The ins and outs of intracellular ion homeostasis: NHX-type cation/H\(^+\) transporters
Elias Bassil and Eduardo Blumwald

The biochemical characterization of cation/H\(^+\) exchange has been known since 1985 [1], yet only recently have we begun to understand the contribution of individual exchangers to ion homeostasis in plants. One particularly important class of exchangers is the NHX-type that is associated with Na\(^+\) transport and therefore salinity tolerance. New evidence suggests that under normal growth conditions NHXs are critical regulators of K\(^+\) and pH homeostasis and have important roles, depending on their cellular localization, in the generation of turgor as well as in vesicular trafficking. Recent advances highlight novel and exciting functions of intracellular NHXs in growth and development, stress adaptation and osmotic adjustment. Here, we elaborate on new and emerging cellular and physiological functions of this group of H\(^+\)-coupled cation exchangers.

Addresses
Department of Plant Sciences, University of California, Davis, CA 95616, United States

Corresponding author: Blumwald, Eduardo (eblumwald@ucdavis.edu)

Introduction
Developmental cues and the need to respond to changes in their environment, require plants to constantly adjust their cellular pH and ion contents. Ion transport plays a defining role in the provision of energy, uptake and sequestration of ions and organic metabolites, as well as cell expansion. Cell expansion, and therefore growth, depends on turgor pressure that is generated by the coordinated regulation of vacuolar ion and water uptake, and the augmentation of membrane area and cell wall components that are provided by trafficking vesicles. In plants, H\(^+\) is the motive ion and H\(^+\) electrochemical gradients are generated by the H\(^+\) translocating enzymes, the H\(^+\)-ATPase at the plasma membrane or the V-ATPase and PPase in intracellular compartments, to energize the secondary active transport of ions and metabolites. Cation/H\(^+\) exchangers use the H\(^+\) gradient to couple the passive transport of H\(^+\) to the movement of cations against their gradient [2]. The coupled exchange of K\(^+\) or Na\(^+\) for H\(^+\) occurs in all organisms and cellular compartments [3,4,5\(^*\)] and is mediated in part by a family of transporters known as Na\(^+\)/H\(^+\) antiporters (NHXs) in plants or Na\(^+\)/H\(^+\) exchangers (NHEs) in animals. Much work has traditionally focused on use of NHXs in salt tolerance but more recent evidence suggests basic cellular roles that go beyond Na\(^+\) transport into vacuoles.

Diversity of plant NHX-type Cation/H\(^+\) antiporters
Plant NHXs belong to the large CPA family of monovalent cation/H\(^+\) transporters (CPA1), together with closely related members that include the CHX and KEA (CPA2) exchangers [5\(^*\)]. Phylogenetic and sequence analysis of available plant genomes (i.e. phytozome.net) indicate that NHXs are ubiquitous to all eukaryotic organisms. Arabidopsis contains eight isoforms belonging to three classes; two divergent members located at the plasma membrane (SOS1/AtNHX7 and AtNHX8); and six intracellular isoforms that are either vacuolar (AtNHX1 to AtNHX4) or in vesicles (AtNHX5, AtNHX6) [6] (Fig. 1). Interestingly, highly similar orthologues to members of each Arabidopsis class are found in genomes ranging from Chlamydomonas to tomato (Table 1). The fact that these NHX classes are represented even in algae, suggests that vacuolar, vesicular and plasma membrane NHXs have unique cellular functions that have been conserved early in evolution [5\(^*\),6].

Biochemical functions and regulation
A generally accepted mode of NHX operation, known as alternating access [7], results in the transport of either K\(^+\) or Na\(^+\) into the vacuole or endosome in exchange for H\(^+\) influx to the cytosol (NHX1–6) and Na\(^+\) influx out of the cell in exchange for H\(^+\) efflux into the cell (plasma membrane-bound NHX7–8) [6]. No available crystallographic structures of plant NHXs are available but biochemical and kinetic studies suggested that NHXs likely contain 9–12 transmembrane (TM) domains [8].

Epitope tagging of heterologously expressed NHX1 revealed that this antiporter has 9 TM domains with an additional 3 ‘buried’ domains that do not entirely span the membrane [8]. The membrane-spanning pores and putative cation binding domains are highly conserved among plants NHXs, yeast Nhx1 and animal NHEs [3,9,10]. NHXs differ most at their C terminus [3,5\(^*\)].
Protein-protein interactions, phosphorylation and/or glycosylation [6] are proposed to be a means by which antiporter activity or localization could be differentially regulated. A unique feature of NHX1, that differed from its mammalian NHE orthologues [4], was the localization of the C-terminus in the vacuolar lumen [8,11]. The Arabidopsis NHX1 C-terminus interacted with a calmodulin like protein15 (AtCaM15) within the vacuolar

Table 1

<table>
<thead>
<tr>
<th>Classification</th>
<th>Species</th>
<th>Vacuolar (Class I)</th>
<th>Endosomal (Class II)</th>
<th>Plasma Membrane</th>
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<tbody>
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<td>Dicot</td>
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<td>2</td>
<td>2</td>
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<td>Medicago truncatula</td>
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<td>2</td>
<td>1</td>
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<td>Phaseolus vulgaris</td>
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<td>Glycine max</td>
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<tr>
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<td>Populus trichocarpa</td>
<td>5</td>
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<td>Zea mays</td>
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<td>Oryza sativa</td>
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<td>Brachypodium distachyon</td>
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<td>Bryophyte</td>
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<td>Chlorophyte</td>
<td>Chlamydomonas reinhardtii</td>
<td>1</td>
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lumen in a Ca\(^{2+}\)-dependent and pH-dependent manner. Under normal physiological conditions, where the vacuole is acidic (pH 5.5) and Ca\(^{2+}\) activity high, AtCaM15 is bound to the AtNHX1 and results in a higher K\(^{+}/H^{+}\) than Na\(^{+}/H^{+}\) activity. At higher pH (6.0–7.5), AtCaM15 binding to AtNHX1 was reduced and the Na\(^{+}/H^{+}\) activity increased relative to the K\(^{+}/H^{+}\) activity. Because salinity causes the alkalization of the vacuole [12,13], the pH dependent change in K\(^{+}\) for Na\(^{+}\) selectivity of NHX1 might constitute a mechanism for Na\(^{+}\) accumulation (at the expense of K\(^{+}\)) into the vacuole. Regulatory effects of phosphorylation are well documented in NHEs [14] and SOS1/NHX7 [15,16] but no direct evidence for any intracellular NHX is available. Under salt stress, the Ca\(^{2+}\) sensor protein SOS3/CBL4, activates the protein kinase SOS2/CIPK24 which in turn phosphorylates and activates SOS1/NHX7 to reduce cytoplasmic Na\(^{+}\) [15–19]. Interestingly, the activity of vacuolar NHX activity was reduced in sos2 and restored with constitutively active SOS2 but no phosphorylation of NHX was found [17]. The possibility that the CBL/CIPK system might regulate intracellular NHX activity should be considered.

**Cation homeostasis and salt tolerance**

Plant NHXs mediate both Na\(^{+}/H^{+}\) and K\(^{+}/H^{+}\) exchange [20,22,23] and therefore affect both salinity tolerance and K\(^{+}\) nutrition. The initial cloning and overexpression of AtNHX1 in Arabidopsis firmly demonstrated the importance of intracellular Na\(^{+}\) compartmentation for salt tolerance [24]. Many additional studies subsequently confirmed that NHX overexpression lead to improved salt tolerance in diverse species [21,24–29] supporting the idea that maintaining a low Na\(^{+}\)/K\(^{+}\) cytosolic ratio by removing excess cytosolic Na\(^{+}\) into the vacuole, in addition to the extrusion of Na\(^{+}\) into the apoplast by SOS1/NHX7, is critical during salt stress. Enhanced expression of vacuolar NHXs in a salt tolerant tomato variety under salt further confirmed a role of vacuolar NHX in salt tolerance [30]. Salt tolerance of NHX overexpressing transgenics does not seem to depend on the source species or NHX isoform used, but probably affected by the regulation of NHX expression, changes in K\(^{+}\) homeostasis brought about by high intracellular Na\(^{+}\), and possible regulation of NHX cation selectivity. For example endosomal/vesicular NHXs may preferentially transport K\(^{+}\) compared to Na\(^{+}\) [31]. The precise mechanisms and interactions by which K\(^{+}\) and Na\(^{+}\) are regulated remain unclear because NHX overexpression has resulted in contrasting ion accumulation between transgenics and wild type plants and may reflect a primary function of vacuolar NHXs in maintaining osmotic adjustment during both normal growth as well as under salinity. Unexpectedly, the addition of moderate salt (30 mM) to the knockout nhx1/nhx2 lacking the two main Arabidopsis vacuolar antiporters, resulted in improved growth, rather than adversely affecting it, as compared to controls [32**]. Knocking out or silencing endosomal/vesicle NHX isoforms resulted in salt-sensitive plants [26,33**]. Knockouts lacking vacuolar V-ATPase activity had reduced capacity to store NO\(_3^{-}\} or toxic concentrations of Zn\(^{2+}\) but did not exhibit sensitivity to high salt [34], while salt sensitivity was observed instead in knockdowns of the endosomal/trans-Golgi network localized V-ATPase. These results point to the importance of the endosomal/vesicle system in ameliorating salt stress as supported by other studies [13,35–37].

Despite the role of NHXs in salt tolerance, NHX cannot simply catalyze Na\(^{+}/H^{+}\) exchange in non-salinized plants. In grape NHX1 expression was significantly upregulated at véraison and during cell expansion where berry vacuolar K\(^{+}\) accumulation and a drop in acidity occur [38]. Genetic studies in Arabidopsis firmly demonstrated the importance of NHXs in the regulation of pH and K\(^{+}\) homeostasis during normal growth and development [10,23,26,32**,39]. The Arabidopsis knockout nhx1 had lower antiport activity, smaller cells [20] and displayed an upregulation of high affinity K\(^{+}\) uptake transporters [40]. Knockout of the closely related isoform nhx2, did not display obvious phenotypes [32**] but the double knockout nhx1nhx2 displayed a dramatic reduction in cell expansion and growth, especially in rapidly elongating tissues as compared to nhx1. Interestingly these plants also had reduced seed set that was attributed to unsuccessful pollination due to a lack of anther dehiscence and filament elongation and in which K\(^{+}\) dependent hydration/dehydration processes have been implicated [41]. Vacuolar K\(^{+}\) in nhx1nhx2 plants was one third that of wild type root cells [32**] as well as leaf cells [39], K\(^{+}\)/H\(^{+}\) exchange of tonoplast vesicles was markedly reduced in the same knockout which also displayed impaired osmoregulation, turgor and delayed stomatal closure, resulting in poor maintenance of water status [39]. Opening of stomata require the accumulation of guard cell vacuolar K\(^{+}\), a process that relies on NHX1 and NHX2 [42**].

Given the importance of K\(^{+}\) as an enzyme cofactor, in charge balance and an osmoticum, cytosolic K\(^{+}\) concentrations must be tightly maintained [43]. At the typical electrochemical potentials of the plasma membrane and tonoplast, transport of K\(^{+}\) into the cytosol is passive but would require energy to accumulate above ~20 mM in the vacuole [44–46]. To maintain constant cytosolic K\(^{+}\), both uptake of K\(^{+}\) from the apoplast and exchange with the vacuole are essential [44]. The sensitivity of nhx1nhx2 plants to added K\(^{+}\), their reduced vacuolar K\(^{+}\) content [32**] and accumulation of cytosolic K\(^{+}\) [39] highlights the importance of vacuolar NHX in intracellular K\(^{+}\) homeostasis. The technical limitation to measurement of K\(^{+}\) in vesicles, due to the lack of targeted K\(^{+}\) specific probes, limit our understanding of the role of endosomal/vesicular NHXs.
in Na⁺/K⁺ homeostasis and their possible functions in trafficking and salt responses.

**pH homeostasis**

Luminal pH is not uniform throughout the cell, but rather maintained within specific values depending on the intracellular compartment and becomes progressively more acidic with maturity along the secretory pathway [47]. The specific luminal pH of plant cellular compartments was only recently measured using targeted genetically encoded pH sensors [48,49]. *In vivo* pH measurements revealed that a gradual acidification of pH, ranging from pH 7.1 in the ER to ~5.5 in the vacuole, except that the trans-Golgi network (TGN) was more acidic than prevacuolar compartments (PVC) [49**]. Vesicles that colocalized with NHX5 were significantly more alkaline than those colocalizing with the endosomal V-ATPase, while the application of V-ATPase or NHX inhibitors, caused either a respective alkalinization or acidification of vesicles. Such data support the idea that vesicle pH homeostasis requires H⁺-pumps to establish the initial acidity, and alkalinizing mechanisms (NHXs), in order to "fine-tune" the luminal pH, as has been suggested in animals cells [4].

The localization of NHX5 and NHX6 to the Golgi, TGN [33**, and PVC (Blumwald et al., unpublished results), the tomato orthologue NHX2 to vesicles [26], as well as the phenotypes of nhx5nhx6, suggest that endosomal/vesicular NHXs like AtNHX5 control vesicle pH and trafficking. Vacular NHXs have been associated with pH homeostasis. For example, morning glory petal requires NHX activity for coloration [50]. In Arabidopsis nhx1nhx2 roots, vacuolar pH was significantly more acidic especially in cortical cells of the elongation and maturation zones [32**]. Nevertheless, the precise role of vacuolar NHXs in pH regulation is difficult to discern from their roles in vacuolar K⁺ accumulation especially since many of the knockout phenotypes can be attributed to altered K⁺ homeostasis.

**Essential for vesicular trafficking**

Firm demonstration of pH regulation and protein trafficking by NHX-like antiporters was provided in yeast Nhx1Δ where cytosolic and vacuolar pH were altered and protein trafficking out of the Golgi was blocked [51,52]. In Arabidopsis, a role of vesicular/endosomal NHXs in endomembrane trafficking was initially provided by the nhx5nhx6 double knockout [33**]. These plants missorted vacuolar destined cargo to the apoplast and displayed a notable delay in labeling of the vacuole with the tracer, FM4-64 [6]. A functional link between the TGN localized v-ATPase complex and NHX5 and NHX6 was proposed based on high colocalization between the TGN localized V-ATPase and NHX5 and NHX6 [33**]. More recent data indicated that the Golgi and trans-Golgi network in nhx5nhx6 were significantly more acidic than wild type (Blumwald, unpublished results). These data suggest that endosomal/vesicular NHXs have important roles in vesicle pH homeostasis that is essential to trafficking.

**Conclusion**

Recent advances highlight novel NHX cellular and physiological roles that go beyond their importance in salt tolerance. Genetic studies provided compelling evidence to support earlier biochemical data and indicate that NHXs regulate a multitude of cellular and physiological processes including cell expansion, cation homeostasis, turgor and osmotic adjustment, pH regulation, vesicle trafficking, stomatal function and plant water status as well as flowering. The development of multiple NHX knockout lines, together with the generation of genetically-encoded ion sensors will facilitate further understanding of how ion and pH homeostasis regulate mechanisms that control vesicular trafficking and protein processing.

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**References and recommended reading**

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest


In this study, the Arabidopsis double knockout mutant nhx1nhx2 was shown to have reduced growth and severe flower phenotypes. Knockouts had a lower concentration of vacuolar K”+" and more acidic vacuoles, compared to wild type. Knockouts also exhibited high sensitivity to external K”+" concentrations but not to high Na”, suggesting that the role of these transporters is to regulate vacuolar pH and the uptake of vacuolar K”+".

33. Bassil E, Ohto MA, Esumi T, Tajiwa H, Zhu Z, Cagnac O, Belmonte M, Peley Z, Yamaguchi T, Blumwald E: The *Arabidopsis thaliana* Na”H” antagonists NHX5 and NHX6 are endosome associated and necessary for plant growth and development. Plant Cell 2011, 23:224-239. This work demonstrates the importance of the two *Arabidopsis* endosomal NHXs, NHX5 and NHX6 to cell expansion and growth as well as salt stress. NHX5 and NHX6 were located to the Golgi and trans-Golgi network. In the double knockout nhx5nhx6 trafficking to the vacuole is compromised. Results suggested that NHX5 and NHX6 activity might regulate vesicular pH, K”+" and Na”+" homeostasis. The paper highlights the importance of vesicle ion homeostasis and trafficking in higher plants.


This study shows that a mutant lacking tonoplast V-ATPase activity is less critical for growth and salt stress responses than is the V-ATPase in the trans-Golgi network/early endosome and provides interesting insights into the importance of the V-ATPase in vesicle function including salt tolerance.


Similar to results in Ref. [32**], this paper shows that nhx1nhx2 knockouts have abnormally high cytosolic K+ impaired osmoregulation and defective stomatal functions. The study also reports similar exchange activities of between NHX1 and NHX2. Results further support that NHX1 and NHX2 are important for vacuolar accumulation of K+.


In this work the authors demonstrate convincingly the role of NHX1 and NHX2 in stomatal opening and closing by regulating guard cell vacuolar dynamics. Vacuolar K+/H+ exchange, mediated by NHX1 and NHX2 is necessary to accumulate K+ and for normal stomatal function.


Using targeted pH sensitive GFP this work reports the luminal pH of the main organelles of plant cells. pH values indicate that the cytosol, nucleus, peroxisomes, the mitochondrial matrix and plastid stroma are alkaline while the pH of the secretory pathway are gradually more acidic.

In this work, the use of genetically encoded pH sensors, targeted to specific cellular compartments, together with colocalization experiments were used to provide the measurements of luminal pH in all main cellular compartments. Interestingly, and different from animal cells, the pH of prevacuolar compartments was more alkaline than the trans-Golgi network. The work also suggests that endosomal pH requires both V-ATPase for acidification and NHX5 for alkalization.

