A voltage-dependent Ca$^{2+}$ homeostat operates in the plant vacuolar membrane

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Summary

- Cytosolic calcium signals are evoked by a large variety of biotic and abiotic stimuli and play an important role in cellular and long distance signalling in plants. While the function of the plasma membrane in cytosolic Ca$^{2+}$ signalling has been intensively studied, the role of the vacuolar membrane remains elusive.
- A newly developed vacuolar voltage clamp technique was used in combination with live-cell imaging, to study the role of the vacuolar membrane in Ca$^{2+}$ and pH homeostasis of bulging root hair cells of Arabidopsis.
- Depolarisation of the vacuolar membrane caused a rapid increase in the Ca$^{2+}$ concentration and alkalinised the cytosol, while hyperpolarisation led to the opposite responses.
- The relationship between the vacuolar membrane potential, the cytosolic pH and Ca$^{2+}$ concentration suggests that a vacuolar H$^+$/Ca$^{2+}$ exchange mechanism plays a central role in cytosolic Ca$^{2+}$ homeostasis. Mathematical modelling further suggests that the voltage-dependent vacuolar Ca$^{2+}$ homeostat could contribute to calcium signalling when coupled to a recently discovered K$^+$ channel-dependent module for electrical excitability of the vacuolar membrane.

Introduction

Temporal elevations of the cytosolic free calcium concentration ([Ca$^{2+}$]$_{cyt}$) of plant cells are evoked by a variety of biotic and abiotic stimuli (McAinsh & Pittman, 2009; Roelfsema & Hedrich, 2010). These Ca$^{2+}$ spikes can serve as signals within single plant cells, or propagate to enable systemic signalling between distant plant tissues (Choi et al., 2014; Evans et al., 2016; Toyota et al., 2018). Such Ca$^{2+}$-based signals have been implicated in a broad range of functions, including pathogen resistance (Jeworutzki et al., 2010; Ranford et al., 2014; Yuan et al., 2015; Toyota et al., 2018; Huang et al., 2019), nutrient sensing (Xu et al., 2006; Tang et al., 2020), as well as adaptive responses to salt stress (Choi et al., 2014; Evans et al., 2016).

In general, Ca$^{2+}$ signals can be considered as a transient disturbance of the Ca$^{2+}$ homeostasis in plant cells, which controls [Ca$^{2+}$]$_{cyt}$ at a low resting level of c. 100–200 nM (Wheeler & Brownlee, 2008; Roelfsema & Hedrich, 2010; Bose et al., 2011; Tang & Luan, 2017; Kudla et al., 2018). This constant low level is stabilised by a multitude of proteins that bind Ca$^{2+}$ and thereby provide a buffer capacity of 0.1–0.5 mM in plant cells (Trewavas, 1999). In the longer term, low [Ca$^{2+}$]$_{cyt}$ is maintained by the energy-dependent transport of Ca$^{2+}$ ions against their electrochemical gradient across the plasma membrane (PM) and across the intracellular membranes of organelles. So far, four groups of transport proteins have been identified in Arabidopsis thaliana that contribute to this process: the autoinhibited Ca$^{2+}$-ATPases (ACAs), ER-type Ca$^{2+}$-ATPases (ECAs), P1-ATPases (as for HMA1) and H$^+$/Ca$^{2+}$ exchangers (CAX) (Kudla et al., 2018). These Ca$^{2+}$ efflux transporters are therefore likely to affect the shape of intracellular Ca$^{2+}$ signals but, so far, this has only been shown for the PM-localised efflux transporters ACA8 and ACA10 (Costa et al., 2017; Yang et al., 2017).

Ca$^{2+}$-permeable ion channels counteract the active transporters mentioned above and enable a rapid influx of Ca$^{2+}$ ions along the steep electrochemical gradient for Ca$^{2+}$ across the PM and endomembranes. In recent years, our understanding of the contribution of PM-localised Ca$^{2+}$ channels to the generation of cytosolic Ca$^{2+}$ signals has expanded considerably. Members of the cyclic nucleotide gated channel (CNGCs), hyperosmolarity-induced [Ca$^{2+}$]$_{cyt}$ channels (OSCAs) and glutamate receptor-like channels (GLRs) families were shown to be involved in several physiological processes such as auxin signalling (Shih et al., 2015; Dindas et al., 2018), plant immunity (Yuan et al., 2014; Tian et al., 2019; Thor et al., 2020), reproduction (Frietsch et al., 2007; Michard et al., 2011; Tunc-Ozdemir et al., 2013; Wudick...
et al., 2018; Pan et al., 2019) and long distance systemic signalling (Nguyen et al., 2018; Toyota et al., 2018). In addition, several classes of mechanically activated Ca$^{2+}$-permeable PM channels in plants are likely to be encoded by the mid1-complementing activity (MCAs), piezos and MSL1 channels (Kurusu et al., 2013; Hou et al., 2014; Hamilton et al., 2015; Li et al., 2020; Mousavi et al., 2020; Radin et al., 2020).

The central vacuole plays an important role in the cytosolic Ca$^{2+}$ homeostasis of plant cells (Martinoia et al., 2012). Calcium is stored in vacuoles at low millimolar concentrations, while its concentration in the cytosol is below one micromolar (Miller & Sanders, 1987; Felle, 1988; Bethmann et al., 1995). Because of this steep concentration gradient, opening of Ca$^{2+}$ channels in the vacuolar membrane (VM) will cause a rapid increase in the cytosolic Ca$^{2+}$ level. However, little information is known about Ca$^{2+}$ channels in the VM. The two-pore channel 1 (TPC1) was initially proposed to act as a Ca$^{2+}$-release channel (Pfeifer et al., 2005), but it is controversially discussed if this channel is able to conduct Ca$^{2+}$ currents under physiological conditions (Hedrich et al., 2018). A recent publication suggested that the voltage-dependent TPC1 channel provides excitability to the VM (Jaslan et al., 2019). By a mechanism in which TPC1 acts in concert with the Ca$^{2+}$-activated K$^+$ channels TPK1 and TPK3 (Isayenkov et al., 2010; Jaslan et al., 2019). However, it is still unknown how the apparent excitability of the VM is related to long distance Ca$^{2+}$ signalling in plant cells.

To elucidate the role of the VM in Ca$^{2+}$ homeostasis, we used a recently developed approach to clamp the VM potential in root hair cells, while simultaneously recording changes in the cytosolic Ca$^{2+}$ level and pH (Wang et al., 2015). Our data suggest that the VM acts as a voltage-dependent Ca$^{2+}$ homeostat, as a depolarisation of the VM causes an increase in the cytosolic Ca$^{2+}$ concentration, while hyperpolarisation provokes the opposite response. It is likely that the voltage pulses affect H$^+$-coupled transport, as depolarising pulses alkalinised the cytosol, while it became more acidic during hyperpolarisation of the VM. Based on these data and the linear relationship between Ca$^{2+}$ flux and voltage, we concluded that a vacuolar H$^+$/Ca$^{2+}$ exchange mechanism plays a central role in cytosolic Ca$^{2+}$ homeostasis. In a mathematical modelling approach the vacuolar Ca$^{2+}$ homeostat is combined with a recently uncovered module for electrical excitability of the VM (Jaslan et al., 2019), generating a new hypothesis for the link between electrical and Ca$^{2+}$ signals.

**Materials and Methods**

Plant material and growth conditions

*Arabidopsis thaliana* seeds were sterilised in 6% NaOCl with 0.05% Triton X-100 and washed with sterile deionised water. Single seeds were subsequently placed on the surface of 1 ml of growth medium (0.12% Murashige & Skoog basal salt mixture including MES, Duchefa; 0.5% sucrose; 1% agarose, pH 5.8 with Tris) filled in small Petri dishes (Ø 35 mm). Sealed Petri dishes were placed in a vertically position in a growth chamber (KBWF 720; Binder, Tuttingen, Germany) 3–5 d before the experiments. Growth chambers operated on a 12 h : 12 h, day : night cycle with temperatures cycling between day : night, 21°C : 16°C, and a light intensity of 120 µmol photons m$^{-2}$ s$^{-1}$. The R-GECO1 line (Keinath et al., 2015) was provided by Rainer Waadt (University of Tartu), the GFP line, transformed with GFP5 (Siemering et al., 1996) and with the mutation S65T (Heim et al., 1995), was kindly provided by Melanie Krebs (University of Heidelberg) and the cpYFP line (Behera et al., 2018) was a gift from Markus Schwarzländer (University of Münster).

**Electrophysiology**

Microelectrodes were prepared from borosilicate glass capillaries (Ø out 1 mm, Ø in 0.58 mm, w/filament, Hilgenberg, Germany). Single-barrelled microelectrodes were pulled with a P-2000 horizontal laser puller (Sutter Instruments, Novato, CA, USA). Double-barrelled microelectrodes were prepared by fusing two glass capillaries through successive heating, turning them by 360° and prepulling them using a customised L/M-3P-A vertical puller (List-Medical-Electronic, Darmstadt, Germany), before the final pull was executed on the horizontal laser puller.

Measurements were conducted in bath solution (5 mM CaCl$_2$, 4 mM KCl, 0.25 mM MgCl$_2$, 0.5 mM NaCl, 1 mM HEPES/KOH pH 7) to which seedlings were accustomed overnight. To this purpose, 2 ml of sterile bath solution was applied to each Petri dish and the Petri dishes were subsequently sealed and placed in a vertical position in the growth chamber. Before measurements, seedling-containing Petri dishes were placed horizontally on an upright microscope and the bath solution was replaced by fresh solution with the same composition (Axioskop 2FS; Zeiss AG, Jena, Germany). Microelectrodes were mounted in a holder of a micromanipulator (MM3A-LMP, Kleindiek Nanotechnik, Reutlingen, Germany) that was used to impale bulging root hair cells. The barrels of the microelectrodes were backfilled with 300 mM KCl and connected with Ag/AgCl half-cells to head stages with input resistance of 100 GΩ, that were linked to a custom-made microelectrode amplifier (Ulliclamp 01). A reference electrode, filled with 300 mM KCl and sealed with an agarose plug (2% w/ 300 mM KCl), was placed in the solution of the Petri dish. The microelectrode amplifier was equipped with a differential amplifier that enabled double-barrelled electrode, voltage clamp experiments. The voltage and current data were filtered with a four-pole low-pass Bessel filter (LPF 202A; Warner Instruments, Holliston, MA, USA) at 200 Hz and sampled at 1 kHz using the PULSE software (v.8.74, HEKA; Lambrecht/Pfalz, Germany) with an LIH-1600 interface (HEKA), or a UBS-6002 interface (NI, Austin, TX, USA) and WinEHDR software (Dempster, 1997). The electrophysiological data were analysed offline using MS EXCEL® (Microsoft, Redmond, WA, USA) and ORIGIN PRO 9 software (OriginLab, Northampton, MA, USA). The microelectrodes were impaled in vacuoles and the electrical potential difference $E_T$ between bath and vacuolar lumen was measured. Thereafter, the cells were clamped to this value, so that the current at the holding potential was close to 0 nA. Cells in which the current changed more than ±0.1 nA during the measurement were discarded, as it is likely that the PM potential of these cells had changed during the experiment.
Imaging

R-GECO1 was excited with light from a mercury lamp (Leistungselektronik, Jena, Germany) filtered at 562 nm with a Brightline single-bandpass filter (562/40 nm, Semrock, Rochester, NY, USA) and reflected with a 590 nm dichroic mirror (Zeiss). Light emitted by R-GECO1 was filtered at 628 nm with a Brightline single-bandpass filter (628/40 nm; Semrock). The pH-sensitive cpYFP was excited with an LED illumination system (pE-4000; CoolLED, Andover, UK) at 405 nm and 470 nm (Behera et al., 2018). The emission signal was passed through a dichroic mirror with a cut-off wavelength of 499 nm (Zeiss) and a band filter at 520/35 nm. The cytosolic pH was monitored by calculating the ratio of fluorescence signals obtained with excitation at 470 nm (pH sensitive) and at 405 nm (low pH sensitivity). A decrease in this ratio value indicates a drop in the cytosolic pH (Behera et al., 2018). GFP5 (S65T) was excited with light filtered at 472 nm with a Brightline single-bandpass filter (472/30 nm, Semrock, USA) and reflected with a 490 nm dichroic mirror (Zeiss). Light emitted by GFP was filtered at 520 nm with a Brightline single-bandpass filter (520/30 nm, Semrock). The excitation light was focused on the sample through an Achromplan ×40/0.80 W objective (Zeiss). Filters and dichroic mirrors described above were placed inside a CARV2 confocal imager (Crest Optics, Rome, Italy) with the spinning disc out of the light path. Filter selection and image acquisition with a charge multiplying CCD camera (QuantEM 512SC; Photometrics, Tucson, AZ, USA) were controlled with Visiview software (Visitron, Puchheim, Germany). For analysis of imaging data, the freeware tool IMAGEJ (image.nih.gov/ij/) was used (Schindelin et al., 2012).

Estimation of the pH-dependent changes in R-GECO1 fluorescence intensity during VM voltages pulses

The fluorescent reporter protein R-GECO1 is sensitive to changes in the cytosolic Ca\(^{2+}\) concentration, as well as the cytosolic pH (Zhao et al., 2011). As a result, part of the change in R-GECO1 fluorescence intensity, observed during manipulation of the VM potential (Figs 1, 2, 4), could be due to changes in the cytosolic pH. The pH-dependent change of R-GECO1, during application of VM voltage pulses, therefore, was estimated by comparison of the R-GECO1 signals with that of cpYFP, which is highly sensitive to the cytosolic pH (Schwarzländer et al., 2011).

Roots of 6-d-old *A. thaliana* seedlings, which expressed either cpYFP (Supporting Information Fig. S1a), or R-GECO1 (Fig. S1b), were exposed to bath solutions with 50 mM CH\(_3\)COONH\(_4\), in which the pH was buffered to pH 6.0 and 6.5 with 2-(N-morpholino)ethanesulfonic acid (MES) and Bis-Tris propane (BTP) and to pH 7.0, 7.5 and 8.0 with BTP and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Behera et al., 2018). The buffer solutions were exchanged at a speed of 2.5 ml min\(^{-1}\), while the volume of the bath was c. 1.5 ml.

An LED illumination system (pE-4000; CoolLED, Andover, UK) was used to excite cpYFP at wavelengths of 405 nm and 470 nm and R-GECO1 at 580 nm. The filter wheels in a spinning disc unit (CARV2; Crest Optics, Italy) served to pass the emission signal of cpYFP through a dichroic mirror with cut-off wavelength of 499 nm (Zeiss) and a band pass filter of 520/35 nm, while the R-GECO1 signal was passed through a 590 nm dichroic mirror (Zeiss) and a single-bandpass filter (628/40 nm; Semrock).

The relationship between the extracellular pH and the cpYFP signal, as well as that of the extracellular pH and R-GECO1, could be described with single exponential functions (Fig. S1c, d). Both relationships between extracellular pH and fluorescent reporters were used to obtain an association between cpYFP and the pH-dependent changes in the R-GECO1 signal intensity (Fig. S1e). Based on this association and the changes in cpYFP ratio during voltage pulses (470\(_{\text{ex}}\)/405\(_{\text{ex}}\); Eq. 3b,c), the expected pH-dependent changes in the R-GECO1 signal were calculated and plotted in Fig. 4b,c (grey curves).

Simulation of thermodynamically ideal transporters

Calculations of the ideal thermodynamic behaviour of transporters were made using the following equations:

\[
\Delta G = RT \cdot \log \left( \frac{[\text{Ca}^{2+}]_{\text{lum}}}{[\text{Ca}^{2+}]_{\text{cyt}}} \right) - z_{\text{Ca}^{2+}} \cdot F \cdot E_{\text{VM}} \]  
Eqn 1

\[
\Delta G = n_{\text{Ca}^{2+}} \cdot \left[ RT \cdot \log \left( \frac{[\text{Ca}^{2+}]_{\text{lum}}}{[\text{Ca}^{2+}]_{\text{cyt}}} \right) - z_{\text{Ca}^{2+}} \cdot F \cdot E_{\text{VM}} \right] - m_{\text{H}^{+}} \cdot \left[ RT \cdot \log \left( \frac{[\text{H}^{+}]_{\text{lum}}}{[\text{H}^{+}]_{\text{cyt}}} \right) - z_{\text{H}^{+}} \cdot F \cdot E_{\text{VM}} \right] \]  
Eqn 2

\[
\Delta G = n_{\text{Ca}^{2+}} \cdot \left[ RT \cdot \log \left( \frac{[\text{Ca}^{2+}]_{\text{lum}}}{[\text{Ca}^{2+}]_{\text{cyt}}} \right) - z_{\text{Ca}^{2+}} \cdot F \cdot E_{\text{VM}} \right] - m_{\text{H}^{+}} \cdot \left[ RT \cdot \log \left( \frac{[\text{H}^{+}]_{\text{lum}}}{[\text{H}^{+}]_{\text{cyt}}} \right) - z_{\text{H}^{+}} \cdot F \cdot E_{\text{VM}} \right] + \left[ \Delta G_{\text{ATP}} + RT \cdot \log \left( \frac{[\text{ADP}]_{\text{cyt}}}{[\text{ATP}]_{\text{cyt}}} \right) \cdot \frac{[\text{Pi}]_{\text{cyt}}}{\mu M} \right] \]  
Eqn 3

In all equations, \( R \) is the universal gas constant (8.3 J mol\(^{-1}\) K\(^{-1}\)). \( T \) is absolute temperature (293 K). \( z \) is the valence of the ion (\( z_{\text{Ca}^{2+}} = 2 \) and \( z_{\text{H}^{+}} = 1 \)). \( F \) the Faraday constant (9.6 \times 10^{4} \text{C mol}\(^{-1}\)). \( E_{\text{VM}} \) is the VM voltage. For simulation of a \( \text{H}^{+}/\text{Ca}^{2+} \) exchanger and a \( \text{Ca}^{2+}-\text{ATPase} \), \( n_{\text{Ca}^{2+}} \) and \( m_{\text{H}^{+}} \) indicate transport stoichiometry. For the \( \text{Ca}^{2+}-\text{ATPase} \) \( \Delta G_{\text{ATP}} \) is the
standard enthalpy for ATP hydrolysis ($-3.3 \times 10^4$ J mol$^{-1}$), the ADP/ATP ratio was set to 1 and $[P_i]_{\text{cyt}} = 70$ µM (Pratt et al., 2009).

Computational cell biology

The electrical behaviour of the TPC1/TPK1/3 module was simulated as described in detail in the supplementary material of Jaslan et al. (2019), resulting in the time course of $E_{\text{VM}}(t)$ in response to an external stimulus. The net Ca$^{2+}$ flux was experimentally found to be linearly correlated to changes in $E_{\text{VM}}$ ($\Delta V_G$; Fig. 4d). At the resting voltage, which is negative of the activation threshold of TPC1, there is no net Ca$^{2+}$ flux across the tonoplast. Upon hyperpolarisation, the VM mediates a Ca$^{2+}$ flux from the cytosol to the vacuole, while it enables a Ca$^{2+}$ influx into the cytosol at depolarising potentials. In our model, we challenged the vacuole with a range of current pulses and determined the changes in $E_{\text{VM}}$ and $[\text{Ca}^{2+}]_{\text{cyt}}$ over time. Relative changes in the cytosolic Ca$^{2+}$ concentration ($\Delta [\text{Ca}^{2+}]_{\text{cyt}}$) were estimated by integrating the net Ca$^{2+}$ flux during tonoplast excitation:

$$\Delta [\text{Ca}^{2+}]_{\text{cyt}} = \alpha \cdot \int (E_{\text{VM}}(t) - E_R) dt$$

Eqn 4

with $E_R$ being the resting voltage. The unknown proportional factor $\alpha$ was eliminated by normalisation.

Results and Discussion

Voltage clamp experiments with guard cells revealed that hyperpolarisation of the PM evokes an increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ (Grabov &
Depolarisation of the vacuolar membrane (VM) increases the cytosolic Ca\(^{2+}\) concentration. (a) An *Arabidopsis thaliana* root hair cell, of which the VM was stimulated with a voltage ramp. The upper panel shows the R-GECO1 signal of the whole cell, while the lower panels display their magnified images, as indicated by the white dashed box. A magnification of the transmitted light image is shown in the lower right panel. The VM of the cell was stimulated with a depolarising step of 100 mV after 1 min, followed by a gradual return to 0 mV in the following 1.5 min (time indicated in the top of the panels and the voltage at the right side of the panels). The calibration bar (upper right) links the colour code to the fluorescence signal of R-GECO1. (b) Voltage protocol (top panel) used to stimulate the VM of root hair cells, averaged VM currents (middle panels) and averaged relative R-GECO1 fluorescence intensity (bottom panel) at the region of interest (ROI, as shown in upper panel of (a)). Error bars show SE (n = 7).

Voltage stimulation of the VM alters Ca\(^{2+}\)_cyt

Double-barrelled microelectrodes were impaled into the body of bulging root hair cells (Fig. 1a), of four 6-d-old Arabidopsis seedlings. At this subcellular position, the tip of the electrodes normally penetrates both the plasma and VMs and enables manipulation of the VM potential (Wang *et al.*, 2015). Microelectrodes with vacuolar localisation measured on average an electrical potential difference of 112 mV between bath and vacuolar lumen (standard error (SE) = 2 mV, n = 15). According to the convention of membrane voltage measurements on endomembranes (Berl *et al.*, 1992) the PM and VM potentials should be considered relative to the cytosol and, therefore, \(E_{PM} = \psi_{cyt} - \psi_{bath}\), while \(E_{VM} = \psi_{cyt} - \psi_{vac}\). The measured voltage \(E_T = \psi_{bath} - \psi_{vac}\) consequently represents \(E_{VM}\) subtracted by \(E_{PM}\); \(E_T = \psi_{bath} - \psi_{vac} = (\psi_{cyt} - \psi_{bath}) - (\psi_{cyt} - \psi_{vac}) = E_{VM} - E_{PM}\).

Wang *et al.* (2015) found that the VM potential is on average ~30 mV, which suggests that the bulging root hair cells had an average PM potential of ~142 mV. After measuring \(E_T\), the cells were clamped to this value and, therefore, the current at the holding potential was close to 0 nA.

Voltage pulses of 30 s were applied with a \(\Delta V\) of 100 mV, which provoked a depolarisation of the VM from ~30 mV to +70 mV (Fig. 1b, upper panel), while a \(\Delta V\) of ~80 mV was applied to hyperpolarise the VM to ~110 mV (Fig. 1c, upper panel). Just as described by Wang *et al.* (2015), such voltage pulses provoked vacuolar currents that slightly decreased during the voltage pulses (Fig. 1b,c, middle panels). On average we determined a VM conductance of 6.5 nS (SE = 0.7 nS, n = 9) and 6.1 nS (SE = 1.1 nS, n = 6), based on the steady-state currents evoked by depolarising and hyperpolarising pulses, respectively. Under the voltage clamp scenario described, the fluorescence signal of the Ca\(^{2+}\) reporter R-GECO1 (Zhao *et al.*, 2011; Keinath *et al.*, 2015) was used to monitor changes in \([Ca^{2+}]_{cyt}\) (Fig. 1d).

As a control, a version of GFP5 was used that has a relative low sensitivity to cytosolic pH changes. In a recent study with Arabidopsis and tobacco guard cells, it was shown that current injection can lead to osmotically driven cytosolic volume changes (Voss *et al.*, 2016). Due to such volume changes the fluorescence intensity of single-wavelength cytosolic reporters may change and potentially produce false signals. However, we found that pulses of \(\Delta V\) of ~100 mV or ~80 mV did not affect the GFP5 signal (Fig. 1b,c, lower traces and Fig. 1d, upper panels). Apparently,
the voltage pulses did not provoke changes in the cytosolic volume of root hairs. By contrast, the depolarising pulses caused a rapid increase in the R-GECO1 signal, while hyperpolarising pulses evoked the opposite response (Fig. 1b,c, lower panels and Fig. 1d, lower panels). This suggests that a depolarisation of the VM causes an influx of Ca\(^{2+}\) into the cytosol, whereas hyperpolarisation results in an increased Ca\(^{2+}\) loading of the vacuole.

Voltage pulses applied to the VM in intact root hair cells, also provoked small potential changes in the PM (Wang et al., 2015), which could have induced activation of hyperpolarisation activated Ca\(^{2+}\)-permeable channels. However, experiments in which only the PM potential was manipulated showed that these small PM potential changes (on average 8 mV) do not affect [Ca\(^{2+}\)]\(_{\text{cyt}}\) (Fig. S2).

The relationship between the VM potential and [Ca\(^{2+}\)]\(_{\text{cyt}}\) was studied in further detail, by applying a voltage protocol in which \(\Delta V_T (\psi_{\text{bath}} - \Delta \psi_{\text{vac}})\) was first stepped to +100 mV and thereafter changed to 0 mV in a voltage ramp with a rate of 1.67 mV s\(^{-1}\) (Fig. 2a,b). The R-GECO1 signal showed that [Ca\(^{2+}\)]\(_{\text{cyt}}\) increased after the depolarising voltage step and returned to the original value during the subsequent voltage ramp.
Are VM-dependent [Ca\(^{2+}\)]\(_{\text{cyt}}\) changes caused by H\(^+\)/Ca\(^{2+}\) exchangers?

Changes in the [Ca\(^{2+}\)]\(_{\text{cyt}}\) of plant cells are often accompanied by pH changes in the same compartment (Behera et al., 2018; Waadt et al., 2020). We therefore used seedlings that expressed cpYFP to monitor the cytosolic pH during manipulation of the VM (Fig. 3). It turned out that depolarisation of the VM in steps of 30 s caused an alkalisation of the cytosol (Fig. 3a,b), whereas hyperpolarising pulses provoked acidification (Fig. 3a,c). The potential of the VM membrane, therefore, appears to affect both the cytosolic pH (Fig. 3d) and Ca\(^{2+}\) concentration.

We set out to elucidate the nature of the transporter that provokes the changes in [Ca\(^{2+}\)]\(_{\text{cyt}}\) upon manipulation of the VM potential. To this purpose, the relationship between [Ca\(^{2+}\)]\(_{\text{cyt}}\) and the VM was determined with voltage pulses ranging from +100 mV to +20 mV (Fig. 4a,b), as well as pulses ranging from −80 mV to −20 mV (Fig. 4c). In this respect, we had to overcome the technical difficulty that the fluorescence intensity of R-GECO1 is sensitive to changes in [Ca\(^{2+}\)]\(_{\text{cyt}}\) as well as cytosolic pH (Zhao et al. 2011). We therefore developed a procedure, to determine the impact of cytosolic pH changes on R-GECO1, using the cpYFP signal as a reference. This approach revealed that the voltage-induced cytosolic pH changes only had a small impact on R-GECO1 (Fig. 4d) and therefore that most of the changes in the R-GECO1 signal during the voltage pulses are due to a change in [Ca\(^{2+}\)]\(_{\text{cyt}}\) (Fig. 4b–d).
The relationship between changes in the VM potential ($\Delta V_T = \psi_{\text{bath}} - \psi_{\text{vac}}$) and the rate by which the Ca$^{2+}$-dependent R-GECO1 signal changed is shown in Fig. 4(b,c). Both the data obtained at depolarising and hyperpolarising pulses were fitted with a linear function, which revealed an $r^2$ of 0.99 and 0.75, respectively. If we assume that the change in the R-GECO1
Fig. 6 Impact of vacuolar membrane (VM) excitation by the TPC1/TPK module on Ca$^{2+}_{\text{cyt}}$ control by the Ca$^{2+}$ homeostat. (a) Schematic representation of the transporters of the vacuolar excitation module (H$^+$-ATPase-dominated background current, the TPC1 and TPK channels) together with the Ca$^{2+}$ homeostat. An external stimulus can excite the tonoplast, which then affects the Ca$^{2+}$ homeostat and alters the cytosolic Ca$^{2+}$ concentration. (b) Current stimuli of varying amplitude excite the VM in a stimulus-dependent manner and provoke a stimulus-dependent release of Ca$^{2+}$ to the cytosol. (c) Current stimuli of varying duration excite the VM in a stimulus-dependent manner and provoke a stimulus-dependent release of Ca$^{2+}$ to the cytosol. (d) If stimulus amplitude and duration are varied in a way that the total charge of the stimulus (green area) is constant, the VM becomes excited to a similar degree and the magnitude of Ca$^{2+}$ current from the vacuole to the cytosol is the same. (e) Relationship between stimulus charge and raise in [Ca$^{2+}$]$_{\text{cyt}}$; 818 different conditions have been simulated. Each black dot represents a tested condition. The coloured dots indicate the final Δ[Ca$^{2+}$]$_{\text{cyt}}$ for the cases presented in b (yellow), c (green) and d (pink).
signal is a measure of the Ca\(^{2+}\) current across the VM, this Ca\(^{2+}\) current, therefore, has a linear dependency on the VM potential (Fig. 4d). The Ca\(^{2+}\) conductance of the VM was compared with that of several groups of Ca\(^{2+}\) transporters (Fig. 5). Among the possibilities tested, we found that an mH\(^{+}/n\)Ca\(^{2+}\) exchanger with a coupling rate m/n > 2 provided the best fit of our experimental observations (compare Fig. 5a,b with Fig. 4d). By contrast, mH\(^{+}/n\)Ca\(^{2+}\) exchangers with a coupling rate of m/n less than 2 (Fig. 5c,d), ion channels (Fig. 5e), or Ca\(^{2+}\)-ATPases (Fig. 5f) showed a thermodynamic behaviour that clearly deviated from that of the Ca\(^{2+}\) current across the VM. We therefore regard it unlikely that the latter groups of Ca\(^{2+}\) transport proteins contributed essentially to the measured voltage-dependent VM Ca\(^{2+}\) conductance.

Altogether, our data suggested the presence of active H\(^{+}/Ca\(^{2+}\) exchangers in the VM. A depolarisation of the VM reduced the driving force for the H\(^{+}\) influx into the cytosol and as a result caused an increase in pH (Fig. 3). The lowered driving force of H\(^{+}\) also reduced the uptake of Ca\(^{2+}\) by H\(^{+}/Ca\(^{2+}\) exchangers into the vacuole and therefore caused a rise in [Ca\(^{2+}\)]\(_{\text{cyt}}\) (Fig. 4). An inverse behaviour was observed upon hyperpolarisation. The CAX genes are obvious candidates to encode these voltage-dependent VM-localised transporters, as most CAX transporters are located in the vacuole and act as H\(^{+}/Ca\(^{2+}\) exchangers (Pittman & Hirschi, 2016). Future studies will have to reveal if CAX-proteins indeed serve as voltage-dependent vacuolar Ca\(^{2+}\) homeostats, or if other metal transporters also contribute to this system that balances [Ca\(^{2+}\)]\(_{\text{cyt}}\).

A model that couples VM excitation to [Ca\(^{2+}\)]\(_{\text{cyt}}\) signals

The physiological function of the voltage-dependent Ca\(^{2+}\) conductance of the VM (called Ca\(^{2+}\) homeostat in the following) was explored using computer-based simulations. Recently, it was shown that the voltage-dependent channel TPC1 and the K\(^{+}\) selective channels TPK1/TPK3 can convert an initial short voltage stimulus into a prolonged depolarisation of the VM (Jaslan et al., 2019). This experimentally observed mechanism was simulated with high accuracy by a computational model (Jaslan et al., 2019) that was now expanded with the VM-based Ca\(^{2+}\) homeostat (Fig. 6a).

As demonstrated by Jaslan et al. (2019) the application of a current pulse depolarised the VM and the length of the depolarisation depended on the strength of the current pulse (Fig. 6b). During the depolarisation, Ca\(^{2+}\)\(_{\text{cyt}}\) increased and stronger current pulses enhanced this response. Likewise, the magnitude of the [Ca\(^{2+}\)]\(_{\text{cyt}}\) change was enhanced, if the length of the current pulses was increased (Fig. 6c). However, when both amplitude and time were varied, but the stimulus charge (product of current and time) was kept constant, both the excitation time and final level of [Ca\(^{2+}\)]\(_{\text{cyt}}\) remained unchanged (Fig. 6d). We screened the [Ca\(^{2+}\)]\(_{\text{cyt}}\) vs charge relationship for a huge set of time/amplitude-combinations (black dots in Fig. 6e) and found an unambiguous relationship between the calcium signals and the stimulus charge.

Therefore, the VM seems capable of integrating the amplitude and length of current pulses and convert these parameters into a voltage change, which in turn provokes a cytosolic Ca\(^{2+}\) signal (Fig. 6e).

The combined Ca\(^{2+}\) homeostat and the TPC1/TPK1/TPK3-based channel network in the VM may, therefore, explain how the voltage-dependent TPC1 cation channels, together with the Ca\(^{2+}\)-dependent K\(^{+}\) channels TPK1 and TPK3, contribute to shaping the Ca\(^{2+}\) signals, while none of these channels directly releases Ca\(^{2+}\) from the vacuole. The proposed Ca\(^{2+}\)-release model is in line with the observed role of TPC1 in long distance signals, which are provoked by high NaCl concentrations in roots (Choi et al., 2014) and herbivory in shoots (Kiep et al., 2015). It is likely that these Ca\(^{2+}\) signals are passed on from one cell to its neighbour by plasmodesmata (Hedrich et al., 2016; Choi et al., 2017). Because of these connections, elevation of Ca\(^{2+}\)\(_{\text{cyt}}\) in a certain cell may cause a small local Ca\(^{2+}\) signal in the neighbouring cell. In return, the localised Ca\(^{2+}\) signal in the neighbour cell can activate TPK channels by the Ca\(^{2+}\)-binding calcineurin B-like (CBL) proteins that regulate CBL-interacting protein kinases (Gobert et al., 2007; Latz et al., 2007; Voelker et al., 2010; Wang et al., 2015; Tang et al., 2020), depolarise the VM and trigger a large rise in [Ca\(^{2+}\)]\(_{\text{cyt}}\) through the Ca\(^{2+}\) homeostat of the VM.

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Author contributions

JD, MRGR, SH and RH designed the wet laboratory experiments. JD and SH conducted and analysed electrophysiological and imaging experiments. JD modelled the transporter thermodynamics. ID designed and conducted mathematical modelling. All authors contributed to writing of the manuscript. JD and ID contributed equally to this work.

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Calcium signals in guard cells enhance the efficiency by which abscisic acid triggers stomatal closure. A ROS-assisted calcium wave propagates the systemic response to salt stress. Calcium channels activated by hydrogen peroxide mediate Ca²⁺-regulated channel TPC1 cation channel gains shape. Potentiates Ca²⁺ influx systems in stress signaling and adaptation in plants.
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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Calibration of pH-induced changes of cpYFP and R-GECo1 fluorescence signals.

Fig. S2 Cytosolic current pulses do not trigger Ca2+ release at the PM.

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