**Beyond the patch-clamp resolution:
functional activity of non-electrogenic vacuolar NHX proton/potassium antiporters
and inhibition by phosphoinositides**

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Running title: Functional activity of NHXs

Key words

NHX antiporters; plant vacuole; patch-clamp; BCECF; fluorescence; proton

Abbreviations

PI(3,5)P₂ phosphatidylinositol-(3,5)-bisphosphate; VM vacuolar membrane; BCECF 2',7'-Bis(2-carboxyethyl)-5(6)-carboxyfluorescein; BTP Bis-Tris Propane.

29 **Summary**

30 We combined the patch-clamp technique with ratiometric fluorescence imaging using the proton-
31 responsive BCECF dye as a luminal probe. Upon application of a steep cytosol-directed K^+
32 gradient in *Arabidopsis* mesophyll vacuoles, a strong and reversible acidification of the vacuolar
33 lumen was detected, while no associated electrical currents were observed, in agreement with
34 electroneutral cation/ H^+ exchange. Our data show that this acidification was generated by NHX
35 antiport activity, since i) it did not distinguish between K^+ and Na^+ ions, ii) it was sensitive to the
36 NHX inhibitor benzamil, and iii) it was completely absent in vacuoles from *nhx1 nhx2* double
37 knockout plants. Our data further show that NHX activity could be reversed, was voltage
38 independent and specifically impaired by the low-abundance signaling lipid phosphatidylinositol-
39 3,5-bisphosphate ($PI(3,5)P_2$), which may regulate salt accumulation in plants by acting as a
40 common messenger to coordinately shut down secondary active carriers responsible for cation
41 and anion uptake inside the vacuole. Finally, we developed a theory based on thermodynamics,
42 which supports the data obtained by our novel experimental approach. This work, therefore,
43 represents a proof of principle that can be applied to the study of proton-dependent exchangers
44 from plants and animals, which are barely detectable using conventional techniques.

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46

47 **Introduction**

48 The patch-clamp technique consists of attaching a glass pipette with a micrometric tip to the cell
49 membrane (Neher & Sakmann, 1976). The application of a light suction induces an extremely tight
50 contact (seal) between the pipette and the membrane with a resistance that can reach values of
51 about ten billion ohms and more. This leads to a drastic reduction of the background noise,
52 inversely proportional to the resistance (Hamill *et al.*, 1981), and to the possibility of recording,
53 through appropriate precautions, signals of the order of femtoamperes (Carpaneto *et al.*, 2005). In
54 the case of plant cells, it is necessary to remove the cell wall, usually by enzymatic treatment.
55 Since it is difficult to control how "clean" the plasma membrane is, the patch clamp on protoplasts
56 is difficult to apply. On the contrary, vacuoles are an ideal preparation, because they are easy to

isolate, large in size, and the incidence of obtaining a giga seal between pipette and tonoplast is very high (Gradogna *et al.*, 2009; Costa *et al.*, 2012; Festa *et al.*, 2016). Recently, using the pH-sensitive fluorescent dye BCECF, it has been possible to determine that the activity of the vacuolar nitrate/proton antiporter CLC-a is reflected in the vacuolar pH value (Carpaneto *et al.*, 2017). We hypothesized that a similar approach could be used for the functional characterization of non-electrogenic secondary transporters, such as NHX transporters.

NHX (Na^+/H^+ eXchangers) is a family of cation/proton antiporters belonging to clade CPA1, one of the two groups that form the CPA superfamily (Cation/Proton Antiporters, Chanroj *et al.*, 2012). They are believed to be involved in various physiological processes such as pH and potassium homeostasis, plant growth and salt stress acclimation (Reguera *et al.*, 2014). In *Arabidopsis*, there are 8 isoforms divided into three groups: AtNHX1-4 are located at the vacuole, AtNHX5/6 in intracellular compartments and AtNHX7/8 at the plasma membrane. With regard to members which are expressed at the vacuole, i.e. AtNHX1-4, experiments on single isoform or multiple knock-out plants (Bassil *et al.*, 2011, 2019; Barragán *et al.*, 2012) have shown that NHX1 and 2 are responsible for the observed phenotypes (plants lacking both NHX1 and 2 were severely impaired in growth and extremely sensitive to external potassium changes), while the role of NHX3 and 4 is secondary, with a likely low functional activity. Here, we used vacuoles isolated from mesophyll cells of *Arabidopsis thaliana* wild-type plants, *clc-a* and *nhx1 nhx2* mutants and, applying the patch-clamp technique combined with the fluorescence signal of the proton indicator dye BCECF, we measured the functional activity of AtNHX1 and 2 and studied their pharmacological properties. Indeed, we show that the signaling lipid phosphatidylinositol-3,5-bisphosphate ($\text{PI}(3,5)\text{P}_2$) strongly inhibits NHX activity. This novel approach, therefore, is capable to investigate electroneutral vacuolar cation/ H^+ exchange processes in unprecedented detail.

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81

82 Results

83 In order to detect H^+ -dependent ion transport across the membrane of vacuoles isolated from
84 *Arabidopsis thaliana* mesophyll cells, we combined the patch-clamp technique with fluorescence

ratio recordings using the ratiometric H^+ indicator dye BCECF (for a schematic overview on experiment preparation and execution see Suppl. Fig. S1). The ionic solution in the patch pipette contained the fluorophore (at a concentration of 10 μM), which is able to detect proton concentration changes. After gaining access to the whole-vacuole configuration, the fluorescent dye diffused into the vacuolar lumen; this loading phase lasted from 10 to 30 min (Suppl. Fig. S2) and was dependent on both the size of the vacuole, monitored by the electrical membrane capacitance (C_m), and on the access resistance (R_a). Recordings were started after this lag period when the pH inside of the vacuole was stable.

To test the reliability of our experimental system, we performed a series of experiments in which inorganic pyrophosphate (PPi) was added to the cytosolic bath solution (Fig. 1a, upper panel). Since it is known that CLC-a is an active player in modulating vacuolar pH (Carpaneto *et al.*, 2017), experiments in Fig. 1 were performed on vacuoles isolated from *clc-a* knock-out plants. Due to the well-known activity of the H^+ -pumping pyrophosphatase, PPi applied at 1, 3 and 10 μM elicited positive currents of increasing amplitudes, as shown in the middle panel of Fig. 1a, together with a contemporary increase in vacuolar proton concentration, Fig. 1a lower panel. We then applied a different experimental protocol in which the standard bath solution (cytosolic side) was changed by completely removing potassium ions, substituted by an equimolar concentration of caesium, (no K^+ + Cs^+), as shown in Fig. 1b upper panel, therefore generating a large potassium gradient (ΔK^+) from the lumen ($[K^+]_{lumen}=100$ mM) to the cytosolic side of the vacuole ($[K^+]_{cytosol}=0$ mM). The diffusion coefficient of caesium, $D(Cs^+)=2.03 \cdot 10^{-5}$ cm²/s, is very similar to that of potassium, $D(K^+)=1.96 \cdot 10^{-5}$ cm²/s (Hille, 2001); this limits unwanted artifactual effects on the recorded currents due to liquid junction potentials. The respective current signal, recorded by voltage-clamping the tonoplast at 0 mV, did not show any significant variation, Fig. 1b middle panel. However, the proton concentration inside the vacuole rose from about 140 nM (pH 6.85) to 450 nM (pH 6.35), Fig. 1b lower panel. The effect was fully reversible and the initial pH was restored after re-application of standard bath solution with 100 mM potassium. The comparison of the average values for the experiments of Fig. 1a and b is presented in Fig. 1c, d: current density

112 changes were significantly different in the two conditions (Fig. 1c), while the corresponding proton
113 concentration changes were similar (Fig. 1d).

114 The simplest explanation for this acidification is to hypothesize the presence of a potassium/proton
115 (K^+/H^+) exchange system driving protons from the cytosol to the lumen of the vacuole at the
116 expense of the potassium gradient generated under 0 K^+ conditions, i.e. a reversible K^+/H^+
117 antiporter acting in the opposite direction than would be expected in physiological conditions in
118 which the vacuole is acidic relative to the cytosol. Moreover, this antiporter is non-electrogenic,
119 with a transport stoichiometry of 1 K^+ :1 H^+ (or multiples), since no current difference could be
120 recorded after potassium substitution.

121

122 *Vacuolar acidification was dependent on the substituting cation and inhibited by benzamil*

123 To further characterize this cation antiporter, we repeated the experiments by varying the cation
124 replacing potassium in the bath solution. In Fig. 2a the use of sodium (no K^+ + Na^+), caesium (no
125 K^+ + Cs^+) or Bis-Tris Propane (no K^+ + BTP^+) had a different impact on vacuolar acidification.
126 Sodium hardly modified $[H^+]_{vac}$, while the large, membrane-impermeable cation BTP^+ induced the
127 largest effect and was chosen for the following experiments. In this condition (no K^+ + BTP^+), we
128 recorded very small currents, Suppl. Fig. S3, which were not affected by the inhibitors (see below)
129 and were also present in vacuoles isolated from *nhx1 nhx2* knock-out plants. These currents are
130 therefore not related to the phenomenology shown so far and can reasonably be attributed to the
131 movement of potassium through K^+ -selective tonoplast channels driven by the steep potassium
132 gradient present across the vacuolar membrane; this is also supported by the fact that no current
133 signal was apparent when cytosolic potassium was replaced by caesium, a well-known blocker of
134 potassium channels, as shown in Fig. 1b, middle panel. Moreover, the experiments in no K^+ + Na^+
135 condition (steep gradients of Na^+ and K^+ of equal magnitude on each side of the tonoplast; Fig. 2a)
136 suggest that, besides being non-electrogenic, the putative cation/ H^+ antiporter did not discriminate
137 between potassium and sodium, which are biophysical features observed in NHX proteins
138 (Venema *et al.*, 2002)(Hernandez *et al.*, 2009; Bassil *et al.*, 2019). Since benzamil is known as a
139 specific inhibitor of these transporters (Venema *et al.*, 2002), we added this compound at 30 μM

140 concentration in the no K^+ + BTP $^+$ solution. As shown in Fig. 2b, a significant, partially reversible,
141 reduction of K^+ -driven vacuolar acidification was apparent. Group data of these experiments are
142 presented in the histogram of Fig. 2c, suggesting that NHX antiporters may be a major player in
143 ΔK^+ -induced vacuolar acidification.

144

145 *AtNHX1 and AtNHX2 are responsible for the ΔK^+ -induced vacuolar acidification*

146 To verify if NHX antiporters played a relevant role in the above effects, we took advantage of
147 Arabidopsis mutant lines lacking the two major forms of tonoplast-localized NHXs, i.e. NHX1 and
148 NHX2 (Bassil *et al.*, 2011; Barragán *et al.*, 2012). By using BCECF-filled pipettes, we patched
149 vacuoles isolated from Arabidopsis double mutant *nhx1 nhx2* mesophyll cells. As shown in Fig. 3a,
150 the removal of cytosolic potassium (no K^+ + BTP $^+$) did not induce any $[H^+]$ increase in the vacuolar
151 lumen. Instead, cytosolic application of PPI (100 μ M) caused a very large, reversible acidification.
152 The highly significant difference between Columbia wild-type and *nhx1 nhx2* double mutant
153 vacuoles in the K^+ -driven luminal proton increases, Fig. 3b, is a strong indication that NHX1 and
154 NHX2 transporters are responsible for the K^+/H^+ exchange reported by our experimental approach.
155 Moreover, we verified that the current density of the endogenous vacuolar pyrophosphatase
156 elicited by 100 μ M PPI is not significantly different in wild-type (1.04 ± 0.11 pA/pF, n=12) and *nhx1*
157 *nhx2* double mutant (0.76 ± 0.09 pA/pF, n=9) vacuoles. Therefore, the large difference in minimum
158 luminal pH obtained by activating the pyrophosphatase, Fig. 3c, is compelling experimental
159 evidence for the strong impact of NHX activity on the vacuolar pH, as suggested by previous
160 studies (Bassil *et al.*, 2011; Andrés *et al.*, 2014).

161

162 *AtNHX1 and AtNHX2 are not voltage-dependent*

163 In order to establish if H^+/K^+ exchange activity was dependent on the tonoplast voltage, we
164 compared the acidification resulting from K^+ replacement with no K^+ + BTP solution at 0 and -40 mV,
165 Fig. 4a, left panel. In vacuoles isolated from Arabidopsis *c/c-a* knock-out mesophyll cells, BCECF
166 signals were similar in both conditions, Fig. 4b, indicating that AtNHXs were not voltage-
167 dependent. However, in vacuoles isolated from Arabidopsis wild-type, applying the same

168 experimental protocol, we detected a strong reduction of acidification at -40 mV compared to that
169 at 0 mV (Fig. 4a, right panel and Fig. 4b), an effect due to the voltage-dependent activity of CLC-a,
170 which acted to counterbalance the NHXs mediated luminal acidification (see Theory in Supporting
171 Information). These results would have been impossible to obtain by using conventional
172 techniques (see Discussion).

173

174 *AtNHX1 and AtNHX2 can mediate vacuolar alkalinisation*

175 Our experimental data suggested that ΔK^+ -induced vacuolar acidification could be associated to
176 NHX exchanger activity; however, in physiological conditions, NHX exchangers at the tonoplast
177 operate to import K^+ into vacuoles and would be expected to drive protons from the acidic vacuole
178 to the cytosol and not vice versa. Consequently, we wondered if we could also monitor this
179 physiologically meaningful transport mode using our experimental approach. Therefore, in the
180 standard pipette solution, which contained the fluorophore BCECF, potassium was replaced by
181 caesium, as described in Materials and Methods. During the experiment, the bath solution
182 (cytosolic side) containing 100 mM caesium was changed with the standard potassium solution
183 thereby generating a steep potassium gradient from the cytosol ($[K^+]_{\text{cytosol}}=100$ mM) towards the
184 lumen ($[K^+]_{\text{lumen}}=0$ mM). In turn, this gradient should drive, through NHX1/2 activity, the movement
185 of protons from the lumen to the cytosol. In agreement with this hypothesis, we detected luminal
186 alkalinisation in vacuoles isolated from wild-type Arabidopsis in contrast to a weak acidification
187 recorded in vacuoles from *nhx1 nhx2* mutant plants (Fig. 5). These results confirmed that NHXs
188 are capable of moving protons into or out of the vacuole depending on the electrochemical
189 potential of the transporter (see Theory in Supporting Information).

190

191 *Phosphoinositide inhibition of NHX activity*

192 Recently, it was hypothesized by different authors (Scholz-Starke, 2017; Pérez-Koldenkova &
193 Hatsugai, 2017) that NHX1/2 activity might be inhibited by the phosphoinositide PI(3,5)P₂, an
194 emerging tonoplast-specific phospholipid acting as a signaling molecule in plant cells (Bak *et al.*,
195 2013; Carpaneto *et al.*, 2017). Our experimental approach offered a direct way to test this idea.

196 The addition of a water-soluble form of PI(3,5)P₂ (Boccaccio *et al.*, 2014, see also Materials and
197 Methods) at a concentration of 200 nM in the no K⁺ BTP⁺ cytosolic solution induced a strong and
198 reversible reduction of the acidification response mediated by NHX1/2, as shown in Fig. 6a. We
199 obtained a similar effect, albeit slightly milder, with PI(3,4,5)P₂, but not with other two
200 phosphoinositide species tested, PI(3,4)P₂ and PI(4,5)P₂ (Fig. 6b), suggesting that the presence of
201 phosphate groups at the positions 3 and 5 of the inositol moiety is essential for NHX inhibition.
202 Therefore, besides CLC-a (Carpaneto *et al.*, 2017), PI(3,5)P₂ is also able to affect NHX1/2 activity
203 at similar, low nanomolar concentrations, as shown schematically in Fig. 6c.

204

205

206 Discussion

207 In this work, we extended the patch clamp technique applied to vacuoles isolated from Arabidopsis
208 wild-type and mutant plants by filling the patch pipette with the proton-sensitive fluorescent dye
209 BCECF. In the whole-vacuole configuration, the proton probe diffused into the vacuolar lumen
210 allowing to continuously monitor [H⁺] variations inside the vacuole. We applied a very simple
211 experimental protocol: starting from symmetrical concentration of KCl (100 mM) in the cytosolic
212 bath and luminal pipette solutions, we substituted cytosolic potassium with caesium therefore
213 generating a steep potassium gradient across the tonoplast. As a consequence, we could record a
214 significant vacuolar acidification without any measurable current change (Fig. 1b). The absence of
215 current signal was not due to a low-resolution limit in our electrophysiological system because
216 when we activated the vacuolar pyrophosphatase by adding PPi in the cytosolic bath solution,
217 proton currents could clearly be detected even with very small acidifications, Fig. 1a. Subsequent
218 experiments using Arabidopsis *nhx1 nhx2* plants definitely demonstrated that the vacuolar
219 acidification evoked by the potassium gradient was due to the reversible activity of NHX1/2
220 potassium/proton antiporters, Fig. 3a, b. Since in physiological conditions NHX1/2 catalyse active
221 K⁺ uptake into vacuoles and protons driven by these K⁺/H⁺ antiporters were expected to move from
222 the acidic vacuole to the cytosol, we verified that NHX1/2 could work in reverse mode and mediate
223 vacuolar alkalinisation (Fig. 5). Our data confirmed that NHX1/2 were non-electrogenic (Fig. 1b

middle panel) with a stoichiometry of 1 proton/ 1 potassium (or multiples), with a similar permeability for sodium and potassium ions (Fig. 2a). We also verified that they were voltage independent (Fig. 4a) and that benzamil was a specific inhibitor (Fig. 2b). Moreover, our results illustrate the capacity of NHX antiporters to significantly impact vacuolar pH, as shown in Fig 3, and in line with previous results with the *nhx1 nhx2* mutant (Andrés *et al.*, 2014).

Different techniques have previously been used to investigate the functional characteristics of NHX transporters. The impact of NHX activity on the formation or dissipation of proton gradients in tonoplast vesicles from plants or yeast (Barkla *et al.*, 1999; Hernández *et al.*, 2009; Barragán *et al.*, 2012) was monitored using the quenching of specific fluorophores. A similar approach was applied by using proteoliposomes (Venema *et al.*, 2002; Hernández *et al.*, 2009), which contained purified reconstituted NHX proteins. Although AtNHX1 was initially described as Na⁺-selective, subsequent studies have shown that tonoplast-localized NHXs are non-selective Na⁺,K⁺/H⁺ exchangers *in vitro* (Venema *et al.*, 2002; Leidi *et al.*, 2010; Barragan *et al.*, 2012; Bassil *et al.*, 2019). However, *in vivo* they are primarily involved in vacuolar K⁺ uptake (Leidi *et al.*, 2010; Bassil *et al.*, 2011; Barragan *et al.*, 2012; Andres *et al.*, 2014; Bassil *et al.*, 2019). In Arabidopsis guard cells and roots, Na⁺ uptake into intact vacuoles was largely independent of NHXs, suggesting that other unknown transporters were responsible for Na⁺ ingress (Andres *et al.*, 2014; Bassil *et al.*, 2019). Heterologous expression in mutant yeast cells represented a further method to perform functional studies on NHX transporters (Quintero *et al.*, 2002; Shi *et al.*, 2002; Yokoi *et al.*, 2002); as an example, complementation of the yeast AXT3K mutant (lacking the endogenous Na⁺ efflux proteins, ENA1-4 and NHA1, as well as the vacuolar Na⁺/H⁺ antiporter NHX1) with AtNHX1 or 2 restored the ability of the yeast cells to grow in a medium enriched with NaCl or supplemented with hygromycin B (Hernández *et al.*, 2009; Barragán *et al.*, 2012). However, these approaches rely on expression in heterologous systems, or the reconstitution in highly artificial proteoliposomes and suffer from experimental constraints inherent to the assaying method. Recently, the kinetics of NHX-mediated cation uptake has been determined using the cation-responsive fluorescent dyes PBFI (for K⁺) and SBFI (for Na⁺) loaded into intact isolated Arabidopsis vacuoles (Bassil *et al.*, 2019), but these studies are also constrained by the limited selectivity and sensitivity of these fluorochromes. In any

case, it has not been possible so far to analyze the voltage-dependence of NHX transporters, the reversibility of agonist/inhibitor effects or the interplay among different proton transporters.

The technique presented here, an extension of patch-clamp, allows an optimal control of the experimental conditions such as luminal/ cytosolic solutions and membrane voltage. The activity of proton-dependent tonoplast proteins can be followed in real time and their modulation by cytosolic factors can be easily investigated through changes in the external bath ionic solutions. By definition, in this technique the luminal pH is not buffered (BCECF, the only proton buffer present in the pipette solution, is at a concentration of 10 μ M) i.e. we do not work under vacuolar pH-clamp condition; variations of luminal pH could activate and/or deactivate other tonoplast proteins besides the transporter under investigation. The use of specific mutant plants can help to interpret data and to correctly dissect the various contributions, which impact on the vacuolar pH. A clear example of the new possibilities offered by our approach compared to the previous techniques is given in Fig. 5, where the voltage-dependence of both NHXs and CLC-a could be investigated.

Besides plant vacuoles, the patch-clamp fluorimetry presented in this work could be extended to isolated protoplasts; however, the presence of a cell wall prevents its application in intact plant cells. The use of multi-barreled microelectrodes (see as an example Wang *et al.*, 2015) together with genetically encoded pH- and ion-selective sensors (Bischof *et al.*, 2017; Behera *et al.*, 2018) could be the way to overcome this limitation.

To simulate our experimental findings, we developed a theoretical framework based on thermodynamics (see Theory in Supporting Information). We found that the kinetics of the measured signals are linked to the time required for bath perfusion and that the slower time of activation than of deactivation of the luminal proton concentration in our recordings was due to the activity of NHXs (for detail see Theory in Supporting Information).

Interestingly, we verified that phosphoinositides (PIs) could reversibly affect NHX activity. Phosphoinositides are low-abundance phospholipids which are dynamically phosphorylated/dephosphorylated at defined positions of the inositol head group by compartment-specific lipid kinases/phosphatases. PI(3,5)P₂, which is present in the membranes of yeast and plant vacuoles as well as of animal lysosomes, was a powerful inhibitor of NHX activity, as shown

in Fig. 6. Further PI species were tested and simultaneous phosphorylation at positions 3 and 5 emerged as fundamental for their effectiveness. In the yeast *S. cerevisiae*, PI(3,5)P₂ levels increased 20-fold within minutes of hyperosmotic stress to elicit changes in the vacuolar volume (Bonangelino *et al.*, 2002). The vacuolar morphology of wild-type cells changed from a single large vacuole to smaller and highly fragmented vacuoles, whereas the vacuole of mutants unable to synthesize PI(3,5)P₂ remained unresponsive. Since the activity of yeast NHX1 is required for the fusion of vacuolar vesicles and *nhx1* mutants have fragmented vacuoles (Qiu & Fratti, 2010), the inhibition of NHX1 by PI(3,5)P₂ would be coherent with the osmotic induced vacuolar fragmentation. A recent study showed that negative regulation of the yeast cation/H⁺ antiporter VNX1 by PI(3,5)P₂ contributes to the control of vacuolar K⁺ fluxes in yeast cells exposed to osmotic stress (Wilson *et al.*, 2018). In the light of the results presented here, it is possible that yeast VNX1 is indeed a direct target of PI(3,5)P₂ signaling. The Arabidopsis exchangers NHX1 and NHX2, which we show here are targeted by PI(3,5)P₂, are critically involved in the dynamic changes of volume and morphology (convolution rather than fragmentation) of guard cell vacuoles during stomatal movements (Andrés *et al.*, 2014). Mutants in the FAB1 kinases that synthesize PI(3,5)P₂ display aberrant vacuolar morphology (Whitley *et al.*, 2009; Hirano *et al.*, 2011), and PI(3,5)P₂ is required for ABA-induced vacuolar convolution and stomata closure (Bak *et al.*, 2013). Together, these data indicate an evolutionarily conserved role of PI(3,5)P₂ in regulating vacuolar dynamics through the regulation of ion exchangers at the tonoplast (Pérez-Koldenkova & Hatsugai, 2017). It remains to be determined whether the endosomal non-vacuolar isoforms NHX5 and NHX6, or the plasma membrane localized NHX7/SOS1 and NHX8, are also responsive to PI(3,5)P₂. In contrast to vacuolar PI(3,5)P₂, PI(4,5)P₂ is preferentially produced at the plasma membrane in response to salinity stress (van Leeuwen *et al.*, 2007), and we have shown here that PI(4,5)P₂ had little effect to modulate the vacuolar proteins NHX1 and NHX2. However, PI(4,5)P₂ is a known regulator of the mammalian plasma membrane Na⁺/H⁺ exchanger NHE1 (reviewed by Hendus-Altenburger *et al.*, 2014), which is the animal ortholog of NHX7/SOS1. It would be interesting to test whether PI(4,5)P₂ specifically regulates the plasma membrane protein NHX7/SOS1. Of note is that phosphatidic acid produced by phospholipase PLD α in response to salt stress activates MPK6,

308 which in turn phosphorylates and likely activates NHX7/SOS1 (Yu *et al.*, 2010), whereas ScNHX1
309 of *S. cerevisiae* and EcNHX1 from the plant *Eschscholzia californica* (California golden poppy) are
310 stimulated by lysophosphatidylcholine, another signaling lipid that is produced by plasma
311 membrane-localized phospholipase A2 (Weigl *et al.*, 2016). These findings suggest that regulation
312 of NHX exchangers by bioactive lipids, either directly or indirectly, is a common and physiologically
313 relevant feature.

314 Since PI(3,5)P₂ applied at similar concentration was recently found to inhibit the activity of the
315 proton/anion antiporter CLC-a (Carpaneto *et al.*, 2017), we can speculate that this protein could
316 play its physiological functions together with NHXs. Their combined activity, driven by the proton
317 motive force created by the two tonoplast proton pumps, the V-ATPase and the H⁺-
318 pyrophosphatase, has the capability to accumulate potassium-anion salts inside the vacuole. We
319 can, then, define a Salt Accumulation Unit (SAU) formed by CLC-a with NHXs, Fig. 6c, whose
320 capacity to increase osmolytes inside the vacuole is modulated by PI(3,5)P₂. We may therefore
321 expect that during ABA-induced stomatal closure, when PI(3,5)P₂ is thought to increase in guard
322 cells (Bak *et al.*, 2013), SAU activity is inhibited to avoid futile osmolyte cycling and to allow for
323 efficient cation and anion release from the vacuole together with water, which would prevent a
324 potentially damaging dehydration of the cytosol. As a consequence of the reduced activity of both
325 CLC-a and NHXs, vacuolar pH decreases during stomatal closure (Bak *et al.*, 2013).

326 In summary, in this work, we show that the parallel use of electrophysiology and H⁺ detection by
327 fluorescence has the potentiality to unravel new physiological features in proton-coupled transport
328 in plant vacuoles.

329

330

331 **Materials and methods**

332 Suppl. Fig. S1 shows a schematic presentation of the materials and methods used in this work.

333 *Plant material*

334 Plants of *Arabidopsis thaliana* *clc-a* knock-out, Columbia-0 wild-type, *nhx1 nhx2* knock-out were
335 grown on soil in a growth chamber at 22 °C and 8 h light/16 h dark regime. For isolation of

mesophyll protoplasts, leaves coming from 3-5 week old plants, were cut in strips, placed in a Petri dish and enzymatically digested for 1 h at 22°C in the dark. The enzyme solution contained: 0.5% Cellulase Onozuka R-10 (Yakult), 0.05% Pectolyase Y-23 (Seishin), 0.25% Macerozyme R-10, 1mM CaCl₂, 500 mM D-sorbitol, 10 mM MES, pH 5.5. The protoplast suspension was filtered through 50 µm nylon mesh, subsequently centrifuged and washed twice in the so-called W5 (see Yoo *et al.*, 2007) solution containing (in mM): 125 CaCl₂, 154 NaCl, 5 KCl, 2 MES-KOH, 5 glucose, pH 5.6 and 640 mOsm. The protoplast pellet was suspended in W5 solution and maintained at 4°C for 1-3 days. Just before patch-clamp experiments, 20-30 microliters of W5 solution containing the protoplasts were placed in the recording chamber and exposed to the vacuolar release solution (VRS) containing (in mM): 100 malic acid, 155 Bis-tris-Propane (BTP), 5 ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), 3 MgCl₂, 200 D-sorbitol, pH 7.33. Vacuoles were efficiently released from the protoplasts within 3-5 min.

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349

350 *Patch-clamp recordings*

351 Since CLC-a is known to affect vacuolar pH, to measure *bona fide* NHX activity, *clc-a* knock-out
352 plants were selected to perform experiments, unless otherwise indicated.

353 Patch-clamp recordings were performed in the whole-vacuole configuration. The standard pipette
354 solution contained (in mM): 100 KCl, 3 MgCl₂, 10 µM 2',7'-bis-(2-carboxyethyl)-5-(and-6)-
355 carboxyfluorescein (BCECF), 240 D-sorbitol, pH 7.0 (with KOH). The standard cytosolic bath
356 solution contained (in mM): 100 KCl, 3 MgCl₂, 5 Hepes, 280 D-sorbitol, pH 7.2 (with KOH). To
357 establish a steep potassium gradient between the vacuolar lumen and the cytosol, potassium in
358 the standard bath solution was replaced by equimolar concentration of sodium (no K⁺ + Na⁺),
359 caesium (no K⁺ + Cs⁺) or Bis-Tris Propane abbreviated to BTP (no K⁺ + BTP⁺) and the relative
360 hydroxide was used for pH buffering. In the case of BTP, which is itself a proton buffer, the solution
361 was: (in mM): 100 HCl, 69 BTP, 3 MgCl₂, 300 D-sorbitol, pH 7.2.

362 To test the reliability of the system to detect patch-clamp currents and pH changes at the same
363 time, vacuolar proton-pumping pyrophosphatase was activated by inorganic pyrophosphate (PPi).

Potassium pyrophosphate was prepared as a 100 mM aqueous stock solution, stored at -20°C and dissolved in the standard bath solution at the desired concentration.

Benzamil hydrochloride was prepared as a 50 mM DMSO stock solution. It was stored at -20°C and freshly dissolved at 30 μ M concentration in no K^+ + BTP⁺ solution. Water-soluble dioctanoyl esters (diC8) of PI(4,5)P₂, PI(3,4)P₂, PI(3,4,5)P₃, PI(3,5)P₂ (Echelon Biosciences Inc, USA) were prepared as 1 mM aqueous stock solutions. They were stored at -20°C and dissolved at 200 nM concentration in no K^+ + BTP⁺ solution just before use.

In the experiments of Fig. 5, the pipette solution contained (in mM): 100 CsCl, 3 MgCl₂, 10 μ M BCECF, 240 D-sorbitol, pH 7.0 (with CsOH). The cytosolic bath solution contained (in mM): 100 CsCl, 3 MgCl₂, 5 Hepes, 280 D-sorbitol, pH 7.2 (with CsOH) and was substituted by the standard bath solution in order to establish a steep potassium gradient between the cytosol and the vacuolar lumen. Patch pipettes were pulled from borosilicate glass capillaries (Harvard Apparatus, United States) and had resistances of 3-5 M Ω . For patch-clamp experiments, vacuoles were placed in the recording chamber and observed by an inverted microscope (Zeiss, Germany) with a 40 $\times$ objective (overall magnification 400 $\times$). Currents were recorded with a List EPC-7 amplifier (Germany) at holding potential of 0 mV or, when specified, -40 mV, filtered with a low pass Bessel filter (Sakmann & Neher, 1995) and controlled by the acquisition program Pulse (Heka, Germany). The perfusion system was optimized to change the bath solution in about 1 min and consisted of a gravity perfusion system for inflow and a peristaltic pump for outflow (Gilson). The effects of perfusion on the kinetics of the luminal proton concentration are treated in detail in the Theory section. In the main figures (from Fig. 1 to Fig. 6), traces of pyrophosphate or cytosolic potassium concentrations (drawn as dotted lines) just indicates the switching of the corresponding bath solution. Membrane capacitance (C_v) and access resistance (R_a) could be estimated by the current transient elicited by a 10 mV voltage pulse (Sakmann & Neher, 1995). By convention, positive currents correspond to cations moving from the cytosolic side to the vacuolar lumen.

BCECF Fluorescence

391 After reaching the whole-vacuole configuration, vacuole loading by BCECF took from 10 to 30 min,
392 as shown in Suppl. Fig. S2 (diffusion from the patch pipette to the whole cell-wall-free protoplasts
393 was also investigated by Goh *et al.*, 2002 and cited papers). Simultaneous whole-vacuole patch-
394 clamp recordings and fluorescence detection were then performed. Excitation light, alternately
395 selected by a monochromator (Cairn Research, UK) at 490 and 440 nm in 100 ms excitation
396 cycles, was generated by a Xenon arc lamp (PTI). Fluorescence emission was detected using a
397 515-nm bandpass emission filter and recorded every 300 ms by a CCD camera (Roper Scientific,
398 Germany) mounted on an inverted microscope (Axiovert, Zeiss, Germany). The fluorescence ratio
399 (490/440) was calculated online in a selected circular ROI (region of interest) placed inside the
400 vacuole, as shown in Suppl. Fig. S4a. Background fluorescence was usually negligible, or,
401 otherwise, was subtracted off-line. In our experimental conditions, BCECF signals were very stable
402 over long period, up to 4500 s after the loading phase, see as an example Suppl. Fig. S4b, thus
403 indicating negligible photobleaching.

404 For calibration of the fluorescence ratios, a 50 mM buffer at a fixed pH was added to the vacuolar
405 solution containing 10 μ M BCECF (sequentially: HEPES at pH 7, MES at pH 6.5, MES at pH 5.5
406 and Citrate at pH 3.5) and the fluorescence of a 500 μ l droplet of the buffered solution was
407 recorded. The surface of the droplet was flat to avoid undesired light scattering and reflection. A
408 plot of proton concentration vs fluorescence ratio was generated and data were fitted with an
409 exponential function, Suppl. Fig. S5. Parameters of the fit were used to convert experimental
410 fluorescence ratios into proton concentration unit. Previous data (see Fig. EV3 in Carpaneto *et al.*,
411 2017) ensure that the same type of calibration performed in whole-vacuole configuration would
412 have produced similar conversion values.

413

414 *Statistical analysis*

415 All results were gained from $\geq$ three independent experiments. Results are given as mean $\pm$
416 standard error (SE). For statistical analysis and graph preparations the software Igor Pro
417 (WaveMetrics, Inc., Lake Oswego, Oregon, USA) and Excel (Microsoft Corp. Redmond,

418 Washington, USA) were used. Student's t test was used for statistical comparison between means
419 where applicable; *P < 0.05; **P < 0.01; ***P < 0.001.

420

421

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428

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537 **Supporting Information**

538 **Theory**

539 The objective of this theoretical part is to highlight the fundamental elements that regulate the
540 proton concentration in the vacuolar lumen. We will use a thermodynamic approach, which avoids
541 complex and detailed mathematical formalism.

542

543 **Proton pyrophosphatase and acidification**

544 We indicate with I_{dp} the current density (pA/pF) associated with H^+ -pyrophosphatase. If the vacuole
545 has a capacitance of C_v (pF) then the current of the pyrophosphatase will be $I_p = I_{dp} C_v$ (pA). The
546 number of H^+ moles entering into the vacuole per second (n_p) is equal to I_p / F (pmol/s), where F is
547 the Faraday constant, $9.65 \cdot 10^4$ (C/mol). The increase of luminal proton concentration per second
548 due to the activity of H^+ -pyrophosphatase is equal to $c_p = n_p / Vol$ (M/s), where Vol is the vacuolar
549 volume. If we assume that the specific capacitance (C_s) of the vacuole is $1 \mu F/cm^2$ or $0.01 pF/\mu m^2$,
550 the surface (S), the radius (r) and the volume of the vacuole will be, respectively: $S = C_v /$
551 C_s (μm^2), $r = 5\sqrt{C_v/\pi}$ (μm), $Vol = \frac{(C_v)^{3/2}}{6\sqrt{\pi}}$ (pL). We obtain: $c_p = 6 \sqrt{\frac{\pi}{C_v}} \frac{I_{dp}}{F} = 110 \frac{I_{dp}}{\sqrt{C_v}}$ ($\mu M/s$). The
552 proton concentration inside the vacuole, which we indicate with c , can be calculated by using the
553 following equation:

$$554 \quad \frac{dc}{dt} = c_p \quad (1)$$

555 To estimate c_p , we consider data shown from Fig. 1. At PPI is $10 \mu M$, the current was $19 pA$, the
556 capacitance of the vacuole was $37 pF$; therefore $I_{dp} = 0.513$ (pA/pF) and $c_p = 9.30$ ($\mu M/s$). If we
557 neglect the time of perfusion and consider c_p as a constant, then the solution of equation (1), for
558 $t_0 \leq t \leq t_1$ (with t_0 and t_1 respectively 100 and 200 s), with $c_{p0} = 63.1$ nM (pH=7.2), is

$$559 \quad c(t) = c_p (t - t_0) + c_{p0} \quad (2)$$

560 and is displayed in Suppl. Fig. S6a (lower panel, $H^+ = c$). The concentration of PPI and the relative
561 current vs time are shown respectively in the upper and middle panels. The luminal concentration
562 of protons increases linearly and reaches a very high value, $930 \mu M$, which corresponds to pH
563 3.03. In Suppl. Fig. S6b, the proton concentration is transformed in pH units.

564

565 **The role of the patch pipette**

566 When we apply the patch-clamp technique in the whole-vacuole configuration and activate the
567 proton current by adding pyrophosphate in the bath solution, protons moving from the cytosol to
568 the lumen are not trapped inside the vacuole, as happened in the previous case, but can escape
569 into the pipette. The equation describing this condition is the following:

$$570 \quad \frac{dc}{dt} + \frac{c}{\tau} = c_P \quad (3)$$

571 where $\tau = R_S V/D$, R_S is the access resistance of the pipette, V the volume of the pipette, D the
572 diffusion coefficient for protons, ρ the resistivity of the pipette solution. For $R_S = 10$ MOhm, $V = 21.7$ pl
573 (for a vacuole with a capacitance of $C_V = 37$ pF, see above), $D = 9.31 \cdot 10^{-5}$ cm²/s, $\rho = 80$ Ohm cm,
574 the time constant, τ , is 28.4 s. If we assume that the pipette has a proton concentration of $c_{pip} =$
575 63.1 nM (pH=7.2), which can be considered as a constant because of the large size of the pipette
576 volume, equation (3) is modified as follow:

$$577 \quad \frac{dc}{dt} + \frac{c - c_{pip}}{\tau} = c_P \quad (4)$$

578 the solution of (4) for $t_0 \leq t \leq t_1$ (with t_0 and t_1 are respectively 100 and 200 s, see Suppl. Fig. S6c)
579 is:

$$580 \quad c(t) = c_{pip} + \tau c_P \left(1 - e^{-\frac{t-t_0}{\tau}}\right) \quad (5)$$

581 for $t > t_1$ we obtain:

$$582 \quad c(t) = (c(t_1) - c_{pip})e^{-\frac{t-t_1}{\tau}} + c_{pip} \quad (6)$$

583 The solutions (5) and (6) of equation (4) are shown in Suppl. Fig. S6c; different from Suppl. Fig.
584 S6a, due to the presence of the pipette, the proton concentration tends to reach a stationary value
585 of about 250 μ M, also apparent in Suppl. Fig. S6d where the concentration of proton c is
586 transformed in pH units.

587

588 **NHX transporters**

589 The difference in Gibbs free energy for NHX antiporters is:

$$\Delta G_N = n_H \Delta \mu_H - n_K \Delta \mu_K \quad (7)$$

where n_H and n_K are the number of moles of proton and potassium exchanged through the tonoplast, $\Delta \mu_H = \mu_H^{cyt} - \mu_H^{lum}$ and $\Delta \mu_K = \mu_K^{cyt} - \mu_K^{lum}$ the difference between the cytosolic and luminal electrochemical potential respectively for proton and potassium ions. The minus sign in equation 7 is justified by the antiporter nature of NHXs and since NHXs are not electrogenic, see Fig. 1b middle panel, we put $n_H=n_K=n$. We obtain:

$$\Delta G_N = nRT \ln \left(\frac{[H^+]_{cyt}[K^+]_{lum}}{[H^+]_{lum}[K^+]_{cyt}} \right) \quad (8)$$

where R and T have the usual meaning. We assume that the change in luminal proton concentration per unit of time (c_N) due to the proton movement mediated by NHXs is proportional to ΔG_N :

$$c_N = k_N \ln \left(\frac{[H^+]_{cyt}[K^+]_{lum}}{c[K^+]_{cyt}} \right) \quad (9)$$

where we consider k_N as a constant (in principle, it should be dependent on vacuolar and cytosolic potassium and protons) and, as before, we use the symbol c to indicate the luminal proton concentration. Equation 9 is the simplest equation for NHX activity, is clearly valid at equilibrium and irrespective of the details of ion permeation and coupling. Any other equation we can write to describe NHX activity must include equation 9. Inserting equation 9 into equation 4, we obtain:

$$\frac{dc}{dt} + \frac{c - c_{pip}}{\tau} = c_P + c_N \quad (10)$$

To evaluate k_N , we observe that in Fig. 1a, when PPI = 10 μ M, the luminal proton concentration reaches a stationary value, c_∞ , of about 400 nM. If we consider equation 10 for $t \rightarrow \infty$ we have:

$$\frac{c_\infty - c_{pip}}{\tau} = c_P + k_N \ln \left(\frac{[H^+]_{cyt}[K^+]_{lum}}{c_\infty[K^+]_{cyt}} \right) \quad (11)$$

Therefore

$$k_N = \frac{\frac{c_\infty - c_{pip}}{\tau} - c_P}{\ln \left(\frac{[H^+]_{cyt}[K^+]_{lum}}{c_\infty[K^+]_{cyt}} \right)} \quad (12)$$

With $c_\infty = 400$ nM, $c_{pip} = 63.1$ nM, $\tau = 28.4$ s, $c_P = 9.3$ μ M/s, $[H^+]_{cyt} = 63.1$ nM, $[K^+]_{cyt} = [K^+]_{lum} = 100$ mM, we can calculate that $k_N = 5.0$ μ M/s. Simulation of equation 10, which was solved numerically

by using a Runge-Kutta algorithm as implemented in Igor Pro software (WaveMetrics, Inc., Lake Oswego, Oregon, USA), is presented in Suppl. Fig. S7a. The luminal proton concentration increased very fast after activation of the PPase. When the pump is switched off, $[H^+]_{lum}$ goes back, again very fast, to its resting value. Suppl. Fig. S7b represents the corresponding pH values. Comparing Suppl. Fig. S6a with Suppl. Fig. S7a shows that the presence of NHXs has two effects: a dramatic reduction of the stationary luminal proton concentration and a faster time of action. From a physical point of view, these differences can be justified by the fact that, when NHXs are absent, protons can escape only inside the pipette while the presence of NHX allows the protons to move through all the vacuolar membrane. From a mathematical point of view, the term $(c-c_{pip})/\tau$, the contribution of the pipette, is much smaller than c_N , the contribution of NHXs. To correctly explain the kinetics of Fig. 1a, we need to insert in our simulations the time of bath perfusion. Vacuoles are not firmly attached to the bottom of the chamber, therefore we designed an experimental chamber capable of maximizing the perfusion rate with minimal perturbation of the vacuole. We estimate that the time constant of our perfusion system ranges from a minimum of 10 s to a maximum of 30 s. If we assume that when we add/remove pyrophosphate in the external bath solution, its concentration increases/decreases according to an exponential time course with the minimum time constant of 10 s and that the corresponding pyrophosphatase current follows the same trend, the luminal concentration of protons becomes time-dependent as shown in Suppl. Fig. S7c (in Suppl. Fig. S7d data were displayed in pH units), similar to the experiment shown in Fig. 1a.

634 *Changing the cytosolic potassium concentration*

To study the functional characteristics of NHX transporters, we imposed a steep cytosol-directed potassium gradient and performed experiments on vacuoles from Arabidopsis *c/c-a* knock out plants. The equation that simulates this condition is:

$$638 \quad \frac{dc}{dt} + \frac{c-c_{pip}}{\tau} = c_N \quad (13)$$

with c_N given by equation 9. To achieve an acidification consistent with our experimental results, we reduce the cytosolic potassium from 100 to 5 mM, as shown in the top panel of Suppl. Fig. S8a. This approximation is necessary because equation 9 predicts a value of c which tends to infinity for

642 $[K^+]_{cyt}$ which tends to zero. When the cytosolic change of potassium is instantaneous, the
 643 concentration of protons in the lumen also changes very rapidly (black traces of Suppl. Fig. S8a).
 644 In the same figure, the blue and red traces were calculated considering the minimum (time
 645 constant of 10 s, blue traces) and maximum (time constant of 30 s, red traces) time of perfusion
 646 necessary to change the cytosolic solution with different potassium concentration. In Suppl. Fig.
 647 S8b, the traces, corresponding to the perfusion time constant of 10 s, of the cytosolic potassium
 648 and the vacuolar proton concentrations were superimposed. It is clear that the variations over time
 649 of the vacuolar concentration of protons are different from those of cytosolic potassium. When the
 650 potassium concentration decreases from 100 to 5 mM (at t between 100 and 200 s), the relative
 651 proton concentration increases from 63.1 nM to 1.2 μ M slower than the decrease in K^+ . On the
 652 other hand, when K^+ increases from 5 to 100 mM (for $t > 200$ s) protons go back to 63.1 nM more
 653 rapidly than the trend of potassium. Therefore the kinetics in our experiments is not only related to
 654 the time of perfusion but depend on the coupling mechanism of potassium and protons mediated
 655 by NHXs transporters. In fact, if we consider equation 8 at equilibrium, we get:

$$656 \quad \frac{[H^+]_{cyt}[K^+]_{lum}}{[H^+]_{lum}[K^+]_{cyt}} = 1 \quad (14)$$

657 Change of cytosolic potassium concentration due to the perfusion system can be described by the
 658 following equation:

$$659 \quad [K^+]_{cyt} = ([K^+]_i - [K^+]_f)e^{-\frac{t-t_0}{\tau}} + [K^+]_f \quad (15)$$

660 where $\tau=10$ s. When $t_0=100$ s and $100 \text{ s} \leq t < 200 \text{ s}$ then $[K^+]_i=100$ mM e $[K^+]_f = 5$ mM; when $t_0 =$
 661 200 s and $t \geq 200$ s then $[K^+]_i=5$ mM e $[K^+]_f=100$ mM. Inserting 15 in 14, we obtain:

$$662 \quad [H^+]_{lum} = \frac{[H^+]_{cyt}[K^+]_{lum}}{([K^+]_i - [K^+]_f)e^{-\frac{t-t_0}{\tau}} + [K^+]_f} \quad (16)$$

663 where $[H^+]_{cyt}=63.1$ nM e $[K^+]_{lum}=100$ mM. This equation, in which $[H^+]_{lum}$ and $[K^+]_{cyt}$ are inversely
 664 proportional, predicts that any concentration increase for one of these two ions corresponds to a
 665 faster decrease for the other ion. Conversely, a decrease of one of the two ions corresponds to a
 666 slower increase of the other ion. A comparison between equation 16 and solution of equation 13 is
 667 presented in Suppl. Fig. S8c. The two curves are very similar; it can be observed that the steady
 668 state of equation 16 is higher than the curve obtained with equation 13, in accordance with the fact

669 that the solution of equation 13 considers that protons that can enter inside the pipette. However,
 670 the contribution of the pipette is small.

671

672 **Effect of the tonoplast voltage: role of the proton/anion antiporter CLCa**

673 When we apply a voltage of -40 mV to the vacuolar membrane and simulate equation 13, there is
 674 no change in the acidification induced by variations of cytosolic potassium, as shown in Suppl. Fig.
 675 S9a. In equation 13, in fact, parameters are all independent of the membrane potential. This
 676 situation indicates the voltage-independent activity of the NHX transporters and describes the
 677 experimental results of Fig. 4a, obtained in *clc-a* plants. To simulate the voltage dependence
 678 observed in Fig. 4b in wild-type Arabidopsis plants, we calculate the difference in Gibbs free
 679 energy (ΔG_C) for the proton/anion CLC-a antiporter:

$$680 \quad \Delta G_C = n_H \Delta \mu_H - n_A \Delta \mu_A \quad (17)$$

681 where n_H and n_A are the number of moles of proton and anions (chloride) exchanged through the
 682 tonoplast, $\Delta \mu_H = \mu_H^{cyt} - \mu_H^{lum}$ and $\Delta \mu_A = \mu_A^{cyt} - \mu_A^{lum}$ the difference between the cytosolic and
 683 luminal electrochemical potential respectively for proton and chloride ions. We obtain:

$$684 \quad \Delta G_C = (n_H + n_A) FV + RT \ln \left[\left(\frac{[H^+]_{cyt}}{[H^+]_{lum}} \right)^{n_H} \left(\frac{[Cl^-]_{lum}}{[Cl^-]_{cyt}} \right)^{n_A} \right] \quad (18)$$

685

686 In our case $[Cl^-]_{lum} = [Cl^-]_{cyt} = 100 \text{ mM}$. If we assume that the change in luminal proton
 687 concentration per unit of time (c_C) due to the proton movement mediated by CLCa is proportional
 688 to ΔG_C , we obtain:

$$689 \quad c_C = k_C \left[\frac{V}{V_0} + \ln \left(\frac{[H^+]_{cyt}}{c} \right) \right] \quad (19)$$

690 If we consider that $n_H/n_A=0.5$ (see De Angeli *et al.*, 2006) we obtain $V_0 = \frac{n_H/n_A}{n_H/n_A+1} \frac{RT}{F} = 8.42 \text{ mV}$ at a
 691 temperature of 20 °C. As usual, we use the symbol c to indicate the luminal proton concentration
 692 and we assume that k_C is a constant. Therefore, in wild-type plants, the equation modelling proton
 693 movement is:

$$694 \quad \frac{dc}{dt} + \frac{c - c_{pip}}{\tau} = c_N + c_C \quad (20)$$

695 If we consider equation 20 for $t \rightarrow \infty$ and $V=0$ mV we have:

$$696 \quad k_C = \frac{c_\infty - c_{pip}}{\tau \ln\left(\frac{[H^+]_{cyt}}{c_\infty}\right)} - k_N \left(1 + \frac{\ln\left(\frac{[K^+]_{lum}}{[K^+]_{cyt}}\right)}{\ln\left(\frac{[H^+]_{cyt}}{c_\infty}\right)} \right) \quad (21)$$

697 We assume that $\tau=28.4$ s, $c_{pip} = [H^+]_{cyt} = 63.1$ nM, $[K^+]_{lum} = 100$ mM, $[K^+]_{cyt}=5$ mM, $c_\infty=630$ nM (see
 698 Fig. 4b) and, therefore, we obtain $k_C = 1.5$ μ M/s. Simulation of equation 20 is presented in Suppl.
 699 Fig. S9b and correctly describes the voltage dependence of the luminal proton concentration in
 700 wild-type plants shown in Fig. 4b.

701

702 **Effect of inhibitors**

703 Since the experiments with inhibitors were performed in *c/c-a* plants, we use equation 13, which
 704 we modify as follows:

$$705 \quad \frac{dc}{dt} + \frac{c - c_{pip}}{\tau} = c_{N,I} \quad (22)$$

706 where

$$707 \quad c_{N,I} = k_N \frac{1}{1+[I]/K_I} \ln\left(\frac{[H^+]_{cyt}[K^+]_{lum}}{c[K^+]_{cyt}}\right) \quad (23)$$

708 The effect of the inhibitor is expressed in the “Michaelis-Menten” term $1/(1+[I]/K_I)$, where $[I]$ is the
 709 concentration of the inhibitor and K_I its apparent affinity constant. Simulation of equation 22 is
 710 presented in Suppl. Fig. S9c; in Suppl. Fig. S9d traces in the absence (control, red trace) and in
 711 the presence of the inhibitor (inhibitor, black trace) were superimposed: in agreement with our
 712 experimental results of Fig. 2b and Fig 6a, the luminal proton concentration in the presence of the
 713 inhibitor has a slower time of deactivation than the correspondent trace recorded in control
 714 condition.

715

716

717

718 **Figure legends**

719

720 **Figure 1 Vacuolar acidification induced by a cytosol-directed potassium gradient.**

721 (a) Upon PPI addition at concentrations of 1, 3 and 10 μM to the bath solution (upper panel), time
722 course of vacuolar membrane current (middle panel) was recorded by patch-clamp and luminal
723 proton concentration (lower panel) was evaluated by BCECF fluorescence ratio signal (see
724 Materials and Methods for detail). Jumps in the dotted lines indicate the switching of the bath
725 solution irrespective to the real change due to the perfusion system (see Theory section and
726 Materials and Methods for a discussion about the effects of the perfusion).

727 (b) When in the cytosolic bath solution potassium was removed (upper panel, no K^+ + Cs^+ , 100 mM
728 K^+ was substituted by an equimolar amount of Cs^+) and patch clamp was applied on a vacuole
729 isolated from Arabidopsis mesophyll cells, no change in background current was apparent (middle
730 panel); the concentration of luminal potassium (upper panel, K^+ 100) was 100 mM. However, the
731 proton sensitive fluorescent dye BCECF loaded inside the vacuole through the patch pipette
732 indicated a significant acidification (lower panel).

733 (c) Average current changes, normalized to the vacuolar capacitance ($\Delta I/C$), induced by removal of
734 cytosolic potassium (no K^+ + Cs^+ , $n=4$) or by the addition of 10 μM pyrophosphate (PPI, $n=3$).
735 *** $P<0.001$.

736 (d) Comparison of the average increase in proton concentration by removal of cytosolic potassium
737 (no K^+ + Cs^+ , $n=4$) and by addition on the cytosolic bath solution of 10 μM pyrophosphate (PPI,
738 $n=3$).

739 Measurements were performed on vacuoles isolated from Arabidopsis *c/c-a* knock-out plants.

740

741 **Figure 2 Modulation of the ΔK^+ -mediated vacuolar acidification**

742 (a) Potassium in the standard cytosolic bath solution was replaced with an equal concentration of
743 sodium (no K^+ + Na^+), caesium (no K^+ + Cs^+) or BTP (no K^+ + BTP^+). The corresponding vacuolar
744 acidification was shown in the lower panel. (b) When 30 μM benzamil was added to the solution
745 without potassium (no K^+ + BTP^+ solution), a significant reduction of the vacuolar acidification was

recorded. The effect was partially reversible as shown by the increase in $[H^+]_{vac}$ after perfusion with the ΔK^+ solution without the inhibitor. (c) Variation in vacuolar proton concentration ($\Delta[H^+]_{vac}$) normalized to the change in vacuolar proton concentration ($\Delta[H^+]_{BTP}$) mediated by application of no K^+ + BTP⁺ solution. Potassium in standard cytosolic bath solution was replaced respectively by sodium (no K^+ + Na^+ , n=8) and caesium (no K^+ + Cs^+ , n=4) or 30 μ M Benzamil was added in no K^+ + BTP⁺ solution (n=5). *** $P < 0.001$. The average value of $\Delta[H^+]_{BTP}$ was $7.3 \pm 0.7 \cdot 10^{-7}$ M (n=23). Measurements were performed on vacuoles isolated from Arabidopsis *c/lc-a* knock-out plants.

Figure 3 The ΔK^+ -induced vacuolar acidification was mediated by AtNHX1 and AtNHX2

(a) No significant change in proton luminal concentration was observed when potassium was replaced by BTP (no K^+ + BTP⁺, blue line) in the solution bathing a patched vacuole isolated from mesophyll cells of Arabidopsis double mutant *nhx1 nhx2* plants; when 100 μ M PPi was added to the standard control solution, a strong acidification was apparent. (b) Change in proton concentration mediated by the application of no K^+ + BTP⁺ solution in vacuoles from Columbia (WT) and double mutant *nhx1 nhx2* (*nhx1 nhx2*) plants (WT n=5, *nhx1 nhx2* n=9). *** $P < 0.001$. (c) Comparison of the minimum pH detected in vacuoles from Columbia (WT) and double mutant *nhx1 nhx2* (*nhx1 nhx2*) plants after addition of 100 μ M PPi to the standard control solution (WT n=12, *nhx1 nhx2* n=9). *** $P < 0.001$.

Figure 4 Voltage-independence of the ΔK^+ -mediated vacuolar acidification

(a) The increase in proton luminal concentration observed when potassium in the bath solution was replaced with BTP did not change varying the holding potential from 0 mV to -40 mV in a patched vacuole isolated from Arabidopsis *c/lc-a* knock-out. (b) A significant reduction of vacuolar acidification was detected in the same conditions of (a) in a vacuole isolated from Arabidopsis WT. (c) Change in vacuolar proton concentration ($\Delta[H^+]_{vac}$) at -40 mV normalized to the change in vacuolar proton concentration at 0 mV in vacuoles respectively from Arabidopsis *c/lc-a* knock-out (*c/lc-a*, n=5) and Columbia (WT, n=5). Values were significantly different (*** $P < 0.001$).

774 **Figure 5 AtNHX1 and AtNHX2 can mediate vacuolar alkalinisation induced by a vacuolar-**
775 **directed potassium gradient**

776 (a) In the absence of vacuolar potassium, a decrease in proton vacuolar concentration was
777 detected when the bath solution containing caesium (no K^+ + Cs^+ , see Materials and Methods) was
778 replaced with the standard, 100 mM potassium, bath solution. Data from vacuoles isolated from
779 Arabidopsis Columbia (WT). (b) When similar experiments were performed in vacuoles from
780 Arabidopsis *nhx1 nhx2* double mutant no alkalinisation was apparent. (c) Change in vacuolar
781 proton concentration mediated by the replacement of caesium with potassium in the bath solution
782 respectively in vacuoles isolated from Arabidopsis Columbia (WT, n=8) and Arabidopsis *nhx1 nhx2*
783 double mutant (*nhx1 nhx2*, n=3). Values were significantly different (** $P < 0.01$).

784

785 **Figure 6 NHX activity was inhibited by phosphoinositides**

786 (a) The presence of 200 nM PI(3,5)P₂ in no K^+ + BTP⁺ solution caused a strong and reversible
787 inhibition of the vacuolar acidification mediated by NHX activity. (b) Change in proton concentration
788 induced by the application of the no K^+ + BTP⁺ solution in which different phosphoinositides were
789 added at a concentration of 200 nM. Data were normalized to control (no K^+ + BTP⁺) condition
790 (PI(4,5)P₂ n=4; PI(3,4)P₂ n=4; PI(3,4,5)P₃ n=6; PI(3,5)P₂ n=7). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.
791 Measurements were performed on vacuoles isolated from Arabidopsis *clc-a* knock-out plants. (c)
792 Schematic representation of the key players in vacuolar salt uptake. NHXs together with CLC-a
793 form a Salt Accumulation Unit (SAU). V-ATPase: vacuolar H⁺-ATPase; V-PPiase: vacuolar H⁺-
794 pyrophosphatase. A⁻, H⁺ and K⁺ indicates respectively anions, protons and potassium ions; the
795 dimension of the letters is proportional to their concentration.

796

797 **Supplemental Figure legends**

798

799 **Supplemental Figure S1 Schematic drawing of experimental preparation and set-up**

800 The various steps of the experimental preparation are schematically shown in the upper and
801 middle panels: the isolation of mesophyll protoplasts from *Arabidopsis thaliana*; the preparation
802 and calibration of the BCECF fluorophore; the pulling of patch pipettes from borosilicate glass
803 capillaries and their back-filling just before the start of the experiment. In the large lower panel, the
804 main phases of the experiment are shown: the isolation of vacuoles from protoplasts by application
805 of the vacuolar release solution (VRS); the execution of the patch clamp experiment. After the seal
806 between the pipette and vacuole, the whole-vacuole configuration is established; the loading
807 phase of the vacuole with the BCECF fluorophore occurs in 10-30'. Finally, patch clamp recordings
808 and fluorescence detection are performed in parallel to characterize transporters.

809

810 **Supplemental Figure S2 Loading phase of the fluorophore BCECF inside the vacuolar**
811 **lumen**

812 (a) Schematic drawing of the loading phase of the vacuole with the fluorophore BCECF. VRS and
813 Con indicate vacuolar release solution and control solution, respectively. (b) Time course of
814 BCECF fluorescence emission signals upon excitation at 490 nm (F_{490}) and (c) at 440 nm (F_{440}),
815 after establishment of the whole-vacuole configuration. (d) Fluorescence ratio (F_{490}/F_{440}) of data
816 shown in A and B. Seal and break-in (time 0 s) were performed in VRS solution, which was
817 changed to standard bath solution (control) after about 250 s.

818

819 **Supplemental Figure S3 Currents recorded upon replacement of cytosolic K^+ with BTP^+**

820 (a) The substitution of cytosolic potassium with BTP^+ (upper panel, no K^+ + BTP^+) elicited small
821 negative currents (middle panel, $V=0$ mV). These currents were independent of the presence of
822 $PI(3,5)P_2$ added at 200 nM in the no K^+ + BTP^+ solution, which, instead, strongly inhibited the
823 relative acidification, as shown in Fig. 4.

(b) No K^{+} BTP⁺- induced currents were not significantly different when recorded in three Arabidopsis plant lines, Columbia wild-type (WT, n=5), AtCLC-a knock-out (*clc-a*, n=24), AtNHX1 and AtNHX2 double knock-out (*nhx1 nhx2*, n=7) or in the presence of two specific inhibitors of the acidification, benzamil (n=4) and PI(3,5)P₂ (n=6), which were investigated in this work.

Supplemental Figure S4 Long-term stability of BCECF fluorescence

(a) The blasting of a protoplast releases its vacuole (left panel). After reaching the whole-vacuole configuration (the patch pipette is visible on the right of the vacuole) and waiting for the loading of BCECF (from 10 to 30 min) the vacuole becomes fluorescent (right panel, 490 nm excitation light). The red circle is the so-called ROI (region of interest) where fluorescence is evaluated. Scale bar, 10 μ m. (b) In the upper panel, raw BCECF signals resulting from alternate excitation at 490 (black trace) and 440 (red trace) nm. PPI (blue bars) and BTP (pink bars) respectively indicates the application in bath solution of pyrophosphate (100 μ M) or the substitution of 100 mM of cytosolic potassium with an equimolar concentration of BTP. The wavelength at 440 nm is very close to the isosbestic point with consequent small variations. In the lower panel, ratio (490/440) of data shown in the upper panel.

Supplemental Figure S5 Calibration of BCECF fluorescence

Proton concentrations (open symbol) vs BCECF fluorescence ratios (F_R). The BCECF fluorescence ratios were determined in large droplets (see Materials and Methods), whose pH values were adjusted respectively to pH 7.0 ($[H^{+}]$ =100 nM), pH 6.5 ($[H^{+}]$ =316 nM), 5.5 ($[H^{+}]$ = 3.16 μ M) and pH 3.5 ($[H^{+}]$ =316 μ M). Data at pH 7.0, 6.5 and 5.5 vs F_R were fitted with the single exponential function $A_1 + B_1 \exp(-b_1 x)$, obtaining A_1 =7.10, B_1 =-4.28 and b_1 =1.36. Then, the value at pH 3.5 was added and a fit with the double exponential function $A_2 + B_2 \exp(-b_2 x) + C_2 \exp(-c_2 x)$ was performed (continuous line), with constraints $A_2=A_1$ =7.10 and $b_2=b_1$ =1.36, obtaining B_2 =-4.17, C_2 =-16.4 and c_2 =7.93. Data fit were used to convert the fluorescence ratios, which were experimentally measured, into proton concentrations ($[H^{+}]$ =10^{-pH}). This procedure was applied at the beginning of each experimental session. Values were stable over time and different

852 calibrations gave similar results (pH 7: 2.80 ± 0.07 , n=5; pH 6.5: 1.45 ± 0.04 , n=5; pH 5.5: 0.71 ± 0.03 ,
853 n=5; pH 3.5: 0.35 ± 0.03 , n=4).

854

855 **Supplemental Figure S6 Simulating the activity of H⁺-PPase in isolated vacuoles and in**
856 **whole-vacuole patch-clamp configuration**

857 (a) In an isolated vacuole, PPI at 10 μ M concentration (upper panel) activates the
858 pyrophosphatase current shown in the middle panel, which, in turn, increases the luminal proton
859 concentration (lower panel). (b) Data in the lower panel are the same of (a, lower panel)
860 transformed in pH units. (c) Simulation of a patch-clamp experiment with a pipette of 10 MOhm
861 access resistance. The activation/deactivation of the pyrophosphatase current shown in upper
862 panel, increases/decreases the luminal proton concentration (lower panel). (d) Data in the lower
863 panel are the same of (c, lower panel) transformed in pH units.

864

865 **Supplemental Figure S7 Simulating the NHX activity: effect of perfusion**

866 (a) Simulation of a patch-clamp experiment in the whole-vacuole configuration. The patch pipette
867 has an access resistance of 10 MOhm. The vacuole harbors on its membrane a PPase pump and
868 NHX1/2 transporters. The pyrophosphatase current displayed in the upper panel induces the
869 change of luminal proton concentration in lower panel. (b) Same data as in (a, lower panel),
870 transformed in pH units. (c) Effect of perfusion simulated as an exponential time course of the
871 PPase current (upper panel) on the luminal proton concentration (lower panel). (d) Same data as
872 in (c, lower panel), transformed in pH units.

873

874 **Supplemental Figure S8 Simulating the vacuolar acidification mediated by NHXs induced by**
875 **a cytosol-directed potassium gradient**

876 (a) Change in cytosolic potassium (upper panel) and correspondent response in luminal proton
877 concentration variation (lower panel). Whole-vacuole configuration, patch pipette with access
878 resistance of 10 MOhm, tonoplast equipped with NHX1/2 transporters (simulating *clc-a* vacuoles).
879 Traces in upper panel were calculated considering instantaneous perfusion (black line) or a

880 perfusion system with a time constant of 10 (blue line) or 30 s (red line). (b) Cytosolic potassium
881 concentration (thick blue line) and luminal proton concentration were superimposed to favor
882 comparison. (c) Comparison between luminal proton concentrations obtained respectively by
883 equation 13 and 16. The inset shows that stationary level of $[H^+]_{lum}$ predicted by 13 is lower than
884 the one calculated by 16.

885

886 **Supplemental Figure S9 Simulating the effect of voltage and inhibitors on NHX activity**

887 (a) Simulation of a change in holding voltage from 0 to -40 mV (upper panel) in vacuoles harboring
888 only NHX1/2 transporters. Voltage does not modify the luminal proton concentration (lower panel).
889 Potassium concentration profile is shown in the middle panel. (b) When CLC-a was added to the
890 model (equation 20), the luminal proton concentration becomes voltage-dependent. (c) The
891 presence of an inhibitor in the bath solution (middle panel) reduces the luminal proton
892 concentration (lower panel) induced by a cytosol-directed potassium gradient. Cytosolic potassium
893 profile shown in the upper panel.

894

Figure 1

Figure 3

Figure 4

Figure 5

(a)

(b)

(c)

906

907 **Figure 6**

THE EXPERIMENT

908

909 **Supplemental Figure S1**

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911 **Supplemental Figure S2**

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914 **Supplemental Figure S3**

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(a)

(b)

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Supplemental Figure S4

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921
922
923

Supplemental Figure S5

isolated vacuole; H^+ -PPase

(a)

(b)

H^+ -PPase; patch-clamp: pipette with $R_a=10$ MOhm

(c)

(d)

924

925 **Supplemental Figure S6**

926

H⁺-PPase; patch-clamp; NHXs

H⁺-PPase; patch-clamp; NHXs; perfusion

927

928 **Supplemental Figure S7**

929

930

931 **Supplemental Figure S8**

932

Supplemental Figure S9

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