

# Monovalent cation/proton antiporters (CPAs) in moso bamboo (*Phyllostachys edulis*): genome-wide identification, molecular evolutionary and functional description

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## Research article

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# Abstract

**Background:** Plant monovalent cation/proton enantiomer (CPA) family is a type of transmembrane transporters that plays an important role in resistance to plant abiotic stresses such as salt, drought and osmotic stress.

**Results:** In this study, 32 CPA family members were firstly identified from moso bamboo, and divided into two subfamilies, including 8 in CPA1 and 24 in CPA2. Members of the same group shared similar structures and subcellular localization predictions. Gene duplication analysis found that the expansion of the CPA genes in moso bamboo may depend on whole genome duplication (WGD) event approximately 11.18 million years ago (MYA). Gene Ontology (GO) analysis revealed that PheCPA proteins are ion transporter localized on the membrane, and the post-translational modifications (PTMs) prediction indicated there are many PTMs sites may be involved in regulating CPA protein functions. Promoter analysis revealed various cis-acting elements related to abiotic and biotic stresses, plant growth and development and phytohormone responses. The significant change of expression levels of eight *PheNHXs*, which belong to CPA1, under the treatment of ABA, NaCl and PEG, suggesting that they may have played an important role in moso bamboo response to these abiotic stresses. Additionally, PPI prediction reveals potential interaction proteins of PheNHX subfamily members.

**Conclusions:** The results suggested that the CPA family may plays an important role in plant responses to stress conditions. The comprehensive identification and analysis of *PheCPAs* indicated that *PheCPAs*, particularly those genes encoding *PheNHXs*, might serve as valuable genetic resources for the improvement of moso bamboo growth and resistance to abiotic stress.

## Background

Plants are more susceptible to various environmental stresses during growth and development, because they are not able to move freely like animals. Among various environmental stresses, high salt stress is one of the main abiotic stresses, which can cause osmotic pressure and ionic stress of plants, thus endangering plant growth [1]. Furthermore, high salinity can interfere with important cellular macromolecular structures, including proteins, lipids and nucleic acids [2], and can cause membrane peroxidation, which lead to loss of membrane integrity and leakage of important cell components, affecting plant normal growth [1]. Nowadays, soil salinity poses a growing threat to plant growth and agricultural productivity around the world [3], and it is estimated that 20% of the world's irrigated land is affected by salt [4]. In saline soil, high salinity stress can lead to imbalance of cell pH and intracellular ion content. So plants constantly adjust their cellular pH and ion content [5], for instance by a range of buffer molecules, such as  $K^+$ ,  $Na^+$ / $K^+$ , cation/proton exchangers like  $Ca^{2+}$ / $H^+$ , sodium-proton anti-carriers, proton/nutrient transporters, and  $H^+$  transporters [6].

Monovalent cation-proton reverse transporter (CPA), mediates the regulation of cation and pH homeostasis by exchanging  $Na^+$ ,  $Li^+$ ,  $K^+$  for  $H^+$  [7], plays an important role in plant resistance to abiotic

stress, especially in salt stress. They can be divided into two groups, namely CPA1 and CPA2 [8], and all members have a conserved  $\text{Na}^+/\text{H}^+$  exchange domain [9]. CPA1, also called  $\text{Na}^+/\text{H}^+$  exchanger (NHX) family, was a class of transmembrane proteins responsible for monovalent cation ( $\text{Na}^+$  or  $\text{K}^+$ )/  $\text{H}^+$  exchange. CPA1 family members, originally found from the ancestral NhaP gene in prokaryotes and subsequently found exist in bacteria, fungi, metazoan and plant cells [7, 10], played important roles in intracellular pH homeostasis [11], vesicle transport [7, 10], cell growth and development [12] as well as salt stress tolerance [13, 14]. In higher plants, eight CPA1 genes (*AtNHX1-AtNHX8*) were first discovered in *Arabidopsis thaliana* [15] and divided into three distinct functional groups (*AtNHX1-AtNHX4* of vacuolar group; *AtNHX5,6* of endosomal group and *AtNHX7,8* of plasma membrane group) based on localization and proposed cellular roles [12, 16]. *AtNHX1*, which locates in the vacuole membrane, allows  $\text{Na}^+$  in the cytoplasm to enter the vacuole for sodium differentiation to increase plant tolerance to NaCl [17]. *AtNHX7* (*SOS1*) locates in the plasma membrane to involve in  $\text{Na}^+$  outflow from the cell [18] and regulated by protein kinase SOS2 and calcium-binding protein SOS3 [19], thereby enhancing plant tolerance to high  $\text{Na}^+$  and low  $\text{K}^+$  environments. The CPA2 family, which includes the  $\text{K}^+$  efflux antiporters (KEAs) and cation/ $\text{H}^+$  exchangers (CHXs), was found in bacteria, fungi and plants, but rarely seen in metazoans [7]. KEA family genes were homologous to bacteria  $\text{K}^+$  efflux transporters (EcKefB and EcKefC) [16], characterized as  $\text{K}^+/\text{H}^+$  reverse transporter, which promotes  $\text{K}^+$  homeostasis and plays a role in maintaining pH and ion homeostasis in intimal network [20, 21]. KEAs in plants were first identified in *Arabidopsis* and named AtKEA1-6 [15]. Among these, AtKEA1-AtKEA3 were identified as the  $\text{K}^+/\text{H}^+$  reverse transporter of chloroplast, played a key role in chloroplast function, osmotic regulation, photosynthesis and pH regulation in plants [22]. The CHX family is one of the largest  $\text{K}^+$  transporter families in plants [23], composed of genes from higher plants and not found in metazoans [16]. The similarity between plant CHX protein and characterized fungal and bacterial CPA2 indicates that plant CHX protein transports  $\text{K}^+$ ,  $\text{Na}^+$  and  $\text{H}^+$  in various catalytic modes [5, 23]. In *Arabidopsis*, CHX was identified having 28 members [23]. Studies in *Arabidopsis* suggest that these proteins participate in  $\text{K}^+$  and pH homeostasis in the dynamic intima [24], membrane transport events [25] and play a potential role in pollen development, survival and tube growth [25]. So far, the research on CPA family is mainly limited to *Arabidopsis*, the function of CPAs in other species is rarely studied, and how it participates in the mechanism of abiotic stress, like high-salt stress, drought stress or osmotic stress, response is still unclear.

Moso bamboo (*Phyllostachys edulis*), one of the most important non-timber forest products in the world, the planting area accounts for two-thirds of the total area of bamboo [26]. In China, moso bamboo accounts for more than 70% of the bamboo cultivation regions, and has highly cultural, ecological and economic value and extensive prospects for development [27]. About 2.5 billion people depend economically on bamboo, and international trade in bamboo amounts to over 2.5 billion US dollars per year [28]. Furthermore, moso bamboo can be used for wood, paper, crafts, and shoots can as delicious food. This requires bamboo to be able to respond and resist adverse external conditions. Previous studies showed that regulatory and functional proteins play an important role in contribute to abiotic stress

resistance in plant [29], and CPA family proteins are the class of proteins putative to be associated with salt tolerance. Furthermore, availability of the more abundant chromosome-level de novo assembly genome sequence [30] provided us a superexcellent opportunity to conduct a genome-wide analysis of the CPA genes in moso bamboo.

In our study, we performed for the first time a comprehensive analysis of the *CPA* family genes in moso bamboo. Total 32 non-redundant *PheCPA* encoding genes were identified and systematically analyzed. Genome-wide bioinformatics analysis was performed to investigate their phylogenetic relationships, genetic structures, protein structure and conserved motifs, posttranslational modifications (PTMs), chromosomal localization and gene duplication, GO annotation and cis-elements. Further, the expression profiles of 8 NHX genes in bamboo leaves treated with NaCl, ABA and PEG were detected by qRT-PCR analysis, and the protein-protein interaction (PPI) prediction of 8 PheNHX members were performed. These study results provided valuable information for the further identification of the functional characteristics of CPA genes and the candidate gene for improving the salt resistance of moso bamboo.

## Results

### Identification and characterization of *CPA* genes

Totally, 32 genes (*PheNHX1-8*, *PheKEA1-6* and *PheCHX1-18*) were identified as PheCPA genes (Table 1), and 99 genes (26 in maize, 25 in sorghum, 24 in rice and 24 in *Brachypodium*) were collected as the CPA members (protein sequences showed in Table S1). Detailed characteristics of PheCPAs, include gene name, gene ID, locational information, physicochemical parameters, secondary structure and subcellular localization, were showed in Table 1. CDS lengths of the identified *PheCPA* genes ranged from 1314 bp to 5352 bp, resulting in amino acid sequences ranging from 437 to 1783 aa. The corresponding predicted molecular weight (MW) ranged from 46.29 to 194.29 kDa and theoretical isoelectric point (pI) varied from 5.10 to 9.21. The numbers of TM regions of each PheCPA family protein ranges 8 to 12 (Table 1), and members of the same subclade have similar TMs (Table 1; Fig S1). The prediction of their subcellular localization revealed that NHX-I PheNHXs mainly located at Vacuolar (except PheNHX1), all NHX-II PheNHXs mainly located at Golgi, KEA-I PheKEAs located at Plasma membrane and KEA-II PheKEAs located at Chloroplast, almost all PheCHXs at Plasma membrane (Table 1). These evidently demonstrated that the structures of members in same subfamily are conserved. In addition, their secondary structure showed that PheCPA proteins were mainly composed of Alpha helix (Hh), Extended strand (Ee) and Random coil (Cc), which had a great relationship with their function.

### Phylogenetic analysis of the *PheCPA* genes

Phylogenetic tree was constructed based on the sequence alignment of full-length amino acid sequences of moso bamboo, maize, rice, sorghum, *Brachypodium* and *Arabidopsis* to explore the phylogenetic relationship of CPAs among different species. Through phylogenetic analysis with other species, the PheCPA gene family was categorized into the CPAI and CPAII subfamilies (Fig 1 A & B). CPAI also named NHX, which could be distributed into two groups, NHX-I and NHX-II; and CPAII could be divided into KEA

and CHX subclade, include seven groups (KEA-I, KEA-II and CHX-I to CHX-V). Interestingly, in previous studies, the CHX subfamily of *Arabidopsis thaliana* was divided into five branches [23], and in our study, we found that CPA family of five grasses were mainly concentrated in group I and IV.

### Gene structures and conserved motifs analysis

The exon/ intron structures of *PheCPA* genes were explored using GSDS online server. The results showed most members in the same subclade share high similar intron/exon structures, but there also are some differences (Fig 2B). For example, among the NHX genes, three NHX-II NHXs all had 22 introns and type I NHXs had 13 introns (except *PheNHX1* and *PheNHX5*). KEA-I type KEAs had 18-19 introns and KEA-II had 17 introns (the *PheKEA6* gene is special, with 33 introns), and the CHX subfamily genes only had 1-4 introns, respectively.

The online MEME tool predicted 15 specific protein motifs of PheCPA proteins by submitting full-length amino acid sequences (Fig 2C; Fig S2). Each motif was verified using the Pfam and SMART, and motif 1, 2, 5, 6, 10, 11, 12 and 15 were fragments of the Na<sup>+</sup>/H<sup>+</sup> exchanger domain. Furthermore, the result showed that the members with highly phylogenetic relationships had common motif composition. For instance, 2 motifs (motif 10 and 15) were existed in all the members in the NHX subfamily with a conserved sequence 'LFFJYLLPPII' in the motif 10 and a conserved sequence 'LYSLVFGEGVVNDA' in the motif 15, at position of the 3rd TM domain and 6rd TM domain (Fig S3). Meanwhile, Motif 11 was only existed in NHX-I (Fig 2C), which contained a conserved sequence 'WWAGLMRGAVSIALA' at putative TM11 domain (Fig S3). Motif 1, 2 and 5 were existed in all members of CPA II family genes, motif 3, 4, 7, 8, 9, 12 and 13 were identified in CHX subfamily. In addition, motif 6 was detected in all three gene subfamilies. These results indicate that the structures of the same subgroup of CPA family members are highly conserved, and they also further validate our division of the CPA genes in moso.

### Posttranslational modifications prediction of PheCPA proteins

Posttranslational modifications, one of the major factors determining protein biological function [31], can regulate protein activity, localization, and protein-protein interactions, leading to fine-tuning of plant responses to various external stimuli to mitigate potential damage from environmental stress [32]. The results of PTMs prediction of PheCPAs family proteins are shown in Table 2. Glycosylation is one of the most abundant PTMs required for plants, the main type being N-/ O-glycosylation occurring at the Asp (D) residue or the S/ T or Y residue, respectively [33]. In the PheCPA proteins, almost all members (except PheCHX4, 10, 16) were predicted to have N-glycosylation sites, with an average of three, whereas the O-glycosylation site was significantly less.

Protein acetylation is the acetyl, provided by acetyl-CoA, linked to either the  $\epsilon$ -amino group of lysine residues or the alpha-amino group of the N-terminus of proteins [33]. Non-histone acetylation has been reported to involve many key cellular processes such as gene transcription, protein folding, cell division, signal transduction and metabolism [34]. The results show that there are 0-3 acetylation sites in the CPA family protein members of the *Phyllostachys pubescens*.

Reversible protein phosphorylation is a ubiquitous form of cell regulation, which can affect a variety of cellular signaling pathways, including metabolism, cell growth, differentiation and membrane transport [35]. Phosphorylation sites mainly present on serine (S), threonine (T) and Tyrosine (Y). The predicted results indicated that each PheCPAs protein has multiple potential phosphorylation sites, of which S is the most, T is the second, and Y is the least.

### **Chromosomal location, gene duplication and selection pressure analysis**

The results of the physical location of *PheCPA* genes revealed that each subfamily genes were mapped onto Moso Bamboo scaffolds unevenly (Fig 3A). Scaffold 7 and Scaffold 8 had the largest number of genes, while only one gene was found on Scaffold 1, 3, 4, 14, 21, 22 and 23. Collinearity analysis shows that 12 pairs of WGD (whole-genome duplication)/ segmental duplicated genes in moso bamboo were found on scaffolds (Fig 3A; Table S2). And interestingly, there none tandem duplicated gene pairs, this result implied that the tandem duplication event may not play a significant role in the expansion of the CPA gene family in moso bamboo. In addition, according to the analysis of the collinearity diagram between moso bamboo each subfamily genes of CPAs and other five plants (*Arabidopsis*, rice, maize, sorghum and *Brachypodium*), 2, 34, 30, 34 and 30 pairs of intergenomic duplications genes were found (Fig 3B; Table S2), respectively. The collinearity relationship between moso bamboo and the others four gramineous plants were closer, this result indicating that they might have come from the same ancestor.

Further, 8 paralogous and 62 orthologous pairs were identified (Table 3) by synteny gene pairs.  $K_a / K_s$  ratio calculation results showed that they all much less than 1 (Fig 4), implying that CPAs experienced a strongly negative or stable selection over the course of evolution among moso bamboo and four other monocotyledons and evolved more slowly. The divergence times (T) of the 8 *PheCPAs* paralogous pairs calculation results indicated that *PheCPAs* probably underwent WGD events approximately 11.18 million years ago (MYA), which is consistent with a whole-genome duplication event 7~12 MYA in moso bamboo genome [36]. Additionally, the calculation results of divergence times about orthologous pairs demonstrated that *PheCPAs* and *OsCPAs* were separated around 17.36 to 34.13 MYA, *ZmCPAs* around 21.40 to 53.96 MYA, *BdCPAs* around 22.48 to 59.87 MYA and *SbCPAs* around 25.78 to 66.55 MYA (Fig 4), respectively.

### **Gene Ontology Annotations**

The GO slim analysis performed using Blast2GO and classified the 32 *PheCPA* genes into three GO categories: biological process (38.1%), cellular component (42.3%) and molecular function (19.6%) (Fig 5; Table S3; Fig S4). In the cellular component category, the most abundant groups were cell (GO:0005623) and cell part (GO:0044464), and organelle (GO:0043226) protein-containing complex (GO:0032991) and membrane (GO:0016020) were the minor groups within this category. The biological processes category, biological regulation (GO:0065007) was the most abundant groups, and the secondary groups in this classification were cellular process (GO:0009987), metabolic process (GO:0008152), cellular component organization or biogenesis (GO:0071840) and response to stimulus (GO:0050896). In the molecular function category, transporter activity (GO:0005215) was the most highly represented GO terms, and

binding (GO:0005488) was only accounts for 1.19% (only two genes, *PheNHX1* and *PheKEA6*) (Fig 5; Table S3).

These results suggest that the members of the *PheCPA* gene family were distributed among several important groups of all three GO categories at distinctive percentages. In addition, we can also predict from the results that the main function of *PheCPA* is to locate proteins on the membrane that perform ion transport function. This prediction is consistent with previous studies in other species [10, 16].

### **Cis-acting element analysis**

Cis-elements, which are non-coding linear DNA sequences, regulate and control gene transcription by binding to transcription factors at plants all developmental stages [37, 38]. The putative promoter regions of 32 *PheCPAs* (2000bp upstream of the transcription initiation site) were identified into three categories cis-regulatory elements (Table S4), including plant growth and development, hormone response, and biologic and abiotic stress responses by using the PlantCARE database.

Numerous biological and abiotic stress-related elements were identified in the *PheCPA* promoters (Fig 6A & B; Table S4). GT1-motif, a type of salt-responsive element, was found among the 14 genes and appearing 24 times. ARE and LTR element, which related to anaerobic induction and low-temperature responsiveness respectively, were comprised 25% and 20%. Drought-related (MBS), defense and stress responsiveness (TC-rich repeats), anoxic-related (GC-motif), wound-responsive (WUN-motif) and MYB binding site involved in light responsiveness elements, also have been identified in the *PheCPA* gene family. In the category of phytohormone responsive, we found ABRE motif, associated with ABA, was represented more than 50%, and appearing 114 times among the 28 genes. Except *PheNHX2*, *PheNHX3*, *PheNHX7*, *PheNHX8*, MeJA-responsiveness elements (TGACG-motif and CGTCA-motif) were identified in *PheCPAs* genes. In addition, gibberellin-responsive elements (GARE-motif) and auxin-responsive elements (TGA-element and AuxRR-core) also were found in *PheCPAs*. In type of plant growth and development, many cis-acting elements related to growth and development, include CCGTCC-box and CAT-box (related to meristem expression), Box-4 and MRE element (involved in light responsiveness) and MSA-like element (related to cell cycle regulation), were found in *PheCPAs*. According to the previous studies and this analysis, salt, drought and ABA were selected for subsequent hormone and stress treatments.

### **Expression patterns of *PheNHXs* after NaCl, PEG and ABA treatment**

Numerous evidences showed that *NHX* genes might participate in stress response of plants [13, 39, 40]. To explore whether the *NHXs* of moso bamboo have similar functions, the expression patterns of eight *PheNHXs* were researched at NaCl (for salinity stress), PEG (for drought stress) and ABA conditions using qRT-PCR method.

Under salt stress, the expression levels of 8 *PheNHXs* were significantly increased, among which three genes (*PheNHX1*, *PheNHX2*, *PheNHX3*) showed the highest expression level at 3h and then showed a tendency of decline, and *PheNHX4*, *PheNHX5* and *PheNHX6* had the highest expression levels at 6h. In

addition, *PheNHX7* presented a low level of expression at the early stage of treatment, and increased at 24 h (Fig 7). After drought stress, the transcription levels of all eight *PheNHXs* genes showed a tendency of up-regulation to different degrees, which were similar to the expression pattern of NHX family genes previously studied [41]. In ABA treatment, the expression of the eight *PheNHXs* were affected, the expression of *PheNHX6*, *PheNHX7* and *PheNHX8* were down-regulated, and the remaining five genes (*PheNHX1-PheNHX5*) were up-regulated (Fig 7), which implied that the *PheNHX* genes might be involved in ABA-dependent signal pathway. These expression data indicated that *PheNHXs* were responsive to certain abiotic stresses (drought, salt stress) at the transcriptional level.

### Protein- protein interaction prediction of PheNHXs

To further probe the putative function of PheNHXs, the predicted interactions of PheNHXs based on homologous proteins in rice were established (fig 8). Results showed that PheNHX3 and PheNHX5 could interact with PH02Gene13937.t1, which homologous to CIPK24 of rice. PheNHX3, PheNHX4, PheNHX5 and PheNHX8 could interact with WAXY (PH02Gene13019.t1) and GBSII (PH02Gene37359.t3), which belong Granule-bound starch synthase 1 and Granule binding starch synthase II respectively. These results suggested that PheNHX family members might involve in the synthesis of amylose in the endosperm by interacting with these two type proteins. In addition, these PheNHXs also interact with cytochrome b5 reductase family members (PH02Gene29105.t1, PH02Gene03732.t1, PH02Gene02406.t1), which imply these proteins possibly involved in the maintenance of cell membrane function through interaction.

## Discussion

Uptake and transport of cations is essential for plants to complete their life cycle and plays an important role in plant nutrition, growth, development and signal transduction. The monovalent cation/proton antiporter (CPA), which contain a characteristic  $\text{Na}^+/\text{H}^+$  exchange domain, divided into three subfamilies in plants (include NHX, KEA and CHX), and previous studies have found that the NHX and KEA were found from algae to angiosperms, while the CHX subfamily was found only in terrestrial plants [42, 43].  $\text{K}^+$  efflux antiporters (KEA) can maintain the steady state of intracellular potassium ions, thus maintaining charge balance, pH homeostasis and regulating enzyme function, which plays an important role in maintaining plant growth and development [20, 44]. CHX members unique to flowering plants may be involved in some important growth and development processes of flowering plants [42].

In our study, a total of 32 CPA members in moso bamboo were identified (Table 1), and compared with 24 *OsCPAs*, 26 *ZmCPAs*, 24 *BdCPAs*, 24 *SbCPAs* and 40 *AtCPAs*, results showed that the number of CPA genes in moso bamboo is similar with other 4 monocotyledon (rice, maize, sorghum and *Brachypodium distachyon*) and lower than *Arabidopsis*. Compared with *Arabidopsis*, 5 gramineous plants contain less CPA members, even though they have the larger genome size, rice for ~372 Mb [45], maize for ~2300 Mb [46], sorghum for ~732 Mb [47], *Brachypodium distachyon* for ~ 272Mb [48], and moso bamboo for ~ 2050Mb [30], which may signify that gene loss events occurred over the course of monocot-dicot split

evolution process. Previous reports of CPA family identification of Rosaceae (*Pyrus bretschneideri* contain 53 genes, *Malus domestica* contain 61 genes and *Fragaria vesca* contain 35 genes etc.) [43] and populus (contain 44 genes) [42] also supported this view. Meanwhile, the similar size of CPA family members in 5 monocotyledons implied that the CPA gene family of these species might be relatively conserved during the evolution. The collinearity and divergence times (T) analysis also indicated that the relationship of CPA gene family between bamboo and another four grasses was closer than between bamboo and *Arabidopsis* and CPA genes had underwent strong purifying selection during the process of evolution. Besides, the sequence alignment and phylogenetic analysis classified the PheNHX and PheKEA subfamilies into 2 groups and PheCHX members into I and IV groups (Fig 1), the classification results are consistent with previous studies [16, 43]. All these evidence suggested that CPA members of moso in the same subgroups may have similar reported functions as other identified species.

Through protein primary and secondary structure analysis, conservative motif distribution and gene exon/ intron analysis analysis, we found that the structure of the members of the same subgroup were very similar (Fig 2), which might indicate that they also had similar functions. For instance, like other species known antiporters, the deduced amino acid sequences of PheNHX subfamily revealed 9-12 putative TM regions (Fig S1 & S3), among them a high consensus of TM3 with the conserved sequence of 'FF...LLPPII' [49] in each PheNHX, which was reported the membrane-spanning pore and cation-binding domain [50]. In KEA1 group, each of the three PheKEA protein located in the chloroplast has a characteristic long C-terminal tail called TrkA\_N domain (Fig 2B), was reported providing a mechanism of gating control to restrict the flux of K<sup>+</sup> in previous study [51]. The N-terminal domains of 18 PheCHX proteins contain ranged from 8 to 12 putative TM regions (Fig S1), and the genes in the same subgroup have highly similar gene and protein structure. In addition, PheCPA family contain multiple post-translational modifications and PTM sites (Table 2). Yeast Nhx1 was previously reported to be an N-linked glycoprotein [52], like Nhx1, multiple N-glycosylation sites were also found in the PheCPA proteins. In the PheCPA family, we found large numbers of phosphorylation sites related to Ca<sup>2+</sup> dependent phosphorylation pathway. Previous study verified that animal NHE-1 protein regulated intracellular pH homeostasis through phosphorylation pathway involving Ca<sup>2+</sup>/ calmodulin-dependent protein kinase II (CaMKII) [53]. PtNHX7 of poplar were predicted to regulate intracellular ion balance by interacting with Calcineurin B- like protein (CBL) and CBL- interacting protein kinases (CIPK) proteins to participate in calcium-dependent phosphorylation pathways [41]. Meanwhile, PPI prediction results also showed that PheNHX3 and PheNHX4 might interact with CIPK24 (PH02Gene13937.t1) (Fig 8), and other PheNHX members also have potential interactions with different family members to participate in different physiological processes. These results provide some guidance for future studies on the topology, signaling pathways and functions of PheCPA family proteins.

Plant cells contain many organelles, such as vacuoles, chloroplasts, and Golgi, which form subcellular compartments that contain proteins associated with specific biological functions. The different subcellular localization of PheCPA (Table 1) suggests that the mechanism of their function may be different in moso bamboo, implying that they may have specific subfunctions or new functions. AtNHX5,

AtNHX6 and OsNHX1-4 proteins were reported functions specifically in Golgi [39, 54], the PheNHX2, 4, 6, 8 as these proteins also predicted location in Golgi (Table 1), implied that their mechanism of functions might be similar. PheKEA2, 3, 6, as well as AtKEA1, 2 [20], are predicted to be located on chloroplasts, suggesting that they may play a role in maintaining electrical balance and pH stability into chloroplasts and maintaining chloroplast structural integrity under osmotic stress [55]. The subcellular localizations of *Arabidopsis* 28 AtCHXs were more extensive in previous studies report. For instance, AtCHX13 [56], AtCHX14 [24], and AtCHX21 [57] are located on plasma membrane, AtCHX17 is reported to be located to PVC/ PM in plant and endomembrane in yeast [58, 59], and AtCHX20/ AtCHX23 are reticulate endomembrane K<sup>+</sup> transporters [60, 61]. However, almost all the PheCHXs were predicted to be located on the plasma membrane, so further experiments are needed to verify this result.

At present, a numerous of studies have shown that *NHXs* plays an important role in plant growth, development and pressure response. For instance, *Arabidopsis nhx1/2* dual mutants have significantly reduced vegetative growth due to significantly reduced cell size; the double knockout *nhx5/6* in *Arabidopsis* showed reduced growth, with smaller and fewer cells and increased sensitivity to salinity; *AtNHX1*, *AtNHX2*, and *AtNHX5* are responsible for NaCl tolerance of plants through different signaling pathways [40]; the expression of *OsNHXs* in different tissues of rice correlates with salt tolerance of rice [39, 54, 62]. In this study, stress response analyses showed that *PheNHXs* were response to abiotic stress include salinity, drought (Fig 7). Noticeably, *PheNHX3* and *PheNHX4* showed significant expression changes under these abiotic stresses. GT1-motif is a cis-acting element related to salt stress [63]. The promoter analysis found that one GT1 -motif element was found in the promoter region of *PheNHX3*, but none in *PheNHX4* (Fig 6; Table S3). However, high transcription level of *PheNHX4* suggested that there may be additional elements involved in its expression that respond to salt stress. An earlier report [41] showed that the *PtNHX* of poplar genes responded to different types of abiotic stress responses, including drought, salt, heat, and low temperature, through the involvement of the ABA signaling pathway. As an important plant hormone, ABA is involved in not only plant development but also response to various stresses [64]. ABREs (ABA responsive cis-acting element) were found in every *PheNHX* (*PheNHX1–8*) promoter region, and it is notably that *PheNHX4* contains 14 ABRE elements (Fig 6; Table S3). In addition, the transcription levels of 8 *PheNHX* genes changed significantly under ABA treatment (Fig 7). This result suggested that *PheNHX1–8* might be involved in ABA signal pathway.

## Conclusions

In this study, we systematically identified and classified 32 PheCPAs in moso bamboo. Gene structures, cis-acting elements, protein primary and secondary structures, conserved motifs and PTMs analysis of the 32 predicted CPAs in the moso bamboo were performed. Intraspecific and interspecies collinear events and divergence time of moso bamboo CPAs were identified, and we found the duplication of PheCPAs from WGD events. In addition, GO analysis revealed divergent roles of CPA proteins in moso bamboo. qRT-PCR test was used to explore the expression patterns of the 8 *PheNHX* genes after ABA, drought and salt treatment, and the prediction of protein interaction provided further research value in

resistance to abiotic stress. PPI prediction revealed potential interaction proteins of PheNHX subfamily members. The results of this study provide a fundamental information for PheCPA genes and increase the understanding of PheCPAs functions in plant abiotic stress response.

## Methods

### Identification of CPA family genes

The published *Arabidopsis thaliana* CPA protein sequences were used as query to obtain CPA members from moso bamboo genome, downloading from Moso Bamboo SMRT (<http://forestry.fafu.edu.cn/db/PhePacBio/>), using BLASTp (E-values<1e-40) method [41]. Subsequently, redundant sequences were removed manually and those lacking >30% residues of the Na<sub>2</sub>H<sub>2</sub>PO<sub>4</sub> Exchanger domain (PF00999) sequences were dropped [16] based on Pfam database (<http://pfam.xfam.org/>) and TMHMM (<http://www.cbs.dtu.dk/services/TMHMM/>) analysis. The detailed information about each putative CPA protein of moso bamboo, including molecular weight (MW), Isoelectric point (pI) and amino acid lengths, were calculated by the ExpASy server ([https://web.expasy.org/compute\\_pi/](https://web.expasy.org/compute_pi/)). Softberry ProtComp 9.0 (<http://www.softberry.com>) and GOR4 [65] was used to predict the subcellular localization and secondary structure, respectively. CPA members sequences of maize (*Zea mays*) (Ensembl-18), rice (*Oryza sativa*) (version 7), sorghum (*Sorghum bicolor*) (version 3.1.1) and *Brachypodium distachyon* (version 3.1) were obtained from Phytozome database (v12.1) (<http://www.phytozome.net/>) according to previous reports [16, 66, 67].

### Phylogenetic, structure and posttranslational modifications analysis of CPAs

A phylogenetic tree of moso bamboo, *Arabidopsis*, rice, maize, sorghum and *Brachypodium distachyon* CPAs were built using MEGA 6.0 with the neighbor-joining method and 1000 bootstrap replicates based on sequences alignment [68]. Exon/intron structures of moso bamboo CPA genes were predicted through Gene Structure Display Server (GSDS: <http://gsds.cbi.pku.edu.cn/>) [69]. The online tool MEME (<http://meme-suite.org/>) was used to identify the conserved motifs of PheCPA proteins (parameter setting: number of repetitions: any; maximum number of motifs: 15; motif width: 30-60) [6], and the function of each motif was verified in Pfam and SMART (<http://smart.embl-heidelberg.de/>) database.

KinasePhos [70] was used to predicate possible phosphorylation sites of PheCPAs. In addition, N-terminal acetylation sites, N-linked glycosylation sites and O-(alpha)-GlcNAc glycosylation sites of PheCPAs were predicated using CBS Prediction Servers (<http://www.cbs.dtu.dk/services>) page.

### Chromosomal location, synteny and gene duplication

The chromosomal location of PheCPAs were constructed according to the genomic position information. Collinearity of PheCPAs were analyzed using MCScanX [71] with default parameters and the results were displayed by Circos [72]. The homolinear analysis maps were constructed using Dual Synteny Plotter software (<https://github.com/CJ-Chen/TBtools>) to reveal the synteny relationships between orthologous

CPA genes of moso and other selected species. BLASTn was used to pairwise align of CPA encoding sequences with two criteria: (a) shared aligned nucleotide sequence covering > 80% of the longer gene and (b) similarity of the aligned regions of sequence  $\geq 80\%$  and then used synteny-based to identify the orthologous and paralogous pairs. Subsequently, Ka, Ks and Ka/Ks values of them were calculated using DnaSP 5 software [73] based on sequences alignment using MEGA 6.0. The formula  $T = Ks/2\lambda$  ( $\lambda = 6.5 \times 10^{-9}$ ) [36] was used to calculate the divergence time (T).

### Gene Ontology and promoter region analysis of CPAs in moso bamboo

Full-length amino acid sequences of PheCPA were uploaded to the Blast2GO program (blast expectation value:  $1e-5$ ; mapping and annotation with default parameters) to perform Gene Ontology (GO) analysis [74]. To predict cis-acting regulatory elements of *PheCPAs*, the cis-acting elements in the 2kb DNA sequences upstream of the transcription start site were analyzed using PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>).

### Plant materials and treatments

Moso bamboo seeds were identified and provided by the Guilin Forestry Bureau, Guilin, Guang Xi Province, China. And the seedlings were cultivated in greenhouse with a constant photoperiod (16 h light/ 8 h dark), an average temperature of 22°C, 80% humidity. Then, the three-month old young leaves were sprayed with 200 mmol/L NaCl (for salt stress), 20% PEG-6000 solution (for drought stress) or 100  $\mu$ M ABA solution, and the leaves were sampled at 0, 1, 3, 6, 12 and 24 h after treatment, respectively. The untreated samples (0 h) were used as the control (CK). Each plant sample was immediately frozen in liquid nitrogen and stored at -80 °C for RNA isolation.

### RNA extraction and quantitative Real-Time PCR analysis of PheNHXs

Total RNA of plant samples was isolated using Trizol (TaKaRa, Dalian, China) methods and then reverse transcribed into cDNA using a PrimeScript™ RT Reagent Kit (TaKaRa, Dalian, China). Primer Express 5.0 was used to design gene-specific primers for each selected gene and *PheTIP41* (tonoplast intrinsic protein 41) was used as the reference control gene [75] ( Table S5). TransStart R Tip Green qPCR Super Mix (TransGen Biotech, Beijing, China) was used to perform qRT-PCR according to instructions. The qRT-PCR program settings were: 94 °C for 30 s; 39 cycles of 94 °C for 5 s, 60 °C for 15 s, and 72 °C for 10 s. A melting curve was generated to analyze the specificity of the reactions, and three biological replicates were made for each biological replicate. The relative expression level was calculated using  $2^{-\Delta\Delta CT}$  [ $\Delta C_T = C_{T, Target} - C_{T, PheTIP41}$ .  $\Delta\Delta CT = \Delta C_{T, treatment} - \Delta C_{T, CK}$  (0 h)] method. SPSS 17.0 was identified significant differences at \* (P < 0.05) and \*\* (P < 0.01) between the treatment group and control group. And the GraphPad5 software was used to visualize the data in histograms.

### Construction of interaction network of PheNHXs

The predicted protein-protein interaction (PPI) map of PheNHXs was generated from the STRING database (<https://string-db.org/>) [76]. Amino acid sequences of PheNHX were used as query sequences and rice as the model. In evidence mode, the online program was run with parameters as follows: 'minimum required interaction score' was set to 0.7 (high confidence) and others used default settings.

## Abbreviations

*CPA*: monovalent cation/proton antiporter; *NHX*: Na<sup>+</sup>/ H<sup>+</sup> exchanger; *KEA*: K<sup>+</sup> efflux antiporter; *CHX*: cation/H<sup>+</sup> exchanger; *GSDS*: Gene Structure Display Server; *WGD*: whole genome duplication; *MYA*: million years ago; *qRT-PCR*: Quantitative real-time PCR; *PEG*: Polyethylene glyco; *CDS*: Coding sequence; *BLAST*: Basic local alignment search tool; *GO*: Gene Ontology; *ABA*: Abscisci acid; *Ka*: Number of non-synonymous substitutions per non-synonymous site; *Ks*: Number of synonymous substitutions per synonymous site

## Declarations

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**Author Contributions:** All authors conceived and designed the experiments. L.W. carried out the main experiments and drafted the manuscript. M.W. collected plant materials and performed the qRT-PCR. H.-L.L. collated and analyzed the experimental data and revised the manuscript, Y.-M.G. helped to write the manuscript and implemented the software, F.C. helped to handle figures and tables. Y.X., as the correspondence author, provided financial support for the article and designed the way and frame of this study. All authors read and approved the final manuscript.

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

**Consent for publication:** No applicable

**Availability of data and materials:** All identified PheCPA gene sequences in this study were deposited into the Moso Bamboo SMRT (<http://forestry.fafu.edu.cn/db/PhePacBio/>). The CPA sequences of *Arabidopsis thaliana*, *Zea mays*, *Oryza sativa*, *Sorghum bicolor* and *Brachypodium distachyon* were deposited into Phytozome database (v12.1) (<http://www.phytozome.net/>).

**Ethics approval and consent to participate:** Our moso bamboo seeds were provided by the Guilin Forestry Bureau, Guilin, Guang Xi Province, China.

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## Tables

Due to technical limitations, the tables are only available as a download in the supplemental files section.

## Additional Files

**Additional files 1: Table S1.** Information about CPA members in rice, maize, *Brachypodium*, sorghum and *Arabidopsis*.

**Additional files 2: Table S2.** Intraspecific and interspecific collinear gene pairs of CPA family.

**Additional files 3: Table S3.** Information about GO classification of PheCPA proteins.

**Additional files 4: Table S4.** Cis-acting elements in *PheCPA* promoters.

**Additional files 5: Table S5.** Specific primers of four PeNHXs for qRT-PCR experiment.

**Additional files 6: Figure S1.** Prediction of the transmembrane regions of 32 PheCPAs.

**Additional files 7: Figure S2.** MEME motif sequences of PheCPA proteins in moso bamboo.

Additional files 8: Figure S3. Multiple sequence alignment and transmembrane region of *PheNHXs*.

Additional files 9: Figure S4. Three kinds of GO enrichment of 32 PheCPAs.

## Figures

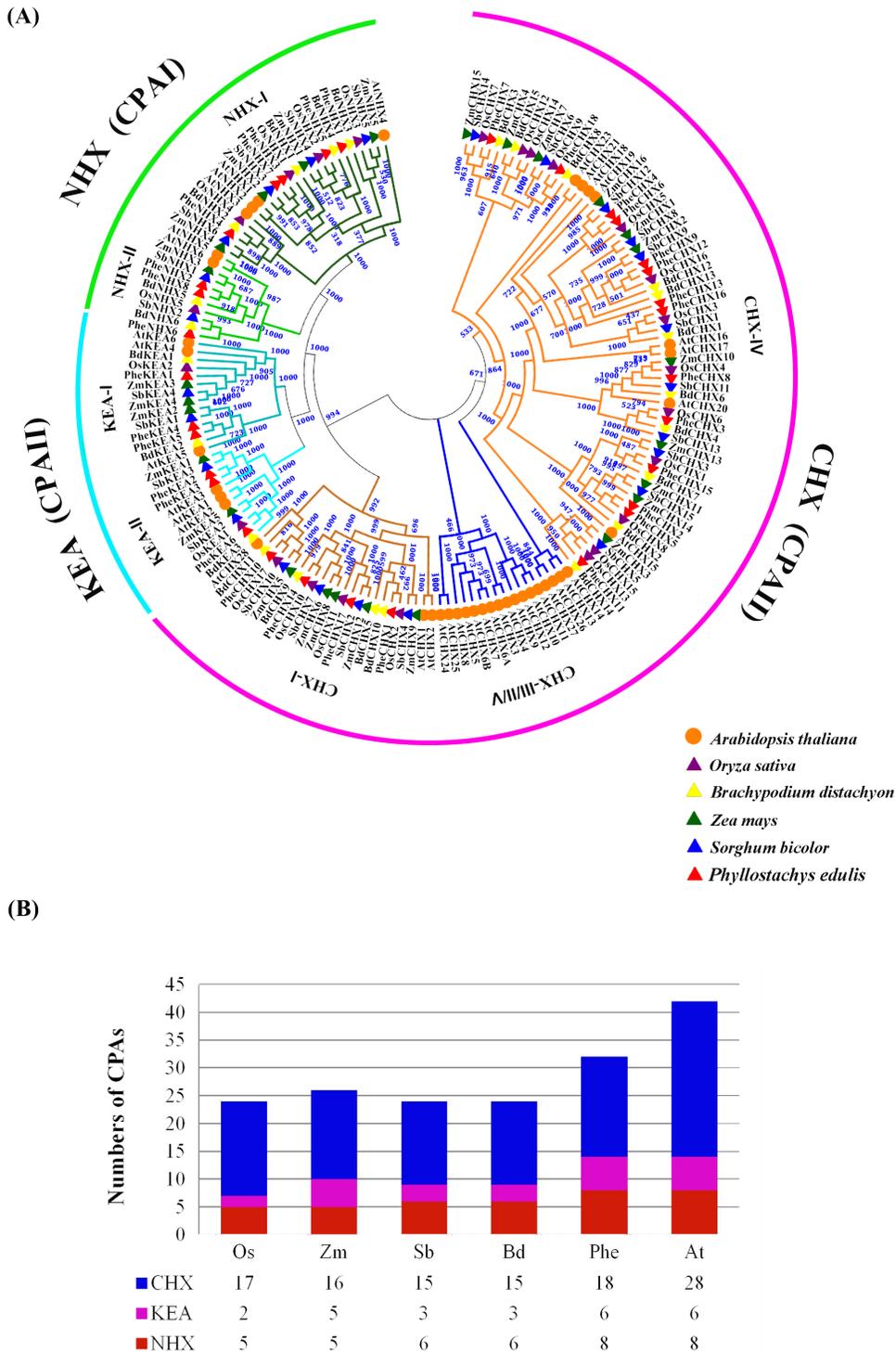
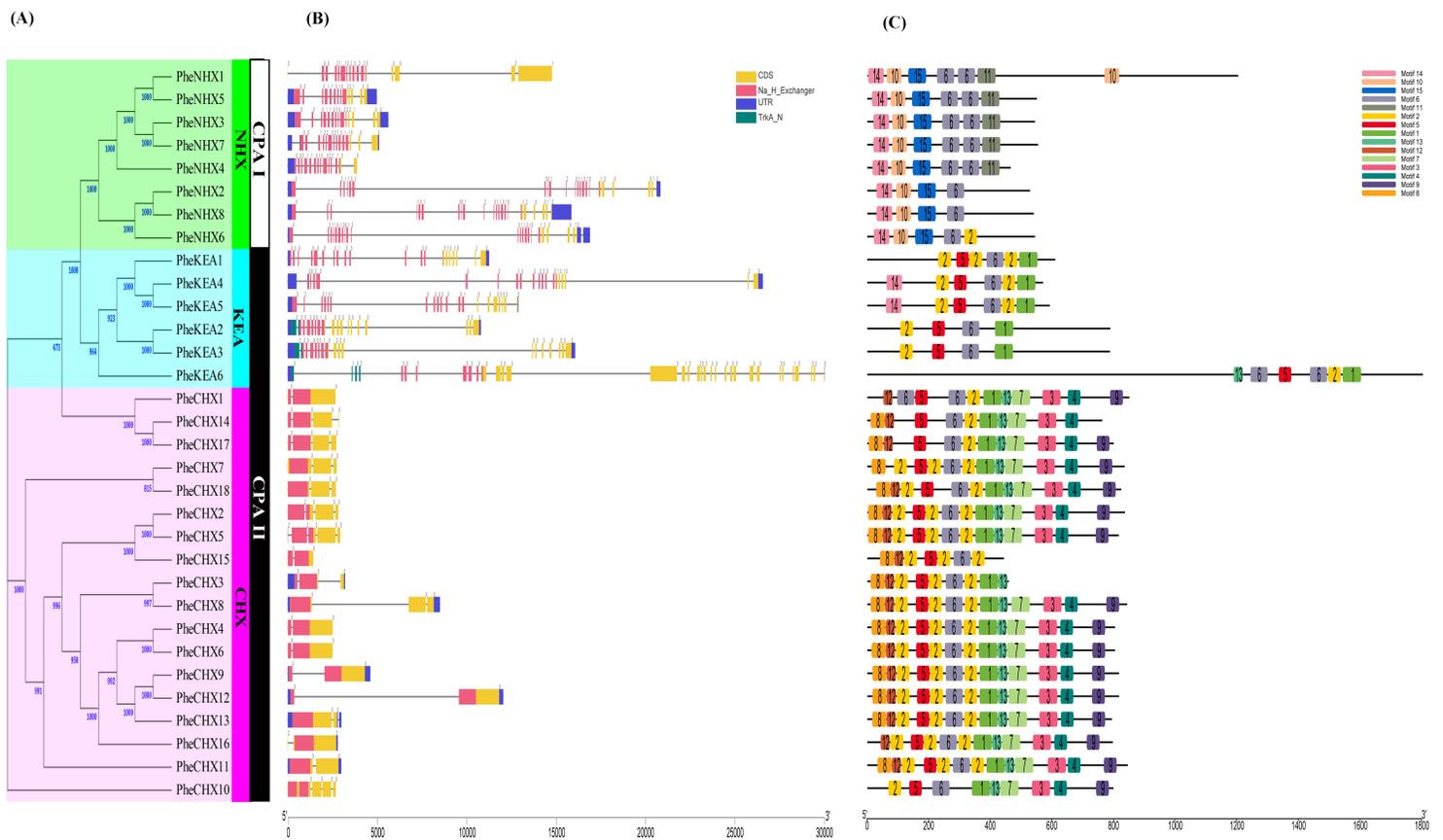


Figure 1

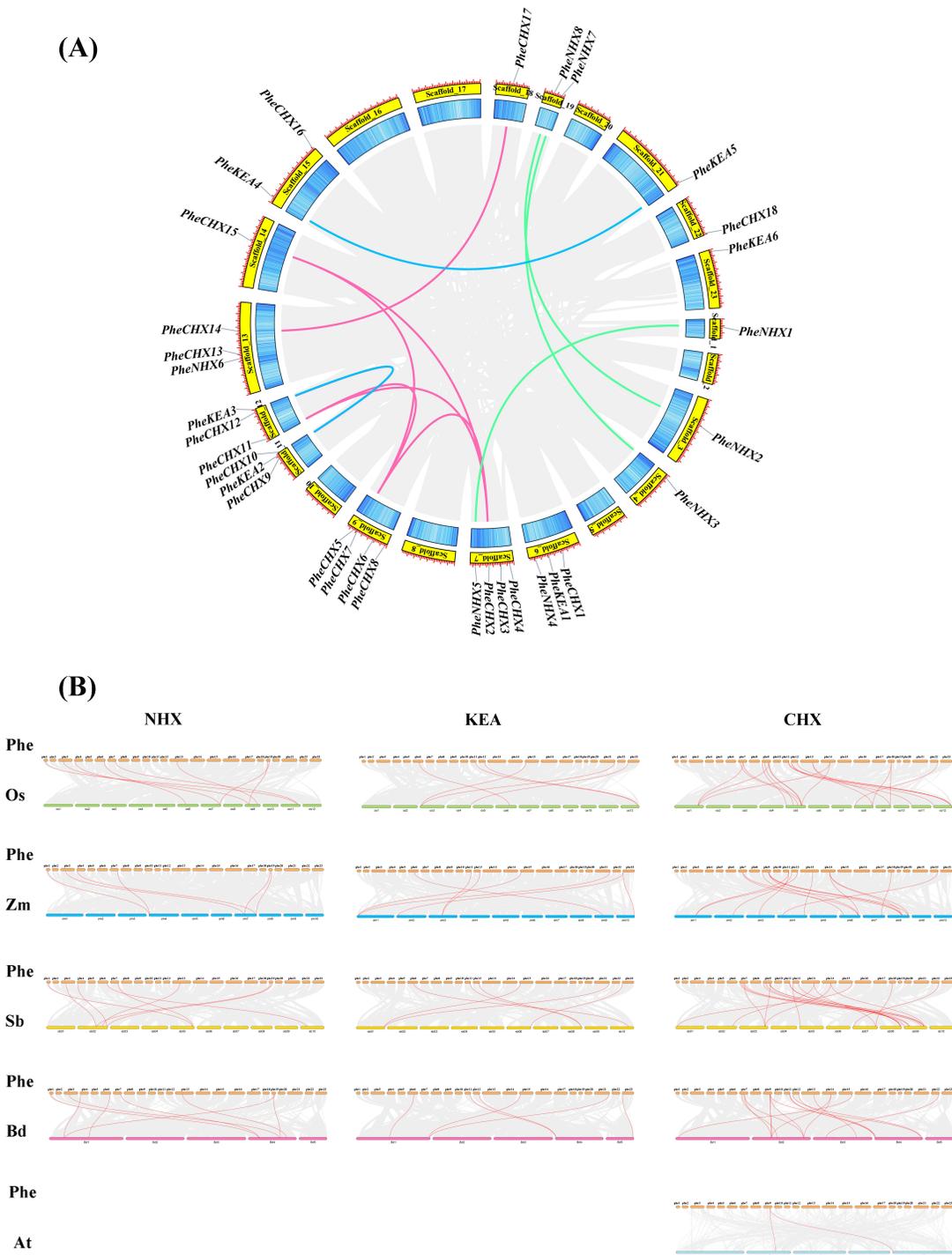
Phylogenetic analysis and statistical analysis of CPA members in different species. (A) Phylogenetic analysis of CPA members from moso bamboo, rice, maize, Brachypodium, sorghum and Arabidopsis. Geometric figures of different colors and shapes are used to mark the CPA members from different species. (B) Statistical analysis of CPA members from moso bamboo, rice, maize, Brachypodium, sorghum and Arabidopsis.



**Figure 2**

The unrooted tree of PheCPA gene family, PheCPAs gene structures and conserved motifs in PheCPA proteins. (A) Phylogenetic tree of PheCPAs constructed by the neighbour-joining method. Bootstrap values from 1,000 replicates are indicated at each node. (B) Exons and introns are indicated by yellow rectangles and grey lines, respectively. Untranslated regions (UTRs) are indicated by blue rectangles. Pink and green rectangles represent exons encoding Na\_H\_Exchange and TrkA\_N respectively. (C) Motifs of the PheCPA proteins were identified using the online MEME program. Different coloured boxes represent different motifs, with their names in the centre of the boxes.





**Figure 4**

Ka, Ks, Ka/Ks value and divergence time of CPA genes in the genomes of moso bamboo, rice, maize sorghum and Brachypodium. (A) Distribution of Ka, Ks, Ka/Ks values and duplication time (MYA) were obtained from paralogous gene pairs in moso bamboo genome. (B) Distribution of Ka, Ks, Ka/Ks values and duplication time (MYA) were obtained from orthologous gene pairs between the moso bamboo and rice, maize, sorghum and Brachypodium genomes.

## GO Distribution Of PheCPAs By Level (2)

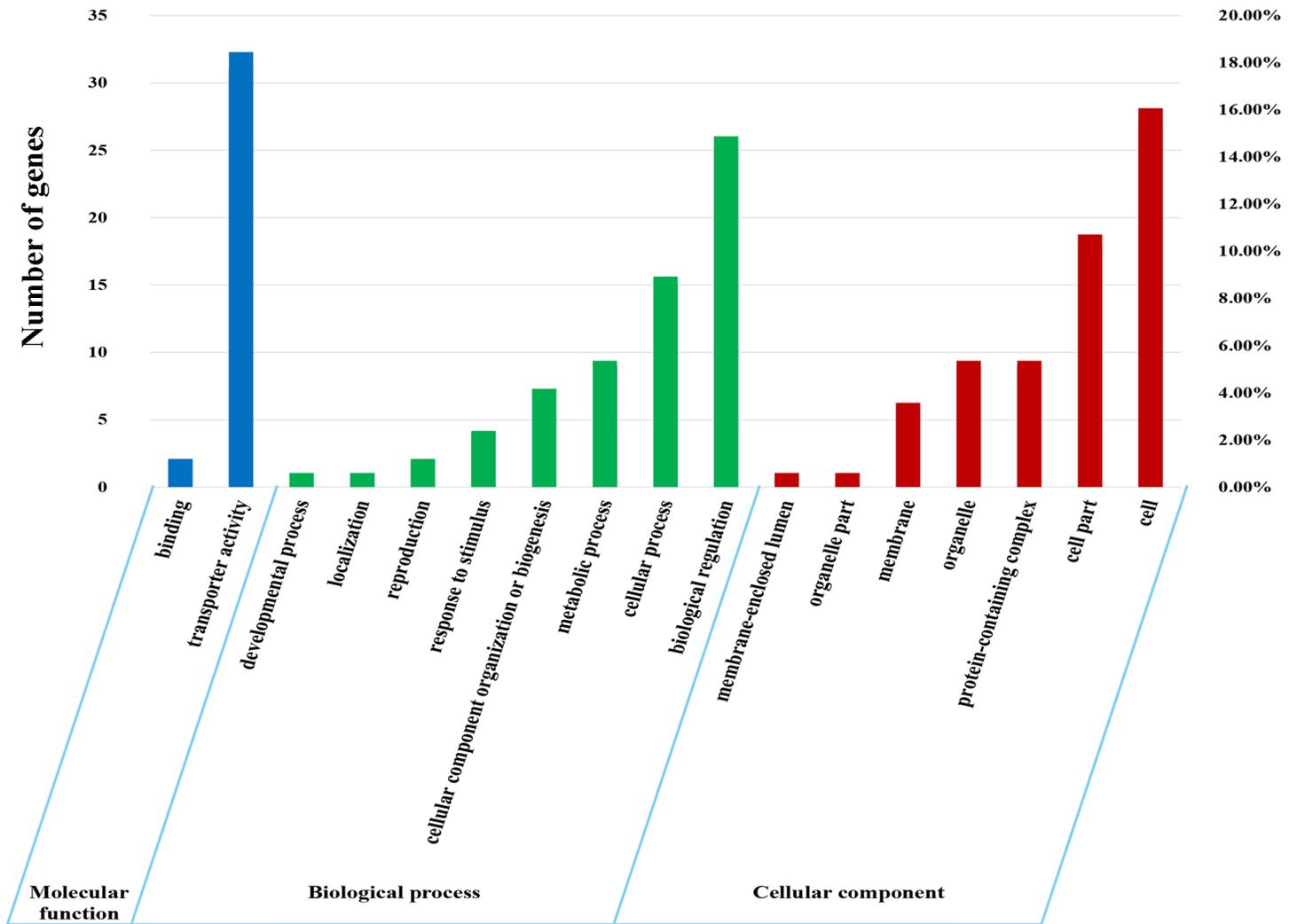
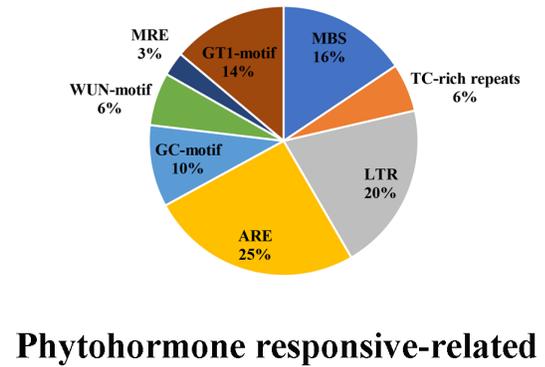
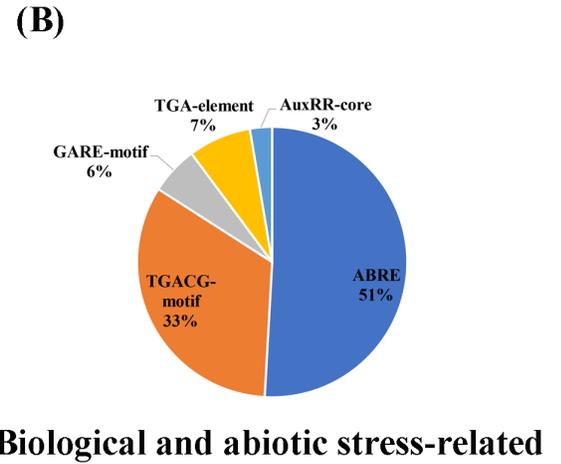
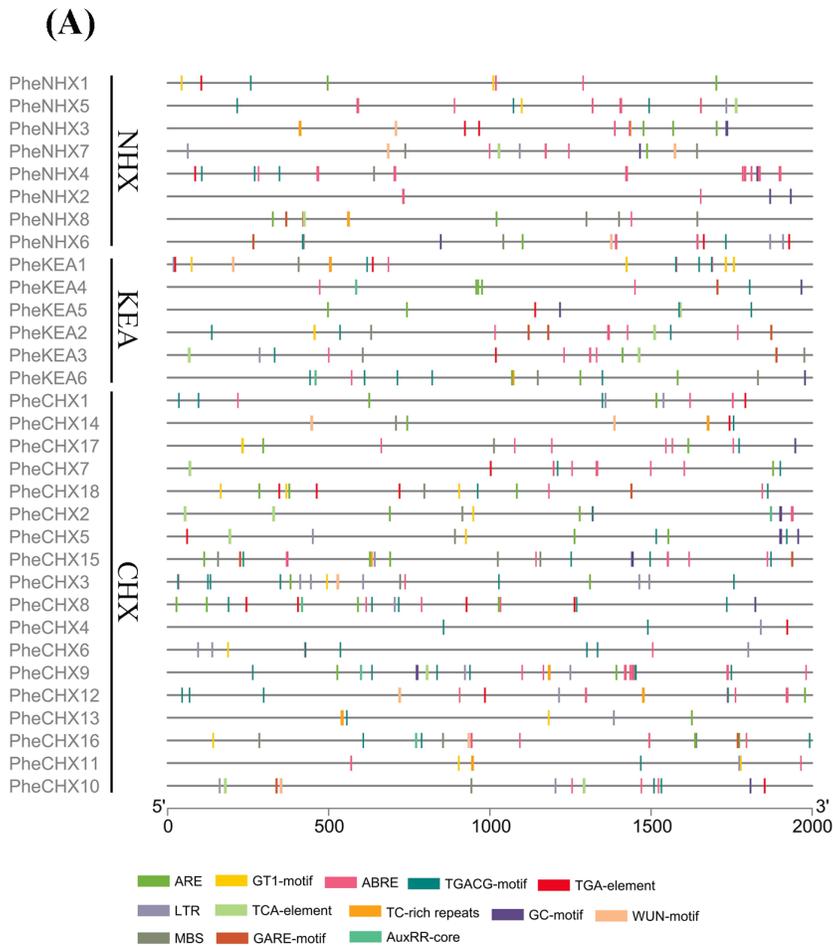


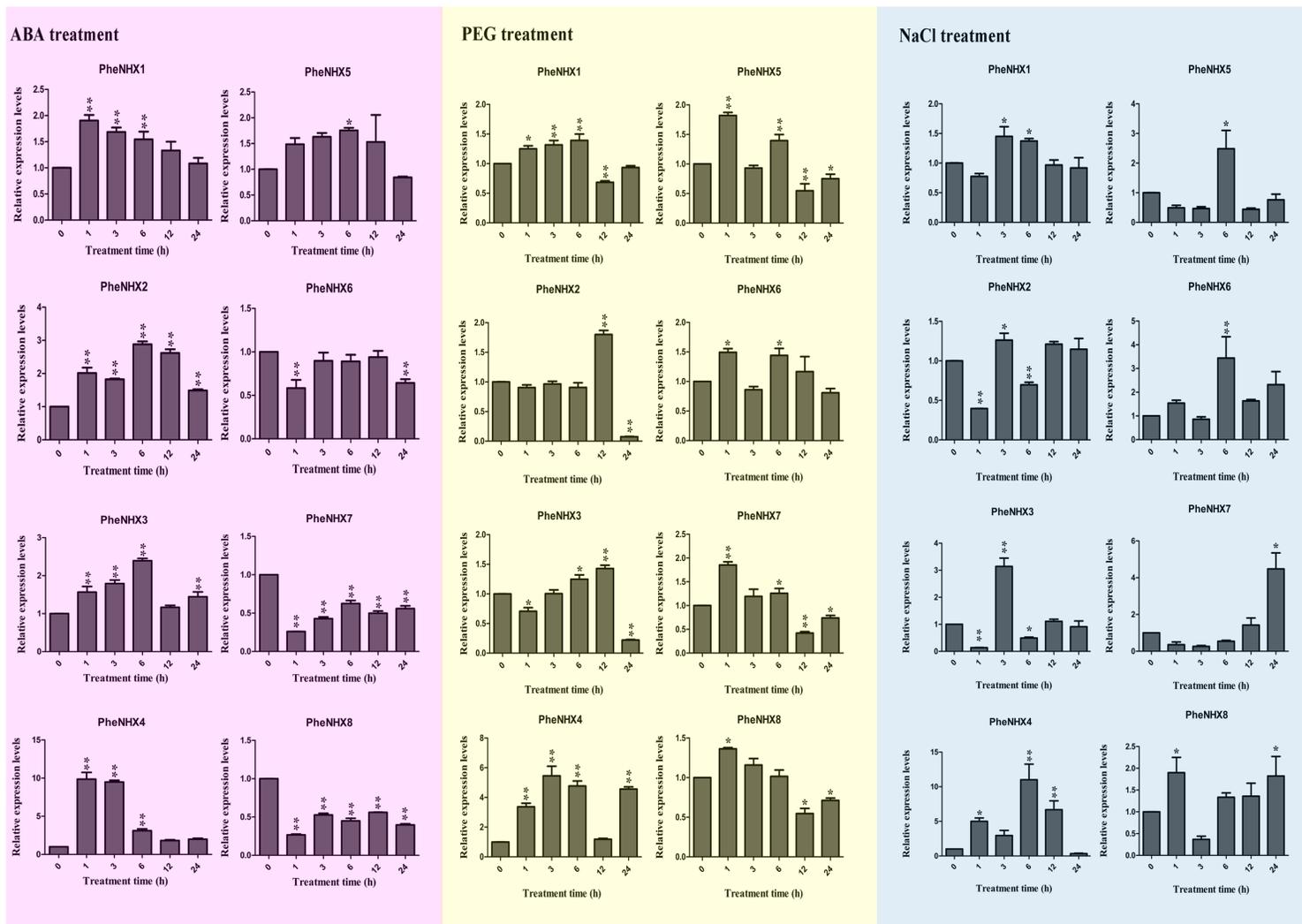
Figure 5

GO annotation of PheCPA transporter proteins. The annotation was performed on three categories: biological process, molecular function and cellular component.



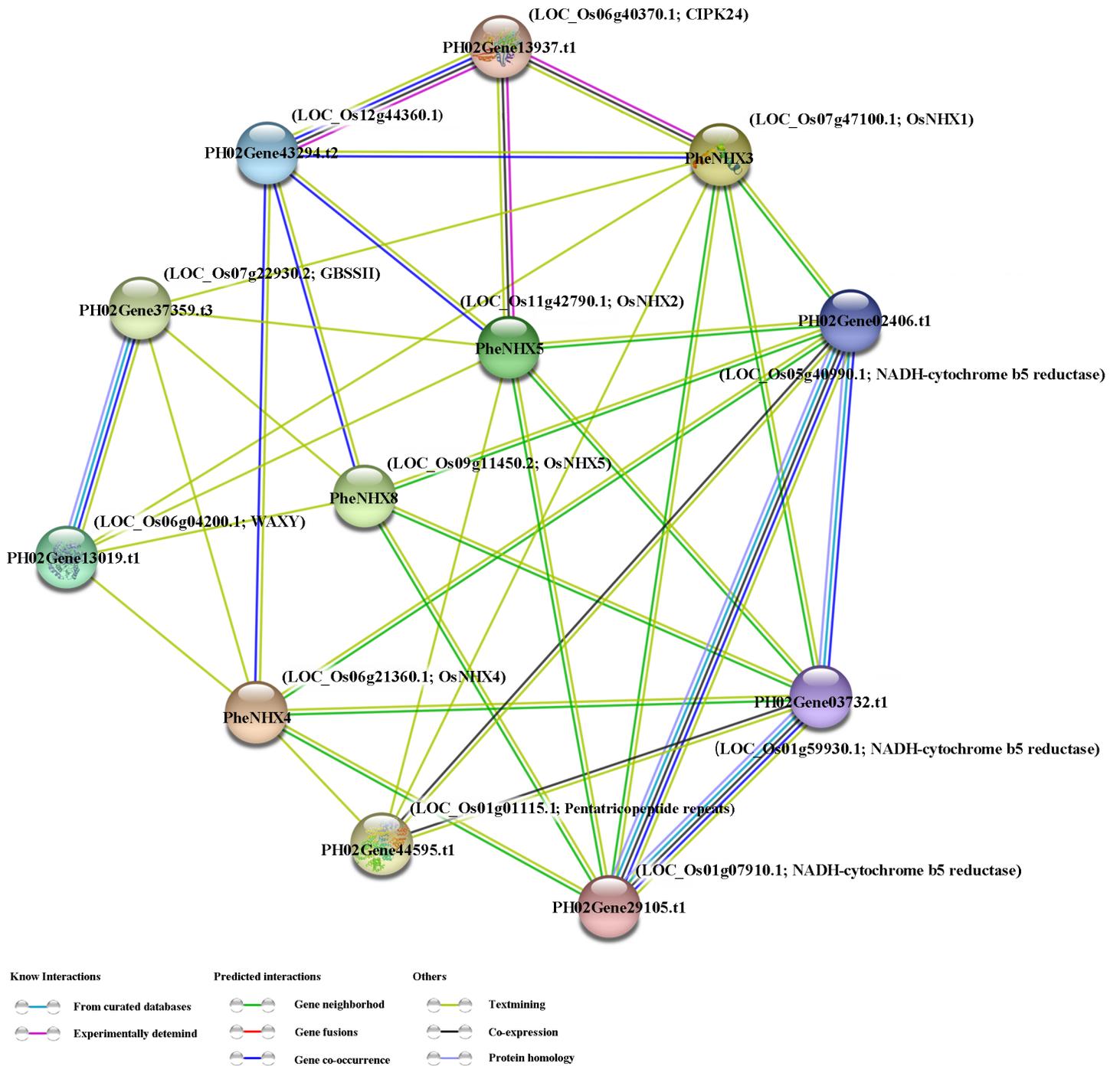
**Figure 6**

Predicted cis-elements in the promoter regions of the maoso bamboo CPA genes. (A) All promoter sequences (-2000 bp) were analyzed. The PheCPA genes are shown on the left side of the figure. Lines of different colors represent different cis-elements. (B) The ratio of each promoter element in biotic and abiotic stress-related and phytohormone responsive-related categories was represented by pie charts.



**Figure 7**

Expression levels of PheCPAs under NaCl (200 mmol/L), PEG-6000 and ABA (100  $\mu$ M) treatments by qRT-PCR. The Y-axis indicates the relative expression levels, and 0, 1, 3, 6, 9, 12, and 24 (X-axis) indicate hours of treatment. Mean values and standard deviations (SDs) were obtained from three biological and three technical replicates.



**Figure 8**

Interaction networks of PheNHXs based on *O. sativa* orthologues in the STRING database. Minimum required interaction score: highest confidence.

## Supplementary Files

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