


# How do vacuolar NHX exchangers function in

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**Key words:** NHX exchangers, vacuole, sodium, potassium, salinity, nutrition

**Abbreviations:** NHX and NHE, sodium-proton exchanger; SOS1, salt overly sensitive 1; CaM15, calmodulin 15;  $V_{\max}$ , maximum velocity;  $K^+_{\text{cyt}}$ , cytosolic potassium activity; R2R3 Myb, transcription factor with structural homology to the R2 and R3 repeats of the Myb oncogene derived from the avian myeloblastosis virus

Potassium ( $K^+$ ) is a major osmoticum of plant cells, and the vacuolar accumulation of this element is an especially crucial feature for plants under high-salt conditions. Emerging evidence indicates that cation/proton transporters of the NHX family are instrumental in the  $H^+$ -linked  $K^+$  transport that mediate active  $K^+$  uptake at the tonoplast for the unequal partitioning of  $K^+$  between vacuole and cytosol. However, and in spite of tenuous supporting evidence, NHX proteins are widely regarded as key players in the sequestration of sodium ( $Na^+$ ) into vacuoles to avert ion toxicity in the cytosol of plants under salinity stress. Here, we propose an updated model positing that NHX proteins fulfill a protective function to minimize salt-related stress mainly through the vacuolar compartmentalization of  $K^+$  and, in some cases, of  $Na^+$  as well thereby preventing toxic  $Na^+$ - $K^+$  ratios in the cytosol while accruing solutes for osmotic balance.

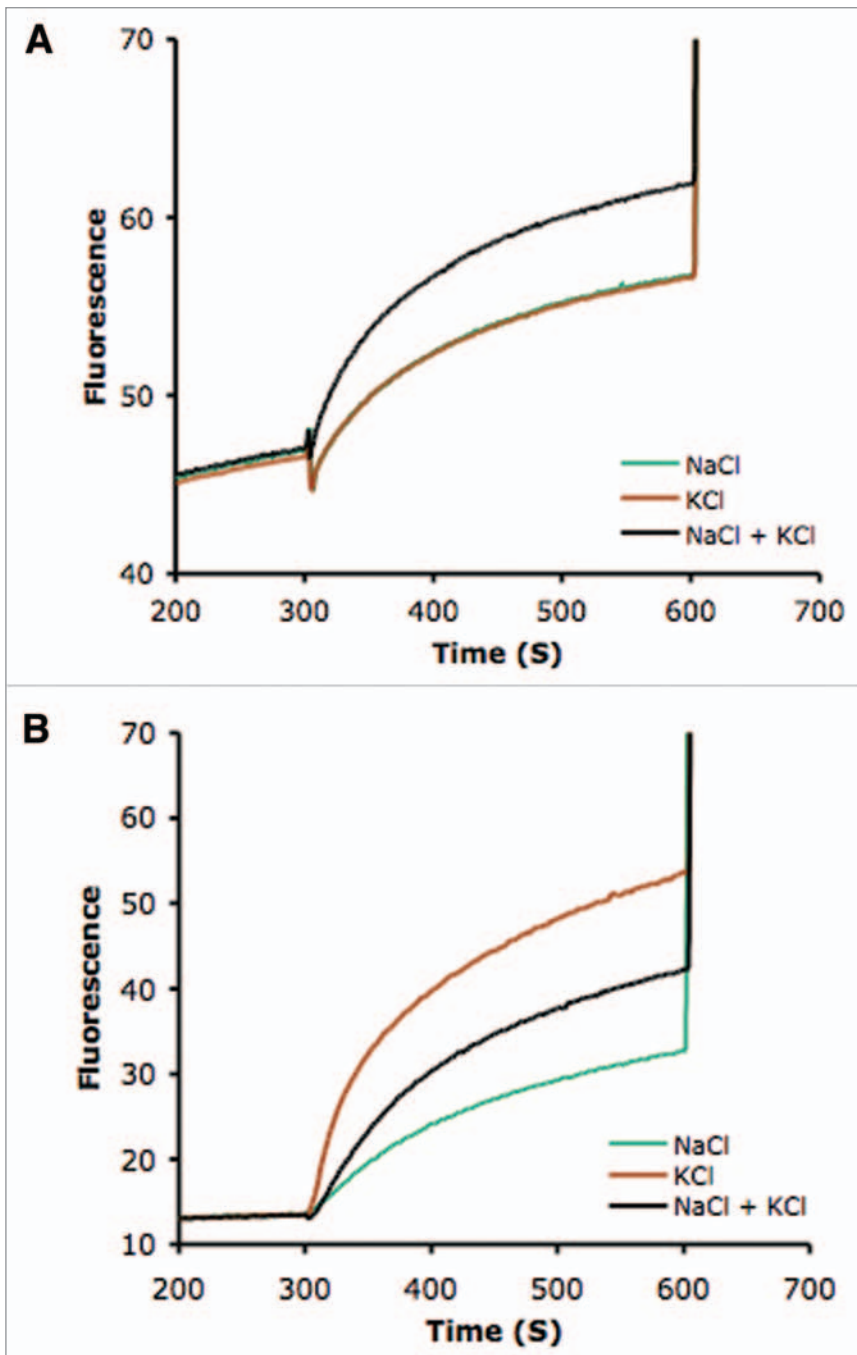
All living cells routinely expel  $Na^+$  ions, maintaining a lower concentration in the cytosol than in the surrounding environment. In plants, this export usually occurs at the expense of the  $H^+$ -motive force created by proton pumps and is driven by  $Na^+/H^+$  exchangers localized in the plasmalemma and endosomal membranes.<sup>1</sup> Given the inside negative electrical membrane potential at the plasma membrane (-120 to -200 mV), a rise in extracellular  $Na^+$  concentrations will establish a large  $Na^+$  electrochemical gradient that will drive the passive transport of  $Na^+$  across the plasma membrane and the potential accumulation of  $Na^+$  in the cytosol to 100- to 1,000-fold greater concentrations than those of the extracellular space (either apoplast or soil solution).<sup>2</sup> Most of the cytosolic  $Na^+$  taken up by roots is extruded back to the soil solution by an energetically costly process. Comparisons of unidirectional  $Na^+$  fluxes and rates of net accumulation of  $Na^+$  in roots of several plant species indicate that 70–95% of the  $Na^+$  permeating into the root symplast is extruded back.<sup>1</sup> To date, the electroneutral exchange of  $Na^+$  for  $H^+$  by plasma membrane  $Na^+/H^+$  antiporter activity is the only mode of transport that has

been measured for  $Na^+$  efflux in vascular plants.<sup>1,3</sup> The SOS1 exchanger, a highly conserved transport protein that seems ubiquitous in plant species, has been shown to mediate  $Na^+$  efflux.<sup>4,5</sup> Plasma membrane vesicles from the Arabidopsis *sos1* mutant retained some  $Na^+/H^+$  exchange activity, suggesting that additional exchangers might be present, although genetic evidence indicates that SOS1 plays a major role in salt tolerance.<sup>4,6</sup>

In spite of the large efflux component in  $Na^+$  metabolism, the ratio of influx vs. efflux rates dictates that, over time,  $Na^+$  will inevitably accumulate in the cytosol of root cells, and eventually in all plant tissues. Hence, the compartmentation of  $Na^+$  ions into vacuoles is regarded as a critical mechanism to avert the toxic effects of  $Na^+$  in the cytosol while providing additional osmoticum for water uptake and turgor maintenance.<sup>3</sup> This function has been attributed to tonoplast localized NHX-like antiporters.<sup>3,7</sup> The founding member of this family of cation exchangers,<sup>8,9</sup> protein AtNHX1 of Arabidopsis, was initially described as a selective  $Na^+/H^+$  antiporter because  $K^+$  ions in the assay medium did not affect the ion exchange driven by AtNHX1.<sup>7</sup> However, subsequent research demonstrated that AtNHX1 mediated both  $Na^+/H^+$  and  $K^+/H^+$  exchange in tonoplast vesicles from transgenic tomato plants,<sup>10</sup> in artificial proteoliposomes in which AtNHX1 was the only transport protein present,<sup>11</sup> and in vacuoles of a yeast mutant strain lacking the endogenous  $Na^+/H^+$  and  $K^+/H^+$  antiporter activities at the tonoplast.<sup>12</sup> Determination of the relative  $Na^+$ - $K^+$  selectivity of AtNHX1 has produced conflicting results, ranging from preferred  $Na^+$  transport over  $K^+$ ,<sup>10</sup> to lack of significant  $Na^+$ - $K^+$  discrimination.<sup>11–13</sup> To complicate matters further, the activity of AtNHX1 is regulated by the binding of the calmodulin-like protein AtCaM15 to its C-terminus.<sup>14</sup> This interaction, which has been suggested to occur upon stress-induced rises in intracellular  $Ca^{2+}$ , modified the  $Na^+$ - $K^+$  selectivity of the antiporter, *lowering* the  $V_{\max}$  of the  $Na^+/H^+$  exchange activity without affectation of the  $K^+/H^+$  exchange activity, thereby *decreasing* the  $Na^+$ - $K^+$  transport ratio. Moreover, mutants of AtNHX1 that conferred improved halotolerance to yeast cells showed greater substrate discrimination, favoring  $K^+$  transport over that of  $Na^+$ .<sup>12</sup>

Plant and fungal NHX antiporters are phylogenetically related to NHE family of mammalian  $Na^+/H^+$  exchangers. On the basis of protein sequence similarity, the NHE/NHX family can be classified into two major groups that have been termed Plasma

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**Figure 1.** Cation transport activity in proteoliposomes. AtNHX1 and LeNHX2 proteins were purified and reconstituted into liposomes to measure their cation/H<sup>+</sup> exchange activity as described.<sup>11,17</sup> An acid-inside pH gradient was created by dilution of proteoliposomes containing (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> into ammonium-free assay buffer at pH 7.5. Fluorescence recovery, indicative of vesicle alkalization, was measured upon addition of NaCl and/or KCl salts to the assay medium. Addition of 25 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> disrupted the pH gradient and ended the assay. Transport activities are given as percent of maximal fluorescence. (A) Proton exchange rates of AtNHX1 were determined in the presence of 50 mM NaCl (green trace), 50 mM KCl (red trace), and 50 mM of each salt (black trace). (B) Transport activity of LeNHX2 with 125 mM NaCl, 125 mM KCl and 125 mM of each salt; trace colors as in (A).

Membrane (PM) and Intra-Cellular (IC) according to their subcellular localization.<sup>15</sup> The PM group is exclusively present in animal cells whereas members of the IC group can be found in

animals, plants and fungi. All plant NHXs characterized to date can be assigned to the IC group and be further subdivided into two subgroups, class-I and class-II.<sup>15,16</sup> Class-I proteins are localized in the vacuolar membrane (reviewed in ref. 16). Although the subcellular localization of only one class-II NHX protein has been reported to date, namely the tomato LeNHX2 protein that is targeted to the prevacuolar compartment (PVC),<sup>17,18</sup> other members of class-II group are also found in non-vacuolar endosomal compartments (Andres Z, Cubero B, Pardo JM, unpublished results). To begin addressing the issue of how the ion selectivity of NHX proteins fits with their suggested cellular function, we have compared the Na<sup>+</sup>-K<sup>+</sup> selectivity of representative members of the two types of plant NHX proteins. Histidine-tagged AtNHX1 (class-I, tonoplast) and LeNHX2 (class-II, PVC) proteins were purified by affinity chromatography, reconstituted into lipid vesicles, and the cation-dependent H<sup>+</sup> exchange was determined with the fluorescent pH indicator pyranine as described.<sup>11</sup> As depicted in Figure 1A, the tonoplast localized AtNHX1 showed equal transport rates for Na<sup>+</sup>/H<sup>+</sup> and K<sup>+</sup>/H<sup>+</sup> exchange, in agreement with our previous reports.<sup>11,12</sup> Furthermore, mixing Na<sup>+</sup> and K<sup>+</sup> salts produced an additive effect, indicating the either ion serves as substrate for transport. By contrast, the non-vacuolar LeNHX2 protein demonstrated a strong preference for K<sup>+</sup>/H<sup>+</sup> exchange over Na<sup>+</sup>/H<sup>+</sup>, and Na<sup>+</sup> salts reduced the net rate of transport when mixed with K<sup>+</sup>, an indication of competitive inhibition of K<sup>+</sup> transport by Na<sup>+</sup> ions (Fig. 1B; Jiang X, Rodriguez-Rosales MP, Venema K, unpublished results). In this regard, it is worth noting that AtNHX1 complemented the lack of endogenous ScNHX1 in the yeast *Saccharomyces cerevisiae*<sup>12,19</sup> and that the ScNHX1/VPS44 protein plays an important role in endosomal trafficking and protein sorting of the late endosome/prevacuolar compartment, presumably by regulating the luminal pH of endosomes.<sup>20,21</sup> Remarkably, mutants of AtNHX1 that conferred greater halotolerance to yeast cells demonstrated improved K<sup>+</sup>-Na<sup>+</sup> selectivity, presumably by averting sodic poisoning of the endomembrane network and preserving endosome/prevacuolar functions. Together, these results strongly suggest that the endosome/prevacuolar compartment of yeast, and likely in plant cells too, is a target for Na<sup>+</sup> toxicity, which can be prevented by improved K<sup>+</sup>-Na<sup>+</sup> discrimination of the resident class-II NHX antiporters, in agreement with previous reports<sup>17,18</sup> and biochemical data shown in Figure 1B.

At face value, insufficient  $\text{Na}^+$ - $\text{K}^+$  discrimination of vacuolar (class-I) NHX exchangers creates a conceptual problem with the prevailing model positing that these proteins are major players in the vacuolar compartmentation of  $\text{Na}^+$ . Cytosolic  $\text{Na}^+$  concentrations of 10–30 mM have been estimated in root cells of salt-treated glycophytes by X-ray microanalysis and ion-sensitive electrodes, while cytosolic  $\text{K}^+$  will often remain above 60 mM.<sup>1,22</sup> Since cytosolic  $\text{K}^+$  concentration will most likely exceed that of  $\text{Na}^+$  under salinity stress, the relevant question is how a non-selective  $\text{Na}^+$ , $\text{K}^+$ / $\text{H}^+$  antiporter could mediate efficient  $\text{Na}^+$  compartmentation in vacuoles at high cytosolic  $\text{K}^+$ - $\text{Na}^+$  ratios. One possibility is that  $\text{K}^+$ / $\text{H}^+$  exchange by NHX1-like proteins would predominate over, but not suppress,  $\text{Na}^+$ / $\text{H}^+$  exchange (see Fig. 1A), and that the backflow of  $\text{K}^+$  from the vacuole to the cytosol were greater than that of  $\text{Na}^+$ . Over time, the net balance of this counteracting, bidirectional transport would result in the accumulation of  $\text{Na}^+$  into the vacuolar lumen, albeit at the expense of a seemingly futile cycle of vacuolar  $\text{K}^+$  import and export. To investigate this issue, we constructed transgenic tomato plants overexpressing AtNHX1. These plants showed improved salt tolerance, although only if the stress was imposed as salt-shock.<sup>13</sup> Importantly, the transgenics had larger  $\text{K}^+$  vacuolar pools in all growth regimes tested, but no consistent enhancement of  $\text{Na}^+$  accumulation was observed under salt stress. Plants overexpressing AtNHX1 had a greater capacity to retain intracellular  $\text{K}^+$  under various salinity regimes, an especially crucial feature for plants under high-salt conditions. They also showed elevated concentrations of soluble sugars before and after salinity stress. Proline concentrations, which were not different among plant lines prior transfer to salt, were also significantly greater in the transgenic plants under stress. These are all well known features that correlate with salinity tolerance in many plant species that may contribute additively to the halotolerance of plants expressing AtNHX1. Similar findings have been reported by others.<sup>23–26</sup>

Strikingly, when transgenic tomato overexpressing AtNHX1 were subjected to  $\text{K}^+$ -limiting conditions, the enhanced  $\text{K}^+$  compartmentation into the vacuole continued at the expense of the cytosolic  $\text{K}^+$  pool, which was 2-fold lower in transgenic plants relative to control plants.<sup>13</sup> The  $\text{K}^+$ <sub>cyt</sub> measured with double-barreled  $\text{K}^+$ -selective microelectrodes in impaled root epidermal cells was  $98 \pm 1.3$  mM in wild-type seedlings and  $55 \pm 2.2$  mM in the transgenics. The drop in  $\text{K}^+$ <sub>cyt</sub> caused the early induction of the  $\text{K}^+$ -starvation responsive gene *HAK5*, triggered the high-affinity mode of  $\text{K}^+$  uptake, enhanced net  $\text{K}^+$  uptake by roots, and increased the  $\text{K}^+$  content in plant tissues and the xylem sap. Transformed plants became much more sensitive to low- $\text{K}^+$  than wild-type plants, presumably as a consequence of compromised  $\text{K}^+$ <sub>cyt</sub>. Likewise, in tomato plants that overexpress the  $\text{K}^+$ / $\text{H}^+$  exchanger LeNHX2, the induction of *HAK5* upon  $\text{K}^+$  starvation is much stronger than in untransformed control plants (Huertas R, Venema K, Rodriguez-Rosales MP, unpublished results). These findings do not lend support to the above proposition that  $\text{K}^+$  ions transported by AtNHX1 are readily fluxed back to the cytosol. Instead, they strongly suggest that NHX proteins are likely candidates for the  $\text{H}^+$ -linked  $\text{K}^+$  transport that is thought

to facilitate active  $\text{K}^+$  uptake at the tonoplast, and that  $\text{K}^+$  remains partitioned between vacuole and cytosol. They also represent a fundamental challenge to the underlying assumptions about the mechanism by which vacuolar NHX exchanger enhance the salt tolerance of plants, namely that the prevailing function of NHX proteins is to drive the efficient compartmentation of  $\text{Na}^+$  into the vacuolar lumen. We posit that the primary function of NHX1-like transporter is to mediate  $\text{K}^+$  compartmentation, with or without concurrent sequestration of  $\text{Na}^+$  depending on the selectivity of the individual NHX protein and the ionic environment in the cytosol of salinized plants.

To be able to extend this new model to other NHX-like proteins, we must determine whether our findings arise from a bizarre behavior of AtNHX1 expressed ectopically in transgenic tomato plants or there is indeed additional evidence supporting this paradigm shift. Numerous reports have shown improved salt tolerance of a variety of plant species expressing vacuolar NHX-like proteins from various sources (reviewed in refs. 3 and 16). Higher  $\text{Na}^+$  contents in tissues of transgenic Arabidopsis and tomato overexpressing AtNHX1 have been reported.<sup>7,10</sup> However, Zhang et al.<sup>10</sup> did not determine the extent to which the ectopic expression of AtNHX1 increased  $\text{Na}^+$  concentration in tissues of transgenic plants relative to non-transformed plants, and neither of these early reports measured  $\text{K}^+$  contents in the transgenics. Subsequent studies found greater accumulation of both  $\text{Na}^+$  and  $\text{K}^+$  in shoots of transgenic plants overexpressing NHX-like proteins.<sup>23,27</sup> By contrast, several reports failed to find a significant correlation between increased salt tolerance and enhanced accumulation of  $\text{Na}^+$ ,<sup>26,28–30</sup> or described greater  $\text{K}^+$  contents, rather than of  $\text{Na}^+$ , in tissues of the transgenic plants.<sup>18,24,25</sup> It should be noted that these reports relied on determinations of total  $\text{Na}^+$  and  $\text{K}^+$  contents in lieu of direct measurements of vacuolar content as in Leidi et al.<sup>13</sup> Therefore, although  $\text{Na}^+$ / $\text{H}^+$  exchange at the tonoplast is generally agreed to play a major role in the salt tolerance of plants, no conclusive evidence has been presented to substantiate that NHX-like antiporters are crucial in this process.

Independent lines of research have also led to the conclusion that vacuolar NHX protein mediate  $\text{K}^+$ / $\text{H}^+$  exchange for turgor regulation and pH control. This is best illustrated by the involvement of NHX exchangers in petal expansion and flower color development. The vacuolar pH (pH<sub>v</sub>) greatly affects coloration of luminal anthocyanin pigments. The sole known structural genes that regulate pH<sub>v</sub> with relevance to flower coloration are antiporters of the NHX family. In Japanese morning glory (*Ipomoea* sp.) NHX1-like proteins are specifically expressed before flower opening and mediate the pH<sub>v</sub> shift from ca. 6.6 to 7.7 that renders blue flowers.<sup>31–33</sup> The *purple* (*pr*) mutation of *I. nil* abolishes the activity of InNHX1, abrogates vacuolar alkalinization and impedes the color shift from red to blue in opening flowers.<sup>32</sup> *Petunia hybrida* flowers normally have a lower pH than *Ipomoea* flowers, and the color of wild-type flowers stays on the reddish (low pH) side of the color spectrum. *Petunia* loci (named *PH1* to *PH7*) that regulate pH<sub>v</sub> have been identified.<sup>34</sup> Gene *PH4*, which activates vacuolar acidification from pH 6 to 5.5, encodes an R2R3 Myb transcription factor whose target genes remain to be identified.<sup>35</sup> These findings indicate that the regulation of pH<sub>v</sub>



is widely used by flowering plants to define the ultimate petal color, and that NHX proteins are instrumental in this process. Modulation of pH<sub>v</sub> has been linked to K<sup>+</sup>/H<sup>+</sup> exchange but not to Na<sup>+</sup>/H<sup>+</sup> exchange.<sup>33</sup>

In summary, a significant and growing body of evidence indicates that NHX antiporters play a fundamental, basic role in the plant cell. At regular growth conditions they contribute to K<sup>+</sup> uptake, capturing K<sup>+</sup> into vacuoles for cellular storage, turgor generation and pH regulation. Under salt or osmotic stress NHX proteins fulfill a protective function through the vacuolar

compartmentalization of K<sup>+</sup> and, in some cases, of Na<sup>+</sup> thereby preventing toxic Na<sup>+</sup>-K<sup>+</sup> ratios in the cytosol while accruing solutes both osmotic balance.

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