Identification of a probable pore forming domain in the multimeric vacuolar anion channel AtALMT9

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Supplementary Figures

Supplemental Figure S1. AtALMT9<sub>WT</sub> and AtALMT9<sub>K193E</sub> are poorly permeable for citrate.

(A, C) Representative traces and normalized mean I-V curves of excised cytosolic-side out patches from <i>N. benthamiana</i> vacuoles overexpressing AtALMT9<sub>WT</sub> (A;
upper traces) and AtALMT9\textsubscript{K193E} (C; upper traces) displayed time dependent malate currents in symmetric ionic conditions (circles; 100 mM malate\textsubscript{vac}/ 100 mM malate\textsubscript{cyt}). When the cytosolic solution was replaced with 100 mM CA\textsubscript{cyt} (squares; 100 mM malate\textsubscript{vac}/ 100 mM CA\textsubscript{cyt}) the inward currents decreased and displayed only weak time dependent currents (A, C; lower traces). The current ratios \(\frac{I_{\text{CA}}}{I_{\text{Malate}}(\text{AtALMT9\textsubscript{WT}})} = 5 \pm 5\%\) and \(\frac{I_{\text{CA}}}{I_{\text{Malate}}(\text{AtALMT9\textsubscript{K193E}})} = 7 \pm 3\%\) show that CA is poorly permeable compared to malate. Currents were evoked in response to 3 s voltage pulses ranging from +60 mV to -120 mV in -20 mV steps followed by a tail pulse at +60 mV. The holding potential was set +60 mV. Error bars represent sd. Each data point corresponds to 4-11 patches. (B) Voltage dependency of the relative open probability of AtALMT9\textsubscript{WT} in control conditions (ctrl; 100 mM malate\textsubscript{vac}/ 100 mM malate\textsubscript{cyt}; circles) and in presence of 10 mM CA\textsubscript{cyt} (100 mM malate\textsubscript{vac}/ 100 mM malate\textsubscript{cyt}+10 mM CA\textsubscript{cyt}; diamonds). The relative open probability was estimated from the initial current amplitude of the tail currents (derived from a mono-exponential fit of the current decay) which followed an activating pulse of various potentials. We were unable to reach the full activation of AtALMT9 channels as it is likely to occur at voltages more negatives than -160 mV, a value at which the vacuolar membrane becomes unstable. Therefore the data (n=8-10) was normalized to the \(I_{\text{max}}\) value that was obtained by fitting each data set with the Boltzmann equation. The solid lines represent the best fits of the mean relative open probability in control and in presence of 10 mM CA\textsubscript{cyt} with a Boltzmann equation in the following form:

\[
P_{\text{O,rel}} = \frac{1}{1 + e^{\left(\frac{zF(V-V_h)}{RT}\right)}}
\]

in which \(P_{\text{O,rel}}\) is the relative open probability, \(z\) the gating charge, \(F\) the Faraday constant, \(R\) the universal gas constant, \(T\) the absolute temperature and \(V_h\) the voltage of half activation. The fit shows that the presence of 10 mM CA\textsubscript{cyt} does not change significantly the voltage dependent gating of the channel since \(V_h = -81 \pm 1\) mV and \(z = 0.6 \pm 2\) under control conditions and \(V_h = -76 \pm 3\) mV and \(z = 0.7 \pm 1\) with 10 mM CA\textsubscript{cyt}. (D) “Kick-out experiment” performed on the mutant AtALMT9\textsubscript{K193E}. Grey traces were obtained under 100 mM malate\textsubscript{vac}/ 100 mM malate\textsubscript{cyt} conditions and black traces in the presence of 10 mM CA\textsubscript{cyt} (100 mM malate\textsubscript{vac}/ 100 mM malate\textsubscript{cyt}+10 mM CA\textsubscript{cyt}). The currents evoked in response to a 2 s voltage pulse at -140 mV. Subsequently, the membrane potential was transiently stepped for 3 ms to +60 mV and then restored to -140 mV for 1 s which was followed by a tail pulse at +60 mV. The holding potential was set to +60 mV. Error bars display sd.
Supplemental Figure S2. Multiple alignment of the ALMT protein family of *Arabidopsis thaliana*.

The alignment was conducted with the Jalview software (Waterhouse *et al.*, 2009). Asterisks and red boxes indicate the residues that were targeted by site-directed mutagenesis in the present study.
Supplemental Figure S3. Intracellular localization of the different mutant channels of AtALMT9-GFP.

Fluorescence images of vacuoles extracted from N. benthamiana protoplasts expressing the different AtALMT9-GFP mutants. None of the introduced mutations altered the tonoplastic localization of AtALMT9. The pictures were obtained with an
Supplemental Figure S4. AtALMT9 point mutants display different channel conductivity and sensitivity to citrate inhibition

Representative traces of current recordings from vacuoles overexpressing AtALMT9\textsubscript{R200N} and AtALMT9\textsubscript{R200E} (A), AtALMT9\textsubscript{R215N} and AtALMT9\textsubscript{R215E} (C) in symmetric malate conditions (100 mM malate\textsubscript{vac} / 100 mM malate\textsubscript{cyt}). Currents were evoked in response to 3 s voltage pulses ranging from +60 mV to -120 mV in -20 mV steps followed by a tail pulse at +60 mV. The holding potential was +60 mV. “Kick-out experiments” were performed with the mutants AtALMT9\textsubscript{R200N} (B) and AtALMT9\textsubscript{R215N} (D). Grey traces were obtained in 100 mM malate\textsubscript{vac} / 100 mM malate\textsubscript{cyt} conditions and black traces in the presence of 10 mM CA\textsubscript{cyt} (100 mM
malatevac/ 100 mM malatecyt + 10 mM CAcyt). The currents evoked in response to a 2 s voltage pulse at -140 mV. Subsequently the membrane potential was transiently stepped for 3 ms to +60 mV and then restored to -140 mV for 1 s which was followed by a tail pulse at +60 mV. The holding potential was set to +60 mV. (E) Representative I-V curves obtained with a voltage ramp (from +60 mV to -160 mV in 1.5 s; holding potential +60 mV) measured in excised cytosolic-side out patches from vacuoles expressing AtALMT9K87E in control conditions (ctrl) and in presence of 10 mM CAcyt. (F) Dose-response of CAcyt concentration-dependent ratio of AtALMT9WT and AtALMT9K87E at -160 mV. To estimate the dissociation constant KdCA, the data points were fitted with a Langmuir isotherm (equation 1). The resulting KdCA values were 5.1 ± 0.3 mM and 16.2 ± 2.3 mM for AtALMT9WT and AtALMT9K87E, respectively. Error bars represent sd.

Supplemental Figure S5. The double mutant AtALMT9K93E/E130K is inhibited by intracellular citrate comparable to AtALMT9WT.

(A) Representative I-V curves obtained with a voltage ramp (from +60 mV to -160 mV in 1.5 s; holding potential +60 mV) measured in excised cytosolic-side out patches from vacuoles expressing the double mutant AtALMT9K93E/E130K in control conditions (ctrl) and in presence of 10 mM CAcyt. (B) Ratio between currents in presence of 10 mM CAcyt and in control conditions at different membrane potentials. Depicted are AtALMT9WT (circles; n = 4) and AtALMT9K93E/E130K (squares; n = 3). Error bars represent sd