DARWIN REVIEW

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

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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 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⁺ exchanger 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., 2005a; 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., 2005a; 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., 2011b; 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., 2011a), 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., 2005a). 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., 2005a) 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., 2005a; 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., 2005a). 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 antipporter (Brett et al., 2005a). 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...
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-terminal 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 ‘FFIYLLPPP’, 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 differently (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⁺ (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 antipporter 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 antipporter 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 Ca2+/calmodulin-dependent protein kinase II (CaMKII) (Vila-Petroff et al., 2010) as well as by the kinase p90RSK (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 NHXs is well documented, evidence demonstrating a similar regulation of NHX activity in plants 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 Ca2+ sensor protein (Liu and Zhu, 1998). Under salt stress, SOS3/CBL4 perceives an increase in cytosolic Ca2+ 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+/Ca2+ 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+/Ca2+ antiporter CAX1 in a SOS3-independent manner (Cheng et al., 2004). Another Ca2+ 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. cb10 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., 2008a, 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 three remaining 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., 2008b) 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 (Molinar, 2007; Desko et al., 2009). ScNHX1 was found to be N-glycosylated at its C-terminus; however, no physiological role for the glycosylation of ScNHX1 has 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 $V_{\text{max}}$ of Na$^+$ exchange activity was repressed by >2-fold in comparison with AtNHX1 activity without AtCaM15. Conversely, the $V_{\text{max}}$ 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 alkalizes (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 (PVs), and pH ~5 in vacuoles and apoplastic/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 for 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 alkalizing 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-transporters 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 (Leaudy 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 Scnhx1Δ, 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., 2005b), 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., 2005b). 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 intra-cellular 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 alkalinization, 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, intra-cellular 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 knock-down 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. (2011a). 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., 2011a). 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 SINVHX2 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., 2011a).
Several lines of evidence indicated that similar to Nhxl1p in yeast, NHX5 and NHX6 are required for trafficking to the vacuole. Using the endocytic 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 nhx3nhx6 double knockout. It did not appear that early endocytic 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., 2011a). 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 CPA s in endomembranes modulate cation and pH homeostasis and might have direct roles in cellular trafficking events (Chanroj et al., 2012).

Vesicular 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 may 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 isoenzyme 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 6.4 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). 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).
mature root zone cells, and the pH in these cells did not differ between the nhxl nhx2 knockout and the wild type. These results suggested that NHX antport 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 nhxl 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 Wy Jones, 1984; Martinoaia et al., 2000; Leigh, 2001). The data of Walker et al., (1996) suggested that assuming no significant 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 of tonoplast vesicles was 3-fold lower in nhxl 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., 2011b), and was also noted in leaf cells (Barragán et al., 2012). K+/H+ exchange activity of tonoplast vesicles was 3-fold lower in nhxl nhx2 and correlated with a significant reduction in leaf cell K+ (Barragán et al., 2012). Leaf turgor and tissue water content in nhxl 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., 2011b; 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., 2011b). 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., 2011b). The response to Na⁺ also suggested that NHX antiporters may not be the only means to transport Na⁺ into the vacuole. Other candidates 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 tissue- and developmental expression, and the growth of nhx1 nhx2 localized to the tonoplast, while functional evidence on KEA is lacking (Pardo et al., 2006; Chanroj et al., 2012). 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 NHxs (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 SINHX2 (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 SINHX2 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 Nhox1 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, 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⁺ 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., 2011b), 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 NO₃⁻ 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 isomorph, 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. (2011a) 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 SINHX2 (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. (2011a), 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., 2011a, 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.

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