DOI: 10.1007/s10535-018-0801-8 BIOLOGIA PLANTARUM 62 (4): 617-629, 2018

REVIEW

# The role of plant cation/proton antiporter gene family in salt tolerance

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## Abstract

Salinity is one of the major abiotic constraints to agriculture. The physiological and molecular mechanisms of salt tolerance have been studied in plants for many years. The regulation of osmosis and ion homeostasis is crucial. A lot of important components involved in plant responses to salt stress have been identified. Among them, ion transporters and channels take an essential role in ion homeostasis, mainly for $Na^+$, $Cl^-$, and $K^+$. Until now, many cation antiporters important for salt tolerance in plants have been characterized. Among them, the monovalent cation/proton antiporters (CPA) family is one of the most important families, including sodium proton exchangers (NHXs), $K^+$-efflux antiporters (KEAs), and cation/$H^+$ exchangers (CHXs). Here, the current knowledge of the plant CPA family in responses to salt stress is reviewed. The regulation mechanisms were also included and discussed.

*Additional key words*: $Na^+/H^+$ exchanger, $K^+$-efflux antiporter, cation/$H^+$ exchanger.

## Introduction

High salinity stress is a major abiotic stress impairing crop production throughout the world. Mainly sodium chloride from irrigation water or seawater contributes to soil salinity (Deinlein *et al.* 2014). Up to 7 % of the total land surface and at least 20 % of the irrigated land is affected by secondary salinization (Munns and Tester 2008, Mansour 2014, Roy *et al.* 2014). Unfortunately, most of the crops belong to glycophytes and are thereby relatively salt sensitive (Munns and Tester 2008). In order to improve the ability of plants to resist salt stress, it is of great interest to study the salt tolerance mechanisms in plants. High concentrations of salts decrease the osmotic potential of soil solution and so water absorption by plant roots results in slow growth of new roots and shoots. Meanwhile, $Na^+$ and $Cl^-$ are taken up and accumulated to toxic concentrations in plants, which causes the generation of reactive oxygen species (ROS) leading to oxidative stress (Zhu 2001, Gupta and Huang 2014) as well as senescence of older leaves (Munns and Tester 2008, Teakle and Tyerman 2010).

The mechanisms of salt tolerance can be divided into three main classes: osmotic adjustment, ion exclusion, and tissue tolerance (reviewed in Munns and Tester 2008, Roy *et al.* 2014). The core of all three mechanisms

*Submitted* 7 November 2017, *last revision* 27 February 2018, *accepted* 14 March 2018.
*Abbreviations:* ABI2 - abscisic acid-insensitive 2; CaM15 - calmodulin-like protein 15; CBL - calcineurin B-like; CDS - coding sequence; CHX - cation/$H^+$ exchanger; CIPK24 - CBL-interacting serine/threonine-protein kinase 24; CPA - cation/proton antiporter; EIN3 - ethylene-insensitive 3; ER - endoplasmic reticulum; ESE1 - ethylene and salt-inducible ERF1; GI - GIGANTEA; GUS - β-glucuronidase; HAK5 - high-affinity $K^+$ transporter 5; HKT - high-affinity $K^+$ transporter; KEA - $K^+$-efflux antiporter; KUP1 - $K^+$ uptake transporter; NaKR3 - sodium potassium root defective 3; NHX - sodium proton exchanger; NSCCs - non selective channels; PM - plasma membrane; PM-ATPase - plasma membrane $H^+$-ATPase; PM-PPase - plasma membrane $H^+$-PPase; PVC - pre-vacuolar compartments; ROS - reactive oxygen species; SCABP8 - SOS3-like calcium binding protein 8; SOS - salt overly sensitive; TF - transcription factor; TGN - *trans*-Golgi network; USP - universal stress protein; UTR - untranslated region; V-ATPase - tonoplast $H^+$-ATPase; V-PPase - tonoplast $H^+$-PPase.
*Acknowledgement*: This work is supported by the National Natural Science Foundation of China (31501232), the China Postdoctoral Science Foundation (2014T70603, 2013M540527), and the FAFU Science Fund for Distinguished Young Scholars (xjq201629). The authors are grateful to Prof. H. Lam and Dr. M. Li (Chinese University of Hong Kong) for the fruitful discussion of the *CHX* gene family.
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is the transport of water and ions (mainly $Na^+$, $Cl^-$, and $K^+$) between the plant and environment, the redistribution inside whole plant, and among the cellular compartments (Craig Plett and Møller 2010). To date, many ion channels and transporters have been shown to play crucial roles in maintaining the ion and pH homeostasis in plants under high salinity (Pardo *et al.* 2006, Ward *et al.* 2009, Barbier-Brygoo *et al.* 2011, Yamaguchi *et al.* 2013, Hamamoto *et al.* 2015). Among them, the cation transporters have been well characterized and most $Na^+$ and $K^+/H^+$ exchangers belong to the monovalent cation/proton antiporters (CPA) family, which is classified into the CPA1 (2.A.36) and CPA2 (2.A.37) subfamilies, according to *Transporter Classification* database (http://www. tcdb.org/) (Pires *et al.* 2013, Saier *et al.* 2014). The *in silico* phylogenic analysis suggested that there are still undefined members of the *CPA* gene family with potential function in salt resistance (Chanroj *et al.* 2012, Ye *et al.* 2013). Studying on cation/proton antiporters would be important to understand the mechanisms of salt tolerance and to seek new solutions for improving salt tolerance in crops. This review will summarize the current knowledge about the roles and the regulation mechanisms of the *CPA* gene family in plant responses to salt stress (Fig. 1).

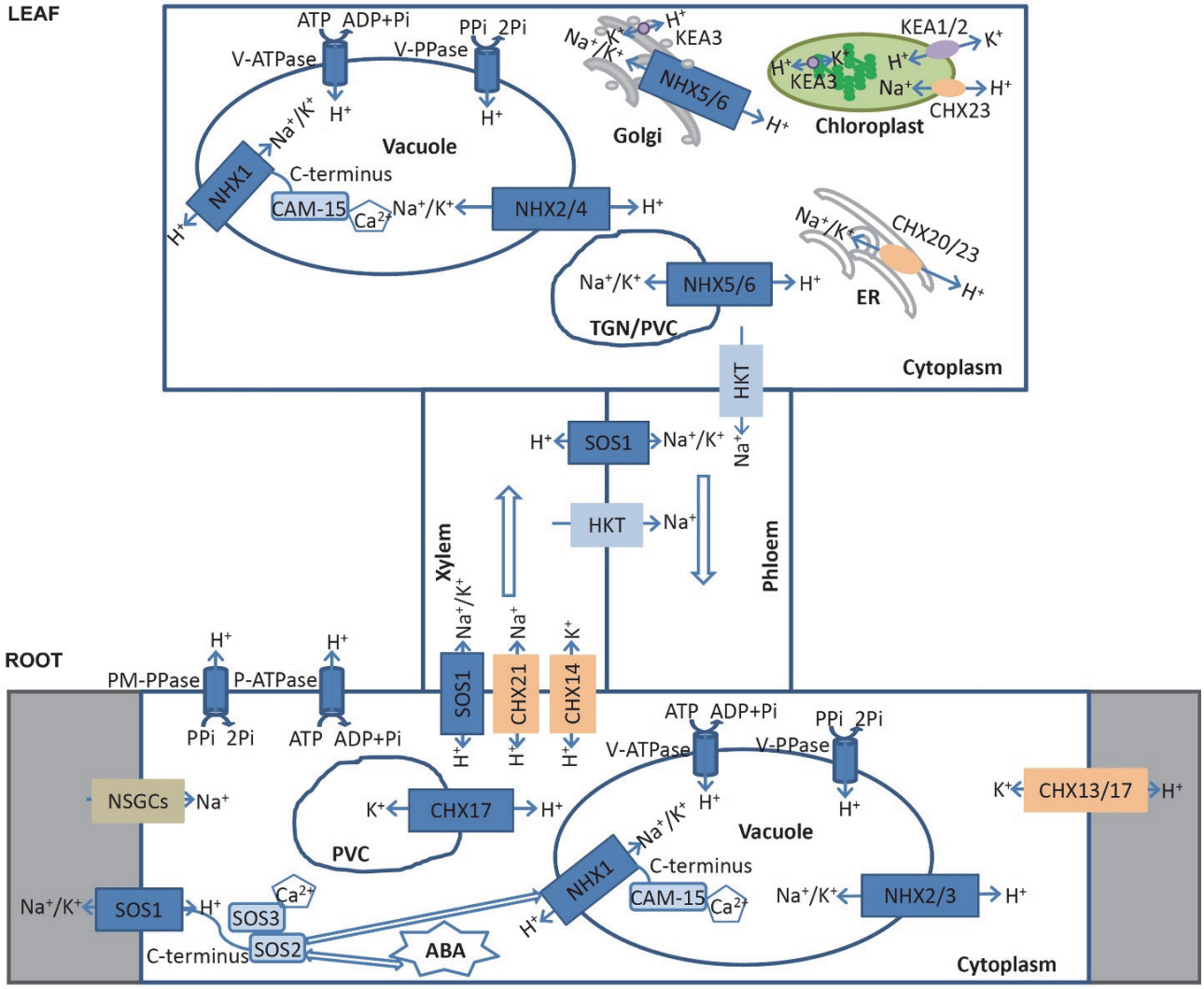


Fig. 1. Schematic representation showing cation-proton antiporter (*CPA*) family members mediating $Na^+$ and $K^+$ homeostasis in plants under salt stress. The subcellular localizations, functions, and regulations of them are indicated. CaM-15 - calmodulin-like protein 15; CHX - cation/$H^+$ exchanger; HKT - high-affinity $K^+$ transporter; KEA - $K^+$-efflux antiporter; NHX - sodium proton exchanger; NSCCs - non selective channels; PM-ATPase - plasma membrane $H^+$-ATPase; PM-PPase - plasma membrane $H^+$-PPase; PVC - prevacuolar compartment; SOS - salt overly sensitive; TGN - *trans*-Golgi network; V-ATPase - tonoplast $H^+$-ATPase; V-PPase - tonoplast $H^+$-PPase.

## CPA1 family

Phylogenetical analysis showed that the plant CPA1-type transporters are predicted to have 10 - 12 membrane-spanning domains and have evolved from the ancestral *NhaP* gene in prokaryotes (Brett *et al.* 2005, Chanroj *et al.* 2012). According to the subcellular localization, the CPA1 family is divided into two main groups, including the intracellular proteins and the plasma membrane-bound proteins (Pires *et al.* 2013). They are drived by the electrochemical $H^+$ gradients generated by $H^+$ pumps at the plasma membrane (PM-$H^+$-ATPase, PM-$H^+$-PPase) or the tonoplast (V-$H^+$-ATPase, V-$H^+$-PPase) to transport $Na^+$ out of the cell or into the vacuole, respectively (Silva and Gerós 2009, Almeida *et al.* 2017). The well-characterized members were proved to be associated with salt tolerance in plants (Table 1 Suppl.), such as the intracellular sodium proton exchangers (NHXs) and the plasma membrane localized salt overly sensitive 1 (SOS1)/NHX7 (Deinlein *et al.* 2014, Almeida *et al.* 2017, Assaha *et al.* 2017). A meta-analysis demonstrated that overexpression of the *CPA1* genes could increase salt tolerance (Ma *et al.* 2017).

**Plasma membrane-bound *NHX* genes:** Two *CPA1* family members, AtSOS1/AtNHX7 and AtNHX8, located in plasma membrane, are grouped into a distinct clade of CPA1 family in *Arabidopsis*, which is closer to the ancestral bacterial NhaP (Mäser *et al.* 2001, Brett *et al.* 2005, Chanroj *et al.* 2012). Topology prediction indicated that AtSOS1 contains 13 transmembrane segments, closer to *Methanocaldococcus jannaschii* NhaP1 and *Thermus thermophile* NapA than to *E. coli* NhaA (Ullah *et al.* 2016). *AtSOS1* was shown to be expressed in all vegetative tissues, and enriched in the root meristem zone and in the parenchyma cells surrounding the vascular tissue (Shi *et al.* 2002, Ward *et al.* 2003, Kronzucker and Britto 2011). The expression of *AtSOS1* is induced by salt stress, but not by abscisic acid (ABA; Shi *et al.* 2000). Salt stress could not only induce the expression of *AtSOS1*, but also increase the stability of its transcripts. The stress-induced mRNA stability is mediated by reactive oxygen species (Jiang and Shi 2008, Chung *et al.* 2008). The expression of *AtNHX8* was detected in the leaves, hypocotyles, and roots by promoter-β-glucuronidase (GUS) analysis (An *et al.* 2007). But the expression of *AtNHX8* was not induced by salt according to real-time PCR detection.

The *SOS1* locus was first identified in *Arabidopsis* using root-bending assay for screening salt-hypersensitive mutants (Wu *et al.* 1996). The *Atsos1* mutants were not only hypersensitive to high $Na^+$ and $Li^+$, but also defective in $K^+$ uptake (Wu *et al.* 1996). The results from the plasma membrane vesicle assay showed that plasma membrane $Na^+/H^+$ exchange activity was reduced by 80 % in the *Atsos1* mutants compared to the wild type (Qiu *et al.* 2002). Except for the activity of $Na^+/H^+$ antiporter, SOS1 was also shown to function in $Li^+$ efflux and low-affinity $K^+$ transport, suggesting its important roles in maintaining intracellular ion and pH homeostasis (Garciadeblás *et al.* 2007, Feki *et al.* 2014). The *Atnhx8* mutants were hypersensitive to $Li^+$ stress, but not to $Na^+$, $K^+$ and $Cs^+$ stresses, suggesting *AtNHX8* encodes a plasma membrane $Li^+/H^+$ antiporter (An *et al.* 2007). The function of AtNHX8 in response to salinity is still indistinct. The study on loss-of-function and gain-of-function of the *Atnhx8* mutants showed that it could play a role in $Li^+$ extrusion and $K^+$ acquisition, but not in $Na^+$ transport (An *et al.* 2007). The expression of *AtNHX8* was induced in the transgenic *Arabidopsis* overexpressing *ZmCBL4* (a putative homolog of the *Arabidopsis* CBL4/SOS3 protein) under LiCl stress (Wang *et al.* 2007). A polymorphism analysis of nine salt-responding genes showed that *NHX8* had more extreme deviation than the other genes, suggesting its putative contribution to local adaptation (Puerma and Aguade 2013).

It is believed that the plasma membrane AtSOS1 could play a role in $Na^+$ extrusion from the cytosol, including the expulsion of $Na^+$ from the root to the surrounding medium and long-distance transport of $Na^+$ inside the whole plant (Shi *et al.* 2000, 2002, Qiu *et al.* 2003, Oh *et al.* 2009, Ji *et al.* 2013, Katschnig *et al.* 2015, Hamam *et al.* 2016). Ectopic overexpression of *AtSOS1* improved salt tolerance in transgenic *Arabidopsis*, which could be achieved by limiting $Na^+$ content in xylem and shoot (Shi *et al.* 2003, Yang *et al.* 2009). A recent study on *SOS1* in barley and *Arabidopsis* showed that the $Na^+$ extrusion mediated by SOS1 was enhanced more in root tips than in bulk roots (Hamam *et al.* 2016), which is different from the earlier findings (Shabala *et al.* 2005). The current reports showed that the major regulation pathway of AtSOS1 is the calcium-dependent SOS signalling pathway containing three key components of SOS1, SOS2, and SOS3 (Qiu *et al.* 2002, Bertorello and Zhu 2009, Kronzucker and Britto 2011, Quintero *et al.* 2011, Ji *et al.* 2013). Comparable to overexpression of *AtSOS1* alone, over-expression of *AtSOS3*, *AtSOS2* + *AtSOS3*, or *AtSOS1* + *AtSOS2* + *AtSOS3* can also improve the salt tolerance of transgenic plants (Yang *et al.* 2009). The salt stress triggers the cytosolic calcium signals, which bind the N-myristoylated calcium binding protein AtSOS3/calcineurin B-like 4 (CBL4) in roots or its ortholog SOS3-like calcium binding protein 8 (SCABP8)/ calcineurin B-like protein 10 (CBL10) in shoots to activate the serine/threonine kinase AtSOS2/CIPK24 (CBL-interacting serine/threonine-protein kinase 24) (Ishitani *et al.* 2000, Kim *et al.* 2007, Quan *et al.* 2007, Monihan *et al.* 2016). Consequently, AtSOS2/CIPK24 is recruited to phosphorylate AtSOS1 located at the plasma membrane, stimulating it to extrude $Na^+$ in exchange for $H^+$. The structure analysis of AtSOS1/AtNHX7 showed that it contains a very long hydrophilic cytoplasmic C-terminal tail, which could play a role in the regulation *via* interaction with the regulators (Shi *et al.* 2000, Kronzucker and Britto 2011, Chanroj *et al.* 2012). The 3D structural analysis by electron microscopy and single-

particle reconstruction techniques showed that AtSOS1 formed a homodimer through the contact between the cytosolic and transmembrane domains (Núñez-Ramírez *et al.* 2012). AtSOS1 would be activated *via* the phosphorylation of the auto-inhibitory domain by *AtSOS2*/*CIPK24* (Halfter *et al.* 2000), and be suppressed when the activation domain and the auto-inhibitory domain of the *AtSOS1* C-terminus were interacted with each other (Quintero *et al.* 2011). The transformation tests in *Schizosaccharomyces pombe* showed that the shortened version of *AtSOS1* without the C-terminus conveyed salt tolerance similarly as the full-length *AtSOS1*, indicating the C-terminal cytosolic tail determines its dimerization, but not the ability of cation transporter (Ullah *et al.* 2016). This is consistent with findings of *TdSOS1* in *Triticum durum* (Feki *et al.* 2014). The truncated *TdSOS1* without the C-terminal domain, only containing the auto-inhibitory and SOS2 phosphorylation site, still could confer super salt tolerance in transgenic *Arabidopsis*. The factors affecting the activity of SOS2 or SOS3 would also influence the function of SOS1 through the SOS pathway. The flowering time regulator *GIGANTEA* (*GI*), a negative regulator of the SOS pathway, suppresses the activation of *AtSOS1* through sequestering *AtSOS2* (Kim *et al.* 2013).

Under salt stress, the SOS pathway would be regulated by other factors, such as phytohormones, transcription factors (TFs), and ROS. AtSOS2 could interact with abscisic acid-insensitive 2 (ABI2) to prevent the binding of AtSOS3 and AtSOS2, suggesting ABA may influence the SOS pathway (Ohta *et al.* 2003). SOS2 activated the expression of *ESE1* (ethylene and salt-inducible ERF1), a target gene of ethylene-insensitive 3 (EIN3) under salt stress, indicating the linkage between the ethylene pathway and the SOS pathway in salt responses (Quan *et al.* 2017). The analysis of *AtSOS1* promoter sequence showed that it contained several transcription factor (TF) binding domains, that is of bZIP, NAC and WRKY, suggesting some unknown TFs might also regulate this pathway (Ji *et al.* 2013). But further understandings on the regulation mechanisms of these TFs and SOS1 under salt stress have not been identified yet. Overexpression of a heavy metal-associated domain protein NaKR3 (sodium potassium root defective 3) would induce the expression of *SOS1* and increase tolerance to salt treatment in *Arabidopsis* (Luo *et al.* 2016). The SOS1 could be important for controlling oxidative stress responses under salt stress (Feki *et al.* 2017). The *SOS1* transcript is unstable under normal growth conditions and the stability is induced by salt stress through ROS production, indicating a potential regulation mechanism of SOS1 exists at the post-transcriptional level (Chung *et al.* 2008).

The homologues of the *SOS1* gene have also been identified in a number of crops, including rice (Martínez-Atienza *et al.* 2007), wheat (Ramezani *et al.* 2013), durum wheat (Feki *et al.* 2014, 2015, 2011, 2017), soybean (Nie *et al.* 2015), and tomato (Olías *et al.* 2009). Most of the *SOS1* homologues were shown to be up-regulated by salt stress, suggesting they probably had a similar function as AtSOS1. Ectopic expression of *OsSOS1* could recover the ability of salt tolerance in yeast AXT3K mutant which lacks 3 $K^+(Na^+)/H^+$ transporters and *Arabidopsis sos-1* mutant. The activity of OsSOS1 could be positively regulated by AtSOS2/AtSOS3 (Martínez-Atienza *et al.* 2007). The homologues of AtSOS2/AtSOS3 have been also identified in rice, so do some other components, OsCIPK24 and OsCBL4 in the SOS pathway, suggesting there is a complete SOS pathway in rice (Martínez-Atienza *et al.* 2007). The durum wheat TdSOS1 could be regulated by phosphorylation and overexpression of a constitutively active form TdSOS1Δ972, which lacks the phosphorylation site and the auto-inhibitory domain, would increase the ability of salt tolerance in transgenic *Arabidopsis* (Feki *et al.* 2014, 2011). The expression of *SOS1* is also induced in leaves and roots of durum wheat after $H_2O_2$ treatment (Feki *et al.* 2017). Salt stress would induce the expression of *GmSOS1* in soybean and the induction was repressed by high external phosphate concentration (Phang *et al.* 2009). A recent report showed that ectopic expression of *GmsSOS1* could alleviate salt tolerance in the *Arabidopsis* mutant *Atsos1-1* (Nie *et al.* 2015). Thus, *SOS1* is considered as an important salt tolerance determinant.

**Intracellular *NHX* genes:** According to their subcellular localization, the intracellular NHX antiporters are divided into two clades, including the tonoplast localized NHXs and the endosome localized NHXs (Rodríguez-Rosales *et al.* 2009). There are six intracellular NHX isoforms identified in *Arabidopsis*: AtNHX1-6 (Yokoi *et al.* 2002). AtNHX1-4 are located in tonoplast membrane and AtNHX5-6 are located in the Golgi, *trans*-Golgi network (TGN), and pre-vacuolar compartment (PVC) (Chanroj *et al.* 2012, Yokoi *et al.* 2002, McCubbin *et al.* 2014, Qiu 2016).

The predominant isoforms are *AtNHX1* and *AtNHX2*. They were shown to be expressed in most tissues throughout all developmental stages in a similar expression pattern, especially highly in vasculature and in guard cells (Shi and Zhu 2002, Apse *et al.* 2003, Barragán *et al.* 2012). But in meristems, the expression was only detected for *AtNHX2*, not for *AtNHX1*. *AtNHX5* is expressed at a lower level in seedling shoots and roots, whereas *AtNHX3* transcripts are found mainly in seedling roots (Yokoi *et al.* 2002). The expression of *AtNHX4* and *AtNHX6* are quite low in seedlings, compared to the other four members of this group (Yokoi *et al.* 2002). In the later developmental stages, *AtNHX3* was also detected in flowers and siliques (Liu *et al.* 2010), *AtNHX4* in stigma and calyces (Li *et al.* 2009), *AtNHX5* and *AtNHX6* in all the tissues (Bassil *et al.* 2011a). It was shown that the expression of *AtNHX1*, *AtNHX2* and *AtNHX4* is induced by salt and ABA, whereas *AtNHX5* is induced only by salt stress (Yokoi *et al.* 2002, Li *et al.* 2009).

Together with the studies on the mutants of *AtNHX*s,

it was suggested that most *AtNHX*s are involved in salt tolerance by maintaining $Na^+$ and $K^+$ homeostasis (Li *et al.* 2009, Liu *et al.* 2010, Bassil *et al.* 2011, Barragán *et al.* 2012, Bassil and Blumwald 2014). Plants can enhance the ability of salt tolerance when *AtNHX1*, *AtNHX2*, *AtNHX3*, *AtNHX5*, or *AtNHX6* were over-expressed, or *AtNHX4* was deficient (Apse *et al.* 1999, Liu *et al.* 2008, Li *et al.* 2009, Wu *et al.* 2016).

Normally, intracellular NHXs play a role in cellular ion homeostasis to tolerate salt stress (Reguera *et al.* 2014). But the regulation mechanism of most NHXs is still ambiguous. AtNHX1 is the first vacuolar $Na^+/H^+$ antiporter identified in plants and one of the best characterized plant $Na^+/H^+$ antiporters (Apse *et al.* 1999, 2003). AtNHX1 was shown to play a role in regulating vacuolar $Na^+$ and $K^+$ homeostasis (Apse *et al.* 1999, Bassil *et al.* 2011b, Barragán *et al.* 2012). AtNHX2 was shown to have identical biochemical activity, and have a redundant function to AtNHX1 (Barragán *et al.* 2012, Bassil *et al.* 2011b). Compartmentalization of $Na^+$ into vacuole is a critical strategy in plants to cope with salt stress. It helps not only to avert the toxic effects of $Na^+$ in the cytoplasm, but also to maintain an osmotic potential for water uptake using $Na^+$ as an osmoticum (Apse and Blumwald 2007, Kronzucker and Britto 2011). The accumulation of $K^+$ in the vacuole could also increase the ability of osmotic regulation and maintain pH homeostasis (Barragán *et al.* 2012). Further studies showed that AtNHX1 and AtNHX2 function primarily as $K^+/H^+$ transporters to mediate vacuolar $K^+$ accumulation, thus affecting transpiration rate *via* regulation of stomatal function (Hernández *et al.* 2009, Barragán *et al.* 2012, Andrés *et al.* 2014, Assaha *et al.* 2017). Studies of NHX1 in tomato suggested that AtNHX1 mediates potassium compartmentalization in vacuole of transgenic tomato to withstand salt shock (Leidi *et al.* 2010). The tomato LeNHX2 mediates $K^+$ accumulation to confer salt resistance as well (Rodriguez-Rosales *et al.* 2008). AtNHX1 was also shown to be involved in vesicle trafficking which might help plant in adaptation to salt stress (Hamaji *et al.* 2009). AtNHX1 localized in vesicular membranes could mediate $Na^+$ influx into the vesicles, which would move and fuse into the vacuole. The hydrophilic C terminal tail of AtNHX1 residing in the vacuolar lumen interacts with calmodulin-like protein 15 (AtCaM15) to regulate its cation selectivity in a $Ca^{2+}$ and pH-dependent manner (Yamaguchi *et al.* 2003, 2005). Salinity stress often causes alkalization of the vacuole. As a consequence, the binding of AtCaM15 to AtNHX1 is decreased, leading an increase of the $Na^+/H^+$ activity to sequester $Na^+$ in vacuole. AtNHX1 activity could be regulated by the salt-overly-sensitive (SOS) pathway through the interaction with the protein kinase SOS2/CIPK24 (Qiu *et al.* 2004). But no phosphorylation form of intercellular AtNHX1 has been detected yet. The complete transcript of *OsNHX1* containing 5'-UTR, 3'UTR, and CDS conferred more salt resistance than the partial transcript containing 5'UTR and CDS in rice, indicating some unknown regulation mechanisms could exist for *OsNHX1* (Amin *et al.* 2016). Overexpression of *AtNHX3* conferred resistance to high salinity in sugar beet (Liu *et al.* 2008). The *Atnhx3* mutant maintained lower $K^+$ and higher $Na^+$ content under normal growth condition and $K^+$-deficient growth condition, suggesting AtNHX3 displayed the activity of $Na^+$, $K^+/H^+$ exchanger (Liu *et al.* 2010). AtNHX4 was also shown to be $Na^+/H^+$ exchanger and its C-terminus could function in regulating its activity (Li *et al.* 2009). The *Atnhx5Atnhx6* double mutant was hyper-sensitive to salt stress comparing with the wild type and the single mutants, indicating endosomal AtNHX5 and AtNHX6 were functionally redundant in salt tolerance probably *via* the maintenance of organelle pH and ion homeostasis (Bassil *et al.* 2011a, Reguera *et al.* 2015, Qiu 2016).

The ion transporters may work synergistically in response to salt stress. As discussed above, the SOS pathway might regulate the activity of NHX1 for the vacuolar compartmentalization of $Na^+$ (Qiu *et al.* 2004). Co-overexpressing *AtNHX1* and *AtSOS1* improved salt tolerance of up to 250 mM NaCl in transgenic *Arabidopsis*, which is much higher than in the transgenic plants with the overexpression of a single gene *AtNHX1* or *AtSOS1*, respectively (Pehlivan *et al.* 2016). The study in the halophytic grass *Puccinellia tenuiflora* showed that the expression of *SOS1*, *HKT1;5*, and *NHX1* was induced under salt stress, indicating the synergistically modulation of $Na^+$ homeostasis (Zhang *et al.* 2017).

The *NHX* genes have been identified and cloned from more than 60 plant species (Bassil and Blumwald 2014, Mishra *et al.* 2014). Most of them are shown to be involved in salt tolerance and overexpression of *NHX* isoforms could usually alter the ability of salt tolerance in many plant species (Rodríguez-Rosales *et al.* 2009, Roy *et al.* 2014). Though further investigation is required for their regulation mechanisms, most of them have been shown to play a role in $Na^+$ and $K^+$ homeostasis to cope with salt stress as their *Arabidopsis* counterparts. Either over-expressing/knocking-out its own NHX-type transporters or making the transgenic plants of the NHX-type transporters from other plants, especially from the halophytes, would increase salt tolerance in most plants with some exceptions. For example, ectopic expression of *AtNHX1* does not improve salt tolerance in barley (Adem *et al.* 2015). There are several putative reasons: low activity of V-ATPase and V-PPase, inability to prevent a passive leak of sodium; insufficient ATP pool, and inactive *Arabidopsis* NHX1 in barley. However, the studies on plant NHXs are quite meaningful for improving plant salt tolerance, especially for crops, such as rice (Fukuda *et al.* 2004, 2011, Zeng *et al.* 2017), wheat (Brini *et al.* 2005, 2007, Xu *et al.* 2013, (Zhang *et al.* 2015), maize (Zörb *et al.* 2005), soybean (Li *et al.* 2006, Sun *et al.* 2006, Chen *et al.* 2015, Wu *et al.* 2016), tomato (Rodriguez-Rosales *et al.* 2008, Leidi *et al.* 2010, Gálvez *et al.* 2012, Huertas *et al.* 2013), and potato (Wang *et al.* 2013).

## CPA2 family

The CPA2 type transporters are predicted to have 8 - 14 transmembrane domains with a Pfam00999 domain for $Na^+$, $K^+/H^+$ exchanger (Chanroj *et al.* 2012). The CPA2 family contains $K^+$-efflux antiporter (KEA) and cation/$H^+$ exchanger (CHX) subfamilies. KEAs contain a long hydrophilic N-terminal domain and a C-terminal transmembrane domain, whereas CHXs usually have an N-terminal transmembrane domain and a C-terminal hydrophilic tail (Chanroj *et al.* 2012). In *Arabidopsis*, there are 6 members of the KEA family and 28 members of the CHX family (Mäser *et al.* 2001). Though a large number of the CPA2 family members have been identified, the biological function of most members has not been well characterized, especially in salt responses (Table 2 Suppl.).

**KEA subfamily:** *AtKEAs* are closely related to the bacterial $K^+$ efflux transporter genes *EcKefB* and *EcKefC*, which are involved in the tolerance to toxic metabolites (Aranda-Sicilia *et al.* 2012, Zheng *et al.* 2013). In contrast to the multiplicity of the *CHX* subfamily, the KEA subfamily just contains several members, suggesting their function may be conserved in plants (Chanroj *et al.* 2012, Ye *et al.* 2013). According to phylogenetic analysis, the six *Arabidopsis* KEA proteins are divided into two groups, KEA1-3 and KEA4-6 with distinct bacterial homologues (Zheng *et al.* 2013). The cellular localization of AtKEAs seemed to be diverse in yeast cells, suggesting each member probably has a different function in $K^+$ homeostasis and osmotic adjustment (Zheng *et al.* 2013). The expression of *AtKEA*s was detected by real-time PCR in both shoots and roots of *Arabidopsis* seedlings (Zheng *et al.* 2013). The expression of *AtKEA1* and *AtKEA3* is much higher in shoots than in roots, whereas the expression of the other *AtKEA*s is similar in shoots and in roots. Further, promoter-driven β-glucuronidase (GUS) staining analyses demonstrated that the expression of all *AtKEA*s was detected in the vascular tissues and in the inflorescence. Besides, their expression was also detected in the guard cells, except for *AtKEA6* (Han *et al.* 2015). The expressions of *AtKEA2* and *AtKEA5* were induced in shoots, whereas those of *AtKEA1*, *AtKEA3*, and *AtKEA5* were induced in roots under NaCl treatments, suggesting they might be involved in salt responses. The expressions of *AtKEA2* and *AtKEA5* were also induced by osmotic stress. They are suggested to be responsive to osmotic stress other than ionic stress and the induction could be dependent on ABA signalling (Zheng *et al.* 2013). Under $K^+$ deficiency, the expressions of *AtKEA1-3* were induced only in shoots (Han *et al.* 2015). AtKEA1 and AtKEA2 are localized in the inner envelop membrane of chloroplasts and AtKEA3 in the thylakoid membrane. Their function is chloroplast osmoregulation, and ion and pH homeostasis (Kunz *et al.* 2014, Aranda-Sicilia *et al.* 2016). The truncated AtKEA2 lacking its N-terminus would alleviate salt tolerance in the yeast mutant which is deficient in a $Na^+(K^+)/H^+$ antiporter Nhx1p (Aranda-Sicilia *et al.* 2012). It was also shown that AtKEA2 has an exchange preference for $K^+ = Cs^+ > Li^+ > Na^+$, *via* an assay using proteoliposomes expressing *AtKEA2* to test their transport activity. The results of this assay indicate that AtKEA2 primarily functions at maintaining $K^+$ and pH homeostasis and that transport of $Na^+$ is not the main function of AtKEA2 (Aranda-Sicilia *et al.* 2012). The mutations of *AtKEA1-3* impaired the rapid intracellular $Ca^{2+}$ elevation during osmotic stress, which would affect the ion homeostasis of chloroplast and reduce the photosynthetic efficiency (Stephan *et al.* 2016). The chloroplasts of the *kea1-1kea2-1* double mutant showed swollen appearance. Interestingly, NaCl treatment, which would cause ion accumulation and decrease water potential, prevented the chloroplasts from swelling and alleviated damage in *kea1-1kea2-1* (Kunz *et al.* 2014).

**CHX subfamily:** The CHX subfamily is ubiquitous in bacteria, fungi, and plants (Chanroj *et al.* 2012). In striking contrast to the other *CPA* subfamilies, flowering plants contain multiple *CHX* genes, representing an interesting model for analyses of gene diversity. *Arabidopsis* possesses 28 *CHX* genes, rice has 17 *CHX* genes, and *Glycine soja* has 34 *GsCHX* genes (Jia *et al.* 2017). Pairs of close homologues exist among the numerous members of the *CHX* family, such as *AtCHX13* and *AtCHX14*, *AtCHX21* and *AtCHX23*, *AtCHX7* and *AtCHX8* in *Arabidospis*. The hydrophobic C-terminal domains of plant CHXs are remarkably similar to *Saccharomyces cerevisiae* KHA1 and *Synechocystis* NhaS4, suggesting their potential role in $K^+$ homeostasis. Most reports so far showed that CHXs play their roles more relying on the modulation of $K^+$ and pH homeostasis (Cellier *et al.* 2004, Padmanaban *et al.* 2007, Zhao *et al.* 2008, Chanroj *et al.* 2011, Mottaleb *et al.* 2013, Zhao *et al.* 2015, Padmanaban *et al.* 2017). By screening a collection of 82 *Arabidopsis* accessions under salt stress, 7 accessions were selected for their stronger ability of salt tolerance. In comparison to Col-0, the 7 salt-resistant accessions had a higher $K^+$ content and a similar $Na^+$ content (Sun *et al.* 2015). In these 7 salt-resistant accessions, the expression of three $K^+$ transporter genes *AtHAK5*, *AtCHX17* and *AtKUP1* was also significantly induced, even under optimal growth conditions. It demonstrated that the potassium retention would be associated with salt tolerance in these plants. Thus, those CHXs proved to keep $K^+$ homeostasis would probably be involved in salt responses as well. Besides, many of the *CHX* genes in *Arabidopsis* have been identified to play an important role in reproductive development (Table 2).

According to phylogenetic analysis, the CHX family members can be divided into five subclades (Sze *et al.* 2004). The largest subclades Group IV, consisting of 8 members (AtCHX15-21 and AtCHX23), has been the best characterized until now. Some *CHX* genes might be

involved in salt responses, such as *AtCHX17*, *AtCHX20*, *AtCHX21*, *AtCHX23*, but their function has not been elucidated well (Table 2). AtCHX17 is an endomembrane transporter localized in pre-vacuolar compartments (PVC) and plasma membrane (PM) (Chanroj *et al.* 2011, 2013). The expression of *AtCHX17* was highly induced in roots by salt stress and $K^+$ starvation. *AtCHX17* transcripts were detected only in the epidermal and cortical cells of roots under salt stress. Besides salt stress and $K^+$ starvation, *AtCHX17* could also be induced by acidic pH, ABA, $Ca^{2+}$ deprivation, osmotic and cold stresses (Maathuis *et al.* 2003, Cellier *et al.* 2004, Pardo *et al.* 2006). Structural modelling and mutagenesis indicated that AtCHX17 has a similar protein fold with similar core residues to bacterial $Na^+/H^+$ antiporters, EcNhaA and TtNapA (Czerny *et al.* 2016). The hydrophilic C-terminus tail of AtCHX17 resembles a tandem universal stress protein (USP) domain, suggesting it could be involved in multiple stress-triggered signal pathways (Chanroj *et al.* 2013, Czerny *et al.* 2016). The functional analyses in yeast and *E. coli* demonstrated that AtCHX17 mediated $K^+$ uptake and had a role in pH homeostasis at the endomembrane (Maresova and Sychrova 2006, Chanroj *et al.* 2011). Though there is no difference identified in the morphological phenotypes when lacking *AtCHX17*, the *Atchx17* mutants accumulated ~20 % less $K^+$ in roots under salt stress or $K^+$ starvation, indicating that CHX17 could play a role in salt tolerance *via* $K^+$ homeostasis (Cellier *et al.* 2004). The analyses using yeast mutants demonstrated that the expression of *AtCHX17*, but not *AtNHX1*, could rescue yeast growth at alkaline pH. Both *AtCHX17* and *AtNHX1* could confer hygromycin B resistance. AtCHX17 and AtNHX1 might have distinct and overlapping functions. AtCHX17 would play a role in membrane trafficking and cargo sorting similarly as AtNHX1.

AtCHX16-20 are clustered in the same branch in the phylogenetic tree of the AtCHX proteins (Sze *et al.* 2004). AtCHX18 and AtCHX19 were co-localized with AtCHX17 in PVC, whereas AtCHX16 and AtCHX20 were localized in endoplasmic reticulum (ER) (Chanroj *et al.* 2011). AtCHX16 and AtCHX18~20 could restore growth of yeast lacking $K^+$ transporter as AtCHX17. The AtCHX20 increased the sensitivity to high $Na^+$ or high $K^+$ stresses in salt-sensitive yeast mutants. In spite of the evidences showing that AtCHX17 and its multiple homologues function together in maintaining $K^+$ and pH homeostasis and play a role in salt responses, the *Arabidopsis* single, double, triple, and even quadruple mutants of *chx16/17/18/19* did not show any consistent differences in vegetative growth from the single mutant *chx17* under salt stress or $K^+$ starvation (Chanroj *et al.* 2013, Padmanaban *et al.* 2017). AtCHX20 has been identified to be expressed preferentially in guard cells and functioned in osmoregulation of guard cells *via* membrane trafficking to increase vacuolar and PM area. The AtCHX20 could facilitate light-induced stomatal opening by regulating $K^+$ flux and by pH modulation (Padmanaban *et al.* 2007).

There are several other *CHX* genes involved in salt responses or $Na^+/K^+$ transport. The knockout mutant of *Atchx21* exhibits retarded root elongation under salt stress, and its $Na^+$ content in leaf sap and xylem is also lower, comparing with the wild type (Hall *et al.* 2006). Immunogold labelling results showed that AtCHX21 is localized in the plasma membrane of root endodermal cells. All these suggest that AtCHX21 could transport $Na^+$ from endodermis to the stele and might regulate the $Na^+$ content in the xylem and in the leaves. AtCHX23, the closest homologue of AtCHX21, is a chloroplast $Na^+(K^+)/H^+$ transporter, playing a role in chloroplast function and salt tolerance *via* cytoplasmic and stromal pH adjustment. The *Atchx23* mutants are more sensitive to salinity than the wild type, suggesting AtCHX23 could sequestrate $Na^+$ into the chloroplast under salt stress (Song *et al.* 2004). But the *Atchx23* mutants have similar root growth as the wild type under salt stress (Evans *et al.* 2012). Besides, the *chx23-1* mutant shows wider stomatal aperture than the wild type in the stomatal opening buffer containing 100 mM KCl. In $K^+$-uptake-deficient mutant of *E. coli*, AtCHX23 could mediate $K^+$ uptake in a pH-dependent manner (Lu *et al.* 2011). Another pair of CHX homologues, AtCHX13 and AtCHX14, are believed to be plasma membrane $K^+$ transporters (Zhao *et al.* 2008, 2015). The expression of *AtCHX13* was induced in roots under $K^+$ deficient conditions, whereas *AtCHX14* was
up-regulated by $K^+$ elevation. AtCHX13 would mediate high affinity $K^+$ uptake in the environment of limited $K^+$. AtCHX14 would be a low $K^+$ affinity transporter functioning in $K^+$ homeostasis and $K^+$ recirculation in plants. Transcriptome analysis of root transporters revealed that AtCHX10 and AtCHX15 were significantly down-regulated by salt stress (Maathuis *et al.* 2003), but no further evidences about their functions in salt responses have been identified yet.

With a potential role in salt responses, CHXs have also been identified in many other plant species. In rice, *OsCHX11,* homologous to *AtCHX20*, is significantly up-regulated in salt tolerant rice cultivar FL478, but not in salt sensitive cultivar IR29 (Senadheera *et al.* 2009). But its function in salt tolerance is still unclear. *OsCHX14*, homologous to *AtCHX19*, might play a role in $K^+$ homeostasis during rice flowering (Chen *et al.* 2016). There are several *CHX* genes identified in soybean taking important roles in salinity tolerance (Guan *et al.* 2014, Qi *et al.* 2014, Jia *et al.* 2017). *GmCHX1* or *GmSALT3* (Glyma03g32900), homologous to *AtCHX20*, was identified to confer salt tolerance in the salt-resistant soybean accessions through *de novo* sequencing/ germplasm re-sequencing and map-based cloning approaches. The *in situ* PCR assay showed that it was expressed in phloem- and xylem-associated primary root cells. Expressing *GmSALT3-GFP* in *Nicotiana benthamiana* protoplasts showed that it is localized in endoplasmic reticulum (Guan *et al.* 2014). Using the gain-of-function tests in BY-2 cells and soybean hairy roots, GmCHX1 was shown to protect the plant from salt

stress (Qi *et al.* 2014). But it still needs further investigation to understand its function in salt tolerance. *GmST1*, overlapped with *Glyma03g32900*, is strongly induced in leaves by salt stress (Ren *et al.* 2016). Overexpression of *GmST1* in *Arabidopsis* confers the tolerance to salt and drought stresses *via* less ROS production. The *GmST1*-transgenic line is sensitive to ABA treatment during seed germination. The promoter analysis of *GmST1* indicates a putative ABRE element and a DRE *cis*-acting element at around 1 kb upstream, suggesting *GmST1* might regulate salt tolerance through an ABA-dependent pathway (Ren *et al.* 2016). Recently, *GsCHX19.3* cloned from wild soybean, homologous to *AtCHX19*, was shown to be highly induced by carbonate alkaline stress (Jia *et al.* 2017). GsCHX19.3, localized in plasma membrane, could confer resistance to low $K^+$ at alkali pH and carbonate alkaline stress in yeast mutant *AXT4K*, which is deficient in 4 cation transporters. The *GsCHX19.3*-transgenic *Arabidopsis* lines could increase tolerance to high salinity and carbonate alkaline stress with lowering $Na^+$ content leading to higher $K^+/Na^+$ ratios. It demonstrated that GsCHX19.3 may mediate $K^+$ uptake and increase salt and carbonate alkaline tolerance. The other two *CHX* genes cloned from *Physcomitrella patens*, *PpCHX1* and *PpCHX2*, are shown to regulate potassium homeostasis (Mottaleb *et al.* 2013). Taken together, CHXs have been shown to have a role in $K^+$ and pH homeostasis in plant development, or in responses to salt stress.

## Conclusions

There are around 40 members in the *CPA* gene family in *Arabidopsis*, and some of their homologues have been also identified in other plant species, suggesting that the role of the *CPA* gene family is conserved in plants. Until now, several members of *CPA* gene family are proved to play a role in salt tolerance, the biological function of the others still remains unclear. NHX1 and NHX2 would sequester $Na^+$ and $K^+$ in the vacuole to keep the cellular ion homeostasis, whereas plasma membrane-binding SOS1 would exclude $Na^+$ out of the cell and also plays a role in the long-term transport of $Na^+$. The SOS signalling pathway is believed in regulating the activity of SOS1 and probably the activity of NHX1 as well. However, there is a paradox about this mechanism and the localization of the components. NHX1 is localized in tonoplast membrane, whereas the major factors of the SOS pathway are localized in cytosol. It still needs further investigation. Several novel soybean *CHX* genes, *GmCHX1/GmSAL1*, *GmST1*, and *GsCHX19.3* have a protective function under salt stress, but the mechanisms are still unknown. In summary, NHX1, NHX2, and SOS1 have crucial roles in salt tolerance at the cellular, organ, and whole-plant levels. The other members of *CPA* gene family would have also a putative role in salt tolerance and need further study. The knowledge of *CPA* gene family could give us a hint of new strategies for improving the ability of salt tolerance in plants.

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