Research Article

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The phosphoinositide PI(3,5)P_2 inhibits the activity of plant NHX proton/potassium antiporters: Advantages of a novel electrophysiological approach

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Abstract: In the present work, we discuss the way in which the parallel application of the patch-clamp technique and the 2′,7′-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) fluorescence detection for recording luminal proton changes allows the functional characterization of nonelectrogenic potassium/proton vacuolar antiporters of the NHX (Na^+/H^+ exchanger) family. Moreover, we review the functional role of the tonoplast-specific phosphoinositide PI(3,5)P_2, able to simultaneously inhibit the activity of NHXs and CLC-a transporters, whose coordinated action can play an important role in the water balance of plant cells.

Keywords: NHX antiporters, plant vacuole, patch-clamp, BCECF, fluorescence, proton

Introduction

NHXs (Na^+/H^+ exchangers) are a family of monovalent cation/proton antiporters involved in various physiological processes, fundamental for plant survival, such as pH and potassium homeostasis, plant growth, and salt stress acclimation [1,2]. In animals, they are referred to as NHEs. They belong to clade CPA1, one of the two groups forming the CPA superfamily, cation/proton antiporters, [3]. Among the eight isoforms present in *Arabidopsis thaliana*, AtNHX1-4 is located at the vacuole, AtNHX5/6 in intracellular compartments, and AtNHX7/8 (AtNHX7 is also indicated as SOS1) at the plasma membrane. Focusing on vacuoles, Arabidopsis plants lacking both NHX1 and 2 are severely impaired in growth and extremely sensitive to external potassium changes [1,4,5], indicating a low functional activity of NHX3 and 4.

Different techniques for the study of NHX transporters

NHXs are potassium/proton antiporters with a 1:1 stoichiometry (or multiples); being nonelectrogenic, they could not be investigated with the usual electrophysiological techniques and their functional characterization is challenging. For this purpose, different approaches were developed. Tonoplast vesicles from plants or yeasts were loaded with specific fluorophores, and NHX activity in the formation or dissipation of proton gradients was monitored measuring their quenching [5–7]. NHX proteins were purified and reconstituted in proteoliposomes and have been studied following an approach similar to the aforementioned one [7,8]. Another method of investigation was that represented by heterologous expression in mutant yeast cells [9–11]: the yeast AXT3K mutant, which lacks the endogenous Na^+ efflux proteins, ENA1-4 and NHA1, and the endogenous vacuolar Na^+/H^+ antiporter NHX1, was complemented with AtNHX members verifying whether its growth could be restored in a selective medium, for example, enriched with NaCl.

In all these techniques, it is not possible to fix the ionic solutions involved, the luminal solution in the case

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of vesicles or proteoliposomes, and the cytosolic and the vacuolar solutions in the case of yeast cells; there is no control of the transmembrane voltage; and it is very difficult to evaluate the interplay among different vacuolar proton transporters (impossible in proteoliposome) and to perform dose-response experiments with agonists/inhibitors (impossible to measure the reversibility of the effects). Furthermore, in addition to the limited intrinsic selectivity and sensitivity of the fluorophores, their photobleaching over time represents a relevant limit to the duration of the experiment.

How to study ion channels and transporters

Patch-clamp is the most important technique for studying ion channels and electrogenic plant transporters [12]. It can be successfully applied to vacuoles of various species, from the model plant *Arabidopsis thaliana* [13] to seagrass [14] and aquatic plants [15]. The use of protoplasts is more demanding, probably because it is difficult to control how clean the membrane is after the enzymatic treatment necessary to remove the cell wall. However, also in this case, protoplasts from many species have been used, such as maize and carrot of agronomic interest [16,17], Arabidopsis [18] and *Posidonia oceanica* the main seagrass in the Mediterranean sea [19].

The patch-clamp technique is usually applied in the whole-cell (or whole-vacuole) configuration. This allows control of both the internal solution, cytosolic in the case of protoplasts and luminal in the case of vacuoles and the bath solution. Although not as firmly attached to the recording chamber as in the case of animal cells [20], it is possible to change the external solution of protoplasts or vacuoles several times on the same preparation [21]. In the patch-clamp technique the membrane voltage is fixed and the application of appropriate potential protocols [22] allows us to study the voltage dependence of channels and transporters in great detail. In the case of *Arabidopsis thaliana*, the whole genome is sequenced and knock-out plants lacking a certain channel or transporter are currently available; wild-type or specific mutant proteins can be overexpressed in the relative KO to perform structure-function correlation studies [23].

Another widely used approach to investigate membrane protein functions is the use of heterologous systems for protein expression. For channels and transporters localized in the plasma membrane, the system of choice is represented by oocytes of *Xenopus laevis* [24]. The RNA is directly injected into a single oocyte, which has a spherical shape of about 1 mm in diameter. Two electrodes are required to perform the voltage-clamp: one measures the potential difference of the oocyte membrane and the other injects current so that the potential is equal to that desired by the operator. The time to obtain the voltage-clamp is less than 1 ms, which is excellent for studying fast mechanisms such as pre-steady-state currents of the maize sucrose/proton symporter ZmSUT1 [25]. Generally, plant channels have much slower activation and deactivation times.

Oocytes are excellent for structure-function studies: to investigate the inward or outward rectification mechanism, Porée et al. [26] generated and expressed in oocytes 250 chimeras from KAT1 and SKOR, respectively, an inward and outward-rectifier channel of the *Shaker* family of *Arabidopsis thaliana*. Furthermore, oocytes may also be used to study protein conformational changes: Derrer et al. [27] applied the voltage-clamp fluorometry technique and solved the first steps in the reaction cycle of ZmSUT1, as shown in Figure 5 of ref. [27]. Recently, oocytes have been used to detect electronic currents flowing in animal and plant cytochromes [28–30]. The oocyte system has also been used to study the effects of plant pathogen toxins on animal channels [31].

Plant channels can also be transiently expressed in tobacco protoplasts. For example, the interaction between two *Shaker*-type subunits of potassium channels was studied, one from carrot (KDC1) and the other from Arabidopsis (AKT1), and both were successfully expressed in tobacco protoplasts [32].

The systems described above are not appropriate to characterize intracellular channels and transporters due to an evident localization problem. Interestingly, Costa et al. [33] showed that the plant vacuole can be an excellent heterologous system for the study of a lysosomal rat transporter, namely, rCLC-7. The human endo-lysosomal channels hTPC1 and hTPC2 were also expressed in vacuoles isolated from the leaf mesophyll of Arabidopsis and characterized in detail [34–38]. From these studies, interesting modulations mediated by the flavonoid naringenin emerged with implications in severe human pathologies, such as cancer and viral infection [39–43].

The plant vacuole can also be used as a system to study the properties of the so-called channel forming peptides (CFPs): SP25A, a toxin produced by the Gram-negative bacterium *Pseudomonas syringae*, a phytopathogenic organism that affects several plants of agronomic interest, is capable of forming pores with anion selectivity on the tonoplast of vacuoles from sugar beet [44,45].

Patch-clamp combined with ion-sensitive fluorescent probes

Plant vacuoles have the so-called slow-activating channels of large unitary conductance from the two-pore channel (TPC) family [46,47]. Arabidopsis has a single gene coding for the AtTPC1 protein, a cation channel activated by cytosolic calcium and modulated by many factors, including reducing and oxidizing agents [48–50], magnesium [51], and heavy metals [52,53]. Since, *in vivo*, the concentration of both cytosolic and luminal calcium is significantly lower than that of potassium, a difficult question that arises is whether under these conditions it is possible to quantify the passage of calcium through the channel. To answer this question Gradogna et al. [54,55] developed the so-called FLEP (fluorescence combined with excised patch) technique, adding the fluorophore fura-2 in the recording pipette as a sensor for calcium changes. Using an excised patch, which was large enough to detect a significant activity of the channel, they could determine the fractional calcium current (approximately 10% of the total currents at elevated positive potentials), a measurement impossible to carry out only with electrophysiology.

Using the ratiometric proton sensitive probe BCECF in place of fura-2 and applying the whole-cell configuration, Carpaneto et al. [56], in parallel with current measurements, could detect an increase in protons in the vacuolar lumen after addition of cytosolic ATP or pyrophosphate and evaluate the impact on the vacuolar pH of the AtCLCa proton/anion antiporter.

To measure the activity of NHX transporters, Gradogna et al. [57] used the same approach, adding BCECF to the pipette (luminal) solution and applying the patch-clamp technique in whole-vacuole configuration. They modified the bath (cytosolic) solution by substituting potassium with an equimolar concentration of cesium, Figure 1a. No current change was recorded, as shown in Figure 1b. However, the proton concentration increased significantly returning to its initial value when the potassium concentration was restored. The increase in luminal proton concentration was absent in *nhx1 nhx2* KO plants, a strong indication that the movement of protons induced by a steep potassium gradient in Figure 1c is mediated by these two transporters (we will refer to both as NHX1/2). Gradogna et al. used a thermodynamic approach, which describes the concentration of luminal protons as a function of time and cytosolic potassium concentration with the following equation:

$$\frac{dc}{dt} + \frac{c - c_{pip}}{\tau} = c_N, \qquad (1)$$

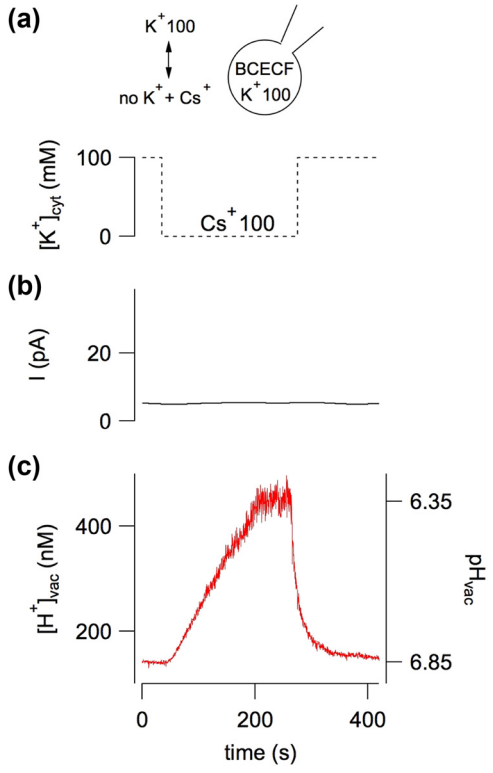

Figure 1: A cytosol-directed potassium gradient induced vacuolar acidification. (a) Schematic representation of cytosolic potassium removal (100 mM K^+) and substitution with an equimolar amount of cesium (no K^+ + Cs^+) versus time. The dotted lines indicate the switching of the bath solution irrespective to the real change rate due to the perfusion system. (b) Current, recorded by the patch-clamp technique applied on a vacuole isolated from Arabidopsis mesophyll cells, did not change. (c) Luminal concentration of potassium, detected by the proton-sensitive fluorescent dye BCECF loaded inside the vacuole through a patch pipette, increased significantly after K^+ removal. Modification of Figure 1, from Gradogna et al. [57], *New Phytologist*, 229:3026–36, reprinted by permission from John Wiley and Sons (license number 5236600128835).

where c is the luminal proton concentration, c_{pip} = 63.1 nM is the proton concentration in the recording patch pipette, τ is the time constant linked to the movement of protons between the pipette and the lumen of the vacuole estimated to be 28.4 s (Supplemental material in ref. [57]), c_N is the luminal proton concentration per unit of time expressed by:

$$c_N = k_N \ln\left(\frac{[H^+]_{cyt}[K^+]_{lum}}{c[K^+]_{cyt}}\right), \qquad (2)$$

where k_N is a constant dependent on the activity of NHX1/2 estimated to be 5.0 µM/s; $[K^+]_{lum}$ = 100 mM and $[H^+]_{cyt}$ = 63.1 nM are, respectively, the luminal potassium and cytosolic proton concentrations.

Taking into account that the cytosolic potassium concentration does not vary instantaneously (black curve in Figure 2a) but its variation depends on the perfusion system (blue and red curves in Figure 2a with the time constant of the perfusion system equal to, respectively, 10 and 30 s), the solution of equation (1) is given by the curves of Figure 2b. The blue curve of Figure 2b is in optimal agreement with the experimental data of Figure 1c and predicts that, as can be seen in Figure 2c, when the cytosolic potassium is decreased, its rate of change is faster than the increase of the luminal protons. Conversely, when potassium is increased, its rate of change is slower than the proton concentration variation. Mathematical modeling, therefore, is able to explain the kinetics of luminal proton variations due to the application or removal of a steep potassium gradient (more details are given in the supplemental material in ref. [57]).

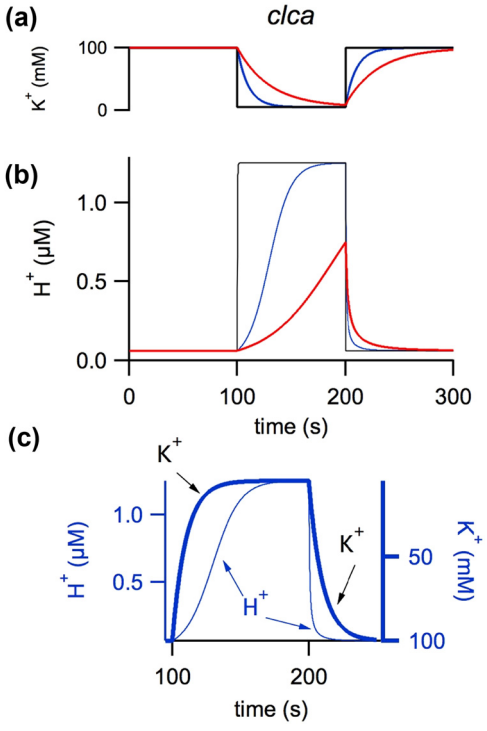

Figure 2: Simulation of the vacuolar acidification mediated by NHXs induced by a cytosol-directed potassium gradient. (a) Change in cytosolic potassium. (b) Corresponding response in luminal proton concentration variation. Traces in A were calculated considering instantaneous perfusion (black line) or a perfusion system with a time constant of 10 (blue line) or 30 s (red line). Traces in B were calculated using equation (1). (c) Superimposition of cytosolic potassium concentration (thick blue line) and luminal proton concentration to favor a direct comparison. Modification of Figure S9 from Gradogna et al. [57], *New Phytologist*, 229:3026–36, reprinted by permission from John Wiley and Sons (license number 5236600128835).

Functional significance of regulation by phosphoinositide $PI(3,5)P_2$

Phosphoinositides are rapidly synthesized, modified, and hydrolyzed in response to numerous stimuli [58,59]. The *myo*-inositol headgroup can be phosphorylated at hydroxyls 1, 3, and 5 by multiple phosphoinositide-kinases, giving rise to an array of signaling compounds unevenly distributed in cell membranes. The most abundant species in plant cells is unphosphorylated phosphatidylinositol (PI), which can be converted to phosphatidylinositol-4-phosphate (PI4P) and phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$) at the plasma membrane. By contrast, PI3P and $PI(3,5)P_2$ are mainly localized to late endosomes and the tonoplast [59].

The application in the cytosolic solution of the phosphoinositide $PI(3,5)P_2$ at 200 nM induced a significant and reversible inhibition of NHX1/2 activity, Figure 3 [57]. This experimental evidence is very intriguing considering that $PI(3,5)P_2$ also inhibits CLC-a [56], which colocalizes with NHX1/2 at the tonoplast. On the contrary, $PI(3,5)P_2$ did not affect the vacuolar ATPase and pyrophosphatase. Stomatal closure is a physiological process where the concentration of $PI(3,5)P_2$ is known to increase [60]. In this case, the simultaneous inhibition of NHX1/2

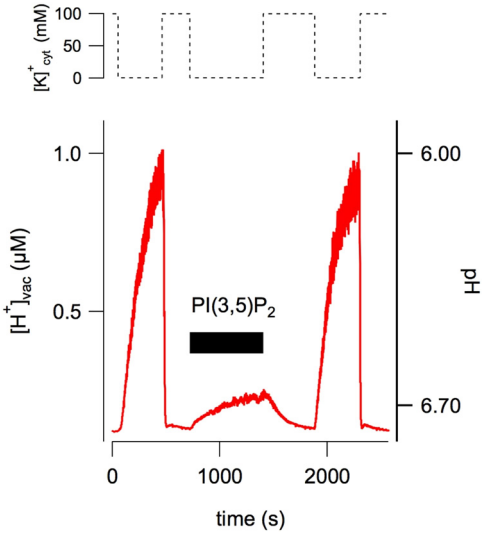

Figure 3: The phosphoinositide $PI(3,5)P_2$ inhibited NHX activity. The presence of 200 nM $PI(3,5)P_2$ in cytosolic solution caused a strong and reversible inhibition of the vacuolar acidification mediated by NHX activity in a cytosol-directed potassium gradient. Modification of Figure 6a from Gradogna et al. [57], *New Phytologist*, 229:3026–36, reprinted by permission from John Wiley and Sons (license number 5236600128835).

and CLC-a can be interpreted as restriction of salt accumulation inside the vacuole. Vacuoles from guard cells must release salt in order to favor the concurrent release of water and stomatal closure. It therefore appears that the regulatory effects of PI(3,5)P_2 identify a salt accumulation unit comprising the combined action of CLC-a and NHX1/2, Figure 4, [57]. Since the activity of both transporters impacts on the vacuolar proton concentration, their simultaneous inhibition should result in a significant increase in vacuolar protons, which is exactly what is measured during stomata closure of wild-type plants [60] and in an exacerbated manner in the *nhx1 nhx2* mutant [61]. As pointed out by Gradogna et al. [57] the coordinated work of CLC-a and NHX1/2 could avoid futile cycles of osmolyte fluxes and mediate the efficient release of cations and anions in physiological processes where this is necessary for optimal water movement.

Interestingly, phosphatidylinositol (PI) has been recently shown to bind and inhibit the plasma membrane H^+-ATPase AHA2 [62]. Phosphorylation of AHA2 at residue T947 leads to 14-3-3 protein binding and activation of the H^+-ATPase, while phosphorylation at the neighboring S931 by kinase PKS5/CIPK11 prevents 14-3-3 binding and inhibits the pump [63]. PI binding to AHA2 was favored by S931 phosphorylation and diminished by phosphorylated T497, suggesting that PI-mediated inhibition is additive to that of PKS5/CIPK11 [62]. In response to salinity stress, PI was rapidly converted to PI(4)P, which then bound and activated the plasma membrane Na^+/H^+ antiporter SOS1/NHX7, while the reduced levels of PI contributed to the coordinated activation of the H^+-ATPase to fuel Na^+/H^+ exchange [62].

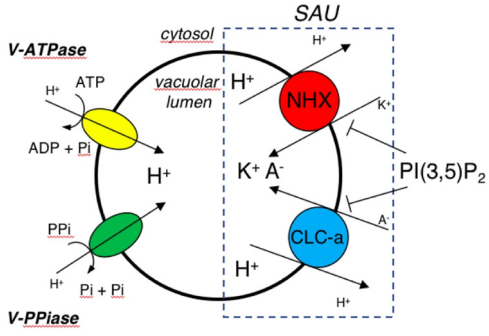

Figure 4: Schematic representation of the key players in vacuolar salt uptake. A salt accumulation unit is formed by NHXs together with CLC-a. V-ATPase: vacuolar H^+-ATPase; V-PPiase: vacuolar H^+-pyrophosphatase. A^-, H^+, and K^+ indicates, respectively, anions, protons, and potassium ions; the dimension of the letters is proportional to their concentration. Modification of Figure 6c from Gradogna et al. [57], *New Phytologist*, 229:3026–36, reprinted by permission from John Wiley and Sons (license number 5236600128835).

Arabidopsis mutants deficient in PI and PI(4)P biosynthesis had altered cellular Na^+ contents, which could result from faulty regulation of SOS1/NHX7 activity [62]. However, NHX1/2 activity contributes to salinity tolerance through the compartmentation of Na^+ and K^+ ions [1,4,5], and it would be interesting to explore further the possibility of coordinated regulation of plasma membrane- and tonoplast-localized NHX proteins by phosphoinositides. Vacuolar NHX1/2 responded strongly to PI(3,5)P_2 and weakly to PI(3,4,5)P_2, whereas PI(3,4)P_2 and PI(4,5)P_2 had no effect on NHX1/2 activity [57]. On the other hand, PI acted on AHA2, but PI3P, PI4P, PI5P, and PI(4,5)P_2 failed to interact. PI4P activated SOS1/NHX7 but PI3P did not [62]. These assays are informative but incomplete. Although different phosphoinositide species show some degree of uneven distribution among cell membranes and compartments, it would be important to investigate the signaling roles of the diverse phosphoinositide species on H^+, Na^+, and K^+ fluxes and transport proteins in a systematic manner to delineate an integrative model [64].

Conclusions

The extension of the patch-clamp technique with the use of fluorescent probes sensitive to ions of physiological interest allows us to measure functional details impossible to detect with the usual experimental techniques, such as the functional characterization of the NHX vacuolar transporters and the quantitative estimation of the inhibition mediated by the tonoplast-specific phosphoinositide PI(3,5)P_2. The application of this approach to other vacuolar transporters can be useful to evaluate their interplay in controlling cytosolic and vacuolar pH.

Abbreviations

PI(3,5)P_2	phosphatidylinositol-(3,5)-bisphosphate
VM	vacuolar membrane

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Conflict of interest: Authors state no conflict of interest.

Data availability statement: The datasets generated and analyzed during the current review are available from the corresponding author on reasonable request.

References

[1] Bassil E, Zhang S, Gong H, Tajima H, Blumwald E. Cation specificity of vacuolar NHX-type cation/H+ antiporters. Plant Physiol. 2019;179:616–29.

[2] Ragel P, Raddatz N, Leidi EO, Quintero FJ, Pardo JM. Regulation of K+ nutrition in plants. Front Plant Sci. 2019;10:281.

[3] Chanroj S, Wang G, Venema K, Zhang MW, Delwiche CF, Sze H. Conserved and diversified gene families of monovalent cation/h(+) antiporters from algae to flowering plants. Front Plant Sci. 2012;3:25.

[4] Bassil E, Tajima H, Liang Y-C, Ohto M-A, Ushijima K, Nakano R, et al. The arabidopsis Na+/H+ antiporters NHX1 and NHX2 control vacuolar pH and K+ homeostasis to regulate growth, flower development, and reproduction. Plant Cell. 2011;23:3482–97.

[5] Barragán V, Leidi EO, Andrés Z, Rubio L, De Luca A, Fernández JA, et al. Ion exchangers NHX1 and NHX2 mediate active potassium uptake into vacuoles to regulate cell turgor and stomatal function in Arabidopsis. Plant Cell. 2012;24:1127–42.

[6] Barkla BJ, Vera-Estrella R, Maldonado-Gama M, Pantoja O. Abscisic acid induction of vacuolar H^+-ATPase activity in mesembryanthemum crystallinum is developmentally regulated. Plant Physiol. 1999;120:811–20.

[7] Hernández A, Jiang X, Cubero B, Nieto PM, Bressan RA, Hasegawa PM, et al. Mutants of the Arabidopsis thaliana cation/H^+ antiporter AtNHX1 conferring increased salt tolerance in yeast: the endosome/prevacuolar compartment is a target for salt toxicity. J Biol Chem. 2009;284:14276–85.

[8] Venema K, Quintero FJ, Pardo JM, Donaire JP. The arabidopsis Na^+/H^+ exchanger AtNHX1 catalyzes low affinity Na^+ and K^+ transport in reconstituted liposomes. J Biol Chem. 2002;277:2413–8.

[9] Quintero FJ, Ohta M, Shi H, Zhu J-K, Pardo JM. Reconstitution in yeast of the Arabidopsis SOS signaling pathway for Na+ homeostasis. Proc Natl Acad Sci U S A. 2002;99:9061–6.

[10] Shi H, Quintero FJ, Pardo JM, Zhu J-K. The putative plasma membrane Na(+)/H(+) antiporter SOS1 controls long-distance Na(+) transport in plants. Plant Cell. 2002;14:465–77.

[11] Yokoi S, Quintero FJ, Cubero B, Ruiz MT, Bressan RA, Hasegawa PM, et al. Differential expression and function of Arabidopsis thaliana NHX Na^+/H^+ antiporters in the salt stress response. Plant J Cell Mol Biol. 2002;30:529–39.

[12] Hille B. Ion Channels of Excitable Membranes. Third edn. Sunderland, Massachusetts, USA: Oxford University Press; 2001.

[13] Gutla PVK, Boccaccio A, De Angeli A, Gambale F, Carpaneto A. Modulation of plant TPC channels by polyunsaturated fatty acids. J Exp Bot. 2012;63:6187–97.

[14] Carpaneto A, Cantu' AM, Busch H, Gambale F. Ion channels in the vacuoles of the seagrass Posidonia oceanica. FEBS Lett. 1997;412:236–40.

[15] Paganetto A, Carpaneto A, Gambale F. Ion transport and metal sensitivity of vacuolar channels from the roots of the aquatic plant *Eichhornia crassipes*. Plant Cell Environ. 2001;24:1329–36.

[16] Bregante M, Carpaneto A, Pastorino F, Gambale F. Effects of mono- and multi-valent cations on the inward-rectifying potassium channel in isolated protoplasts from maize roots. Eur Biophys J. 1997;26:381–91.

[17] Costa A, Carpaneto A, Varotto S, Formentin E, Marin O, Barizza E, et al. Potassium and carrot embryogenesis: are K^+ channels necessary for development? Plant Mol Biol. 2004;54:837–52.

[18] Carpaneto A, Ivashikina N, Levchenko V, Krol E, Jeworutzki E, Zhu J-K, et al. Cold transiently activates calcium-permeable channels in Arabidopsis mesophyll cells. Plant Physiol. 2007;143:487–94.

[19] Carpaneto A, Naso A, Paganetto A, Cornara L, Pesce E-R, Gambale F. Properties of ion channels in the protoplasts of the Mediterranean seagrass Posidonia oceanica. Plant Cell Environ. 2004;27:279–92.

[20] Carpaneto A, Accardi A, Pisciotta M, Gambale F. Chloride channels activated by hypotonicity in N2A neuroblastoma cell line. Exp Brain Res. 1999;124:193–9.

[21] Festa M, Lagostena L, Carpaneto A. Using the plant vacuole as a biological system to investigate the functional properties of exogenous channels and transporters. Biochim Biophys Acta. 2016;1858:607–12.

[22] Gambale F, Cantu AM, Carpaneto A, Keller BU. Fast and slow activation of voltage-dependent ion channels in radish vacuoles. Biophys J. 1993;65:1837–43.

[23] Rocchetti A, Sharma T, Wulfetange C, Scholz-Starke J, Grippa A, Carpaneto A, et al. The putative K(+) channel subunit AtKCO3 forms stable dimers in *Arabidopsis*. Front Plant Sci. 2012;3:251.

[24] Stühmer W. Electrophysiologic recordings from Xenopus oocytes. Methods Enzymol. 1998;293:280–300.

[25] Carpaneto A, Koepsell H, Bamberg E, Hedrich R, Geiger D. Sucrose-and H^+-dependent charge movements associated with the gating of sucrose transporter ZmSUT1. PLoS One. 2010;5:e12605.

[26] Porée F, Wulfetange K, Naso A, Carpaneto A, Roller A, Natura G, et al. Plant K(in) and K(out) channels: approaching the trait of opposite rectification by analyzing more than 250 KAT1-SKOR chimeras. Biochem Biophys Res Commun. 2005;332:465–73.

[27] Derrer C, Wittek A, Bamberg E, Carpaneto A, Dreyer I, Geiger D. Conformational changes represent the rate-limiting step in the transport cycle of maize sucrose transporter1. Plant Cell. 2013;25:3010–21.

[28] Picco C, Scholz-Starke J, Naso A, Preger V, Sparla F, Trost P, et al. How are cytochrome b561 electron currents controlled by membrane voltage and substrate availability? Antioxid Redox Signal. 2014;21:384–91.

[29] Picco C, Scholz-Starke J, Festa M, Costa A, Sparla F, Trost P, et al. Direct recording of trans-plasma membrane electron currents mediated by a member of the cytochrome b561 family of soybean. Plant Physiol. 2015;169:986–95.

[30] Trost P, Picco C, Scholz-Starke J, Festa M, Lagostena L, Costa A, et al. Electron current recordings in living cells. Biophys Chem. 2017;229:57–61.
[31] Nicastro G, Orsomando G, Ferrari E, Manconi L, Desario F, Amici A, et al. Solution structure of the phytotoxic protein PcF: the first characterized member of the Phytophthora PcF toxin family. Protein Sci. 2009;18:1786–91.
[32] Bregante M, Yang Y, Formentin E, Carpaneto A, Schroeder JI, Gambale F, et al. KDC1, a carrot Shaker-like potassium channel, reveals its role as a silent regulatory subunit when expressed in plant cells. Plant Mol Biol. 2008;66:61–72.
[33] Costa A, Gutla PVK, Boccaccio A, et al. The Arabidopsis central vacuole as an expression system for intracellular transporters: functional characterization of the Cl^-/H^+ exchanger CLC-7. J Physiol. 2012;590:3421–30.
[34] Boccaccio A, Scholz-Starke J, Hamamoto S, Larisch N, Festa M, Gutla PVK, et al. The phosphoinositide $PI(3,5)P_2$ mediates activation of mammalian but not plant TPC proteins: functional expression of endolysosomal channels in yeast and plant cells. Cell Mol Life Sci CMLS. 2014;71:4275–83.
[35] Lagostena L, Festa M, Pusch M, Carpaneto A. The human two-pore channel 1 is modulated by cytosolic and luminal calcium. Sci Rep. 2017;7:43900.
[36] Kirsch SA, Kugemann A, Carpaneto A, Böckmann RA, Dietrich P. Phosphatidylinositol-3,5-bisphosphate lipid-binding-induced activation of the human two-pore channel 2. Cell Mol Life Sci CMLS. 2018;75:3803–15.
[37] Milenkovic S, Bodrenko IV, Lagostena L, Gradogna A, Serra G, Bosin A, et al. The mechanism and energetics of a ligand-controlled hydrophobic gate in a mammalian two pore channel. Phys Chem Chem Phys PCCP. 2020;22:15664–74.
[38] Milenkovic S, Bodrenko IV, Carpaneto A, Ceccarelli M. The key role of the central cavity in sodium transport through ligand-gated two-pore channels. Phys Chem Chem Phys. 2021;23:18461–74.
[39] Pafumi I, Festa M, Papacci F, et al. Naringenin impairs two-pore channel 2 activity and inhibits VEGF-induced angiogenesis. Sci Rep. 2017;7:5121.
[40] Benkerrou D, Minicozzi V, Gradogna A, et al. A perspective on the modulation of plant and animal two pore channels (TPCs) by the flavonoid naringenin. Biophys Chem. 2019;254:106246.
[41] Filippini A, D'Amore A, Palombi F, Carpaneto A. Could the inhibition of endo-lysosomal two-pore channels (TPCs) by the natural flavonoid naringenin represent an option to fight SARS-CoV-2 infection? Front Microbiol. 2020;11:970.
[42] Clementi N, Scagnolari C, D'Amore A, et al. Naringenin is a powerful inhibitor of SARS-CoV-2 infection in vitro. Pharmacol Res. 2020;105255.
[43] D'Amore A, Gradogna A, Palombi F, Minicozzi V, Ceccarelli M, Carpaneto A, et al. The discovery of naringenin as endolysosomal two-pore channel inhibitor and its emerging role in SARS-CoV-2 infection. Cells. 2021;10:1130.
[44] Carpaneto A, Dalla Serra M, Menestrina G, Fogliano V, Gambale F. The phytotoxic lipodepsipeptide syringopeptin 25A from Pseudomonas syringae pv syringae forms ion channels in sugar beet vacuoles. J Membr Biol. 2002;188:237–48.
[45] Dalla Serra M, Menestrina G, Carpaneto A, Gambale F, Fogliano V, Ballio A. Molecular mechanism of action of syringopeptins, antifungal peptides from Pseudomonas syringae pv. syringae. Pore-Form Pept Proteins Toxins. 2003;272–95.
[46] Peiter E, Maathuis FJM, Mills LN, Knight H, Pelloux J, Hetherington AM, et al. The vacuolar Ca^{2+}-activated channel TPC1 regulates germination and stomatal movement. Nature. 2005;434:404–8.
[47] Scholz-Starke J, Naso A, Carpaneto A. A perspective on the slow vacuolar channel in vacuoles from higher plant cells. J Chem Inf Model. 2005;45:1502–6.
[48] Carpaneto A, Cantù AM, Gambale F. Redox agents regulate ion channel activity in vacuoles from higher plant cells. FEBS Lett. 1999;442:129–32.
[49] Scholz-Starke J, De Angeli A, Ferraretto C, Paluzzi S, Gambale F, Carpaneto A. Redox-dependent modulation of the carrot SV channel by cytosolic pH. FEBS Lett. 2004;576:449–54.
[50] Scholz-Starke J, Gambale F, Carpaneto A. Modulation of plant ion channels by oxidizing and reducing agents. Arch Biochem Biophys. 2005;434:43–50.
[51] Carpaneto A, Cantù AM, Gambale F. Effects of cytoplasmic Mg2+ on slowly activating channels in isolated vacuoles of Beta vulgaris. Planta. 2001;213:457–68.
[52] Carpaneto A. Nickel inhibits the slowly activating channels of radish vacuoles. Eur Biophys J EBJ. 2003;32:60–6.
[53] Corem S, Carpaneto A, Soliani P, Cornara L, Gambale F, Scholz-Starke J. Response to cytosolic nickel of slow vacuolar channels in the hyperaccumulator plant Alyssum bertolonii. Eur Biophys J EBJ. 2009;38:495–501.
[54] Gradogna A, Scholz-Starke J, Gutla PVK, Carpaneto A. Fluorescence combined with excised patch: measuring calcium currents in plant cation channels. Plant J. 2009;58:175–82.
[55] Carpaneto A, Gradogna A. Modulation of calcium and potassium permeation in plant TPC channels. Biophys Chem. 2018;236:1–7.
[56] Carpaneto A, Boccaccio A, Lagostena L, Di Zanni E, Scholz-Starke J. The signaling lipid phosphatidylinositol-3,5-bisphosphate targets plant CLC-a anion/H^+ exchange activity. EMBO Rep. 2017;18:1100–7.
[57] Gradogna A, Scholz-Starke J, Pardo JM, Carpaneto A. Beyond the patch-clamp resolution: functional activity of nonelectrogenic vacuolar NHX proton/potassium antiporters and inhibition by phosphoinositides. New Phytol. 2021;229:3026–36.
[58] Heilmann I. Phosphoinositide signaling in plant development. Dev Camb Engl. 2016;143:2044–55.
[59] Han X, Yang Y. Phospholipids in salt stress response. Plants Basel Switz. 2021;10:2204.
[60] Bak G, Lee E-J, Lee Y, Kato M, Segami S, Sze H, et al. Rapid structural changes and acidification of guard cell vacuoles during stomatal closure require phosphatidylinositol 3,5-bisphosphate. Plant Cell. 2013;25:2202–16.
[61] Andrés Z, Pérez-Hormaeche J, Leidi EO, et al. Control of vacuolar dynamics and regulation of stomatal aperture by tonoplast potassium uptake. Proc Natl Acad Sci U S A. 2014;111:E1806–814.
[62] Yang Y, Han X, Ma L, Wu Y, Liu X, Fu H, et al. Dynamic changes of phosphatidylinositol and phosphatidylinositol 4-phosphate levels modulate H^+-ATPase and Na^+/H^+ antiporter activities to maintain ion homeostasis in Arabidopsis under salt stress. Mol Plant. 2021;14:2000–14.
[63] Fuglsang AT, Guo Y, Cuin TA, et al. Arabidopsis protein kinase PKS5 inhibits the plasma membrane H^+-ATPase by preventing interaction with 14-3-3 protein. Plant Cell. 2007;19:1617–34.
[64] Shabala S, Yu M. Phosphoinositides: emerging players in plant salinity stress tolerance. Mol Plant. 2021;14:1973–5.