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Guard Cell-Specific Calcium Sensitivity of High Density and Activity SV/TPC1 Channels

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The slow vacuolar (SV) channel, a Ca^{2+}-regulated vacuolar cation conductance channel, in *Arabidopsis thaliana* is encoded by the single-copy gene *AtTPC1*. Although loss-of-function *tpc1* mutants were reported to exhibit a stoma phenotype, knowledge about the underlying guard cell-specific features of SV/TPC1 channels is still lacking. Here we demonstrate that *TPC1* transcripts and SV current density in guard cells were much more pronounced than in mesophyll cells. Furthermore, the SV channel in motor cells exhibited a higher cytosolic Ca^{2+} sensitivity than in mesophyll cells. These distinct features of the guard cell SV channel therefore probably account for the published stomatal phenotype of *tpc1-2*.

Keywords: Ca^{2+} and pH sensitivity • Guard cell • Mesophyll cell • SV/TPC1 channel.

Abbreviations: EDX, energy dispersive X-ray; SV, slow vacuolar.

Introduction

The slow vacuolar (SV) channel and the ATP-dependent proton pump represent the major conductances of the vacuolar membrane (Hedrich et al. 1986, Hedrich and Schroeder 1989; for a review, see Pottosin and Schönknecht 2007). The SV channel discovered in barley mesophyll vacuoles (Hedrich et al. 1986) was identified as a cation channel with preference for monovalent alkali metal ions in several cell types and species including Arabidopsis cell culture vacuoles (Ivashikina and Hedrich 2005, Ranf et al. 2008). Its unitary channel conductance ranges from 26 pS in *Hordeum vulgare* aleurone cells, to 40–90 pS in *Arabidopsis thaliana* and *Nicotiana tabacum* mesophyll cells, 54 pS in *A. thaliana* guard cells and up to 200–280 pS in *Allium cepa* and *Vicia faba* guard cells (Hedrich and Neher 1987, Hedrich et al. 1988, Amodeo et al. 1994, Bethke and Jones 1994, Schulz-Lessdorf and Hedrich 1995, Peiter et al. 2005, Beyhl et al. 2009). The SV channel is activated upon depolarizing voltages and elevated cytosolic Ca^{2+} (Hedrich and Neher 1987, Bethke and Jones 1994, Pottosin et al. 1997). In the absence of Ca^{2+} in the cytosol, the voltage threshold for SV channel activation is located far positive to the physiological membrane voltage range (Hedrich and Neher 1987, Pottosin et al. 1997, Pei et al. 1999). With increasing cytosolic Ca^{2+} concentrations, however, the voltage gate of the SV channel shifts towards the physiological range of potentials (Hedrich and Neher 1987, Schulz-Lessdorf and Hedrich 1995, Beyhl et al. 2009, Pottosin et al. 1997). In addition to cytosolic Ca^{2+}, the voltage sensor of the SV channel responds to changes in the K^+ gradient across the vacuolar membrane (Ivashikina and Hedrich 2005).

Among plant species and cell types the SV channel is ubiquitously expressed (for a review, see Pottosin and Schönknecht 2007). In *A. thaliana*, the SV channel is encoded by the single-copy gene *AtTPC1* (Peiter et al. 2005). TPC1 is structurally related to voltage-dependent cation channels with two *Shaker*-like monomers arranged in tandem (Furuchi et al. 2001). Consistent with this, the gain-of-function *fou2* mutant harbors a point mutation in TPC1 (D454N) at a channel site that is most probably facing the vacuolar lumen. This mutation reduced SV channel sensitivity towards luminal Ca^{2+} (Bonaventure et al. 2007, Beyhl et al. 2009). While loss of TPC1 function seems not to result in a mesophyll phenotype (Ranf et al. 2008), the *tpc1-2* mutant has been reported to result in the incapability of stomatal closure in response to high external Ca^{2+} loads (Peiter et al. 2005). Islam et al. (2010) very recently showed that this guard cell phenotype of *tpc1-2* mutants is not related to a function of TPC1 channels as Ca^{2+}-induced Ca^{2+} release (CICR) elements in guard cell vacuoles. Instead, lack of guard cell TPC1 properties seems to feed back on plasma membrane S-type

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channel activation in guard cells. This and other findings point to the physiological importance of cell type-specific transporter properties in general and guard cell function in particular. The properties of the SV/TPC1 channels in *A. thaliana* guard cells, however, have not yet been elucidated. Therefore, we determined the salt composition and content of *A. thaliana* guard cells and explored the SV/TPC1 channel features in comparison with mesophyll cells. Cell types differed in the level of *TPC1* expression, activation kinetics and sensitivity of SV/TPC1 channels to cytosolic Ca^{2+} and luminal pH. The guard cell-specific SV channel properties are discussed with respect to their physiological impact.

Results

Under salt stress, potassium and sodium dominate the guard cell cation pool

To gain insights into the salt composition and content of intact guard cells, *A. thaliana* leaves were excised and frozen in liquid nitrogen. Following freeze-drying the vacuolar K, Na and Cl content was determined by energy dispersive X-ray (EDX) analysis. In motor cells of leaves from *A. thaliana* plants grown in the absence of additional soil sodium supply, potassium dominated the elements analyzed (**Fig. 1**). Under these conditions the luminal sodium content reached <10% of the K equivalents. Cl was only weakly represented in guard cells. This halide and the dicarboxylate malate are known to counterbalance the potassium ion moiety in guard cells (Raschke and Hedrich 1987, Negi et al. 2008). Thus our EDX analysis suggests that under the given growth conditions malate rather than Cl^- compensates a large fraction of cellular K^+ ions.

In salt stress experiments with Arabidopsis, in general sodium loads of 150–300 mM have been used (Qiu et al. 2002). Therefore, we exposed plants for 3 d to 200 mM NaCl to test whether *A. thaliana* guard cell vacuoles are capable of taking advantage of Na (ions) to promote stomatal movement under salt stress. Under salt stress the reference elements Mg, P and S analyzed remained unaffected (**Supplementary Fig. S1**). The amount of Na in guard cells, however, increased by a factor of 7 at the expense of K, which dropped by about 50% (**Fig. 1**). In the presence of the metabolically 'cheap' Cl, this halide reached approximately the sum of Na and K content, indicating that in the presence of excess Cl the impact of metabolically expensive malate is reduced.

Unique responses of guard cell SV channels to cations

EDX analyses suggested that K^+ and Na^+ might represent potential physiological substrates for the guard cell SV channel. Previous studies have shown that the SV channel from *A. thaliana* mesophyll cell vacuoles under symmetrical potassium operates as an outward-rectifying cation channel (Beyhl et al. 2009). Upon voltage stimulation (+70 mV pulses) of wild-type SV channels, in mesophyll cell vacuoles outward currents gradually

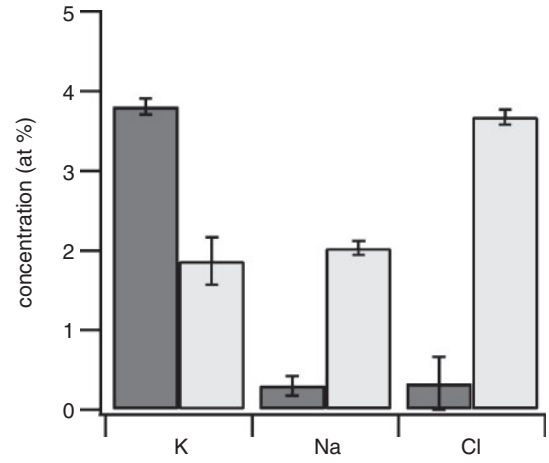

Fig. 1 Semi-quantitative EDX analysis of ion concentrations in guard cell vacuoles. Data were obtained from leaves of four plants each grown under control conditions (dark gray) and NaCl stress (gray). Columns show mean values with the standard error of six recorded spectra. The scale on the left gives the atomic% of recorded X-ray signals. Data were statistically analyzed with a *t*-test ($P < 0.05$). Note that differences in the relative K^+, Cl^- and Na^+ concentrations in the vacuole were statistically significant in plants treated with NaCl.

increased with time, reaching steady state after about 7 min in the whole-vacuole mode (**Supplementary Fig. S2**; cf. Beyhl et al. 2009). In contrast, in guard cell vacuoles outward currents already appeared as soon as the whole-vacuole configuration was established due to the faster equilibration of the guard cell vacuole sap with the pipet medium (**Supplementary Fig. S2**). These voltage-induced guard cell currents appear to be solely carried by SV channels, because they were absent with guard cell vacuoles of *tpc1-2* loss-of-function mutant plants (**Fig. 3A**).

Potassium and protons. Interestingly, in symmetrical K^+ media at pH 7.5, SV currents activated at +70 mV about five times faster, and current densities were nine times higher in guard cells than in mesophyll cells (**Figs. 2, 3A, B**). Vacuolar acidification with the guard cell system led to a 7-fold change in channel activation kinetics, while changes with mesophyll cell vacuoles were only 2-fold (**Fig. 2A, B**; **Supplementary text 1**), pointing to a higher sensitivity of guard cell SV channels towards luminal acidification. The distinct pH effect on the SV channel activation kinetics in both cell types (**Fig. 2A, C**) probably originates from different voltage-dependent gating behavior (**Supplementary text 1**).

Sodium. SV channels of cultured *A. thaliana* cells conduct Na^+ into the vacuole lumen but not its release into the cytosol (Ivashikina and Hedrich 2005). When Na^+ accumulates in the vacuole of these cultured cells, this alkali metal ion even blocks SV channel-mediated K^+ release from the vacuole lumen into the cytosol. In contrast to cultured *A. thaliana* cells (cf. **Fig. 3** of Ivashikina and Hedrich 2005), Na^+ seems to permeate through

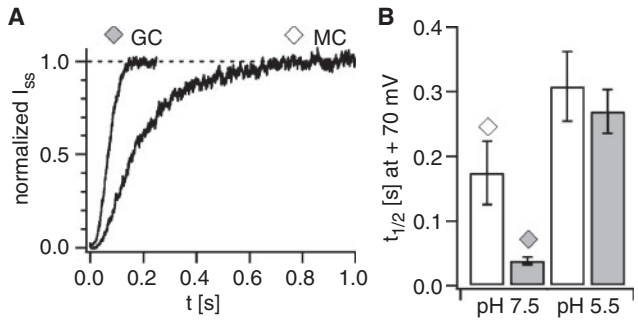

Fig. 2 Different activation kinetics of SV channels in guard cell and mesophyll cell vacuoles. (A) SV currents elicited upon a voltage pulse to +70 mV were normalized to the maximal current level as indicated by the dotted line. The holding voltage was −60 mV. The currents were recorded under symmetrical 150 mM K^+ and pH 7.5. (B) The half-activation time $t_{1/2}$ was determined at +70 mV in the presence of symmetrical 150 mM K^+ with pH 7.5 in the bath solution (cytosol) and either pH 7.5 or pH 5.5 in the pipet solution (vacuolar lumen) as indicated. The number of experiments was $n = 5–9$ and $n = 5–7$ for guard cell and mesophyll cell vacuoles, respectively. Filled bars and symbols, guard cell vacuoles, GC; open bars and symbols, mesophyll cell vacuoles, MC. Error bars give the standard deviation. In A and B the bath solution contained 1 mM free Ca^{2+} and no Mg^{2+} while the pipet solution was nominally Ca^{2+} free in the presence of 2 mM Mg^{2+}.

guard cell SV channels without affecting the voltage gate much (**Supplementary Figs. S3, S4; Supplementary text 2**). The elevated inward currents in the presence of a Na^+ gradient, however, suggest that other transport characteristics (e.g. single channel conductance) have been altered. Due the ability of TPC1 to conduct Na^+ across the tonoplast in both directions one might predict that under salt stress guard cells could drive stomatal movement, with sodium permeating through this guard cell transporter.

Specific interaction of guard cell SV channels with Ca^{2+} ions

Calcium uptake. When the potassium gradient was directed out of the vacuolar lumen (high $[K^+]_{lumen}$/low $[K^+]_{cytosol}$), mesophyll cell- and guard cell-expressed SV channels conducted inward and outward K^+ currents (**Fig. 3C**; cf. Ivashikina and Hedrich 2005, Beyhl et al. 2009). Under these asymmetrical K^+ conditions the guard cell SV channels activated at about 30 mV less depolarized voltages than the mesophyll cell SV channels and mediated pronounced inward currents. After replacement of cytosolic 30 mM K^+ by 15 mM Ca^{2+} with pH 7.5 at both membrane sides, voltage stimulation (+70 mV) of mesophyll cell vacuoles did not elicit outward Ca^{2+} currents (**Fig. 4**; cf. Beyhl et al. 2009). Under the same experimental conditions, however, outward Ca^{2+} currents of 60 pA/pF were recorded in guard cell vacuoles. Introduction of luminal acid loads even caused these Ca^{2+} currents in guard cells to increase 2.5-fold (**Fig. 4**). At luminal pH 5.5 outward Ca^{2+} currents were observed in mesophyll cells too, but characterized by 3-fold smaller amplitude (**Fig. 4**). This finding implies that in the presence of an acidic vacuolar sap and Ca^{2+} as the sole cytosolic cation, guard cell SV channels could catalyze Ca^{2+} fluxes into the vacuolar lumen of higher amplitude than mesophyll cell SV channels. Nevertheless this SV-mediated Ca^{2+} influx into guard cell vacuoles—gained non-physiological cytosolic Ca^{2+} concentrations—is unlikely to be of physiological importance in planta because it counters the thermodynamics of physiological Ca^{2+} gradients and tonoplast potentials (Felle 1988, Bethke and Jones 1994, Walker et al. 1996, Miller et al. 2001, Cuin et al. 2003, Perez et al. 2008). The calcium dependence was determined here only to develop settings suited to provide a cell-type specific fingerprint of the SV channel. Thus the direction and magnitude of the SV channel-mediated Ca^{2+} currents measured under the given conditions have no physiological relevance.

Given the fact that the luminal Ca^{2+} concentration feeds back on SV channel-mediated transport of mesophyll cell vacuoles (Beyhl et al. 2009), we examined at the single channel level whether the vacuolar Ca^{2+} concentration affects the guard cell SV channel in a similar way. We exposed excised patches with the vacuolar membrane side facing nominally 0 or 1 mM Ca^{2+} under symmetrical K^+ and H^+ concentrations and measured the single channel activity at −30 mV. In line with the SV channel behavior in mesophyll cells, the open probability P_o of single SV channels in guard cells vanished with an increase in the luminal Ca^{2+} concentration (**Supplementary Fig. S5**).

Calcium sensitivity. SV channels are characterized by pronounced sensitivity to cytosolic Ca^{2+} (Hedrich and Neher 1987; for a review, see Pottosin and Schönknecht 2007). To test whether the *TPC1* gene product in mesophyll and guard cells differs in its Ca^{2+} sensitivity, SV current densities were quantified in both vacuole types in the presence of different cytosolic Ca^{2+} concentrations. The derived dose–effect curves could be described with a Michaelis–Menten function (**Fig. 5**). Thereby, apparent K_m values of 130 and 1,170 µM Ca^{2+} were determined for the guard cell and mesophyll SV channels, respectively. The difference in apparent K_m^{Ca2+} indicates that the guard cell SV channel is more sensitive towards regulatory cytosolic Ca^{2+}.

Higher TPC1 transcript and SV channel numbers in guard cells than in mesophyll cells

On the basis of the vacuolar surface area represented by the membrane capacitance, the wild-type SV current density was about 9-fold higher in guard cells than in mesophyll cells (**Fig. 3B**). This difference could result from a higher number of SV channel proteins, an increased unitary conductance and/or gating behavior. When we analyzed the single channel properties of the *TPC1* gene product with symmetrical 150 mM K^+ (**Fig. 6A**), the unitary conductance of the SV channel was similar in both cell types (**Table 1**). Note that pH changes affected the unitary SV conductance in a cell type-independent manner (**Table 1**). However, single SV channel gating was different in guard cell and mesophyll cells (**Fig. 6A**) leading to slightly reduced open probability P_o of single SV channels in guard cells

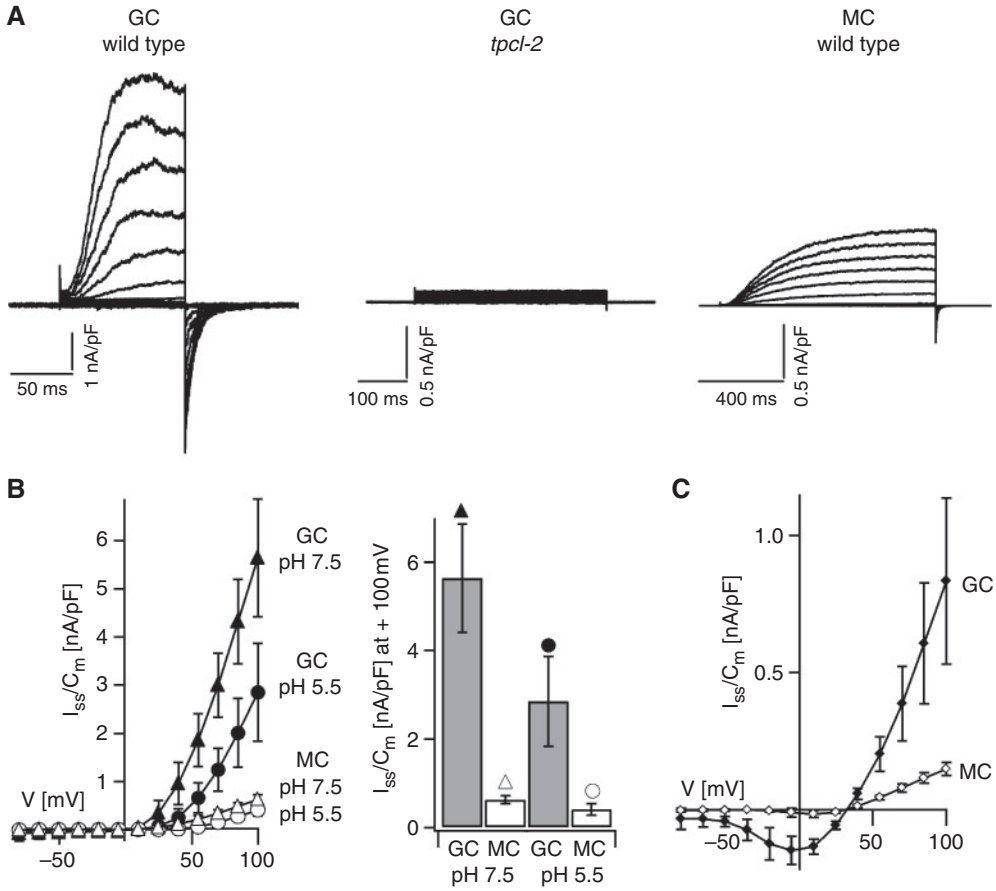

Fig. 3 Comparison of macroscopic SV channel currents from guard cell (GC) and mesophyll cell (MC) vacuoles. (A) Representative current traces recorded from GC and MC vacuoles of wild-type and *tpc1-2* mutant plants under symmetrical 150 mM K^+ and pH 7.5 are shown. For channel activation, voltage pulses were applied in the range from −80 to +100 mV in 15 mV steps from a holding voltage of −60 mV. (B, C) Steady-state SV current densities determined at different luminal pH for both cell types were plotted against the respective voltage. The right-sided bar diagram in B shows the current densities at +100 mV. Data points represent the mean ± SE. The number of experiments in B was $n_{GC\ pH\ 7.5} = 15$, $n_{GC\ pH\ 5.5} = 9$, $n_{MC\ pH\ 7.5} = 5$, $n_{MC\ pH\ 5.5} = 8$ and in C $n_{GC} = 5$ and $n_{MC} = 3$. In B current measurements were performed under symmetrical 150 mM K^+ with pH 7.5 in the bath solution and either pH 7.5 or pH 5.5 in the pipet solution. In C macroscopic currents were recorded with pH 7.5 on both sides of the vacuolar membrane and in the presence of a K^+ gradient (30 mM K^+ in the cytosol and 150 mM K^+ in the vacuolar lumen). In A–C the bath solution contained 1 mM free Ca^{2+} while the pipet solution was nominally Ca^{2+} free.

compared with those in mesophyll cells (cf. **Fig. 6A**, **Supplementary Fig. S5**; **Fig. 5** of Beyhl et al. 2009). Instead quantitative real-time PCR analysis showed that the number of *TPC1* transcripts was about 4-fold higher in guard cells than in mesophyll cells (**Fig. 6B**). Thus the difference in whole-vacuole SV current amplitude very probably results from a higher SV channel density in the vacuole of the motor cell type (**Figs. 3, 6**).

Do SV channel properties originate from differential RNA splicing?

Differential post-transcriptional (e.g. RNA splicing, RNA editing) and/or post-translational modifications could account for the cell-type specific TPC1 features identified. To differentiate between these possibilities, we searched for potential splicing products with *TPC1* transcripts isolated from guard cells and mesophyll cells (**Supplementary Fig. S6**). Seven different primer sets were designed for the generation of putative PCR fragments with an expected size of 444–591 bp distributed all over the whole non-spliced *TPC1* mRNA (**Supplementary Fig. S6A**). When these primers were employed with mRNA purified from guard cells and mesophyll cells in PCR assays, PCR products of the same base pair length and sequence were obtained from the *TPC1* transcripts of both cell types (**Supplementary Fig. S6B**). Thus the unique features of the guard cell SV channels are most probaby not related to post-transcriptional modifications.

Discussion

Initial SV channel studies were performed on mesophyll vacuoles from *H. vulgare* (Hedrich et al. 1986). Differences in SV channel conductance between 26 and 280 pS suggested

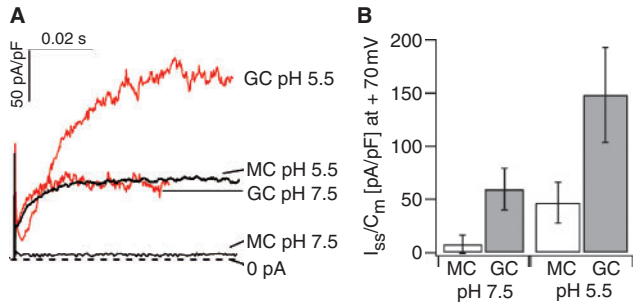

Fig. 4 SV channel-mediated Ca^{2+} transport into guard cell (GC) and mesophyll cell (MC) vacuoles. (A) Representative Ca^{2+} current traces were evoked upon a voltage pulse to +70 mV from a holding voltage of −60 mV in the whole-vacuolar configuration. (B) Averaged steady-state current densities were determined at +70 mV from current traces shown in A. The number of experiments was for luminal pH 7.5 $n_{MC} = 3$ and $n_{GC} = 5$ and for luminal pH 5.5 $n_{MC} = 7$ and $n_{GC} = 5$. Error bars the give standard deviation. (A, B) The pipet medium contained 150 mM K^+ and was nominally Ca^{2+} free. The bath medium contained 15 mM Ca^{2+} in the absence of K^+. The experiments were performed with pH 7.5 in the bath solution and either pH 7.5 or pH 5.5 in the pipet solution as indicated.

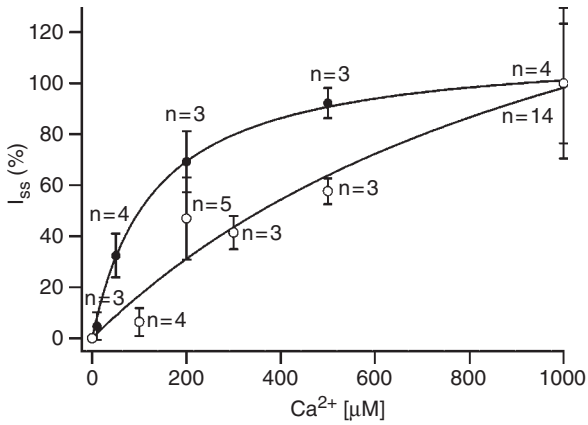

Fig. 5 Cytosolic Ca^{2+} dependence of the SV channels from mesopyll cell and guard cell vacuoles. Experiments were performed with 100 mM KCl and pH 7.5 on both sides of the vacuolar membrane in the presence of 0.1 mM luminal free Ca^{2+} and different free cytosolic Ca^{2+} concentrations. SV current densities measured at +100 mV were normalized to the value recorded with guard cell vacuoles in the presence of 1 mM Ca^{2+} and plotted against the respective free cytosolic Ca^{2+} concentration. Open and filled symbols represent data obtained from mesophyll and guard cell vacuoles, respectively, in the whole-vacuole configuration. Solid curves give the fit of the data points with a Michaelis–Menten function. Error bars represent the standard deviation.

variability in the encoding genes (Hedrich and Neher 1987, Hedrich et al. 1988, Amodeo et al. 1994, Bethke and Jones 1994, Schulz-Lessdorf and Hedrich 1995, Peiter et al. 2005, Beyhl et al. 2009). Peiter *et al.* (2005), however, recognized that the *A. thaliana* SV channel is encoded by the single copy gene *TPC1*. The diversity of ion channel properties and function

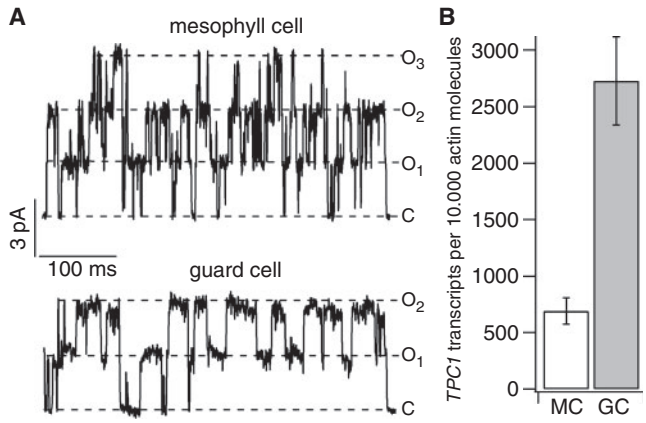

Fig. 6 Single SV channel fluctuations (A) and mRNA level of *TPC1* (B) in guard cells (GC) and mesophyll cells (MC). (A) Opening and closing of single SV channels were monitored at +30 mV in excised membrane patches with the cytoplasmic side of the vacuolar membrane facing the bath medium. C indicates the current baseline where all channels were closed. $O_{1, 2, 3}$ give the current levels at which one, two or three SV channels were simultaneously open. The experiments were performed under symmetrical K^+ (100 mM) and H^+ concentrations (pH 7.5). The free Ca^{2+} concentration of the bath and pipet medium was the same as in **Figs. 2** and **3**. (B) The *TPC1* transcript level in guard cells and mesophyll cells was analyzed with quantitative real-time PCR. The number of experiments was $n = 4$ (±SE) for both cell types.

Table 1 Single channel conductance γ of SV channels from vacuoles of guard cells and mesophyll cells

	γ_{GC} in pS	γ_{MC} in pS
$pH_{cytosol/vacuole}$ 7.5	76±10 (10)	70±11 (3)
$pH_{cytosol}$ 7.5/$pH_{vacuole}$ 5.5	116±5 (3)	110±8 (4)

The unitary conductance was determined from single channel fluctuations recorded at 3–6 different membrane voltages in 10 mV steps in the range of −10 to −60 mV under symmetrical or asymmetrical pH. Otherwise the same solutions were used as in **Fig. 2**. Current measurements were performed from excised membrane patches with the cytoplasmic side of the vacuolar membrane facing the bath medium. Data represent the mean±SD. The number of experiments is given in parentheses.

could also be broadened by the presence of more than one alternatively spliced exon along with RNA editing at multiple sites. Our detailed fragment analyses with *TPC1* mRNA, however, revealed that the size and sequence of *TPC1* transcripts in mesophyll and guard cells of *A. thaliana* were identical (**Supplementary Fig. S6**). These findings indicate that (i) interaction with regulatory proteins or other mediators differentially expressed in guard and mesophyll cells, and/or (ii) cell type-specific post-translational modifications [it should be noted that cell type-specific differences in surface charge densities might also be involved (Pottosin et al. 2005)] rather than *TPC1* RNA splicing and editing, most probably account for the different features of the most prominent vacuolar cation channel in the two *A. thaliana* cell types studied.

SV channels operate as outward rectifiers with physiological luminal Ca^{2+} concentration, apart from conditions where the K^+ gradient is directed out of the vacuole into the cytosol (Hedrich and Neher 1987, Hedrich et al. 1988, Bethke and Jones 1994, Schulz-Lessdorf and Hedrich 1995, Gambale et al. 1996, Pottosin et al. 1997, Ivashikina and Hedrich 2005, Beyhl et al. 2009). In the latter scenario in *A. thaliana* cell culture and mesophyll cell vacuoles with K^+ as the sole monovalent cation in the solutions, TPC1 channels conduct time- and voltage-dependent inward and outward K^+ currents (**Fig. 3C**) (Ivashikina and Hedrich 2005, Beyhl et al. 2009). Under the same conditions wild-type guard cell TPC1 channels were characterized by similar unitary conductance and voltage-dependent gating (**Fig. 3**, **Table 1**; **Supplementary Fig. S5**). Wild-type guard cell SV channels also respond to a rise in luminal Ca^{2+} with a strong decline in single channel activity pointing to a similar luminal Ca^{2+} sensitivity of TPC1 in mesophyll and guard cell vacuoles (**Supplementary Fig. S5**). However, in comparison with mesophyll cells, guard cell SV channels displayed faster activation kinetics, higher luminal pH and cytosolic Ca^{2+} sensitivity and lower absolute single-channel open probability (**Figs. 2, 3, Supplementary Fig. S5**; cf. Beyhl et al. 2009). Consequently, the 9-fold higher SV current density detected in guard cells than mesophyll cells may arise primarily from the 4-fold higher *TPC1* transcript number (**Figs. 3, 6B**).

Previous studies demonstrated that cytosolic Ca^{2+} acts as a gating modifier of SV channels. With increasing cytosolic Ca^{2+} levels SV channels are stimulated by shifting the activation threshold to more negative voltages (Hedrich and Neher 1987, Schulz-Lessdorf and Hedrich 1995, Pottosin et al. 1997, Pei et al. 1999, Beyhl et al. 2009). In the present work, the *A. thaliana* guard cell-expressed SV/TPC1 channel exhibited a 10-fold higher Ca^{2+} affinity than the mesophyll-expressed channel (apparent $K_{m\ GC} = 130\ \mu M$ vs. $K_{m\ MC} = 1{,}170\ \mu M$; **Fig. 5**). The K_m values were determined in the absence of cytosolic Mg^{2+} and would point to a very low cytosolic Ca^{2+} sensitivity of the *A. thaliana* TPC1 channels. Future studies of the Mg^{2+} and ATP dependency (Pei et al. 1999, Carpaneto et al. 2001, Bethke and Jones 1997) will have to prove whether and how protein phosphorylation/dephosphorylation events (Allen and Sanders 1995, Bethke and Jones 1997, Xu et al. 2006, Geiger et al. 2009a) affect the cytosolic Ca^{2+} sensitivity of TPC1.

In contrast to guard cells, no significant TPC1-mediated Ca^{2+} currents into the vacuole were monitored in mesophyll cells at luminal pH 7.5, probably because of the lower channel density and Ca^{2+} sensitivity in this cell type (**Fig. 4**) (Beyhl et al. 2009). The guard cell-specific properties of the SV/TPC1 channel identified very probably account for the external Ca^{2+} stomatal phenotype of the loss-of-function mutant *tpc1-2* (Peiter et al. 2005, Islam et al. 2010). However, cytosolic Ca^{2+} oscillations induced in guard cells of epidermal leaf fragments during external calcium up-shocks appear to be not affected by *tpc1-2* loss-of-function (Allen et al. 2001, Islam et al. 2010). Instead, anion channel activation seems to be reduced in *tpc1-2* plants. These findings suggest that TPC1 is involved in mediating rather than generating cytosolic Ca^{2+} signals during external Ca^{2+}-induced stomatal closure (Islam et al. 2010). Future studies will thus have to explore how the guard cell-specific SV channel features, for example via the cytosolic Ca^{2+} sensitivity, are linked to anion channel activation during external calcium loads.

Materials and Methods

Plant material and vacuole isolation

Growth conditions of *A. thaliana* ecotype Columbia (Col-0) and the *tpc1-2* mutant, as well as preparation of mesophyll cell vacuoles were essentially as described previously (Beyhl et al. 2009). Guard cell protoplasts were isolated and used for release of vacuoles essentially as described in Beyhl et al. (2009) and Geiger et al. (2009b).

Electrophysiology

Patch–clamp experiments on vacuoles were performed either in the whole-vacuole or in the excised patch configuration essentially as described previously (Ivashikina and Hedrich 2005, Beyhl et al. 2009), and according to the convention for electrical measurements on endomembranes (Bertl et al. 1992). Further details of data acquisition and analysis are given in the **Supplementary Materials and Methods**.

Patch–clamp solutions

Bath and pipet solutions were composed of 2 mM dithiothreitol (DTT), varied $KCl/CaCl_2$ concentrations and set to an osmolality of 400 mosmol kg^{-1} with D-sorbitol. Vacuolar side media usually also contained 2 mM $MgCl_2$. pH values were adjusted to pH 7.5 with 10 mM HEPES/Tris or to pH 5.5 with 10 mM MES/Tris. The designated free Ca^{2+} concentration of the media was adjusted upon certain Ca^{2+} and EGTA concentrations obtained from the calculation with webmaxc standard (http://www.stanford.edu/~cpatton/webmaxc/webmaxcS.htm). Details about the composition of the solutions are given in the figure legends.

Supplementary data

Supplementary data are available at PCP online.

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