Genome-wide identification, characterization and expression analysis of the monovalent cation-proton antiporter superfamily, and their function analysis in maize salt tolerance.

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Abstract

**Background:** Sodium toxicity and potassium insufficient are important factors affecting the growth and development of maize in saline soil. The monovalent cation proton antiporter (CPA) superfamily comprises Na⁺/H⁺ exchanger (NHX), K⁺ efflux antiporter (KEA), and cation/H⁺ exchanger (CHX) subfamily proteins, which play vital functions in maize salt tolerance.

**Results:** A total of 35 ZmCPA genes were identified in maize, and they were phylogenetically classified into 13 ZmNHXs, 16 ZmCHXs and 6 ZmKEAs. ZmCPA genes have a conserved gene structure, with the determined introns range from 11 to 25, 0 to 5 and 16 to 19 in ZmNHXs, ZmCHXs, ZmKEAs, respectively. All proteins have transmembrane domains, with an average transmembrane number of 8, 10, and 10 in ZmNHX, ZmCHX and ZmKEA proteins, respectively. Transient expression in maize protoplasts showed that ZmCHX16 and ZmNHX8 are located in the cell membrane. All ZmCHX subfamily genes showed lower expression compared to ZmNHX and ZmKEA subfamilies. Diverse expression in the 60 tissues and modulated expression in response to salt stress suggested ZmCPAs' role in maize development and salt stress. Yeast complementary experiment revealed the function of ZmNHX8, ZmCHX8, -12, -14, -16 and ZmKEA6 in salt tolerance. Maize mutants zmnhx8 and zmkea6 further validated the important function of ZmNHX8 and ZmKEA6 in salt tolerance. Phosphorylation sites and cis-acting regulation elements analyses indicated that phosphorylation and transcriptional regulation may be involved in salt tolerance of ZmCPA genes.

**Conclusions:** Our study provides comprehensive information about ZmCPA gene superfamily, which would be useful in their future functional characterization.

**Keywords:** CPA, maize, Salinity, ZmNHX, ZmCHX, ZmKEA
Background

High salinity stress is a major abiotic stress affecting crop production worldwide. High concentration of salt can reduce the osmotic potential of soil solution and affect the absorption of water by plant roots, resulting in slow growth of new roots and shoots. Meanwhile, Na\(^+\) and Cl\(^-\) are absorbed and accumulated to toxic concentrations in plants, which causes the generation of reactive oxygen species (ROS) leading to oxidative stress [1, 2] and senescence of older leaves [3, 4]. 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 [5-9]. Among them, the cation transporters have been well characterized and most Na\(^+\) and K\(^+\)/H\(^+\) exchangers belong to the monovalent cation/proton antiporter (CPA) superfamily, which is classified into the CPA1 and CPA2 families, according to Transporter Classification database (http://www.tcdb.org/) [10, 11]. The CPA1 consists of Na\(^+\)/H\(^+\) exchanger (NHX), while CPA2 consists of K\(^+\) efflux antiporter (KEA) and cation/H\(^+\) exchanger (CHX) subfamilies [12, 13].

The CPA1 family is divided into two main groups, including the intracellular proteins and the plasma membrane-bound proteins, according to the subcellular localization [10]. In Arabidopsis, six intracellular NHX isoforms AtNHX1-6 were identified [14], which localized into the plant vacuole and endosomes, while other two deviating members (AtSOS1/AtNHX7 and AtNHX8) are localized into the plasma membrane (PM) [15]. The NHXs consist of 9-12 transmembrane domains (TMs) [16] and are reported to be involved in numerous functions including salt tolerance, pH regulation, ion homeostasis, turgor generation, vesicular trafficking, protein processing and flower development. For instance, ectopic expression of AtNHX1 causes dramatic salt tolerance in Arabidopsis [17,18]. Further, AtNHX1 and AtNHX2 are associated with K\(^+\) homeostasis, vacuolar pH control, floral development, reproduction, cell turgor, and regulation of stomata [19]. Endosomal AtNHX5 and AtNHX6 play key roles in cell proliferation and growth in Arabidopsis [20]. Under salt stress, the expression of AtSOS1 was induced and increase the stability of its
transcripts [21]. The expression of SOS1 is also induced in leaves and roots of durum wheat after H₂O₂ treatment [22]. Ectopic expression of GmsSOS1 could alleviate salt tolerance in Arabidopsis mutant at sos1-1 [23]. However, AtNHX8 is Li⁺ specific and performs Li⁺ detoxification in Arabidopsis [24].

The CPA2 type transporters are predicted to have 8-14 transmembrane domains with a Pfam00999 domain for Na⁺, K⁺/H⁺ exchanger [12]. In Arabidopsis, there are 6 members of the KEA subfamily and 28 members of the CHX subfamily [25]. AtKEAs are closely related to the bacterial K⁺ efflux transporter genes EcKefB and EcKefC, which are involved in the tolerance to toxic metabolites [26]. The AtKEA subfamily contains six genes forming two subgroups in the cladogram: AtKEA1-3 and AtKEA4-6. 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 [27]. AtKEA1 and AtKEA2 are localized in the inner envelop membrane of chloroplasts and AtKEA3 in the thylakoid membrane. Their functions are chloroplast osmoregulation, and ion and pH homeostasis [28, 29]. AtCHXs regulate K⁺ and pH homeostasis, and function in controlling membrane trafficking, osmoregulation, and pollen growth and development [30, 31]. In the AtCHX subfamily with 9-12 TMs, the expression of 18 AtCHX genes is either pollen specific or pollen enhanced, and only 6 are highly expressed in vegetative tissues. This indicates that the multiple CHX gene plays an important role in the development, survival, and function of the male gametophyte [32]. AtCHX14 is located in the PM and regulates K⁺ redistribution in Arabidopsis [33]. AtCHX21, AtCHX23, and AtCHX24 have role in salt tolerance [34], chloroplast development and pH homeostasis [35], and leaf senescence, respectively [36]. Moreover, AtCHX21 and AtCHX23 are also engaged in guidance of pollen tube to the target ovules [31]. OsCHX14 played an important role in K⁺ homeostasis during flowering in rice [37]. PbrCHX16 of pear also plays significant role in pollen tube growth [38].

In this study, genome-wide identification of ZmCPA genes was firstly conducted in maize. The identified proteins were classified into ZmNHX, ZmKEA and ZmCHX
subfamilies, and used for the analysis of various physicochemical properties like molecular weight (MW), isoelectric point (pI), sub-cellular localization, transmembrane (TM), motifs, structure and evolutionary relationship. The protein-encoding genes were analyzed for the occurrence of splice variants, exon-intron structure and intron phase. Phosphorylation sites and cis-acting regulatory elements were analyzed to investigate the relationship between phosphorylation and transcriptional regulation and the salt tolerance of the ZmCPA genes. The ZmCPA genes were also analyzed for their expression during numerous developmental stages and in the presence of salinity stresses. Further, six ZmCPA genes were cloned and used for functional characterization in the presence of salt. Two maize mutants zmnhx8 and zmkea6 were obtained, which further verified the important functions of ZmNHX8 and ZmKEA6 in salt tolerance.

Results

Genome-wide identification and characterization of the CPA superfamily genes in maize

An extensive BLAST search identified a total of 35 ZmCPA superfamily proteins in the genome of maize (Table S1), which were further confirmed through Pfam database (http://pfam.xfam.org/search) search for the existence of signature Na+/H+ exchanger (PF00999) domain. These genes were classified into three subfamilies including 13 ZmNHX, 16 ZmCHX and 6 ZmKEA genes based on protein similarity with the earlier reported the CPA genes in Arabidopsis. The identified CPA genes were named ZmNHX1-ZmNHX13, ZmCHX1-ZmCHX16 and ZmKEA1-ZmKEA6 based on their order on maize chromosomes (Table S1). The ZmCPAAs were distributed on all chromosomes of maize, with 5, 3, 3, 4, 1, 3, 5, 4, 3 and 4 ZmCPA genes on chromosome 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10, respectively (Figure S1). Each ZmCPA gene model was validated by analyzing the similarity between the ZmCPA genes and their orthologs of relative species, such as Arabidopsis, rice, Sorghum, Brachypodium distachyon and Setaria italica and by analyzing cDNA and DNA sequences of each
ZmCPA gene which were obtained using reverse transcription polymerase chain reaction (RT-PCR) assays with the gene-specific primers listed in Table S2, as most of the ZmCPA genes had more than one transcript annotated in MaizeGDB database (Table S1 and Figure S2). By cDNA and DNA sequence alignment analysis, we found that 5 ZmCPA genes had alternative splicing events, resulting in multiple transcripts for one gene (Figure S3), and the transcripts with the conserved gene structure similar to that of their orthologs were selected for further analysis. The gene structures of the ZmCPA genes were constructed by aligning their genomic sequences with cDNA sequences obtained by RT-PCR (Figure 1). The number of introns determined in ZmNHXs, ZmCHXs, ZmKEAs ranges from 11 to 25, 0 to 5 and 16 to 19, respectively (Table 1). The majority of introns in each family were in 0 phase. The ZmCPA genes from the same subfamily share the conserved gene structure (Figure 1).

The average protein length of ZmNHX, ZmCHX and ZmKEA proteins were 640, 824 and 815 amino acid (aa) residues, respectively. The average molecular weight (MW) of ZmNHX, ZmCHX and ZmKEA proteins were 70.8, 88.4 and 87.4 kDa, respectively. The isoelectric point (pI) value of ZmNHX, ZmCHX and ZmKEA proteins ranged from 5.28 to 9.07, 5.95 to 9.88, and 5.22 to 6.01, respectively (Table 1).

**Phylogenetic analysis of maize CPA genes**

In order to analyze the evolutionary relationships among the identified ZmCPA genes, we aligned their protein sequences with 26 OsCPA and 42 AtCPA proteins to constructed the neighbor-joining (NJ) phylogenetic tree. The detailed information of AtCPA and OsCPA genes was shown in the Table S3. Based on the topology of the phylogenetic tree, the CPA gene superfamily in plants can be subdivided into 3 subfamilies, NHX, CHX and KEA subfamilies. NHX and CHX subfamilies can be further divided into groups N1, N2 and N3, and C1, C2, C3 and C4, respectively. KEA subfamily was formed by K1 and K2 groups (Figure 2). In addition, we performed a phylogenetic analysis only with ZmCPA proteins. As described in Fig. 3,
ZmCPA genes were also divided into three subfamilies of ZmNHX, ZmCHX, and ZmKEA genes, which is consistent with the classification in Fig. 1. (Figure 3). All groups contain the genes from the three species of Arabidopsis, rice, and maize, indicating that the homologous genes in each group have similar conservative functions.

Subcellular localization and motif analysis of ZmCPA genes

The transmembrane (TM) helices within ZmCPA proteins were predicted in TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). The results showed that all of ZmCPA proteins have transmembrane domains, and different subfamilies have different transmembrane domains (Figure S4). A maximum of 12 TM regions were predicted in ZmNHX2,-8, ZmCHX3,-11,-16 and ZmKEA4. The average occurrence of TM regions was 8, 10 and 10 in ZmNHX, ZmCHX and ZmKEA proteins, respectively (Table S1). In the NHX and CHX subfamilies, the transmembrane domain is mainly located at the C-terminal, while the transmembrane domain of ZmKEAs is mainly located at the N-terminal. But ZmKEA1, -4, -5 have a single transmembrane domain at the C-terminal. Proteins with different transmembrane domains may have different functions.

We performed the transient expression assay to investigate subcellular localization of ZmCHX16 and ZmNHX8 protiens with in maize protoplasts. The ZmNHX8-GFP and ZmCHX16-GFP fusion proteins were co-localized with OsSCMP1-RFP protien which was rice SECRETORY CARRIER MEMBRANE PROTEIN 1 fused with RFP as a membrane protein control in this study [39], suggesting that ZmNHX8 and ZmCHX16 were localized to plasma membrane (Figure 4). The tertiary stuctures of nine ZmCPA proteins were analyzed by the SWISS-MODEL server [40] and the results showed that similar 3D structures existed among the same subfamily (Figure S5).

Known conserved domains of ZmCAP proteins were identified by screening Pfam database (http://pfam.xfam.org/). All proteins contained the Na⁺/H⁺ exchanger domain
(PF00999), and 3 ZmKEAs and 5 ZmNHXs also contained TrkA_N domain and TatD-related DNase domain, respectively (Figure S6). Meanwhile, putative motifs of the ZmCPA proteins were mined with the MEME server (http://meme-suite.org/tools/meme). All of 15 motif sequences identified here were listed in Table S4. Generally, ZmNHX proteins contained 5-8 conserved motifs, and motifs 3,7,9,15 was found in most of them. The ZmCHX and ZmKEA proteins had 6-7 and 2-3 conserved motifs, respectively. Motifs 1 and 11 were present most frequently in ZmCHX and ZmKEA proteins (Figure S6). The conserved domains of the ZmCPA proteins corresponded to partial motifs and covers the transmembrane domain (Figure S4, Figure S6). The results revealed that most closely related members in the same subfamily have common motifs, which indicates functional similarity among the ZmCPA proteins.

Phosphorylation modification of ZmCPA proteins was analyzed with GPS 5.0 (http://gps.biocuckoo.cn/online.php). Most phosphorylated sites residues of all ZmCPA proteins are evenly distributed throughout the protein (Figure S7). On average, 20.5, 23 and 18.6 phosphorylated sites were found in each member of the three subfamilies, respectively.

**Cis-elements in promoter sequences of ZmCPA genes**

The *cis*-acting regulatory elements interacted with specific transcriptional factors (TFs) are essential for gene expression regulation [41]. The *cis*-acting regulatory elements in promoter sequences of the *ZmCPA* genes were predicted in PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Figure S8). On basis of functions annotations, the identified *cis*-acting elements were divided into light, stress, and hormone responsive responsive categories. In light responsive category, the G-box, Sp1, ARE, GT1-motif, the MRE, ATCT-motif and the GATA-motif were the common elements in all *ZmCPA* genes. The LTR, ARE, GC-motif, MBS and TC-rich repeats were common elements in the stress response category. The hormone responsive category contained CGTCA-motif, ABRE,
AuxRR-core, TGA-element, P-box, GARE-motif, TATC-box, TCA-element and MBSI.

Expression analysis in different tissues and developmental stages

The NimbleGen maize microarray data [42] (ZM37) including 60 tissues representing 11 major organ systems and various developmental stages of the B73 maize inbred line was employed to analyze the expression pattern of the ZmCPA genes. The gene expression data of 31 ZmCPA genes including 10 ZmNHXs, 16 ZmCHXs and 5 ZmKEAs was used for cluster analysis. As revealed by the heatmap, all ZmNHX genes except ZmNHX6 and ZmNHX13 were highly expressed in all 60 tissues (Figure 5). ZmNHX6 had a much higher expression level in anthers and leaf than other 58 tissues, and ZmNHX13 was low in all 60 tissues. In case of ZmKEAs, all genes were highly expressed in all 60 tissues. All ZmCHX genes showed low expression compared to ZmNHX and ZmKEA subfamilies, and ZmCHX genes only exhibited high expression in anthers (Figure 5).

To confirm the organ-specific expression of ZmCPA genes revealed by the microarray data, semi-quantitative reverse transcription polymerase chain reaction (semiq-RT-PCR) of 9 ZmCPA genes was performed with total RNA isolated from the roots, leaves, ears, immature tassel, pollens, anthers, silk and whole seed (20 days after pollinated) of the B73 inbred line, and the primers for semiq-RT-PCR were listed in Table S5. The semiq-RT-PCR analysis showed that the expression of 8 ZmCPA genes was consistent with that of microarray (Figure 6). The ZmNHX4, -5 and -8 showed specific expression in roots and leaves, while ZmCHX6, -14, -16 were specifically expressed in pollens and anthers. ZmKEA1 and ZmKEA4 were predominately expressed in all tested tissues except seed (20 days after pollinated). The ZmKEA6 was not included in the microarray data, and it had the same expression pattern as other ZmKEA subfamily genes.

Expression analysis of ZmCPA genes under salinity stress
In order to understand the expression response of ZmCPA genes to salt stress, two
gene subfamilies including 4 ZmNHXs and 4 ZmKEAs were chosen for expression
profile analysis by real-time quantitative reverse transcription polymerase chain
reaction (qRT-PCR) with the primers listed in Table S6 (Figure 7). This study
analyzed the gene expression response at 1h, 2h, 4h, and 24h after salt stress. Under
100 mM KCl stress condition and in root, ZmKEA4, -5, -6 and ZmNHX4, -5, -11 were
downregulated, while other genes were upregulated at first then downregulated. In
leaf, all 8 ZmCPAs were upregulated. When treated with 100mM NaCl, expression of
ZmKEA1, -5 and ZmNHX4, -5, -11 were downregulated, and ZmNHX8 were
upregulated after 24h, while that of the other genes did not change much in root. In
leaf, ZmKEA4, -6 and ZmNHX4, -8, -11 were upregulated, while other genes were
downregulated. In conclusion, these results implied that ZmCPAs might play a role in
salinity stress tolerance through expression regulation.

Functional analysis of ZmCPA genes in yeast under salt stress

To test the function of ZmCPAs in salt tolerance, the coding sequences of ZmNHX8,
ZmCHX8, -12, -14, -16 and ZmKEA6 were cloned into the yeast expression vector
pDR196 with the promoter PMA1 and then vectors were introduced into a
Saccharomyces cerevisiae mutant strain AXT3K. The strain AXT3K lacks the
function of plasma membrane Na\(^+\)-ATPases (ScENA1-4), plasma membrane Na\(^+\),
K\(^+\)/H\(^+\) antiporter ScNHA1, and vacuolar Na\(^+\), K\(^+\)/H\(^+\) antiporter ScNHX1 [43].
Therefore, it is sensitive to high Na\(^+\). The transformed yeast was grown on Arg
phosphate (AP) medium with different levels of NaCl (Figure 8). AXT3K mutants
failed to grow in medium containing 20 mM NaCl. Expression of ZmNHX8, ZmCHX8,
-12, -14, -16 and ZmKEA6 enhanced AXT3K salt tolerance (Figure 8). These results
indicate that ZmCPAs have the function of salt tolerance.

Functional analysis of ZmNHX8 and ZmKEA6 under salt stress

We obtained two maize mutants of zmnhx8 (EMS4-0a18d8) and zmkea6
(EMS4-02c2af), which were produced by EMS mutagenesis of B73 inbred line, from the Maize EMS induced Mutant Database (MEMD) [44]. The zmnhx8 and zmkea6 mutants had pre-termination mutation in ZmNHX8 (Zm00001d022504) and ZmKEA6 gene (Zm00001d026645), causing production of truncated proteins (Figure 9).

Phenotype of inbred lines B73 and two maize mutants were analyzed after four days of growth under salt stress. Under normal conditions, the growth status of B73 and mutants was not significantly different. However, the seedling length and dry weight of zmnhx8 mutant under 100 mM KCl condition were significantly lower than B73 without salt treatment (P<0.05). Similarly, the seedling length and dry weight of zmkea6 mutant under 100 mM NaCl treatment were significantly lower than B73 without salt treatment (P<0.05) (Figure 9). These results further verified that ZmNHX8 and ZmKEA6 are important salt tolerance-related genes.

Discussion

Ion homeostasis is an essential process for the survival of plants [34] A number of cation transporters have been known to play pivotal functions in plant growth, development, nutrition, and signal transduction [25]. Cation/proton antiporters (CPAs) superfamily comprises an important group of proteins, which are responsible for the exchange of monovalent cations in bacteria, fungi, animals and plants [45]. Up to now, the function of a number of the CPA genes have been studied in Arabidopsis [14, 17, 18, 20], rice [37], wheat [41], Soybean [23], and Arachis hypogea [46]. Most of them shown to play crucial roles in maintaining the ion and pH homeostasis in plants under high salinity [47].

In this study, 35 ZmCPAs were identified to analyze the function of this gene family in maize. Earlier six NHX genes of maize have been reported in various studies [48, 49], which were probably named on the basis of their sequence similarity to known plant CPA genes. To avoid the ambiguity, we performed nomenclature of each ZmCPA gene following their order on the chromosomes. Analysis of chromosomal distribution revealed that ZmCPAs were evenly distributed on the 10 chromosomes of
maize. Similarly, the CPA genes were derived from all chromosomes in wheat [41], and 15 out of 17 chromosomes in pear [38], respectively. Phylogenetic tree was generated using full-length CPA protein sequences of maize, rice and Arabidopsis. The homologous proteins were found tightly clustered due to high homology among them. Classification of the CPA superfamily genes into NHX, KEA and CHX subfamilies and their further categorization into various groups such as N1-N3, K1-K2 and C1-C4 has also been previously performed in Arabidopsis, pear [13, 38].

The sub-cellular localization predicted of different species was consistent up to some extent. AtNHXs exhibited vacuole, endosome and plasma membrane localization [12]. Majority of ZmNHX proteins were also predicted for similar localization. ZmKEA2 was predicted chloroplast localization, which similar with AtKEA1, AtKEA2 and AtKEA3. Most of the ZmCHX proteins were predicted to be localized in plasma membrane, which same as reported for AtCHX13 and AtCHX14 [33, 50]. Since, various tools predicted different localizations of different ZmCPA proteins, we chose ZmNHX8 and ZmCHX16 for transient expression in maize protoplast cells, and the results proved that they were expressed on the plasma membrane.

The expression pattern of the ZmCPA genes from the NimbleGen maize microarray data showed ZmNHX6 was highly expressed in leaf and anthers, ZmNHX9 was found to be grain specific, other ZmNHX and ZmKEA genes exhibited significant expression during multiple developmental stages. However, ZmCHXs were specific expression in anthers. Similar expression trend has been reported for the CPA genes in other plant species. AtNHX1 and AtNHX2 are required for growth and floral development in Arabidopsis [19], AtNHX5-6 are essential for normal growth and development in Arabidopsis [20]. TaNHX2, TaNHX5 and TaNHX8 genes exhibited significant expression during multiple developmental stages, which suggested their crucial role in growth and development. TaKEA6 and TaKEA3 group genes were prominently expressed in certain developmental stages of root, leaf, stem and spike, which suggested their function in tissue development [41]. TaCHX family genes showed
distinct expression pattern where most of the genes were relatively highly expressed
in anthers [41], which similar to maize suggested their role in reproductive organ
development. At the same time, specific expression of genes was verified by semi-
qRT-PCR. We studied expressions of the ZmCPA genes in the control and salinity
treatments using qRT-PCR. Four ZmKEAs in high concentration of KCl were
upregulated with salinity treatment in leaf, but in root, ZmKEA4, -5, -6 were
downregulated in high K+. In Arabidopsis, AtKEA1, AtKEA3 and AtKEA4 expression
was enhanced significantly under low K+ stress (1mM KCl), but AtKEA2, -5, and -6
were not [27]. The differential expression in response to K+ stress suggests that
ZmKEA1 involved in K+ acquisition under K+ conditions in maize, whereas ZmKEA4,
5 and 6 may have different functions. ZmNHX8 was upregulated in NaCl treatment,
but ZmNHX4, -5, and -11 were downregulated in root. NHX7/SOS1 is critical for
excluding Na+ from plant roots [51] and ZmNHX2 is associated with a major
quantitative trait locus (QTL), qST1, which confers salt tolerance on maize plants [52,
53]. Upregulated expressions of ZmNHX8 in our salinity treatment support the idea
that their roles in response to salt stress may be conserved in maize.

Six ZmCPAs were cloned in the yeast expression vector pDR196 and introduced
into a yeast mutant strain AXT3K. They restored AXT3K mutant resistance to Na+.  
ZmNHX8 had been verified again for its role in salt stress. AtNHX5 and AtNHX6
recovered tolerance to salt using a yeast expression system [20]. These results suggest
that ZmNHXs share a common mode of action and are involved Na+ transport in
maize. Nevertheless, neither AtCHXs nor AtKEAs have been found to improve yeast
growth in salt stress [12, 27]. In this study, ZmCHX8, -12, -14, -16 and ZmKEA6
recovered tolerance to high Na+. This found was different from Arabidopsis,
suggesting that the CHX and KEA subfamilies are also resistant to salt stress in maize.
Growth inhibition of maize EMS mutants zmnhx8 and zmkea6 under salt stress once
again validated their important functions on salt stress tolerance.

Conclusions

In the present study, we performed identification and characterization of ZmCPA
superfamily comprising ZmNHX, ZmKEA and ZmCHX subfamily proteins in the genome of maize. Gene and proteins structure analyses suggested conserved nature of evolutionary related molecules in each subfamily, however they significantly differed from the members of other groups. The occurrence of high composition of helices and coils in tertiary structure, and numerous TM regions supported hydrophobic membrane bound nature of these proteins. Diverse occurrence of differential expression in various tissues and under abiotic stress conditions indicated the importance of these genes in growth and development and stress management. The prediction of phosphorylation sites and cis-acting regulatory elements indicates that phosphorylation and transcriptional regulation may be related to the salt tolerance of ZmCPA genes. Characterization of ZmNHX8, ZmCHX8, -12, -14, -16 and ZmKEA6 in yeast established their role in monovalent cation homeostasis and abiotic stress tolerance. This study verified the function of ZmNHX8 and ZmKEA6 by phenotypic analysis of mutants. The study provided numerous features of ZmCPA genes, and extended the opportunities for functional validation of each gene in future studies. Further, these genes will also be useful in future crop improvement programs for stress tolerance.

Materials and methods

Identification and bioinformatic analysis of ZmCPA gene superfamily.

The known CPA genes of Arabidopsis were used to query the maize AGPv4 gene set (https://download.maizegdb.org/Zm-B73-REFERENCE-GRAMENE-4.0/) using a local BLASTP program with an E-value <1e-10. The putatively identified sequences were further confirmed by HMMER search for the presence of signature Na⁺/H⁺ exchanger (PF00999) domain.

The phylogenetic tree was constructed using full length CPA protein sequences of maize, Arabidopsis, and rice. Alignment of the sequences was done using MUSCLE v3.8.31 program [54], and a phylogenetic tree was built employing neighbor-joining (NJ) method using MEGA 6.0 [55] with the following sets, bootstrap value of 1000,
Poisson model for amino acid substitution model. The transmembrane domains were predicted using the TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). The Predotar [56], TargetP [57] and WoLF PSORT (https://www.genscript.com/wolf-psort.html), three in silico programs, were used to predict the putative organellar localization of ZmCPA proteins. All the ZmCPA proteins were modeled using SWISS-MODEL (https://swissmodel.expasy.org/) [40] to simulate their 3D structures. Putative conserved motifs in maize CPA proteins were identified using the MEME Suite 5.1.1 (http://meme-suite.org/tools/meme) with the following sets, motif length of 10-50 aa, maximum number of motifs to find is 15.

**Gene structure analysis**

The DNA and transcript sequences of ZmCPA genes obtained from the maize sequence annotation database MaizeGDB were used to design gene-specific PCR primers with Primer3 (http://primer3.ut.ee/). DNA and cDNA sequences were validated using PCR and RT-PCR with B73 genomic DNA and total RNA as templates and gene-specific primers shown in Table S2. Alignment of validated DNA and cDNA sequences of each maize CPA gene was performed to analyze the gene structure of ZmCPA genes. Gene structure display server (GSDS 2.0) was used to display the exon-intron structure, and intron phases [58].

**Plant materials and treatments**

The maize B73 inbred lines was used in this study. For qRT-PCR, the sterilized seeds were plant in a hydroponic equipment described previously [59] with sterile water in a greenhouse at 27/23°C with day/night of 12/12h. Four days later, the plants were incubated in 1× Hoagland solution (PhytoTech, USA) until fully trifoliate leaves. For controls (CK), excessive potassium stress and NaCl stress, maize seedlings were planted in 1× Hoagland solution without treatment, containing 100 mM KCl and containing 100 mM NaCl, respectively. The concentrations were maintained until the
end of the experiments. For RT-PCR analysis, the corresponding genes were detected of CK plants to exclude the effects of plant development.

For maize mutants, the sterilized seeds were cultured hydroponically in a greenhouse at 27/23°C with day/night of 12/12h as above. The 1× Hoagland solution was exchanged every two days. Ten days later, 100 mM KCl and 100 mM NaCl were added. Four days after salt stress, phenotypic analysis was performed.

Expression analysis of ZmCPA genes in different tissues.

To investigate the spatiotemporal expression patterns of ZmCPA genes, the log2-transformed and RMA-normalized data for ZmCPA genes were downloaded from PLEXdb (http://www.plexdb.org/) [60]. A heat map was produced using Lianchuan Bio Cloud Platform (https://www.lc-bio.cn/overview).

RNA isolation and cDNA synthesis

Total RNA was isolated from different tissues of the B73 inbred lines, including seedling roots, leaves, 5-cm ears, immature tassels, anthers, pollens, silks, and seeds of 20 days after pollination, using the Trizol reagent (Invitrogen, USA) according to the manufacturer’s protocol. All RNA was purified using the DNase I (Thermo Scientific, China). First-strand cDNA was synthesized from 1µg of total RNA (20 µL reaction volume) using PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara, Japan) according to the manufacturer’s protocol.

Semi-quantitative reverse transcription PCR

All gene-specific primers were designed as shown in Table S5. Specific primers for the maize Actin1 gene (GRMZM2G126010) were used as an internal control. Reactions were performed with 2xTaq Master Mix (Vazyme, China) on a Bio-Rad Thermal Cycler (Bio-Rad, USA) using the following procedure: 5 min at 94 °C to start; 33 cycles of 30 s at 94 °C, 30 s at 59 °C and 2 min at 72 °C; and a final extension step of 72 °C for 10 min to complete the reaction, and the Actin1 transcript
was amplified with 29 PCR cycles. Each PCR pattern was performed in triplicate, mixtures without a template were employed as negative controls, and the maize Actin1 amplicon served as an internal control for each gene investigated.

**Real-time PCR**

Real-time PCR was performed using TB Green **Premix Ex Taq**™ II (Takara, Japan). ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) was used with the following thermal cycling conditions of 95°C for 5 min followed by 40 cycles of 95°C for 15 s, 60°C for 5 s, and 72°C for 34 s. The maize Actin1 gene (GRMZM2G126010) was used as an endogenous control to normalize the samples. Based on the cDNA sequences of ZmCPA genes, real-time PCR primers (Table S6) were designed with primer 3 (http://primer3.ut.ee/). The experiment was performed with three technical replicates for each sample. The specificity of the PCR reaction was confirmed by melting curve analysis of the amplicons. Comparative $2^{-\Delta\Delta CT}$ method was used to calculate the relative quantities of each transcript in the samples [61].

**Prediction of phosphorylation sites and cis-regulatory elements**

The GPS5.0 (http://gps.biocuckoo.cn/online.php) software was used to predict the phosphorylation site of ZmCPA proteins. The study selected the 2kb sequence in front of the gene coding region (ATG) as the gene's promoter region sequence. Promoter cis-regulatory elements were predicted using PlantCARE [62] (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) and displayed using DOG2.0 [63].

**Subcellular localization**

The full-length cDNAs of ZmCHX16 and ZmNHX8 were amplified using the primers listed in Table S7 and then were introduced into pM999-EGFP vector to construct the
GFP fusion proteins, ZmCHX16-GFP and ZmNHX8-GPF with the ClonExpress II One Step Cloning Kit (Vazyme, China). Constitutive expression of the fused constructs, ZmCHX16-GFP and ZmNHX8-GPF, were driven by the cauliflower mosaic virus 35S promoter. The maize mesophyll protoplasts were isolated and prepared from etiolated leaves according to the established protocols[64]. The plasmids harboring the ZmCHX16-GFP and ZmNHX8-GPF fusion constructs each was co-transfected with the OsSCAMP1-RFP construct into the protoplast cells. OsSCAMP1 is a known rice secretory carrier membrane protein and used here as a membrane protein control [39]. The transformed protoplast cells were cultured at room temperature overnight and were observed using an Leica SP8 confocal microscope (Leica, USA).

**Functional expression of ZmCPAs in yeast**

The coding sequences of ZmNHX8, ZmCHX8, ZmCHX12, ZmCHX14, ZmCHX16, ZmKEA6 were cloned into the PDR196 vector, and then transformed into the yeast strain AXT3K (ena1-4::HIS3, nha1::LEU2, nhx1::KanMX). The transformed yeast cells were cultured overnight at 29°C in YPDA medium containing 1 mM KCl. Cells were normalized in water to A600 of 0.8. For cation tolerance testing, 5μL aliquots from yeast cultures or 10-fold serial dilutions were spotted onto AP [65] plates supplemented with 1 mM KCl with or without NaCl.
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Authors’ contributions

YST, YXZ(Yanxin Zhao) and JRZ conceived the experiment. MSK, YXZ(Yunxia Zhang), ZF, WS, JNL, RYZ, RHW and YDW performed bioinformatic analysis and data acquisition. MSK, MJL, and YXZ(Yanxin Zhao) analyzed the data and wrote the manuscript. All authors revised and approved the final manuscript, and agreed to be accountable for this work.

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Availability of data and materials

The datasets and materials used and/or analyzed during the current study are available from the corresponding author on reasonable request.
Ethics declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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Figure Legends

Figure 1. Gene structures of ZmCPA genes.
The schematic diagram of the NJ tree of maize CPA genes at left was drawn based on Fig. 3. The exon-intron organization of ZmCPA genes (right) was analyzed by aligning the DNA and cDNA sequences of one CPA gene within GSDS 2.0 server (http://gsds.gao-lab.org/index.php). Introns and exons are shown as thin lines and yellow boxes, respectively. Numbers 0, 1 and 2 represent the different intron phases. Blue boxes at 5′ and 3′ ends represent untranslated regions (UTRs).

Figure 2. Phylogenetic tree of the CPA genes of maize, Arabidopsis and rice.
The NJ tree was constructed using MEGA 6.0 based on alignment of a total of 103 CPA proteins including 35 ZmCPAs, 42 AtCPAs and 26 OsCPAs. NHX, KEA and CHX subfamilies are divided into three (N1, N2 and N3), three (C1, C2, C3and C4) and two (K1 and K2) groups, respectively.

Figure 3. Phylogenetic tree of ZmCPA genes.
All of 35 ZmCPA proteins were aligned by the MUSCLE v3.8.31 program [54] and the alinment was used to construct the NJ tree with MEGA 6.0 [55]. ZmCPA genes are divided into three subfamilies, ZmNHXs, ZmKEAs and ZmCHXs.

Figure 4. Subcellular localization of ZmCHX16 and ZmNHX8 proteins.
The ZmCHX16-GFP (the upper panel) and ZmNHX8-GFP (the lower panel) fusion proteins were transiently expressed in maize protoplast cells co-transformed with the OsSCAMP1-RFP fusion construct as a membrane protein marker. The GFP and RFP signals were detected by Leica SP8 confocal fluorescent microscopy. Scale bar = 5μm.

Figure 5. Gene expression profile of ZmCPA genes in different tissues.
The normalized microarray expression data of ZmCPA genes was download from PLEXdb (http://www.plexdb.org/). Cluster analysis and the heatmap production was performed using Lianchuan BioCloud Platform (https://www.lc-bio.cn/overview).

Figure 6. Expression analysis of ZmCPA genes in the eight different tissues of B73 inbred line by semiq-RT-PCR.
The total RNA of eight tissues including seedling roots, seedling leaves, 5-cm immature ears, tassels, anthers, pollen, silk and whole seeds (20 days after pollination, DAP), were isolated and used to perform the semiq-RT-PCR of ZmCPA genes with the primers shown in Table S. The maize Actin1 gene was an internal control.
Figure 7. Expression analysis of ZmCPA genes under salt stress conditions by qRT-PCR.

Expression analysis of ZmCPA genes under 100 mM NaCl or 100 mM KCl was carried out by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The y-axis represents the relative expression levels of ZmCPA genes compared with that of actin1. The x-axis represents different time points after salt treatment in each group. Error bars represent standard deviations for three replicates. qRT-PCR data were analyzed using the 2^{−\Delta\Delta C_{t}} method as described previously [65].

Figure 8. ZmCPA genes facilitate growth of yeast mutant strain AXT3K under salt stress.

The full length CDS sequences of ZmNHX8, ZmCHX8, ZmCHX12, ZmCHX14, ZmCHX16, ZmKEA6 were cloned into the PDR196 vector, and then transformed into the yeast strain AXT3K (ena1-4::HIS3, nha1::LEU2, nhx1::KanMX). The yeast transformant cells cultured overnight were normalized in water to A_{600} of 0.8. Aliquots (5 \mu L) of 10-fold serial dilutions were spotted on AP plates supplemented with 20 mM NaCl at pH 7.5 (right panel) and normal AP plates as a control (left panel). The strains were grown at 29°C for 5 days and were photographed.

Figure 9. Growth performance of zmnhx8 and zmkea6 mutant seedlings under salt stress.

The point mutation sites of the EMS mutants zmnhx8 and zmkea6 were identified by PCR sequencing with the primers shown in Table S7. (a) The stop codons induced by EMS resulted in truncated proteins. Growth status (b), seedling length and dry weight (c) of the mutant seedlings under 100 mM NaCl treatment for 4 days were measured (n = 6, means ± SEM). Significant difference was calculated using Student’s t-test. * indicants p-value < 0.05.
Table 1. Information of maize CPA genes identified in this study.

| Gene Name | Gene Model | Chromosomal Location | CDS (bp) | Protein Length (aa) |
|-----------|------------|----------------------|----------|---------------------|
| ZmNHX1    | Zm00001d028330 | Chr1: 31041489-31049951 | 1599     | 532                 |
| ZmNHX2    | Zm00001d031232 | Chr1: 182925112-182945175 | 3411     | 1136                |
| ZmNHX3    | Zm00001d003728 | Chr2: 56632216-56643296 | 2367     | 788                 |
| ZmNHX4    | Zm00001d048732 | Chr4: 4469601-4475393 | 1641     | 546                 |
| ZmNHX5    | Zm00001d019978 | Chr7: 81486383-81509317 | 1611     | 536                 |
| ZmNHX6    | Zm00001d020892 | Chr7: 135679272-135687679 | 1491     | 496                 |
| ZmNHX7    | Zm00001d021844 | Chr7: 164344371-164361391 | 2820     | 939                 |
| ZmNHX8    | Zm00001d022504 | Chr7: 179047417-179052457 | 1620     | 539                 |
| ZmNHX9    | Zm00001d045883 | Chr9: 45473833-45477106 | 1638     | 545                 |
| ZmNHX10   | Zm00001d048459 | Chr9: 156705048-156709982 | 1440     | 479                 |
| ZmNHX11   | Zm00001d024832 | Chr10: 90134129-90141743 | 1680     | 559                 |
| ZmNHX12   | Zm00001d020502 | Chr10: 101802712-101807654 | 1565     | 520                 |
| ZmNHX13   | Zm00001d026118 | Chr10: 138832710-138862665 | 2115     | 704                 |
| ZmCHX1    | Zm00001d031077 | Chr1: 176192733-176196049 | 2388     | 795                 |
| ZmCHX2    | Zm00001d031078 | Chr1: 176200721-176205899 | 2061     | 686                 |
| ZmCHX3    | Zm00001d005032 | Chr2: 155039785-155042337 | 2553     | 850                 |
| ZmCHX4    | Zm00001d041198 | Chr3: 105028259-105030854 | 2529     | 842                 |
| ZmCHX5    | Zm00001d044623 | Chr3: 233503244-233505658 | 2415     | 804                 |
| ZmCHX6    | Zm00001d049663 | Chr4: 39074035-39076527 | 2493     | 830                 |
| ZmCHX7    | Zm00001d050509 | Chr4: 94925858-94928987 | 2232     | 743                 |
| ZmCHX8    | Zm00001d053237 | Chr4: 221575337-221578465 | 2478     | 825                 |
| ZmCHX9    | Zm00001d017805 | Chr5: 207302222-207308368 | 2484     | 827                 |
| ZmCHX10   | Zm00001d035631 | Chr6: 37793506-37814621 | 2571     | 856                 |
| ZmCHX11   | Zm00001d038517 | Chr6: 159098609-159101687 | 2598     | 865                 |
| ZmCHX12   | Zm00001d021461 | Chr7: 152741608-152744549 | 2424     | 807                 |
| ZmCHX13   | Zm00001d009889 | Chr8: 87464523-87467367 | 2391     | 796                 |
| ZmCHX14   | Zm00001d010601 | Chr8: 121526743-121529490 | 2475     | 824                 |
| ZmCHX15   | Zm00001d010629 | Chr8: 122278283-122281316 | 2874     | 957                 |
| ZmCHX16   | Zm00001d012521 | Chr8: 176021482-176024817 | 2628     | 875                 |
| ZmKEA1    | Zm00001d027466 | Chr1: 5893819-5906194 | 1791     | 596                 |
| ZmKEA2    | Zm00001d001788 | Chr2: 935639-948444 | 3480     | 1159                |
| ZmKEA3    | Zm00001d041308 | Chr3: 111434917-111440097 | 2397     | 798                 |
| ZmKEA4    | Zm00001d036981 | Chr6: 108202268-108211871 | 1848     | 615                 |
| ZmKEA5    | Zm00001d046231 | Chr9: 73621310-73635421 | 1872     | 623                 |
| ZmKEA6    | Zm00001d026645 | Chr10: 149326566-149340249 | 3315     | 1104                |
