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MINIREVIEW

The functions of plant cation/proton antiporters

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Abstract

The cation/H^+ exchange is a basic process in transmembrane transport. The acquisition of genome sequences has now established that plants possess genes encoding a large number of cation/proton antiporter 1 (CPA1) proteins, few of which have been characterized with respect to their contribution to ion homeostasis. The CPA1s comprise plasma membrane, vacuolar, and endosomal forms, and they have been identified as important for a salinity tolerance. They are, however, also involved in both the control of cellular pH and K^+ homeostasis, and regulate processes over a wide range of physiological events, from vesicle trafficking to development.

Additional key words: pH and K^+ homeostasis, salinity tolerance, vesicle trafficking.

Introduction

The cation/proton antiporter1 (CPA1) class of transmembrane antiporters contribute to the transmembrane exchange of Na^+ or K^+ ion for H^+, exploiting electrochemical gradients generated by various proton translocating enzymes such as H^+-ATPase in the plasma membrane and vacuolar ATPase and pyrophosphatase (PPase) within intracellular compartments (Pardo *et al*. 2006, Rodríguez-Rosales *et al*. 2009). They are present in bacterial, plant, fungal, and animal cells. With the exception of yeast, all eukaryotic genomes sequenced to date contain multiple isoforms of them (Brett *et al*. 2005, Chanroj *et al*. 2012, Ford *et al*. 2012). Plant *CPA1* genes have been assigned to either the Na^+/H^+ antiporter (NhaP) or the $Na^{(+)}$-$H^{(+)}$ exchanger (NHX)) clade (Brett *et al*. 2005, Chanroj *et al*. 2012). Genes allocated to the former clade encode proteins which localize to the plasma membrane and share similarity with the human sodium-hydrogen exchanger isoform-1 (NHE-1) proteins (Qiu *et al*. 2002, Chanroj *et al*. 2012), while those of the NHX clade localize to intracellular (IC) membranes. The IC class I NHXs are deposited in the tonoplast and are plant-specific; IC class IIs are found in the endosome and are closely related to the yeast protein ScNHX1 and the human proteins NHE6 and NHE7 (Bowers *et al*. 2000, Brett *et al*. 2005, Chanroj *et al*. 2012). The *Arabidopsis thaliana* and rice *CPA1* gene families are almost identical in size: there are two isoforms of NhaP in *A. thaliana* (NHX7=SOS1 and NHX8) and one in rice salt overly sensitive1 (SOS1). The NHX clade is represented by six genes in *A. thaliana* and by five in rice: *AtNHX1-4* and *OsNHX1-4* encode intracellular-Is (IC-Is), while *AtNHX5, AtNHX6*, and *OsNHX5* encode IC-IIs. The *A. thaliana* and rice IC-Is share 54 - 87 % similarity at the polypeptide level, while the similarity between the three IC-IIs ranges from 72 to 79 %. The IC-I and IC-II sequences share only about 22 % similarity.

Ion and pH homeostasis is critical for the operation of many cellular processes underlying plant development and growth. CPA1 activity is a major determinant of the cellular osmotic environment and hence of cell pressure

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Abbreviations: AKT1 - *Arabidopsis* K^+ transporter1; CPA1 - cation/proton antiporter 1; ER - endoplasmic reticulum; IC - intracellular; NhaP - Na^+/H^+ antiporter; NHE - sodium-hydrogen exchanger isoform; NHX - $Na^{(+)}$-$H^{(+)}$ exchanger; PPase - proton pumping pyrophosphatase; PVC - pre-vacuolar compartment; SOS1 - salt overly sensitive1; TGN - *trans*-Golgi network.

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potential. Coupled cation/proton exchange is central to the regulation of vacuolar and endosomal pH and ionic composition, thereby affecting protein processing and trafficking, as well as vesicular cargo composition and movement (Pardo *et al*. 2006, Rodriguez-Rosales *et al*. 2009). The conservation across species of the three classes of CPA1s (plasma membrane, endosomal, and vacuolar) implies an early appearance of these distinct functional classes during evolution (Bassil and Blumwald 2014). The suggestion is that they retain their compartment specificity. This review focuses on the physiological significance of the three classes of plant CPA1s.

Salinity tolerance

When exposed to a saline environment, plants inevitably accumulate Na^+ ions (although to a varying extent), driven by the major gradient in Na^+ concentration between the soil solution and the interior of the plant. A significant component of plant salinity tolerance is the capacity to limit the uptake of Na^+ into the root. Interspecific comparisons of Na^+ flux and the rate of Na^+ accumulation suggest that 70 - 95 % of the Na^+ entering the root symplast is actively returned to the rhizosphere *via* an energetically costly processes (Tester and Davenport 2003). To date, only the charge neutral exchange achieved by the plasma membrane Na^+/proton exchanger SOS1 has been subjected to rigorous quantification (Apse and Blumwald 2007, Jiang *et al*. 2010, Zhang *et al*. 2017). The over-expression of its encoding gene (or certain homologs) enhances salinity tolerance in both *A. thaliana* and tobacco (Shi *et al*. 2003, Yang *et al*. 2009, Yue *et al*. 2012). Plasma membrane vesicles formed by the *A. thaliana* loss-of-function *sos1* mutant have some Na^+/proton exchange activity (Qiu *et al*. 2002, Shi *et al*. 2002). The closely related protein AtNHX8 is thought to be a plasma membrane localized Li^+/proton antiporter which responds specifically to the stress imposed by an excess of Li^+ ions, since T-DNA insertion mutants of its encoding gene are more sensitive to Li^+ than the wild type is; the effect does not extend to the other monovalent cations Na^+, K^+, or Cs^+ (An *et al*. 2007). The implication is that additional plasma membrane exchangers are likely present; nevertheless, the genetic evidence confirms that SOS1 makes a major contribution to salinity tolerance.

The export of Na^+ only exacerbates the osmotic and ionic imbalance, thereby aggravating the primary stress. Returning Na^+ to the medium can therefore be only an interim solution and cannot on its own confer a prolonged tolerance to soil salinity. Since the efficiency of Na^+ removal is not 100 %, over time Na^+ ions will inevitably accumulate, initially in the root and later throughout the plant. As a second line of defense, plants use the IC-I enzyme NHX to sequester Na^+ ions within their cellular vacuoles, thereby both protecting the cytosol from Na^+ toxicity and encouraging the osmotically driven uptake of water (Apse and Blumwald 2007). The over-expression of either *AtNHX1* or genes encoding isoforms of NHX increase salinity tolerance in a variety of plant species (Apse *et al*. 1999, Hamada *et al*. 2001, Ohta *et al*. 2002, Brini *et al*. 2007, Zhang *et al*. 2012). The simultaneous over-expression of *AtNHX1* and *AtSOS1* significantly mitigates loss of biomass induced by salinity stress (Pehlivan *et al*. 2016). In contrast, both tomato plants deficient for NHX2 (IC-II NHX) and the *A. thaliana nhx5nhx6* double mutant are hypersensitive to salinity (Rodríguez-Rosales *et al*. 2008, Bassil *et al*. 2011a). Plants lacking vacuolar V-ATPase show a reduced capacity to store nitrate and do not over-accumulate Zn^{2+}, and they are not over-sensitive to salinity. On the other hand, plants with increased activity of endosomal or *trans*-Golgi network (TGN) localized V-ATPase are sensitive to salinity (Krebs *et al*. 2010). The implication is that the endosomal/vesicle system provides an important means of protecting plants against the damage generated by salinity stress as supported by other studies (Mazel *et al*. 2004, Hamaji *et al*. 2009, Hernandez *et al*. 2009). The salinity tolerance of *NHX* over-expressing plants seems to be independent of both the species origin of the transgene and the identity of the encoded isoform: both IC-I and IC-II antiporters appear to have a similar role in salinity tolerance.

As NHX transporters also transport K^+ ions, they are expected to exert an effect on intercellular K^+ content, especially in the case of K^+ specific IC-II antiporters. The over-expression of either *AtNHX1* or *AtNHX2* has been shown to increase the content of both K^+ and Na^+ in ENA (the main Na^+ efflux system) and *ScNHX1*-disrupted yeast cells grown in the presence of NaCl (Quintero *et al*. 2000, Yokoi *et al*. 2002). The constitutive expressions of *AtNHX5* and *LeNHX2* encoding the IC-II antiporter raise the cellular content of K^+, but reduce the content of Na^+ (Yokoi *et al*. 2002, Venema *et al*. 2003). SOS1 appears to lack the capacity to transport K^+ both in its basal state and after its activation by SOS2/SOS3 kinase. Plants experiencing a high concentration of Na^+ in the soil solution are not able to take up K^+ due to competition for the transporter binding sites involved in K^+ uptake (Hasegawa *et al*. 2000). Therefore, the removal of Na^+ from the cytoplasm achieved by SOS1 protects cells against loss of the membrane capacity to transport K^+. This is particularly in the case of the AKT1 (*Arabidopsis* K^+ transporter1) K^+ channel (Qi *et al*. 2004). The inference is that plant CPA1-mediated salinity tolerance is more than just a consequence of the accumulation of Na^+ inside the vacuole and its expulsion from the cell; rather, at least a part of this tolerance reflects the effect of CPA1 on the cytoplasmic content of K^+.

Homeostasis of K^+

Some CPA1s, and especially the NHXs, have been suggested to participate in K^+ homeostasis under normal growth conditions (Adem *et al*. 2014). In addition to being an essential nutrient, the K^+ acts to balance intracellular charge and also represents a co-factor for certain cytosolic enzymes. The majority of cellular K^+ is found in the vacuole, where it maintains pressure potential and so indirectly drives cell expansion (Bassil *et al*. 2012). The acidification of the cytoplasm may serve as a signal to induce either high affinity K^+ uptake from the soil or K^+ efflux from the vacuole (Walker *et al*. 1996). A reduction in the pH gradient across the tonoplast membrane may attenuate the accumulation of vacuolar K^+ driven by IC-I NHX. In grape, NHX1 expression was significantly upregulated at veraison and during cell expansion where berry vacuolar K^+ accumulation and a drop in acidity occur (Hanana *et al*. 2007). Antiport activity is reduced in *A. thaliana nhx1* null mutants, which form smaller cells and display a reduced expansion of highly vacuolated cells; these effects may be related to the vacuolar K^+ deficit needed to ensure pressure potential for cell expansion (Apse *et al*. 2003). A microarray-based transcriptomic analysis has indicated that genes encoding high affinity K^+ uptake transporters are up-regulated in the absence of a functional copy of *NHX1*, which supports the notion that AtNHX1 is involved in K^+ homeostasis (Sottosanto *et al*. 2004). While no clear phenotype is induced when the closely related isoform *NHX2* is knocked out, the double *nhx1nhx2* mutant suffers a substantial reduction in cell expansion and growth, especially in the rapidly elongating filament (Bassil *et al*. 2011b). The vacuolar K^+ content in the double mutant is only one third of that in wild type plants, both in the root and in the leaf (Bassil *et al*. 2011b, Barragán *et al*. 2012). Stomatal opening depends on an increase in the guard cell vacuolar K^+ content, a process which relies on vacuolar NHXs (Andrés *et al*. 2014). These observations highlight the importance of vacuolar NHX for cellular K^+ homeostasis.

The knockout/knockdown of NHX results in severe growth defects. For example, in tomato, the knockdown of *LeNHX2* induces growth retardation (Rodríguez-Rosales *et al*. 2008), and similarly in *A. thaliana*, the simultaneous loss of *NHX5* and *NHX6* reduces cell size, and slows both floral development and root growth (Bassil *et al*. 2011a). K^+ content in the *nhx5nhx6* double mutant is greatly reduced compared to that present in wild type tissue (Wang *et al*. 2015). The constitutive expression of either *NHX5* or *NHX6* in the double mutant rescues root growth. Thus endosomal NHXs make a significant contribution to growth and development, possibly *via* their support of K^+ homeostasis. However, the lack of targeted K^+ specific microprobes inhibits measurement of the K^+ content in the vesicles, preventing the identification of the role of endosomal/vesicular NHXs in K^+ homeostasis.

Homeostasis of pH

Cellular pH homeostasis is highly critical for cellular function. Cytoplasmic pH is controlled primary by proton pumps and metabolic processes producing protons or hydroxyl ions. Luminal pH is not uniform throughout the cell; it is dependent on the intracellular compartment (Paroutis *et al*. 2004). *In vivo* measurement has revealed that the pH ranges from pH 7.1 in the endoplasmic reticulum (ER) to about 5.5 in the vacuole; the TGN is more acidic than the pre-vacuolar compartments which are intermediate organelles where secretory and endocytic traffic leads to vacuole (Yu *et al*. 2006, Martinière *et al*. 2013). Given the involvement of CPA1s in proton leakage, it is hardly surprising that they are able to regulate pH in the cytoplasm or in vesicles depending on their sub-cellular localization. Evidence for intracellular NHX-dependent pH regulation first arose from a study of the pigmentation of the petals of *Ipomea* sp. (Yoshida *et al*. 2005). During development, petals begin to accumulate anthocyanins in their vacuoles: this results in red colour at low vacuolar pH and blue colour as the pH increases. The increase of petal vacuolar pH from 6.5 to 7.5 is accompanied by an enhanced activity of V-ATPase, proton-PPase, and NHX1. Direct measurements of vacuolar pH in *nhx* mutants strongly suggest that vacuolar NHX antiporters are important for the regulation of vacuolar pH. In the roots of the *A. thaliana nhx1nhx2* double mutant, the vacuolar environment is markedly more acidic than in the wild type in the cells of elongation and maturation zones, especially in the cortical cells (Bassil *et al*. 2011b). Cells in the root tips tend to have more acidic pH than mature root zone cells. However, there is little difference between the cellular pH of *nhx1nhx2* and wild type plants in root tip cells (Barragán *et al*. 2012). The implication is that NHX antiporter activity is more pronounced in cells which need to raise their vacuolar volume to drive their elongation. Reguera *et al.* (2015) have used luorin-based pH sensors to measure the luminal pH within the Golgi bodies, the TGN, and the late pre-vacuolar compartment (LPVC); their finding is that pH in these compartments in the *nhx5nhx6* double mutant is lower than in the wild type, which is taken to imply that endosomal/vesicular NHXs increase vesicle pH. This observation was supported by Wang *et al*. (2015) who showed that

nhx5nhx6 has a reduced vacuolar pH as measured with the semimicro-electrode. Although the involvement of plant NHX transporters in cytoplasmic pH regulation has yet to be demonstrated, the use of fluorescent probes has shown that the loss of a functional copy of *AtSOS1* results in an altered pH homeostasis within both the root cell cytosol and vacuole, probably due to an alteration in the proton flux through the plasma membrane (Shabala *et al*. 2005, Oh *et al*. 2010).

Potential functions of plant *CPA1*s

The recently uncovered functionality of the endosome-localized AtNHX5 and AtNHX6 proteins is particularly intriguing. Not unlike the situation in yeast (Bowers *et al*. 2000) and animal cells (Casey *et al*. 2010, Ohgaki *et al*. 2011), differential gene expression in the *nhx5nhx6* double mutant is largely associated with vesicular trafficking and with defects in trafficking to the vacuole (Bassil *et al*. 2011a). Using the endocytotic tracer, the lipophilic styryl dye (FM4-64), and monitoring the progressive fluorescence labelling of endomembranes, it was shown that labelling of the vacuole was severely delayed in nhx5nhx6. In addition, trafficking of newly synthesized carboxypeptidase Y-green fluorescent protein (CPY-GFP), which normally accumulates in the vacuole, was transported to the apoplast in *nhx5nhx6* plants. (Bassil *et al*. 2012). A functional link between the V-ATPase complex and NHX5 and NHX6 has been proposed, based on the extensive co-localization of the TGN-localized V-ATPase with NHX5 and NHX6 (Bassil *et al*. 2011a). The proposal is that endosomal NHXs control vesicular trafficking, probably *via* their regulation of endosomal ionic and pH homeostases. However, pH is not the only maintainer of endosomal enzyme activity and protein stability, as it is also involved in the determination of vesicle identity, the regulation of receptor and cargo interactions, and ultimately endomembrane trafficking (Paroutis *et al*. 2004). *In vivo* measurements of the vesicular luminal pH status of endomembrane compartments and their contribution to the regulation of protein maturation should aid in establishing the biochemical basis of these processes.

Seed storage proteins are synthesized as precursors in the ER and then they are transported into the protein storage vacuoles (PSVs), and converted to mature forms. Studies have shown that proteins are transported to the vacuole through a vesicle-mediated trafficking pathway that includes the ER, Golgi, TGN, and multivesicular bodies (MVB) in PVC. Hence, the Golgi, TGN, and MVB/PVC are major protein sorting stations in vesicular transport (Qiu *et al*. 2016). AtNHX5 and AtNHX6 are localized to the Golgi, TGN, and PVC, where they overlap with the protein trafficking pathway. The IC-I antiporters may also participate in intracellular vesicle trafficking, since the transcription of a large number of genes encoding proteins associated with intravesicular trafficking, trafficking to the nucleus, and processing in the Golgi bodies are altered in an *nhx1* T-DNA insertional mutant (Sottosanto *et al*. 2004).

So far, the major focus of *CPA1* expression has been in the context of salinity stress, for example, the salt-induced *AtCAPE1* negatively regulated salt tolerance by suppressing several salt-tolerance genes functioning in the production of osmolytes, detoxification, stomatal closure control, and cell membrane protection. *AtNHX1* and *AtNHX2* play an important role in cell expansion and flower development by regulating intravacuolar $K^{(+)}$ content and pH (Fukuda *et al*. 1999, Quintero *et al*. 2000, Brini *et al*. 2005, Zahran *et al*. 2007, Chien *et al*.2015, Bassil *et al*. 2011b). However, some members of this gene family appear to be inducible by abscisic acid (Venema *et al*. 2003, Yokoi *et al*. 2002), KCl (Fukuda *et al*. 1999, 2004a, 2004b), dehydration (Li *et al*. 2006) and/or hyper-osmotic stresses (Fukuda *et al*. 1999, 2004a, 2004b, Yokoi *et al*. 2002). The isolation of certain *NHX* genes from *Morus atropurpurea* has recently shown that they are not only inducible by salinity, drought, and abscisic acid, but also by salicylic acid, hydrogen peroxide, and methyl jasmonate (Cao *et al*. 2016).

Conclusions

Genetic analysis provides compelling evidence supporting the idea that the three classes of CPA1 (plasma membrane, vacuolar, and endosomal) regulate a range of cellular and physiological processes, including cell expansion, cation homeostasis, osmotic and pressure potential, pH homeostasis, vesicle trafficking, stomatal function, as well as floral development. The availability of multiple *CPA1* knockout lines together with the development of platforms able to measure *in vivo* pH and ion content in various intracellular compartments has enabled deeper understanding the functions of these proteins. The identification of the protein partners regulating activities of these transporters are required to elucidate the underlying mechanisms. In response to salinity stress, the three classes of CPA1 seem to operate in tandem. In addition, they are important for the maintenance of cellular pH and K^+ content. They act cooperatively to regulate a range of processes from vesicle trafficking and cell expansion to plant development.

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