Journal of Experimental Botany Advance Access published September 17, 2012

Journal of Experimental Botany
doi:10.1093/jxb/ers250

Journal of Experimental Botany
www.jxb.oxfordjournals.org

DARWIN REVIEW

Cellular ion homeostasis: emerging roles of intracellular NHX Na^+/H^+ antiporters in plant growth and development

Elias Bassil, Ardian Coku and Eduardo Blumwald*

Department of Plant Sciences, University of California, One Shields Ave, Davis, CA 95616, USA

* To whom correspondence should be addressed. E-mail: eblumwald@ucdavis.edu

Received 26 April 2012; Revised 13 August 2012; Accepted 14 August 2012

Abstract

Recent evidence highlights novel roles for intracellular Na^+/H^+ antiporters (NHXs) in plants. The availability of knockouts and overexpressors of specific NHX isoforms has provided compelling genetic evidence to support earlier physiological and biochemical data which suggested the involvement of NHX antiporters in ion and pH regulation. Most plants sequenced to date contain multiple NHX members and, based on their sequence identity and localization, can be grouped into three distinct functional classes: plasma membrane, vacuolar, and endosomal associated. Orthologues of each functional class are represented in all sequenced plant genomes, suggesting conserved and fundamental roles across taxa. In this review we seek to highlight recent findings which demonstrate that intracellular NHX antiporters (i.e. vacuolar and endosomal isoforms) play roles in growth and development, including cell expansion, cell volume regulation, ion homeostasis, osmotic adjustment, pH regulation, vesicular trafficking, protein processing, cellular stress responses, as well as flowering. A significant new discovery demonstrated that in addition to the better known vacuolar NHX isoforms, plants also contain endosomal NHX isoforms that regulate protein processing and trafficking of cellular cargo. We draw parallels from close orthologues in yeast and mammals and discuss distinctive NHX functions in plants.

Key words: Homeostasis, ion exchanger, membrane transport, Na^+/H^+ antiporter, NHX, pH, potassium, sodium, vacuole, vesicle trafficking

Introduction

Ion and pH homeostasis are fundamental regulators of cellular processes that determine and control plant growth. Vital to the establishment and maintenance of cellular ion and pH balance are the H^+-translocating enzymes that generate the H^+ electrochemical potential gradients, and cation/H^+ exchangers that use these gradients to couple the passive transport of H^+ to the movement of cations against their electrochemical potential (Blumwald, 1987). The activity of these cation/H^+ antiporters (CPAs) is critical to cellular osmotic adjustment, regulation of cell turgor, plant growth, and development. In addition, coupled cation/H^+ exchange plays a paramount role in the regulation of the pH and ionic composition of the internal milieu of vacuoles and endosomes affecting protein processing, vesicular cargo composition, vesicular movement, and protein trafficking (Pardo *et al*., 2006; Rodriguez-Rosales *et al*., 2009).

The coupled exchange of K^+ or Na^+ for H^+ is known to occur across membranes of all organisms, from prokaryotes to higher eukaryotes (Brett *et al*., 2005*a*; Pardo *et al*., 2006; Rodriguez-Rosales *et al*., 2009; Orlowski and Grinstein, 2011; Chanroj *et al*., 2012). This $K^+(Na^+)/H^+$ exchange is mediated by members of a family of transporters referred to as Na^+/H^+ antiporters (NHXs) in plants or Na^+/H^+ exchangers (NHEs) in animals. Although in animals NHEs are better characterized, recent advances shed light on the diverse physiological roles of the NHX antiporters.

In plants, a number of monovalent cation/H^+ transporters have been identified and classified into the large CPA family.

A number of recent reviews described in eloquent detail the different subfamilies comprising monovalent cation/H^+ exchangers, their function, and their evolution (Brett *et al*., 2005*a*; Rodriguez-Rosales *et al*., 2009; Chanroj *et al*., 2012). Here, we focus on the intracellular NHX antiporters. In *Arabidopsis*, this group is comprised of six members (NHX1–NHX6) located to the plant vacuole and endosomes. Two other divergent members that localized to the plasma membrane, AtNHX7 and AtNHX8, have been reviewed previously (Chinnusamy *et al*., 2005) and will not be discussed here.

Phylogenetic analysis of several sequenced plant genomes has revealed that intracellular NHX antiporters are ubiquitously encoded and that functionally distinct classes appeared early in evolution (Chanroj *et al*., 2012). Recent genetic evidence has demonstrated that two of the most abundant vacuolar NHX antiporters in *Arabidopsis* primarily catalyse K^+/H^+ exchange under normal growth conditions, and are required for many processes associated with growth and development (Apse *et al*., 2003; Rodriguez-Rosales *et al*., 2008; Bassil *et al*., 2011*b*; Barragán *et al*., 2012). In addition, endosomal NHX antiporters have been shown to be critical regulators of vesicle trafficking, especially to the vacuole. A number of recent publications have made significant contributions to our understanding of the roles that the operation of the NHX-type of Na^+/H^+ antiporters play in the regulation of vesicular trafficking (Bassil *et al*., 2011*a*), cell expansion, growth, and development. In this review we seek to highlight and discuss these recent advances.

Phylogeny of NHX-type cation/proton antiporters

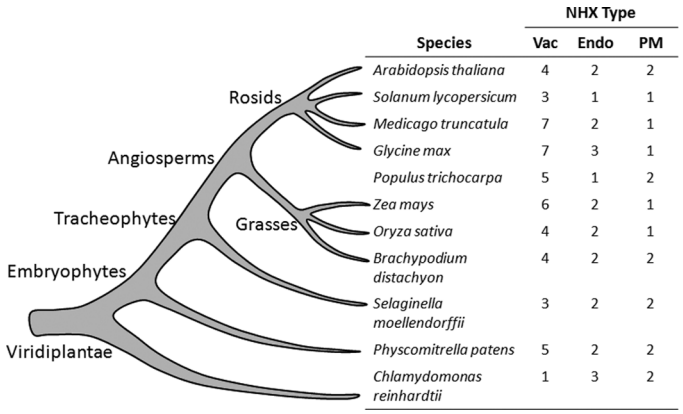
Plant NHXs belong to a large super family of monovalent CPAs that is made up of two subgroups, CPA1 and CPA2 (Saier *et al*., 1999; Brett *et al*., 2005*a*). The CPA2 family includes members of the lesser known cation/H^+ antiporters (CHXs), which contains 28 genes divided into eight subclades in *Arabidopsis*. Little is known about CHX functions in plants, and information is limited to *Arabidopsis* (Chanroj *et al*., 2012). Some CHX isoforms localize to endosomal compartments where they may have roles in pH and ion homeostasis and trafficking (Chanroj *et al*., 2011) or osmoregulation in stomata (Padmanaban *et al*., 2007). The diversity of CHX isoforms, especially in flowering plants, is surprising and not adequately understood (Chanroj *et al*., 2012), but their expression in guard cells and reproductive organs (Sze *et al*., 2004), as well as an involvement in pollen tube guidance (Lu *et al*., 2011), suggested that CHX proteins may have been important to the evolution of land plants. Another less characterized group of CPAs of the CPA2 family are the K^+ efflux antiporters (KEAs). Recently, AtKEA2 was shown to be localized to the plastids and to mediate cation/H^+ exchange, and rescue the Nhx1p mutant in yeast (Zybailov *et al*., 2008; Aranda-Sicilia *et al*., 2012). CPA2 genes probably evolved from prokaryotic NhaA/NapA genes (Chang *et al*., 2004; Brett *et al*., 2005*a*) and will not be discussed in great detail in this work. Other reviews which discuss in detail the diversity of CPA genes, their representation in plants and animals, as well as their possible functions are available (Chang *et al*., 2004; Brett *et al*., 2005*a*; Chanroj *et al*., 2012).

The eukaryotic CPA1 family includes members of the NHX type together with the prokaryotic NhaP antiporters that are probably the early progenitors from which NHX-type antiporters evolved (Brett *et al*., 2005*a*). CPA1 members are widely distributed among species from all kingdoms. In *Arabidopsis*, the NHX family comprises eight members which are divided into distinct functional groups. The intracellular members NHX1–NHX6 are further divided into a vacuolar group (NHX1–NHX4) and an endosomal group (NHX5 and NHX6) based on localization and proposed cellular roles. Previously these two groups have been referred to as class I and class II, respectively (Pardo *et al*., 2006), but we prefer the more informative terms 'vacuolar' and 'endosomal'. The two remaining members, NHX7/SOS1 and NHX8, are plasma membrane bound, more divergent in sequence and function than the intracellular members, and more closely resemble the prokaryotic NhaP antiporter (Brett *et al*., 2005*a*). The sequencing of multiple plant genomes now enables the identification of NHX orthologues across taxa. Most plant species sequenced to date appear to possess multiple NHX isoforms and, interestingly, contain representative members from each of the three functional groups; that is, plasma membrane SOS1 like, vacuolar NHX1 like, and endosomal NHX5/6 like (Rodriguez-Rosales *et al*., 2009; Chanroj *et al*., 2012). Using *Arabidopsis* NHX1, NHX5, and SOS1 as representatives of each functional class; that is, plasma membrane SOS1 like, vacuolar NHX1 like, and endosomal NHX5/6 like, a BLAST search of different genomes (http://www.phytozome.net/) indicated that these species contain multiple NHX-like sequences and that orthologues of each NHX functional class exist in species ranging from *Chlamydomonas* to tomato (Fig. 1). Another interesting outcome of this search was that the number of isoforms from each functional NHX grouping did not differ appreciably (compare *Physcomitrella* with *Populus*, for example). Therefore, it appears that NHX functional groups appeared early in evolution and have conserved and fundamental cellular roles in plants.

Structure and mode of action

Topology

To date, no X-ray crystallographic structures for animal NHEs, or yeast or plant NHX antiporters are available. Nonetheless, other experimental evidence has contributed to our understanding of NHE- and NHX-like topology. Biochemical and kinetic analyses of NHEs strongly suggested that they may function as homodimers and contain 10–12 transmembrane domains (Orlowski and Grinstein, 2007). These arguments were further supported by the relatively high resolution (3.45 Å) crystal structure of the evolutionarily divergent *Escherichia coli* Na^+/H^+ antiporter, NhaA (Padan *et al*., 2009). A study whose aim was to characterize the topological features of plant vacuolar NHXs found that the global structural features of AtNHX1 differ from all those of known NHX-like antiporters, but hydrophobicity plots indicated that AtNHX1 also contains 10–12 transmembrane domains (Yamaguchi *et al*., 2003). Epitope tagging and protease protection assays, applied to the expression of full-length AtNHX1 in yeast as a heterologous system, revealed that AtNHX1 is comprised of nine transmembrane domains that



Species	NHX Type Vac	Endo	PM
Arabidopsis thaliana	4	2	2
Solanum lycopersicum	3	1	1
Medicago truncatula	7	2	1
Glycine max	7	3	1
Populus trichocarpa	5	1	2
Zea mays	6	2	1
Oryza sativa	4	2	1
Brachypodium distachyon	4	2	2
Selaginella moellendorffii	3	2	2
Physcomitrella patens	5	2	2
Chlamydomonas reinhardtii	1	3	2

Fig. 1. Schematic representation of the evolutionary tree of the main plant families with several representative species. The table lists species and the number of orthologous NHX genes belonging to each of the three NHX functional classes: vacuolar (Vac), endosomal (Endo), or plasma membrane (PM) antiporters. Only sequenced genomes (http://www.phytozome.net/) were used to BLAST for orthologues in the PHYTOZOME database using *Arabidopsis* sequences for NHX1, NHX5, or SOS1/NHX7 as representatives for each class. Each entry in the table corresponds to the branch end-point of the tree.

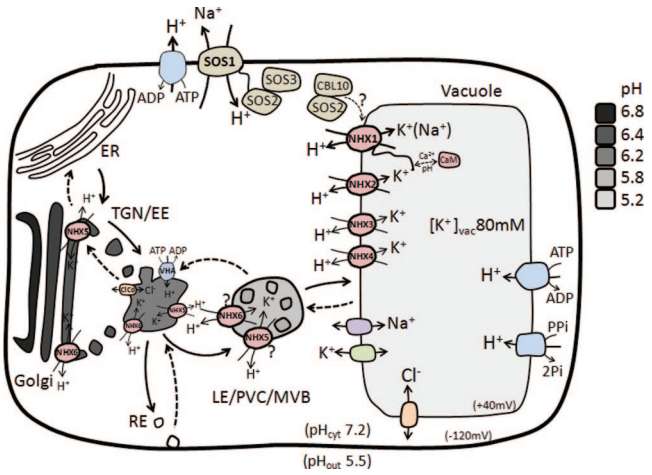


Fig. 2. Schematic diagram of a plant cell and the subcellular localization of the family of NHX proteins in *Arabidopsis*. Anterograde and retrograde trafficking pathways are also highlighted to show the roles of NHX5 and NHX6, in trafficking to the vacuole. Except for cytosolic pH (pH_{cyt}), the pH of individual cellular organelles and compartments is represented according to the pH scale indicated at the right of the figure. ER, endoplasmic reticulum; TGN/EE, *trans*-Golgi network/early endosome; LE/PVC/MVB, late endosome/pre-vacuolar compartment/multivesicular body; RE, recycling endosome.

traverse the membrane, with another three hydrophobic regions that, although they are membrane associated, do not span both sides of the membrane (Yamaguchi *et al*., 2003). Another striking structural feature of AtNHX1 that differed from the structure of animal NHEs was the orientation of the hydrophilic C-terminus in the vacuolar lumen rather than the cytosol (Yamaguchi *et al*., 2003). However, another study, using only fragments of the protein, found that AtNHX1 contained 11 transmembrane domains, a cytosolic C-terminus, and an overall membrane topology similar to that of human NHE (Sato and Sakaguchi, 2005). These conclusions should be treated with caution however, since AtNHX1 fragments, rather than the full-length protein, were used, and this probably altered the association of the protein fragments with the membrane. Several NHX features have been well established in yeast, animals, and plants. Significant sequence homology between the different species isoforms exists in the N-terminal domain. For example, the putative cation-binding domain and membrane-spanning pore are highly conserved in plants (Yokoi *et al*., 2002; Aharon *et al*., 2003) as well as animal NHEs and yeast Nhx1, and comprises the amino acid sequence 'FFIYLLPPI', a region where the diuretic drug amiloride and its derivatives bind and inhibit cation/H^+ exchange (Kinsella and Aronson, 1981; Blumwald and Poole, 1985). The C-terminus of members of the NHX family from yeast, plants, and animals is highly diverged even among closely related isoforms within the same species. Because the C-terminus regulates the antiport activity, it is thought that heterogeneous C-terminal sequences might constitute a novel means to regulate each member differentially (Orlowski and Grinstein, 2007). Mechanisms by which the C-terminus modulates the activity and localization of the antiporters via protein–protein interactions, phosphorylation, and glycosylation are discussed below.

Kinetics and structural model

The generally accepted mechanistic model for the operation of Na^+/H^+ antiporters is known as 'ping–pong', or 'alternating access' (Post and Dawson, 1994; Hunte *et al*., 2005; Mager *et al*., 2011). This model proposes a single binding site that is accessible to only one side of the membrane (either inwards or outwards). H^+s (or cations) bind to this site, and open binding induces a conformational change that exposes the bound cation (or H^+) to the other side of the membrane, where it is released, based on the concentration gradient. The binding site is now accessible to the environment containing the cations (or H^+s), so that it can be bound, translocated, and released on the opposite side of the membrane. Thus, the antiporter has only two chief conformations, and an alternation between these two conformations can only occur when the binding site is interacting with either a H^+ or a cation. Although no complete crystal structure of a yeast, plant, or animal NHX exists, the crystal structure of the distantly related *E. coli* NhaA has provided some insights into the structural mechanism of Na^+/H^+ antiporters (Padan *et al*., 2009). The cytoplasmic funnel contains negatively charged residues to both attract and increase the local concentration of cations while simultaneously repelling anions from the cytosolic ion passage (Hunte *et al*., 2005). Based upon this crystal structure, the cation (H^+) binding to the negatively charged active site at the centre of the antiporter would result in the displacement/bending of one of the helices that make up the core structure of NhaA, most probably as a result of the electrostatic destabilization of the active site (Hunte *et al*., 2005; Padan *et al*., 2009; Mager *et al*., 2011). This conformational shift would result in exposing the cation (H^+) to the periplasmic/cytosolic side of the membrane,

respectively. The change in the electrostatic environment of the active site and negative charges lining the periplasmic/cytosolic funnel results in the translocation of the cation (H^+) out of the antiporter into the aqueous environment. While the generation and analysis of the crystal structure of NhaA was a major milestone in H^+-coupled transport research, care should be taken when applying the proposed NhaA Na^+/H^+ exchange mechanism to plant NHXs. First, NhaA is very distantly related to the plant NHXs and animal NHEs, and thus shares little sequence homology, although structural similarities are still plausible. Secondly, NhaA is electrogenic, moving 1 Na^+ for every 2 H^+, while NHXs are electroneutral (Blumwald and Poole, 1985; Apse *et al*., 2003), as shown by the independence of Na^+/H^+ exchange from the electrical potential difference (membrane potential) (Blumwald and Poole, 1985; Apse *et al*., 2003). Thirdly, in order to provide well-ordered and static protein necessary for crystallization, NhaA was crystallized at the non-physiological pH of 4 which resulted in a 'pH-locked state' that did not allow the determination of specific conformations during ion binding, translocation, and release (Hunte *et al*., 2005). Lastly, intracellular NHXs mediate the H^+-coupled transport of both Na^+ and K^+ (Zhang and Blumwald, 2001; Apse *et al*., 2003) while NhaA does not mediate K^+ transport; thus, the structure of NHXs must accommodate the relatively larger K^+ ion.

Regulation

Post-translational modifications

One of the most simple and yet key post-translational modifications that proteins undergo is phosphorylation and dephosphorylation via kinases and phosphatases (Maathuis, 2008). This type of modification is known to modulate the activity and interaction of many proteins in plants, and at least 30% of all proteins undergo phosphorylation (Maathuis, 2008). It has been shown that phosphorylation can regulate the activity of NHE antiporters. Both HsNHE1 and HsNHE3 undergo phosphorylation both *in vivo* and *in vitro*, particularly at their C-terminus. Phosphorylation of HsNHE1 via a Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (Vila-Petroff *et al*., 2010) as well as by the kinase $p90^{RSK}$ (Manhas *et al*., 2010) enhanced its activity, whereas HsNHE3 phosphorylation by the protein kinase A resulted in the inhibition of its exchange activity (Zhao *et al*., 1999).

While the regulatory effects that phosphorylation has on the activity of the animal NHEs is well documented, evidence demonstrating a similar regulation of NHX activity is more modest. SOS2/CIPK24 is a serine/threonine protein kinase with an N-terminal catalytic domain (Liu *et al*., 2000) and SOS3/CBL4 is a Ca^{2+} sensor protein (Liu and Zhu, 1998). Under salt stress, SOS3/CBL4 perceives an increase in cytosolic Ca^{2+} and activates SOS2/CIPK24, which is subsequently recruited to and activates SOS1 by phosphorylation to reduce cytosolic Na^+ (Quintero *et al*., 2002). The same group later found that the activity of a vacuolar Na^+/H^+ antiporter was significantly reduced in *sos2* null mutants compared with wild-type controls and was restored when constitutively active SOS2 was added to the assay (Qiu *et al*., 2004). Although the phosphorylation of NHX by SOS2 was not shown, it is plausible that the *in vitro* phosphorylation reaction conditions may not have been optimal (Qiu *et al*., 2004). Interestingly, at least 25 SOS-like protein kinases, in part similar to CaMKII kinases, exist in *Arabidopsis* (Luan *et al*., 2009), and a homologue of this family was shown to phosphorylate HsNHE1 (Vila-Petroff *et al*., 2010). Moreover, SOS2 was shown to regulate the activity of the vacuolar H^+/Ca^{2+} antiporter CAX1 in a SOS3-independent manner (Cheng *et al*., 2004). Another Ca^{2+} sensor protein (calcineurin B like) CBL10 also interacts with SOS2/CIPK24 and targets the CBL10–SOS2/CIPK24 complex from an endosomal localization to the tonoplast. *cbl10* mutants are salt sensitive but accumulate less Na^+ than the wild type (Kim *et al*., 2007). Given the interaction at the tonoplast, and the Na^+ phenotypes, it is possible that CBL10 together with SOS2/CIPK24 may affect processes leading to sequestration of Na^+ into the vacuole and might involve NHX members. Since CBL10 is endosomal, it is possible that with CIPK interactors, CBL/CIPK modules may affect endosomal NHX5 and/or NHX6 functions, especially given their role(s) in salt responses. The CBL/CIPK module may constitute a complex yet versatile system to regulate responses of different Na^+/K^+ transporters, including perhaps intracellular NHX members (Luan *et al*., 2009).

Phosphoproteomic studies of *Arabidopsis*, *Hordeum vulgare* (barley), and *Oryza sativa* tonoplast preparations further suggested the regulation of NHX antiporters by phosphorylation (Whiteman *et al*., 2008*a*, *b*; Endler *et al*., 2009). In rice, the vacuolar OsNHX3 was phosphorylated at S471 in the C-terminus, a residue that is conserved among the remaining three vacuolar rice isoforms (OsNHX1, 2, and 4). Sequence comparisons between the OsNHX members and the AtNHX isoforms (AtNHX3, 5, and 6) indicated that the *Arabidopsis* isoforms contained the same serine residue at a similar position. Phosphorylation prediction programs (http://www.cbs.dtu.dk/services/NetPhos/) suggested that these residues have a high probability of undergoing phosphorylation. In AtNHX1 and AtNHX2, a threonine (itself a putative phosphorylation site) is substituted at this serine residue. Interestingly, this serine residue is also conserved in the only NHX in yeast (ScNHX1). While a literature search yielded no studies that characterized this putative phosphorylation site in either yeast or plants, it is possible to speculate that this serine/threonine phosphorylation site arose early in the evolution of NHXs as an important regulatory residue. A second serine phosphorylation site (S471) has also been suggested by a phosphoproteome analysis of *Arabidopsis* (Whiteman *et al*., 2008*b*) as well as barley tonoplast preparations (HvNHX1) (Endler *et al*., 2009). Moreover, sequence comparisons indicated that three vacuolar OsNHX isoforms (OsNHX1–OsNHX3) also contained this putative phosphorylation site.

Protein glycosylation has been shown to be crucial for proper folding, protection against degradation, localization, activity, selectivity, and protein–protein interactions (Molinari, 2007; Desko *et al*., 2009). ScNHX1 was found to be *N*-glycosylated at its C-terminus; however, no physiological role for the glycosylation of ScNHX1has been suggested (Wells and Rao, 2001). Glycosylation was also shown in both HsNHE1 and HsNHE3. Inhibition of HsNHE3 glycosylation resulted in significantly decreased antiporter activity, a 3-fold rise in HsNHE3 transcript

levels, and the abolishment of HsNHE-3 transport and integration into the plasma membrane (Soleimani *et al*., 1996). Although no experimental evidence is available, *in silico* analysis suggests that the glycosylation of NHXs, most probably at the C-terminus, might occur.

Calmodulin (CaM) is a small and ubiquitous Ca^{2+} sensor protein found in all eukaryotes that must bind calcium in order to interact with and activate or repress its target protein (Bouche *et al*., 2005). Human NHE1 was found to contain two CaM-binding domains (CaMBDs), with one binding domain having high affinity for CaM and the other site having intermediate affinity for CaM (Bertrand *et al*., 1994). Activation of NHE1 was found to be dependent on CaM binding to the high affinity CaMBD when exposed to stimuli such as osmotic stress (Bertrand *et al*., 1994). The precise mechanism of NHE1 activation by CaM occurs by the binding of CaM to an autoinhibitory domain, which overlaps with the high affinity CaMBD of NHE1, resulting in the release of NHE1 autoinhibition (Wakabayashi *et al*., 1994). In plants, the C-terminus of AtNHX1 was found to interact with a specific CaM-like protein (AtCaM15) (Yamaguchi *et al*., 2005). This interaction was dependent on both calcium and pH, as the interaction decreased with high vacuolar pH and low Ca^{2+} concentrations. Upon binding of AtCaM15 to AtNHX1, the V_{max} of Na^+ exchange activity was repressed by >2-fold in comparison with AtNHX1 activity without AtCaM15. Conversely, the V_{max} of the K^+/H^+ exchange activity with AtCaM15 was not significantly altered, resulting in a nearly 50% reduction in the Na^+/K^+ selectivity ratio. This was correlated with a 35% decrease in the antiporter's K_m for Na^+, indicating the presence of structural changes that modified the cation-binding site and altered Na^+ without affecting K^+ binding. It was proposed that under normal physiological conditions [i.e. an acidic vacuole (pH ~5.5) with a high Ca^{2+} concentration], AtCaM15 is bound to AtNHX1, resulting in a higher K^+/H^+ as compared with Na^+/H^+ exchange activity. However, under salt stress, the vacuole alkalinizes (Okazaki *et al*., 1996; Leshem *et al*., 2006) and binding of AtCaM15 to AtNHX1 is reduced, resulting in an increased Na^+/H^+ exchange activity and enhanced vacuolar Na^+ sequestration (Yamaguchi *et al*., 2005).

NHX functions in cell and plant growth and development

Vesicular trafficking

Cells synthesize, modify, and deliver molecular cargo in and between distinct endomembrane compartments through a complex and coordinated system of intracellular trafficking of cargo via vesicles. Vesicular trafficking depends on numerous molecular players and biochemical and biophysical factors (Jürgens, 2004; Šamaj *et al*., 2005). Principal among these factors is a vesicular luminal pH that must be maintained within a narrow range that is unique to specific intracellular compartments of the endomembrane system. In animals, intracellular compartments along the secretory and endocytotic pathways are known to be progressively more acidic with maturity (i.e. the anterograde route), with a typical representation of the luminal pH of the main endomembrane compartments ranging from pH ~7.2 in the endoplasmic reticulum (ER), pH ~6.8–6.2 in the medial Golgi, pH ~6.0–6.2 in the *trans*-Golgi network (TGN), pH 5.5–6.0 in the late endosome (LE)/multivesicular bodies (MVBs)/pre-vacuolar compartments (PVCs), and pH ~5 in vacuoles and apoplast/extracellular matrix (Paroutis *et al*., 2004; Casey *et al*., 2010; Ohgaki *et al*., 2011). In plants, however, other than the pH of the large cellular compartments such as vacuoles, the cytosol, or the apoplast, where measurements are relatively easier to obtain, little is known about the pH of the remaining endosomal compartments (Felle, 2005). pH is critical not only for the compartmentation of specific biochemical reactions but also for maintaining vesicular identity (through receptor association), sorting of newly synthesized or modified cargo, endocytosis, coat protein formation, energizing secondary transport systems, as well as the degradation or recycling of molecules (Paroutis *et al*., 2004). Changes in endosomal pH can lead to diverse cellular phenotypes such as an alteration of the protonation state of cargo proteins and their receptors, which in turn would alter the charge and structure of their binding domains and affect proper protein folding (Casey *et al*., 2010). The establishment of vesicular acidification is achieved by the action of V-type H^+-ATPase (V-ATPase) and H^+-inorganic pyrophosphatase (PPiase) pumps (Schumacher, 2006; Marshansky and Futai, 2008). Although no evidence exists to support the operation of a PPiase in intracellular compartments other than the vacuole (Schumacher, 2006), subunits of the V-ATPase have been localized to the TGN/early endosome (EE) (Dettmer *et al*., 2006). The localization of a V-ATPase in the plant TGN lends strong support to the notion that the basic mechanisms underlying vesicular trafficking are conserved within eukaryotes. The application of the V-ATPase-specific inhibitor concanamycin A leads to changes in Golgi morphology, defects in vesicular trafficking, and dissipation of the proton gradient in intracellular compartments, indicating the critical role of pH homeostasis in vesicular trafficking (Marshansky and Futai, 2008). If intravesicular pH were solely controlled by the action of V-type H^+-ATPase, the vesicular luminal pH could reach its theoretical maximum of ≤3.0 (Casey *et al*., 2010); therefore, vesicular pH homeostasis is controlled by the active H^+ translocation mediated by the H^+ pump and luminal H^+ leaks that establish optimal endosomal pH (Orlowski and Grinstein, 2011). Although additional regulation of the V-ATPase through subunit assembly, regulation of pump activity, or pump abundance might all contribute to the establishment of compartment-specific pH (Forgac, 2007; Marshansky and Futai, 2008), evidence in yeast, plants, and animals supports the role of H^+-coupled cation antiporters as alkalinizing agents that, together with V-ATPases, establish and regulate the luminal pH of intracellular compartments (Orlowski and Grinstein, 2007). Evidence to support these roles is summarized below.

pH homeostasis relies not only on the action of H^+ pumps and NHX-type antiporters, but also on the proper regulation of membrane potential that would otherwise build up across endomembrane compartments. Because the V-ATPase is electrogenic, luminal acidification could not proceed without counterion balance. In particular, chloride (Cl^-) might be the main counterion that can reduce the electrical potential difference generated across endosomal membranes (Maeda *et al*., 2008; Ohgaki *et al*., 2011). A Golgi/TGN-localized anion channel GPHR (Golgi pH

regulator) is required for the acidification of the Golgi/TGN because mutated GPHR resulted in an increase in Golgi/TGN pH, a deterioration of Golgi integrity, and disruption of vesicular trafficking and glycosylation. However, a knockout of ScGef1p in yeast, a putative voltage-gated chloride channel, did not result in an obvious trafficking phenotype as seen in ScNhx1p (Bowers *et al*., 2000). Although the information regarding plant endosomal Cl^- requirements is limited (De Angeli *et al*., 2009), it was shown recently that AtCLC-d co-localized with the V-type ATPase subunit a1 to the TGN (von der Fecht-Bartenbach *et al*., 2007). The *clc-d* mutant showed impaired root growth under normal conditions and had a significantly higher sensitivity to concanamycin A, suggesting that AtCLC-d might function as an 'electrical shunt' to increase the acidity of the TGN (von der Fecht-Bartenbach *et al*., 2007). The generation of multiple knockout lines of anion transporters together with VHA and NHX mutants should further our understanding of possible co-regulation and co-functionality of these transporters in endosomal compartments. Nevertheless, CLC co-transproters may also couple the transport of H^+ to nitrate instead of Cl^- (De Angeli *et al*., 2006), thus a role for nitrate in charge compensation across endomembranes should also be considered. A number of K^+ channels have been identified (Lebaudy *et al*., 2007) and a role for these channels in the regulation of endosome membrane potential also cannot be ignored.

The dependency of trafficking on endosomal pH and NHX-type antiporters is well exemplified in yeast. Bowers *et al*. (2000) first reported on the requirement of yeast ScNhx1p for vesicle trafficking and showed that in the knockout Sc*nhx1Δ*, trafficking out of the Golgi/PVC was blocked (Bowers *et al*., 2000). The knockout also had acidic cytoplasmic and vacuolar pH as well as growth sensitivity to acid media (Ali *et al*., 2004; Brett *et al*., 2005*b*), high Na^+, and hygromycin (Gaxiola *et al*., 1999). In addition, protein processing and mis-sorting also occurred because ~35% of the newly synthesized carboxypeptidase Y (CPY) was secreted to the apoplast (Bowers *et al*., 2000). Interestingly, the application of a weak base alleviated both the protein trafficking defects and the sensitivity to hygromycin B in *nhx1Δ*, while weak acid treatments in wild-type plants mimicked the *nhx1Δ* phenotypes (Brett *et al*., 2005*b*). Because both cytoplasmic and vacuolar pHs were significantly lower in *nhx1Δ* when grown at low pH, lack of Nhx1p was likely to have pleiotropic and indirect effects in yeast pH homeostasis. The data supported the idea that endosomal CPAs are required for intracellular pH homeostasis especially when challenged by growth conditions that altered intracellular pH.

More recent data indicated that Nhx1p might be involved in homotypic vacuolar fusion in yeast. *Nhx1Δ* had fragmented vacuoles that could be induced to fuse by complementation with functional Nhx1p (but not a mutant protein lacking transport activity), by treatment with the weak base chloroquine, as well as by addition of the SNARE Vam7p (Qiu and Fratti, 2010). It is possible that in *Nhx1Δ* mutants, vesicles might lack the necessary components needed to complete vacuole fusion.

In animal cells, four isoforms of endosomal NHE exchangers localize to post-Golgi endosomal compartments. NHE8 is found in the mid- to *trans*-Golgi, but also in MVBs; NHE7 in mid- to *trans*-Golgi stacks and the TGN; and NHE6 and NHE9 in early and late recycling endosomes. Overexpression of NHE6, NHE8, or NHE9 caused vesicular alkalanization, whereas suppression of either NHE6 or both NHE6 and NHE9 expression lowered the luminal pH of endosomal compartments where these NHEs reside (Nakamura *et al*., 2005; Ohgaki *et al*., 2010). Evidence also suggests that depending on the tissue and the cell type, intracellular NHEs cycle between intracellular compartments and are affected by proteins interacting with the NHE C-terminus (Ohgaki *et al*., 2011). For example, the secretory membrane carrier protein SCAMP2, which is implicated in vesicular trafficking and localized in Golgi and post-Golgi compartments, interacts with NHE7 at the C-terminus, causing a shuttling of NHE7 from recycling endosomes to the TGN. A disruption of this interaction, using a dominant-negative mutant of SCAMP2, restricted NHE7 localization to recycling endosomes alone (Lin *et al*., 2005). Another C-terminus interaction of NHE6 with a receptor for activated C kinase 1 (RACK1) affected the distribution of NHE6 between plasma membranes and an endosomal population (Ohgaki *et al*., 2008). The overexpression of NHE8 resulted in a 7-fold increase in proton concentration in the mid-to *trans*-Golgi stacks (Nakamura *et al*., 2005), while the knockdown of NHE8 resulted in enlarged and more densely packed late MVBs, likely to be a result of trafficking defects between the endosomal compartments brought about the acidification of the Golgi (Lawrence *et al*., 2010). Interestingly, NHE8 knockdown did not significantly alter the pH of MVBs, suggesting that the trafficking defects were a consequence of ion (K^+) imbalance rather than aberrant pH (Lawrence *et al*., 2010). Overexpression of human NHE9, which is localized to late recycling endosomes, caused an increase in the luminal pH of NHE9 compartments, suggesting that *in vivo*, NHE9 regulates the pH and the cation concentration (Nakamura *et al*., 2005). Collectively, data from the mammalian endosomal NHEs mirror results obtained from yeast and point to a role for NHX-type antiporters in controlling endosomal pH by mediating vesicular luminal H^+ efflux.

The most direct evidence demonstrating a requirement for endosomal NHX antiporters in vesicular trafficking in plants was provided by Bassil *et al*. (2011*a*). Localization experiments indicated that both NHX5 and NHX6 reside in the TGN and co-localized with the TGN markers syntaxin SYP61 and the V-ATPase subunit VHA-a1 (Bassil *et al*., 2011*a*). Nevertheless, additional localization studies are needed using additional markers to determine whether NHX5 and NHX6 are also distributed in other cellular compartments, whether they completely overlap in localization or participate in distinct trafficking pathways, or whether they exist in distinct compartment and participate in unique trafficking pathways. The closely related tomato antiporter SlNHX2 also localized to small vesicles when transiently expressed in onion epidermal cells and also co-localized with PVC and Golgi markers in yeast and tomato protein fractions (Venema *et al*., 2003; Rodriguez-Rosales *et al*., 2008). Plants lacking both NHX5 and NHX6 displayed severe growth retardation, while the single knockouts *nhx5* or *nhx6* did not result in any obvious phenotype, suggesting that NHX5 and NHX6 have redundant roles. Supporting this conclusion, the single knockouts and wild-type plants displayed highly similar gene expression profiles compared with those between the *nhx5nhx6* double knockout and wild type that differed significantly (Bassil *et al*.,

2011*a*). Several lines of evidence indicated that similar to Nhx1p in yeast, NHX5 and NHX6 are required for trafficking to the vacuole. Using the endocytotic tracer dye, FM4-64, and monitoring the progressive fluorescence labelling of endomembranes, it was shown that labelling of the vacuole was severely delayed in *nhx5nhx6*, suggesting that a step from the TGN or PVC was compromised in the *nhx5nhx6* double knockout. It did not appear that early endocytotic events specifically required NHX5 and NHX6 because uptake of FM4-64 did not differ between the *nhx5nhx6* mutant and wild-type root cells. In addition, trafficking of CPY–green fluorescent protein (GFP), which normally accumulates in the vacuole, was missorted to the apoplast in *nhx5nhx6* plants (Bassil *et al*., 2011*a*). Also, transcriptional analysis indicated an abundance of trafficking-related transcripts that were differentially expressed in the double knockout *nhx5nhx6*.

Although functional studies are limited, closely related endosomal K^+/H^+ antiporters have also been implicated in K^+ and pH regulation, and endosomal trafficking in yeast and plants. Two CHX members, CHX17 and CHX20, co-localized with PVC and ER endomembrane markers in diverse tissues (Chanroj *et al*., 2011). The heterologous expression of CHX17 and CHX20 rescued alkaline pH sensitivity and trafficking defects in yeast mutants. Furthermore, CHX20, but not CHX17, caused the alkalization of the vacuole and the acidification of the cytosol, while both CHX17 and CHX20 were implicated in K^+ transport (Cellier *et al*., 2004; Chanroj *et al*., 2011). These data support the idea that in addition to endosomal NHX members, other CPAs in endomembranes modulate cation and pH homeostasis and might have direct roles in cellular trafficking events (Chanroj *et al*., 2012).

Vacuolar NHX antiporters could also have a role in vesicular trafficking because transcriptional analysis of *nhx1* mutants revealed that a significant number of vesicular trafficking- and protein targeting-related transcripts were differentially expressed (Sottosanto *et al*., 2004). Immunolocalization of NHX1 in salt-treated cells clearly showed a punctate pattern indicative of vesicles in the cytoplasm, suggesting that at least under salt stress, NHX1 maybe have functions in vesicular trafficking (Hamaji *et al*., 2009).

Collectively these data indicate that endosomal NHX-type antiporters are critical regulators of endosomal trafficking by controlling endosomal pH. A limitation in obtaining a direct demonstration between vesicular pH and endosomal processes is the lack of pH or ion measurements in endosomal compartments *in planta*. The development of targeted and/or genetically encoded pH sensors should advance our understanding of the role(s) played by endosomal pH homeostasis.

pH regulation

Given the role of Na^+/H^+ antiporters in pH regulation, it is perhaps not surprising that the antiporters could themselves be regulated by pH. The 'pH-locked' crystal structure of NhaA provided evidence to support the notion that NhaA transport activity was regulated by a peptide-encoded pH sensor which would 'sense' the proton concentration and result in transporter conformational changes (Hunte *et al*., 2005; Padan *et al*., 2009). Based on its crystal structure, NhaA was proposed to be inactive at pH <6.5 as a result of the partial occlusion of the ion-binding site on the cytosolic side of the antiporter, resulting in the inaccessibility of hydrated Na^+ to the binding site as well as a complete obstruction of the periplasmic side of the antiporter. At pH >6.5 the antiporter's ion-binding site would reorient to allow the ion-binding site to be fully exposed to the cytosolic passage as well as the elimination of the periplasmic barrier (Hunte *et al*., 2005). However, a recent study found that while NhaA is indeed nearly completely inactive at pH ~7, this is only the case at symmetrical pH, namely when the pH was the same on both sides of the membrane (Mager *et al*., 2011), while the opposite resulted under asymmetrical pH, and NhaA was active even at pH 5 (Mager *et al*., 2011). This was the case even when the proposed pH sensor at the cytosolic-facing side of the antiporter was exposed to pH 5 (Mager *et al*., 2011). Instead of H^+ acting allosterically to inactivate NhaA, it is now proposed that H^+ and Na^+ ions compete for the ion-binding site (Mager *et al*., 2011); thus, under high H^+ concentrations, Na^+ ions are blocked from binding to the active site (Mager *et al*., 2011).

In the case of NHE1, lower cytosolic pH allosterically activated the protein activity whereas at normal cytosolic pH (pH ~7.3) NHE1 was inactive (Putney *et al*., 2002). Although the precise mechanism of H^+ allosteric activation is unknown, two possibilities can be postulated: (i) the direct protonation of certain amino acid side chains results in conformational changes that alter the antiporter activity; or (ii) the action of interactors 'sensing' changes in cytosolic pH which subsequently bind to NHE1 and regulate its activity (Putney *et al*., 2002). In plants, the activity of the *Beta vulgaris* Na^+/H^+ antiporter activity was affected by changes in vacuolar pH, but not cytosolic pH (Blumwald and Poole, 1985). While maintaining a constant pH difference across the tonoplast, a change in vacuolar pH from 6.4 to 7.2 resulted in a >3-fold increase in K_m for H^+-coupled Na^+ transport, while the V_{max} of the exchange remained constant (Blumwald and Poole, 1985). These results indicated that protons allosterically increased, either directly or indirectly, the affinity of the vacuolar antiporter for Na^+ ions and suggested that plant vacuolar antiporters 'sense' changes in vacuolar pH, altering their cation affinity(ies). This notion was supported by the pH-dependent interaction of the vacuolar lumen-localized NHX1 C-terminus and vacuolar AtCaM15 which affected the K^+/Na^+ selectivity of NHX1 (Yamaguchi *et al*., 2005). Clear evidence for the role of NHXs in pH regulation was provided by studies of morning glory (*Ipomea*) flower petal coloration. During flower development, *Ipomea* petals begin to accumulate anthocyanins in vacuoles which are red at low vacuolar pH but turn blue as the pH increases. During the colour transition, petal vacuolar pH increased from 6.5 to 7.5 and was accompanied by increased V-ATPase, H^+-PPiase, and NHX1 expression and activity (Yoshida *et al*., 1995, 2009; Yamaguchi *et al*., 2001). The pH change and parallel change in colour was only partial in *nhx1* mutants of *Ipomea* and may depend on another abundantly expressed isoform, *InNHX2* (Ohnishi *et al*., 2005).

Another vacuolar member, NHX2, is also implicated in pH homeostasis. Recently it was shown that a knockout mutant lacking both NHX1 and NHX2 had more acidic vacuoles in hypocotyl cells of etiolated seedlings as well as root cells of the mature zone of roots. Interestingly, root tip cells were more acidic than

mature root zone cells, and the pH in these cells did not differ between the *nhx1 nhx2* knockout and the wild type. These results suggested that NHX antiport activity is more pronounced in elongating cells that require a significant increase in vacuolar volume to drive that expansion. Despite the measurement of acidic pH in *nhx1 nhx2* plants, the precise effect of altered vacuolar pH is not clear given the concomitant reduction in vacuolar K^+ and clear K^+-associated phenotypes observed (see below).

Cation homeostasis

Potassium is an essential plant nutrient and the most abundant cation in plants, comprising up to 10% of plant dry matter. It is an important cofactor in many biosynthetic processes, for charge balance and enzyme activity in the cytosol, and in the vacuole K^+ plays key roles in cell volume regulation (Maathuis and Podar, 2011). Given its importance in many biochemical functions, cellular K^+ homeostasis requires that a tightly controlled cytosolic K^+ concentration be maintained. Under variable K^+ supply, and unless the tissue concentration falls below 25 mM, the cytosolic K^+ concentration does not change appreciably and is kept between 80 mM and 100 mM. On the other hand, the vacuolar K^+ concentration fluctuates dramatically with K^+ supply and tissue content (Walker *et al*., 1996; Leigh, 2001). At the typical tonoplast electrical potential difference (~30 mV positive inside), K^+ transport across the tonoplast must be energized to accumulate K^+ in the vacuole above ~20 mM (Leigh and Wyn Jones, 1984; Martinoia *et al*., 2000; Leigh, 2001). The data of Walker *et al*., (1996) suggested that assuming no signifcant changes in cell turgor or transport at the plasma membrane, cytosolic K^+ might be homeostatically maintained in part by exchange with the vacuole, a role that could be satisfied by NHX-type antiporters.

Although the operation of vacuolar H^+-coupled cation transport was demonstrated >25 years ago (Blumwald and Poole, 1985), the molecular identification of AtNHX1, the first member of the NHX type of intracellular Na^+/H^+ antiporters (Apse *et al*., 1999; Gaxiola *et al*., 1999), was only attained after the completion of the *Arabidopsis* genome. Since then, the roles for NHX-type antiporters in vacuolar K^+ homeostasis have been convincingly demonstrated. Under normal growth conditions, NHX1 mediates both the Na^+/H^+ and K^+/H^+ exchange activity (Zhang and Blumwald, 2001; Apse *et al*., 2003). NHX1 catalysed K^+/H^+ exchange with affinities in the order of 12 mM K^+ (Yamaguchi *et al*., 2005). In the knockout *nhx1*, the reduced antiporter exchange activity was correlated with smaller cells, especially the expansion of highly vacuolated cells, and it was suggested to be caused by low intravacuolar K^+ required for turgor (Apse *et al*., 2003). Transcript analysis indicated that *nhx1* knockouts up-regulated transcipts for high affinity K^+ uptake transporters (Sottosanto *et al*., 2004). *In situ* hybridization of AtNHX1 indicated that the protein was ubiquitously expressed in all major organs including petals, anthers, and leaves (Apse *et al*., 2003). During grape berry development, where ripening requires a sustained uptake of vacuolar K^+, the expression of VvNHX1 was grealty enhanced during the veraison and post-veraison fruit growth stages, indicating the need for vacuolar K^+ for fruit growth (Hanana *et al*., 2007). The overexpression of AtNHX1 in tomato resulted in increased vacuolar Na^+/H^+ and K^+/H^+ exchange (Zhang and Blumwald, 2001; Leidi *et al*., 2010). Under control conditions, *NHX1*-overexpressing tomato also exhibited K^+ deficiency symptoms and an up-regulation of K uptake transcripts despite having a high tissue K^+ content compared with the wild type (Leidi *et al*., 2010). The overexpression of the tomato LeNHX2 in *Arabidopsis* also resulted in an increased endosomal K^+/H^+ exchange and demonstrated that the regulation of K^+ homeostasis by endosomal compartments is also essential for plant growth and development (Venema *et al*., 2003; Rodriguez-Rosales *et al*., 2008).

More direct evidence for the role of vacuolar NHX1 and NHX2 in K^+ homeostasis was recently provided using reverse genetics (Bassil *et al*., 2011b; Barragán *et al*., 2012). In *Arabidopsis*, the single knockout of *NHX2* did not display any growth phenotype and co-localized with NHX1, suggesting that this isoform is at least partially redundant to NHX1 (Bassil *et al*., 2011*b*). Nevertheless, additional functional studies are required to better understand whether NHX2 functions completely overlap those of NHX1. In *Arabidopsis* lacking both NHX1 and NHX2, growth was significantly reduced compared with either *nhx1*, which showed a mild growth phenotype (Apse *et al*., 2003), or wild-type plants. *nhx1 nhx2* plants had reduced cell expansion in all tissues, but cell size was much more reduced in rapidly elongating organs such as flower filaments and hypocotyls of etiolated seedlings (Bassil *et al*., 2011*b*). The double *nhx1 nhx2* knockout exhibited poor seed set because its filaments did not extend far enough to position the anther at the stigma. In addition, these plants had non-dehiscent anthers, but flowers could be artificially pollinated. These results implicated NHX1 and NHX2 in processes involving K^+-dependent hydration/dehydration during dehiscence (Heslop-Harrison and Heslop-Harrison, 1996; Rehman and Yun, 2006). In root and hypocotyl cells, the vacuolar K^+ concentration was ~30 mM, about one-third that of comparable wild-type cells (Bassil *et al*., 2011*b*), and was also noted in leaf cells (Barragán *et al*., 2012). K^+/H^+ exchange activity of tonoplast vesicles was 3-fold lower in *nhx1 nhx2* and correlated with a significant reduction in leaf cell K^+ (Barragán *et al*., 2012). Leaf turgor and tissue water content in *nhx1 nhx2* was also significantly lower, with impaired osmoregulation and delayed stomatal closure resulting in poor regulation of plant water status (Barragán *et al*., 2012). Interestingly, another cation/H^+ exchanger, CHX20, which is endosome localized, was also implicated in guard cell osmoregulation or pH homeostasis (Padmanaban *et al*., 2007). Collectively, these data illustrate the importance of NHX antiporters in the maintenance of turgor for cell expansion, affecting not only growth but also specific developmental processes such as flowering and stomatal regulation.

Interestingly, plants lacking *NHX1* and *NHX2* had extreme sensitivity to external K^+ concentrations >5 mM and displayed poor leaf expansion, root curling, and yellowing of leaves (Bassil *et al*., 2011*b*; Barragán *et al*., 2012). The response was specific to K^+ because replacing the anion of the salt did not alter the response to K^+ (Bassil *et al*., 2011*b*). Treatment of rice with high KCl induced the expression of OsNHX1 and OsNHX2 in rice shoot and roots, suggesting that these two isoforms may be necessary to transport excess K^+ from the cytosol into the vacuole (Fukuda *et al*., 2011). The expression of other vacuolar NHX isoforms (OsNHX3) remained unaffected by excess

K^+, possibly because particular isoforms may alter their expression only under specific conditions or may not catalyse significant K^+ transport. This might be the case in *Arabidopsis* as well (see below). However, the addition of ≥10 mM Na^+ instead of K^+ partly rescued the growth of the *nhx1 nhx2* knockout plants, suggesting that Na^+ uptake into the vacuole substituted the lack of turgor driven by K^+ needed for cell expansion (Bassil *et al*., 2011*b*). The response to Na^+ also suggested that NHX antiporters may not be the only means to transport Na^+ into the vacuole. Other candiates such as CHX or KEA are unlikely to be major contributors because of the 28 CHX members, none has been localized to the tonoplast, while functional evidence on KEA is lacking (Pardo *et al*., 2006; Chanroj *et al*., 2012). It is also unlikely that the two other vacuolar members of *Arabidopsis*, NHX3 or NHX4, would be significant contributors to vacuolar Na^+ uptake. First, AtNHX3 and AtNHX4 isoforms have different tissue- and developmental-specific expression, and the growth of *Arabidopsis* knockout mutants lacking all four vacuolar NHX members (i.e. NHX1–NHX4) improved under external Na^+, but not K^+ (E. Bassil *et al*., unpublished results). Collectively, these results indicate that NHX1 and NHX2 are the main transporters mediating K^+ uptake into the vacuole. The sensitivity of plants lacking NHX1 and NHX2 to even moderate amounts of K^+ in the growth medium could be a consequence of the 'toxic' accumulation of K^+ in the cytosol, since the K^+ electrochemical potential would favour the uptake of K^+ into the cell, while the lack of K^+ exchange at the tonoplast of *nhx1 nhx2* vacuoles would maintain a low vacuolar K^+ content (Bassil *et al*., 2011*b*). Consistent with this notion, a high cytosolic K^+ activity was reported in *nhx1 nhx2 Arabidopsis* knockout mutants (Barragán *et al*., 2012).

Although much is known about limiting K^+ uptake at the plasma membrane, little is known about the possible consequences of increasing cytosolic K^+ (Gierth and Maser, 2007). This is probably because under normal conditions, an influx of cytosolic K^+ is efficiently modulated by compartmentation into the vacuole. NHX mutants are therefore particularly useful to decipher the mechanisms of homeostatic control of cellular K^+, and the feedback that controls uptake at the plasma membrane and cytosol/vacuole distribution. The response of *nhx1 nhx2* also raises interesting questions about the 'compatibility' of cytosolic K^+, since it would appear that both high and low cytosolic K^+ concentrations affect cell function. A relatively high cytosolic K^+ might result in secondary effects, for example inhibiting the activity of the H^+-ATPase (Buch-Pedersen *et al*., 2006).

Less is known about functions of endosomal NHX antiporters in K^+ homeostasis, mainly because the direct measurement of endosomal ion contents is technically challenging (Orlowski and Grinstein, 2011). Nevertheless the selectivity of intracellular NHX-type antiporters is known to be higher for K^+ than for Na^+ for both plant NHXs and animal NHEs (Numata and Orlowski, 2001; Venema *et al*., 2003; Nakamura *et al*., 2005; Rodriguez-Rosales *et al*., 2008). The first endosomal NHX to be identified was SlNHX2 (Rodriguez-Rosales *et al*., 2008) which is orthologous to NHX5 of *Arabidopsis* with ~70% identity on a nucleotide basis (Pardo *et al*., 2006). Activity assays indicated that this antiporter contributes significantly to endosomal K^+ and pH exchange (Venema *et al*., 2003; Rodriguez-Rosales *et al*., 2008). Although overexpression of SlNHX2 did not affect plant growth in normal growth conditions, it reduced growth at low K^+, possibly caused by a reduction in cytosolic K^+ (Rodriguez-Rosales *et al*., 2008).

One possible consequence of altered cation (K^+) homeostasis in endosomes might also be related to the KEX2/furin family of proteases of the Golgi/secretory pathway that are required for post-translational modification of proteins in yeast. The activity of KEX2 *in vitro* is cation dependent and, although not examined in the context of Nhx1 function, K^+ is known to act as a regulatory cofactor of the KEX2/furin family of endoprotease activity. These endoproteases are required for maturation of newly synthesized proteins in the secretory pathway of yeast as well as animal cells (Rockwell and Fuller, 2002).

Salt tolerance

Under typical physiological conditions, the cytosol of higher plant cells contains between 60 mM and 100 mM K^+, and lower Na^+ concentrations (1–10 mM) (Blumwald, 2000). An abnormally high cytosolic Na^+/K^+ ratio has an inhibitory effect on protein synthesis and cytosolic enzymatic activity (Glenn *et al*., 1999). A saline environment with high extracellular Na^+ would create a steep Na^+ electrochemical gradient favouring Na^+ influx into the cell. Unless actively extruded out of the cell, Na^+ ions will accumulate to detrimental levels within the cytosol.

Once Na^+ enters the cytosol, cytosolic Na^+ content is controlled by the action of plasma membrane-bound Na^+/H^+ antiporters (SOS1, and possibly other CPAs) that actively extrude Na^+ out of the cell (Shi *et al*., 2002; Pardo *et al*., 2006; Apse and Blumwald, 2007), and NHX-type vacuolar Na^+/H^+ antiporters that mediate the sequestration of Na^+ ions into the vacuole (Blumwald and Poole, 1985; Apse *et al*., 1999). Transport studies to date also indicated that NHXs catalyse both Na^+/H^+ and K^+/H^+ exchange (Zhang and Blumwald, 2001; Venema *et al*., 2002; Apse *et al*., 2003; Leidi *et al*., 2010). The initial cloning and subsequent overexpression of AtNHX1 firmly demonstrated that the overexpression of AtNHX1 improved salt tolerance by mediating the sequestration of Na^+ into the vacuole (Apse *et al*., 1999; Zhang and Blumwald, 2001). This has been confirmed in numerous species such as *Arabidopsis*, tomato, canola, cotton, rice, wheat, tobacco, and sugar beet (Apse and Blumwald, 2002, 2007; Kronzucker and Britto, 2011). Recently, the ability of NHX1 to mediate Na^+ compartmentation into the vacuole has been challenged. Leidi *et al*. (2010) showed that transgenic tomato plants expressing AtNHX1 mediated the accumulation of vacuolar K^+ and not Na^+. Nevertheless, these conclusions should be treated with caution for the following reasons: (i) it is not clear whether the overexpression of AtNHX1 resulted in increased protein in the vacuolar membrane, since the work only showed increased NHX1 in a microsomal fraction; and (ii) vacuolar ion contents were estimated using EDX (energy dispersing X-ray analysis) in fast-frozen samples and not rapid freezing combined with slow substitution with chemical fixatives. Thus, the method used was prone to elemental redistribution of soluble elements, in particular Na^+ (Smart *et al*., 2010). Recently, the role of NHX-type vacuolar antiporters as determinants of salt tolerance in tomato was confirmed by Galvez *et al*. (2012), who compared the expression of NHX proteins (LeNHX1, LeNHX2, LeNHX3,

and LeNHX4) in salt-sensitive and salt-tolerant tomato varieties. Their results showed that during salt stress, the salt-tolerant varieties displayed enhanced expression of LeNHX3 and LeNHX4 (orthologues of AtNHX1 and AtNHX2) with a concomitant increase in Na^+ in their tissues (Galvez *et al*., 2012).

The K^+-related phenotypes seen in the *nhx1 nhx2* double knockouts clearly indicated that these antiporters mediate K^+/H^+ exchange under normal conditions (Bassil *et al*., 2011*b*), corroborating previous observations (Zhang and Blumwald, 2001; Venema *et al*., 2002; Apse *et al*., 2003; Leidi *et al*., 2010). Nevertheless, ion specificity could be altered when overexpressing *NHX1* and/or *NHX2* since the concomitant increase in protein would contribute to overcome the endogenous mechanisms controlling the antiporter selectivity. For example, the binding of AtCaM15 to the C-terminus of AtNHX1 would decrease the Na^+ selectivity of the antiporter, and the increase in protein content in the AtNHX1-overexpressing plants would by-pass this inhibition (Yamaguchi *et al*., 2005). Results showing that the increase in vacuolar Na^+/H^+ activity of transgenic *Arabidopsis* overexpressing *AtNHX1* was much higher than the relative increase in AtNHX1 abundance (Apse *et al*., 1999) support this notion.

Knockout plants lacking two vacuolar V-ATPase subunits exhibited reduced tonoplast V-ATPase activity, had a reduced capacity to store NO_3^- or toxic amounts of Zn, but did not show increased sensitivity to high salinity (Krebs *et al*., 2010). Salt sensitivity was observed instead in the knockdown of the endosomal (EE/TGN) V-ATPase isoform, demonstrating the critical importance of the endosomal system for salt tolerance (Krebs *et al*., 2010). The critical role of V-ATPase in the endosomal system might be due to a lack of H^+-PPiase (Schumacher, 2006). These results suggested a role for endosomal NHX Na^+/H^+ antiporters in the responses of plants to salt. Support for this notion was provided by Bassil *et al*. (2011*a*) who demonstrated that two endosomal NHX members, NHX5 and NHX6, were required for salt tolerance, since the double knockout plants lacking these two isoforms were extremely sensitive to low salt. Silencing of endosomal SlNHX2 (an orthologue of AtNHX5) in tomato led to plant growth inhibition, as well as increased sensitivity to salt (Rodriguez-Rosales *et al*., 2008). *Nhx1Δ* yeast mutants were similarly sensitive to salt stress (Nass *et al*., 1997). From the work of Bassil *et al*. (2011*a*), it was not possible to assess whether NHX5 and NHX6 were required for Na^+ uptake into the endosomes or whether their main role was associated with pH homeostasis and vesicular trafficking, needed for the response of the plants to salt stress. Endosomal trafficking, and vesicle fusion to the vacuole, are increasingly considered to be an important aspect of the cellular responses to abiotic stresses (Mazel *et al*., 2004; Leshem *et al*., 2006; Hamaji *et al*., 2009). The rapid increase in vacuolar volume occurring during salt stress (Mimura *et al*., 2003; Hamaji *et al*., 2009) would require enhanced vesicular trafficking to the vacuole for membrane augmentation and the allocation of transporters.

Few studies have reported an improvement in salt tolerance in several species overexpressing isoforms of endosomal NHX antiporters such as NHX5 (Shi *et al*., 2008; Li *et al*., 2011*a*, *b*) and might be related to altered endosomal cation homeostasis (Rodriguez-Rosales *et al*., 2008). Although the mechanisms by which endosomal pH and/or ion homeostasis affect salt tolerance are not yet clear, the possibility that excess cytosolic Na^+ is also sequestered within vesicles that subsequently fuse to the vacuole, thus contributing to reduce high cytosolic Na^+ concentrations, should also be considered.

Concluding remarks

There is extensive evidence on the importance of the establishment and maintenance of ion and pH homeostasis in all compartments of plant cells. The generation of vacuolar and endosomal pH is attained by the action of H^+ pumps and the operation of cation/H^+ antiporters that, in addition to regulating the H^+ electrochemical gradient in each compartment, also mediate cation uptake. During recent years, critical functions of NHX-type antiporters in cell growth and development have been highlighted. Nevertheless, the mechanisms regulating NHX function(s) remain largely unknown. Genetic studies using single and multiple knockouts are most needed to understand the contribution of each individual NHX isoform, their physiological significance, or their apparent redundancy. Although vacuolar-bound NHX antiporters have been characterized in some detail, their roles in osmoregulation, cell growth, and development are now emerging. The discovery that endosomal-bound NHX antiporters are critical for cell growth and play important roles in vesicular trafficking, protein processing, and cargo delivery raises important questions. How do endosomal ion and pH homeostasis regulate vesicular trafficking? What are the protein partners that regulate NHX functions and influence their localization? An improvement of methodologies for the isolation and purification of the different endosomes, together with the development of ion-specific molecular sensors for the precise measurement of endosomal pH and ion content, should unravel answers to these questions.

Acknowledgements

This work was supported in part by grants from the National Science Foundation (MCB-0343279; IOS-0820112) and the Will W. Lester Endowment, University of California, to E. Blumwald.

References

Aharon GS, Apse MP, Duan S, Hua X, Blumwald E. 2003. Characterization of a family of vacuolar Na^+/H^+ antiporters in *Arabidopsis thaliana*. *Plant and Soil* **253,** 245–256.

Ali R, Brett CL, Mukherjee S, Rao R. 2004. Inhibition of sodium/proton exchange by a Rab-GTPase-activating protein regulates endosomal traffic in yeast. *Journal of Biological Chemistry* **279,** 4498–4506.

Apse MP, Aharon GS, Snedden WA, Blumwald E. 1999. Salt tolerance conferred by overexpression of a vacuolar Na^+/H^+ antiport in Arabidopsis. *Science* **285,** 1256–1258.

Apse MP, Blumwald E. 2002. Engineering salt tolerance in plants. *Current Opinion in Biotechnology* **13,** 146–150.

Apse MP, Blumwald E. 2007. Na^+ transport in plants. *FEBS Letters* **581,** 2247–2254.

Apse MP, Sottosanto JB, Blumwald E. 2003. Vacuolar cation/H^+ exchange, ion homeostasis, and leaf development are altered in a T-DNA insertional mutant of AtNHX1, the Arabidopsis vacuolar Na^+/H^+ antiporter. *The Plant Journal* **36,** 229–239.

Aranda-Sicilia MN, Cagnac O, Chanroj S, Sze H, Rodríguez-Rosales MP, Venema K. 2012. Arabidopsis KEA2, a homolog of bacterial KefC, encodes a K^+/H^+ antiporter with a chloroplast transit peptide. *Biochimica et Biophysica Acta* **1818,** 2362–2371.

Barragán V, Leidi EO, Andrés Z, Rubio L, De Luca A, Fernández JA, Cubero B, Pardo JM. 2012. Ion exchangers NHX1 and NHX2 mediate active potassium uptake into vacuoles to regulate cell turgor and stomatal function in Arabidopsis. *The Plant Cell* **24,** 1127–1142,

Bassil E, Ohto MA, Esumi T, Tajima H, Zhu Z, Cagnac O, Belmonte M, Peleg Z, Yamaguchi T, Blumwald E. 2011*a*. The Arabidopsis intracellular Na^+/H^+ antiporters NHX5 and NHX6 are endosome associated and necessary for plant growth and development. *The Plant Cell* **23,** 224–239.

Bassil E, Tajima H, Liang Y-C, Ohto M-a, Ushijima K, Nakano R, Esumi T, Coku A, Belmonte M, Blumwald E. 2011*b*. The Arabidopsis Na^+/H^+ antiporters NHX1 and NHX2 control vacuolar pH and K^+ homeostasis to regulate growth, flower development, and reproduction. *The Plant Cell* **23,** 3482–3497.

Bertrand B, Wakabayashi S, Ikeda T, Pouyssegur J, Shigekawa M. 1994. The Na^+/H^+ exchanger isoform 1 (NHE1) is a novel member of the calmodulin-binding proteins: identification and characterization of calmodulin-binding sites. *Journal of Biological Chemistry* **269,** 13703–13709.

Blumwald E. 1987. Tonoplast vescicles as a tool in the study of ion-transport at the plant vacuole. *Physiologia Plantarum* **69,** 731–734.

Blumwald E. 2000. Sodium transport and salt tolerance in plants. *Current Opinion in Cell Biology* **12,** 431–434.

Blumwald E, Poole RJ. 1985. Na^+/H^+ antiport in isolated tonoplast vesicles from storage tissue of Beta vulgaris. *Plant Physiology* **78,** 163–167.

Bouche N, Yellin A, Snedden WA, Fromm H. 2005. Plant-specific calmodulin-binding proteins. *Annual Review of Plant Biology* **56,** 435–466.

Bowers K, Levi BP, Patel FI, Stevens TH. 2000. The sodium/proton exchanger Nhx1p is required for endosomal protein trafficking in the yeast *Saccharomyces cerevisiae*. *Molecular Biology of the Cell* **11,** 4277–4294.

Brett CL, Donowitz M, Rao R. 2005*a*. Evolutionary origins of eukaryotic sodium/proton exchangers. *American Journal of Physiology-Cell Physiology* **288,** C223–C239.

Brett CL, Tukaye DN, Mukherjee S, Rao RJ. 2005b. The yeast endosomal $Na^+(K^+)/H^+$ exchanger Nhx1 regulates cellular pH to control vesicle trafficking. *Molecular Biology of the Cell* **16,** 1396–1405.

Buch-Pedersen MJ, Rudashevskaya EL, Berner TS, Venema K, Palmgren MG. 2006. Potassium as an intrinsic uncoupler of the plasma membrane H^+-ATPase. *Journal of Biological Chemistry* **281,** 38285–38292.

Casey JR, Grinstein S, Orlowski J. 2010. Sensors and regulators of intracellular pH. *Nature Reviews Molecular and Cellular Biology* **11,** 50–61.

Cellier F, Conéjéro G, Ricaud L, Luu DT, Lepetit M, Gosti F, Casse F. 2004. Characterization of AtCHX17, a member of the cation/H^+ exchangers, CHX family, from Arabidopsis thaliana suggests a role in K^+ homeostasis. *The Plant Journal* **39,** 834–846.

Chang AB, Lin R, Studley WK, Tran CV, Saier MH. 2004. Phylogeny as a guide to structure and function of membrane transport proteins (Review). *Molecular Membrane Biology* **21,** 171–181.

Chanroj S, Lu Y, Padmanaban S, Nanatani K, Uozumi N, Rao R, Sze H. 2011. Plant-specific cation/H^+ exchanger 17 and its homologs are endomembrane K^+ transporters with roles in protein sorting. *Journal of Biological Chemistry* **286,** 33931–33941.

Chanroj S, Wang G, Venema K, Zhang MW, Delwiche CF, Sze H. 2012. Conserved and diversified gene families of monovalent cation/H^+ antiporters from algae to flowering plants. *Frontiers in Plant Science* **3,** 25.

Cheng NH, Pittman JK, Zhu JK, Hirschi KD. 2004. The protein kinase SOS2 activates the Arabidopsis H(+)/Ca(2+) antiporter CAX1 to integrate calcium transport and salt tolerance. *Journal of Biological Chemistry* **279,** 2922–2926.

Chinnusamy V, Jagendorf A, Zhu J-K. 2005. Understanding and improving salt tolerance in plants. *Crop Science* **45,** 437–448.

De Angeli A, Monachello D, Ephritikhine G, Frachisse JM, Thomine S, Gambale F, Barbier-Brygoo H. 2006. The nitrate/proton antiporter AtCLCa mediates nitrate accumulation in plant vacuoles. *Nature* **442,** 939–942.

De Angeli A, Monachello D, Ephritikhine G, Frachisse JM, Thomine S, Gambale F, Barbier-Brygoo H. 2009. CLC-mediated anion transport in plant cells. *Philosophical Transactions of the Royal Society B: Biological Sciences* **364,** 195–201.

Desko MM, Gross DA, Kohler JJ. 2009. Effects of N-glycosylation on the activity and localization of GlcNAc-6-sulfotransferase 1. *Glycobiology* **19,** 1068–1077.

Dettmer J, Hong-Hermesdorf A, Stierhof YD, Schumacher K. 2006. Vacuolar H^+-ATPase activity is required for endocytic and secretory trafficking in Arabidopsis. *The Plant Cell* **18,** 715–730.

Endler A, Reiland S, Gerrits B, Schmidt UG, Baginsky S, Martinoia E. 2009. *In vivo* phosphorylation sites of barley tonoplast proteins identified by a phosphoproteomic approach. *Proteomics* **9,** 310–321.

Felle HH. 2005. pH regulation in anoxic plants. *Annals of Botany* **96,** 519–532.

Forgac M. 2007. Vacuolar ATPases: rotary proton pumps in physiology and pathophysiology. *Nature Reviews Molecular and Cellular Biology* **8,** 917–929.

Fukuda A, Nakamura A, Hara N, Toki S, Tanaka Y. 2011. Molecular and functional analyses of rice NHX-type Na^+/H^+ antiporter genes. *Planta* **233,** 175–188.

Galvez FJ, Baghour M, Hao GP, Cagnac O, Rodriguez-Rosales MP, Venema K. 2012. Expression of LeNHX isoforms in response to salt stress in salt sensitive and salt tolerant tomato species. *Plant Physiology and Biochemistry* **51,** 109–115.

Gaxiola RA, Rao R, Sherman A, Grisafi P, Alper SL, Fink GR. 1999. The Arabidopsis thaliana proton transporters, AtNhx1 and Avp1, can function in cation detoxification in yeast. *Proceedings of the National Academy of Sciences, USA* **96,** 1480–1485.

Gierth M, Maser P. 2007. Potassium transporters in plants—involvement in K^+ acquisition, redistribution and homeostasis. *FEBS Letters* **581,** 2348–2356.

Glenn EP, Brown JJ, Blumwald E. 1999. Salt tolerance and crop potential of halophytes. *Critical Reviews in Plant Sciences* **18,** 227–255.

Hamaji K, Nagira M, Yoshida K, *et al.* 2009**.** Dynamic aspects of ion accumulation by vesicle traffic under salt stress in Arabidopsis. *Plant and Cell Physiology* **50,** 2023–2033.

Hanana M, Cagnac O, Yamaguchi T, Hamdi S, Ghorbel A, Blumwald E. 2007. A grape berry (Vitis vinifera L.) cation/proton antiporter is associated with berry ripening. *Plant and Cell Physiology* **48,** 804–811.

Heslop-Harrison Y, Heslop-Harrison JS. 1996. Lodicule function and filament extension in the grasses: potassium ion movement and tissue specialization. *Annals of Botany* **77,** 573–582.

Hunte C, Screpanti E, Venturi M, Rimon A, Padan E, Michel H. 2005. Structure of a Na^+/H^+ antiporter and insights into mechanism of action and regulation by pH. *Nature* **435,** 1197–1202.

Jürgens G. 2004. Membrane trafficking in plants. *Annual Review of Cell and Developmental Biology* **20,** 481–504.

Kim B-G, Waadt R, Cheong YH, Pandey GK, Dominguez-Solis JR, Schültke S, Lee SC, Kudla J, Luan S. 2007. The calcium sensor CBL10 mediates salt tolerance by regulating ion homeostasis in Arabidopsis. *The Plant Journal* **52,** 473–484.

Kinsella JL, Aronson PS. 1981. Amiloride inhibition of the Na^+/H^+ exchanger in renal microvillus membrane-vesicles. *American Journal of Physiology* **241,** F374–F379.

Krebs M, Beyhl D, Gorlich E, Al-Rasheid KAS, Marten I, Stierhof YD, Hedrich R, Schumacher K. 2010. Arabidopsis V-ATPase activity at the tonoplast is required for efficient nutrient storage but not for sodium accumulation. *Proceedings of the National Academy of Sciences, USA* **107,** 3251–3256.

Kronzucker HJ, Britto DT. 2011. Sodium transport in plants: a critical review. *New Phytologist* **189,** 54–81.

Lawrence SP, Bright NA, Luzio JP, Bowers K. 2010. The sodium/proton exchanger NHE8 regulates late endosomal morphology and function. *Molecular Biology of the Cell* **21,** 3540–3551.

Lebaudy A, Véry A-A, Sentenac H. 2007. K^+ channel activity in plants: genes, regulations and functions. *FEBS Letters* **581,** 2357–2366.

Leidi EO, Barragan V, Rubio L, *et al.* 2010**.** The AtNHX1 exchanger mediates potassium compartmentation in vacuoles of transgenic tomato. *The Plant Journal* **61,** 495–506.

Leigh RA. 2001. Potassium homeostasis and membrane transport. *Journal of Plant Nutrition and Soil Science* **164,** 193–198.

Leigh RA, Wyn Jones RG. 1984. A hypothesis relating critical potassium concentrations for growth to the distribution and functions of this ion in the plant cell. *New Phytologist* **97,** 1–13.

Leshem Y, Melamed-Book N, Cagnac O, Ronen G, Nishri Y, Solomon M, Cohen G, Levine A. 2006. Suppression of Arabidopsis vesicle-SNARE expression inhibited fusion of H_2O_2 containing vesicles with tonoplast and increased salt tolerance. *Proceedings of the National Academy of Sciences, USA* **103,** 18008–18013.

Li MR, Li Y, Li HQ, Wu GJ. 2011*a*. Overexpression of AtNHX5 improves tolerance to both salt and drought stress in Broussonetia papyrifera (L.) Vent. *Tree Physiology* **31,** 349–357.

Li MR, Lin XJ, Li HQ, Pan XP, Wu GJ. 2011*b*. Overexpression of AtNHX5 improves tolerance to both salt and water stress in rice (Oryza sativa L.). *Plant Cell, Tissue and Organ Culture* **107,** 283–293.

Lin PJC, Williams WP, Luu Y, Molday RS, Orlowski J, Numata M. 2005. Secretory carrier membrane proteins interact and regulate trafficking of the organellar $(Na^+,K^+)/H^+$ exchanger NHE7. *Journal of Cell Science* **118,** 1885–1897.

Liu J, Ishitani M, Halfter U, Kim C-S, Zhu J-K. 2000. The Arabidopsis thaliana SOS2 gene encodes a protein kinase that is required for salt tolerance. *Proceedings of the National Academy of Sciences, USA* **97,** 3730–3734.

Liu J, Zhu J-K. 1998. A calcium sensor homolog required for plant salt tolerance. *Science* **280,** 1943–1945.

Lu Y, Chanroj S, Zulkifli L, Johnson MA, Uozumi N, Cheung A, Sze H. 2011. Pollen tubes lacking a pair of K(+) transporters fail to target ovules in Arabidopsis. *The Plant Cell* **23,** 81–93.

Luan S, Lan W, Chul Lee S. 2009. Potassium nutrition, sodium toxicity, and calcium signaling: connections through the CBL–CIPK network. *Current Opinion in Plant Biology* **12,** 339–346.

Maathuis FJM, Podar D. 2011. Uptake, distribution, and physiological functions of potassium, calcium, and magnesium. In: Hawkesford MJ, Barraclough P, eds. *The molecular and physiological basis of nutrient use efficiency in crops*. Wiley-Blackwell, 265–293.

Maeda Y, Ide T, Koike M, Uchiyama Y, Kinoshita T. 2008. GPHR is a novel anion channel critical for acidification and functions of the Golgi apparatus. *Nature Cell Biology* **10,** 1135–1145.

Mager T, Rimon A, Padan E, Fendler K. 2011. Transport mechanism and pH regulation of the Na(+)/H(+) antiporter NhaA from *Escherichia coli*: an electrophysiological study. *Journal of Biological Chemistry* **286,** 23570–23581.

Manhas N, Shi YJ, Taunton J, Sun DD. 2010. p90(RSK) activation contributes to cerebral ischemic damage via phosphorylation of Na(+)/H(+) exchanger isoform 1. *Journal of Neurochemistry* **114,** 1476–1486.

Marshansky V, Futai M. 2008. The V-type H^+-ATPase in vesicular trafficking: targeting, regulation and function. *Current Opinion in Cell Biology* **20,** 415–426.

Martinoia E, Massonneau A, Frangne N. 2000. Transport processes of solutes across the vacuolar membrane of higher plants. *Plant and Cell Physiology* **41,** 1175–1186.

Mazel A, Leshem Y, Tiwari BS, Levine A. 2004. Induction of salt and osmotic stress tolerance by overexpression of an intracellular vesicle trafficking protein AtRab7 (AtRabG3e). *Plant Physiology* **134,** 118–128.

Mimura T, Kura-Hotta M, Tsujimura T, Ohnishi M, Miura M, Okazaki Y, Mimura M, Maeshima M, Washitani-Nemoto S. 2003. Rapid increase of vacuolar volume in response to salt stress. *Planta* **216,** 397–402.

Molinari M. 2007. N-glycan structure dictates extension of protein folding or onset of disposal. *Nature Chemical Biology* **3,** 313–320.

Nakamura N, Tanaka S, Teko Y, Mitsui K, Kanazawa H. 2005. Four Na^+/H^+ exchanger isoforms are distributed to Golgi and post-Golgi compartments and are involved in organelle pH regulation.

Nass R, Cunningham KW, Rao R. 1997. Intracellular sequestration of sodium by a novel Na^+/H^+ exchanger in yeast is enhanced by mutations in the plasma membrane H^+-ATPase. Insights into mechanisms of sodium tolerance. *Journal of Biological Chemistry* **272,** 26145–26152.

Numata M, Orlowski J. 2001. Molecular cloning and characterization of a novel $(Na^+,K^+)/H^+$ exchanger localized to the trans-Golgi network. *Journal of Biological Chemistry* **276,** 17387–17394.

Ohgaki R, Fukura N, Matsushita M, Mitsui K, Kanazawa H. 2008. Cell surface levels of organellar Na^+/H^+ exchanger isoform 6 are regulated by interaction with RACK1. *Journal of Biological Chemistry* **283,** 4417–4429.

Ohgaki R, Matsushita M, Kanazawa H, Ogihara S, Hoekstra D, van Ijzendoorn SCD. 2010. The Na^+/H^+ exchanger NHE6 in the endosomal recycling system is involved in the development of apical bile canalicular surface domains in HepG2 cells. *Molecular Biology of the Cell* **21,** 1293–1304.

Ohgaki R, van Ijzendoorn SCD, Matsushita M, Hoekstra D, Kanazawa H. 2011. Organellar Na(+)/H(+) exchangers: novel players in organelle pH regulation and their emerging functions. *Biochemistry* **50,** 443–450.

Ohnishi M, Fukada-Tanaka S, Hoshino A, Takada J, Inagaki Y, Iida S. 2005. Characterization of a novel Na^+/H^+ antiporter gene InNHX2 and comparison of InNHX2 with InNHX1, which is responsible for blue flower coloration by increasing the vacuolar pH in the Japanese morning glory. *Plant and Cell Physiology* **46,** 259–267.

Okazaki Y, Kikuyama M, Hiramoto Y, Iwasaki N. 1996. Short-term regulation of cytosolic Ca^{2+}, cytosolic pH and vacuolar pH under NaCl stress in the charophyte alga Nitellopsis obtusa. *Plant, Cell and Environment* **19,** 569–576.

Orlowski J, Grinstein S. 2007. Emerging roles of alkali cation/proton exchangers in organellar homeostasis. *Current Opinion in Cell Biology* **19,** 483–492.

Orlowski J, Grinstein S. 2011. Na^+/H^+ exchangers. *Comprehensive Physiology* **1,** 2083–2100.

Padan E, Kozachkov L, Herz K, Rimon A. 2009. NhaA crystal structure: functional–structural insights. *Journal of Experimental Biology* **212,** 1593–1603.

Padmanaban S, Chanroj S, Kwak JM, Li X, Ward JM, Sze H. 2007. Participation of endomembrane cation/H^+ exchanger AtCHX20 in osmoregulation of guard cells. *Plant Physiology* **144,** 82–93.

Pardo JM, Cubero B, Leidi EO, Quintero FJ. 2006. Alkali cation exchangers: roles in cellular homeostasis and stress tolerance. *Journal of Experimental Botany* **57,** 1181–1199.

Paroutis P, Touret N, Grinstein S. 2004. The pH of the secretory pathway: measurement, determinants, and regulation. *Physiology* **19,** 207–215.

Post MA, Dawson DC. 1994. Basolateral Na^+–H^+ antiporter: mechanisms of electroneutral and conductive ion transport. *Journal of General Physiology* **103,** 895–916.

Putney LK, Denker SP, Barber DL. 2002. The changing face of the Na^+/H^+ exchanger, NHE1: structure, regulation, and cellular actions. *Annual Review of Pharmacology and Toxicology* **42,** 527–552.

Qiu Q-S, Fratti RA. 2010. The Na^+/H^+ exchanger Nhx1p regulates the initiation of *Saccharomyces cerevisiae* vacuole fusion. *Journal of Cell Science* **123,** 3266–3275.

Qiu QS, Guo Y, Quintero FJ, Pardo JM, Schumaker KS, Zhu JK. 2004. Regulation of vacuolar Na^+/H^+ exchange in Arabidopsis thaliana by the salt-overly-sensitive (SOS) pathway. *Journal of Biological Chemistry* **279,** 207–215.

Quintero FJ, Ohta M, Shi H, Zhu JK, Pardo JM. 2002. Reconstitution in yeast of the Arabidopsis SOS signaling pathway for Na^+ homeostasis. *Proceedings of the National Academy of Sciences, USA* **99,** 9061–9066.

Rehman S, Yun SJ. 2006. Developmental regulation of K accumulation in pollen, anthers, and papillae: are anther dehiscence, papillae hydration, and pollen swelling leading to pollination and fertilization in barley (*Hordeum vulgare* L.) regulated by changes in K concentration? *Journal of Experimental Botany* **57,** 1315–1321.

Rockwell NC, Fuller RS. 2002. Specific modulation of Kex2/furin family proteases by potassium. *Journal of Biological Chemistry* **277,** 17531–17537.

Rodriguez-Rosales MP, Jiang XY, Galvez FJ, Aranda MN, Cubero B, Venema K. 2008. Overexpression of the tomato K^+/H^+ antiporter LeNHX2 confers salt tolerance by improving potassium compartmentalization. *New Phytologist* **179,** 366–377.

Rodriguez-Rosales MP, Galvez FJ, Huertas R, Aranda MN, Baghour M, Cagnac O, Venema K. 2009. Plant NHX cation/proton antiporters. *Plant Signaling and Behavior* **4,** 265–276.

Saier MH Jr, Eng BH, Fard S, *et al.* 1999**.** Phylogenetic characterization of novel transport protein families revealed by genome analyses. *Biochimica et Biophysica Acta* **1422,** 1–56.

Šamaj J, Read ND, Volkmann D, Menzel D, Baluška F. 2005. The endocytic network in plants. *Trends in Cell Biology* **15,** 425–433.

Sato Y, Sakaguchi M. 2005. Topogenic properties of transmembrane segments of Arabidopsis thaliana NHX1 reveal a common topology model of the Na+/H+ exchanger family. *Journal of Biochemistry* **138,** 425–431.

Schumacher K. 2006. Endomembrane proton pumps: connecting membrane and vesicle transport. *Current Opinion in Plant Biology* **9,** 595–600.

Shi HZ, Quintero FJ, Pardo JM, Zhu JK. 2002. The putative plasma membrane Na^+/H^+ antiporter SOS1 controls long-distance Na^+ transport in plants. *The Plant Cell* **14,** 465–477.

Shi LY, Li HQ, Pan XP, Wu GJ, Li MR. 2008. Improvement of Torenia fournieri salinity tolerance by expression of Arabidopsis AtNHX5. *Functional Plant Biology* **35,** 185–192.

Smart KE, Smith JAC, Kilburn MR, Martin BGH, Hawes C, Grovenor CRM. 2010. High-resolution elemental localization in vacuolate plant cells by nanoscale secondary ion mass spectrometry. *The Plant Journal* **63,** 870–879.

Soleimani M, Singh G, Bookstein C, Rao MC, Chang EB, Dominguez JH. 1996. Inhibition of glycosylation decreases Na^+/H^+ exchange activity, blocks NHE-3 transport to the membrane, and

increases NHE-3 mRNA expression in LLC-PK1 cells. *Journal of Laboratory and Clinical Medicine* **127,** 565–573.

Sottosanto JB, Gelli A, Blumwald E. 2004. DNA array analyses of Arabidopsis thaliana lacking a vacuolar Na^+/H^+ antiporter: impact of AtNHX1 on gene expression. *The Plant Journal* **40,** 752–771.

Sze H, Padmanaban S, Cellier F, *et al.* 2004**.** Expression patterns of a novel AtCHX gene family highlight potential roles in osmotic adjustment and K^+ homeostasis in pollen development. *Plant Physiology* **136,** 2532–2547.

Venema K, Belver A, Marin-Manzano MC, Rodriguez-Rosales MP, Donaire JP. 2003. A novel intracellular K^+/H^+ antiporter related to Na^+/H^+ antiporters is important for K^+ ion homeostasis in plants. *Journal of Biological Chemistry* **278,** 22453–22459.

Venema K, Quintero FJ, Pardo JM, Donaire JP. 2002. The Arabidopsis Na^+/H^+ exchanger AtNHX1 catalyzes low affinity Na^+ and K^+ transport in reconstituted liposomes. *Journal of Biological Chemistry* **277,** 2413–2418.

Vila-Petroff M, Mundina-Weilenmann C, Lezcano N, Snabaitis AK, Huergo MA, Valverde CA, Avkiran M, Mattiazzi A. 2010. Ca^{2+}/calmodulin-dependent protein kinase II contributes to intracellular pH recovery from acidosis via Na^+/H^+ exchanger activation. *Journal of Molecular and Cellular Cardiology* **49,** 106–112.

von der Fecht-Bartenbach J, Bogner M, Krebs M, Stierhof YD, Schumacher K, Ludewig U. 2007. Function of the anion transporter AtCLC-d in the trans-Golgi network. *The Plant Journal* **50,** 466–474.

Wakabayashi S, Bertrand B, Ikeda T, Pouyssegur J, Shigekawa M. 1994. Mutation of calmodulin-binding site renders the Na^+/H^+ exchanger (NHE1) highly H^+-sensitive and Ca^{2+} regulation-defective. *Journal of Biological Chemistry* **269,** 13710–13715.

Walker DJ, Leigh RA, Miller AJ. 1996. Potassium homeostasis in vacuolate plant cells. *Proceedings of the National Academy of Sciences, USA* **93,** 10510–10514.

Wells KM, Rao R. 2001. The yeast Na^+/H^+ exchanger Nhx1 is an N-linked glycoprotein—topological implications. *Journal of Biological Chemistry* **276,** 3401–3407.

Whiteman S-A, Nuhse TS, Ashford DA, Sanders D, Maathuis FJM. 2008*a*. A proteomic and phosphoproteomic analysis of Oryza sativa plasma membrane and vacuolar membrane. *The Plant Journal* **56,** 146–156.

Whiteman S-A, Serazetdinova L, Jones AME, Sanders D, Rathjen J, Peck SC, Meathuis FJM. 2008*b*. Identification of novel proteins and phosphorylation sites in a tonoplast enriched membrane fraction of Arabidopsis thaliana. *Proteomics* **8,** 3536–3547.

Yamaguchi T, Aharon GS, Sottosanto JB, Blumwald E. 2005. Vacuolar Na^+/H^+ antiporter cation selectivity is regulated by calmodulin from within the vacuole in a Ca^{2+}- and pH-dependent manner. *Proceedings of the National Academy of Sciences, USA* **102,** 16107–16112.

Yamaguchi T, Apse MP, Shi HZ, Blumwald E. 2003. Topological analysis of a plant vacuolar Na^+/H^+ antiporter reveals a luminal C terminus that regulates antiporter cation selectivity. *Proceedings of the National Academy of Sciences, USA* **100,** 12510–12515.

Yamaguchi T, Fukada-Tanaka S, Inagaki Y, Saito N, Yonekura-Sakakibara K, Tanaka Y, Kusumi T, Iida S. 2001. Genes encoding the vacuolar Na^+/H^+ exchanger and flower coloration. *Plant and Cell Physiology* **42,** 451–461.

Yokoi S, Quintero FJ, Cubero B, Ruiz MT, Bressan RA, Hasegawa PM, Pardo JM. 2002. Differential expression and function of *Arabidopsis thaliana* NHX Na^+/H^+ antiporters in the salt stress response. *The Plant Journal* **30,** 529–539.

Yoshida K, Kondo T, Okazaki Y, Katou K. 1995. Cause of blue petal color. *Nature* **373,** 291–291.

Yoshida K, Miki N, Momonoi K, Kawachi M, Katou K, Okazaki Y, Uozumi N, Maeshima M, Kondo T. 2009. Synchrony between flower opening and petal-color change from red to blue in morning glory, Ipomoea tricolor cv. Heavenly Blue. *Proceedings of the Japan Academy, Series B* **85,** 187–197.

Zhang HX, Blumwald E. 2001. Transgenic salt-tolerant tomato plants accumulate salt in foliage but not in fruit. *Nature Biotechnology* **19,** 765–768.

Zhao H, Wiederkehr MR, Fan LZ, Collazo RL, Crowder LA, Moe OW. 1999. Acute inhibition of Na/H exchanger NHE-3 by cAMP—role of protein kinase A and NHE-3 phosphoserines 552 and 605. *Journal of Biological Chemistry* **274,** 3978–3987.

Zybailov B, Rutschow H, Friso G, Rudella A, Emanuelsson O, Sun Q, van Wijk KJ. 2008. Sorting signals, N-terminal modifications and abundance of the chloroplast proteome. *PLoS ONE* **3,** e1994.