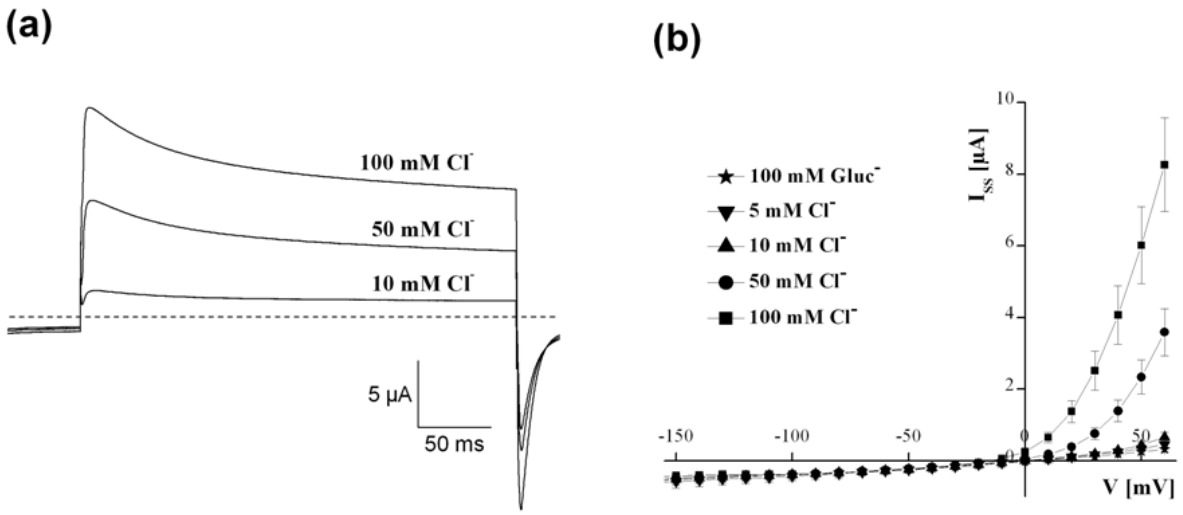
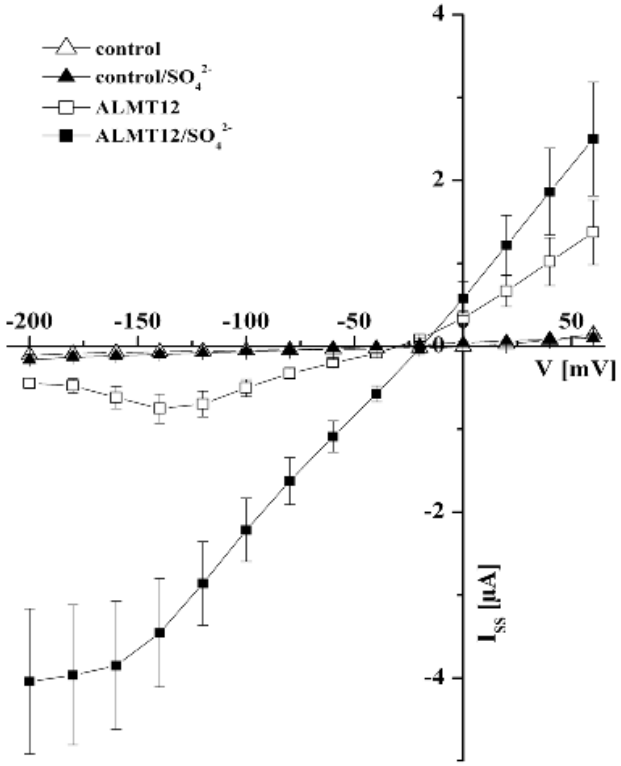

(a)
100 mM Cl-
50 mM Cl-
10 mM Cl-
5 µA
50 ms
(b)
100 mM Gluc-
5 mM Cl-
10 mM Cl-
50 mM Cl-
100 mM Cl-
I_SS [µA]
V [mV]
10
8
6
4
2
-150
-100
-50
0
50

Figure SI5


control
control/SO4 2-
ALMT12
ALMT12/SO4 2-
4
2
-2
-4
-200
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-50
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50
V [mV]
ISS [µA]