Transcriptomic profiling identifies candidate genes involved in salt tolerance of the xerophyte Pugionium cornutum

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Abstract

Background: Pugionium cornutum is a xerophytic plant that primarily adapts to salt stress by accumulating inorganic ions (e.g., Cl -) for osmoregulation, improving its reactive oxygen species (ROS)-scavenging ability and maintaining high photosynthetic carbon assimilation efficiency, but the associated molecular mechanisms still remain unclear. Results : Here, we present an analysis of gene responses to salt stress based on the transcriptome of P. cornutum exposed to 50 mM NaCl treatment. The data revealed that, after NaCl treatment for 6 or 24 h, the transcript levels of multiple genes encoding proteins facilitating Cl - accumulation and NO 3 - homeostasis such as SLAH1, CLCg, CCC1, and NPF6.4, as well as the transport of other major inorganic osmoticums were significantly upregulated in roots and shoots, which should be favorable to enhancing osmotic adjustment capacity and maintaining the plant uptake and transport of nutrient elements; a large number of genes related to ROS-scavenging pathways were also significantly upregulated, which should be beneficial for mitigating salt-induced oxidative damage to the cell metabolism. Meanwhile, many genes encoding components of the photosynthetic electron transport and carbon fixation enzymes were significantly upregulated in shoots after salt treatment, possibly resulting in a high carbon assimilation efficiency in P. cornutum . Additionally, numerous salt-inducible transcription factor genes probably regulating the abovementioned processes were found. Conclusion : Candidate genes involved in salt tolerance of P. cornutum were identified, which lays a preliminary foundation for clarifying the molecular mechanism of the xerophytes adapting to harsh environments.

Background

Soil salinity is one of the primary abiotic stresses that limits the sustainable development of agriculture worldwide, and approximately one-fifth of the world's cultivated land has been affected by salinity [1-3]. Salt tolerance in plants is a complex trait involving responses to cellular osmotic and ionic stresses and their consequent secondary stresses (e.g., oxidative stress), which are all polygenic processes [4]. Most conventional and staple crop and forage species are very sensitive to high concentrations of salt in soils and have limited genetic potential for salt tolerance because of their long-term growth under favorable cultivation conditions [5, 6]. The breeding of crops with higher yield and quality under saline conditions by using molecular genetics is an effective strategy to promote food security [3, 7, 8]. Despite abundant molecular knowledge have derived from model plants such as *Arabidopsis thaliana* and *Oryza sativa*, a low capacity for stress tolerance limits their usefulness in current breeding programs [9]. Certain wild plant species, including halophytes and xerophytes, however, have evolved multiple protective mechanisms to successfully thrive in extremely harsh environments and, as a result, harbor prominent abiotic stress tolerance genes [10, 11]. Hence, it is possible to genetically improve salt tolerance in crops and forage through a better understanding of the molecular basis underlying the adaptive strategies to soil salinity employed by these halophytes and xerophytes [6, 12].

Pugionium cornutum, a xerophytic desert plant in the genus *Pugionium* Gaertn belonging to Brassicaceae, is primarily found in arid and semi-arid regions of northwestern China, with strong

adaptability to various environmental stresses including salinity and drought [13, 14]. In local areas, this species plays an important role in sand fixation and soil and water conservation due to its well-developed root systems; moreover, the higher medicinal property and nutrient content make it attractive as a traditional Chinese herb and vegetable [14, 15]. Previous studies have found that *P. cornutum* possesses a strong ability to reduce oxidative damage under salt and drought stresses by increasing the activities of enzymes involved in reactive oxygen species (ROS) scavenging such as superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) [16-18]. It was also demonstrated that *P. cornutum* is a typical Cl^--tolerant species and can tolerate high tissue Cl^- concentrations that were even toxic to some other Cl^--tolerant species; moreover, *P. cornutum* can directly use Cl^- as an efficient and beneficial osmoticum for enhancing its leaf turgor and hydration under salt stress [19, 20]. Meanwhile, a substantially increased accumulation of other major inorganic osmoticums, such as Na^+, SO_4^{2-}, and PO_4^{3-}, as well as relatively stable concentrations of K^+ and NO_3^-, were observed in roots and shoots of *P. cornutum* under NaCl treatments [19, 20]. Therefore, the large absorption of inorganic osmoticums, especially Cl^-, and the maintenance of K^+ and NO_3^- homeostasis are vital physiological mechanisms in the adaptation of *P. cornutum* to salt stress. In addition, the dry biomass of *P. cornutum* did not decrease under 50 mM NaCl stress despite the significantly suppressed leaf net photosynthetic rate [19], indicative of a high carbon assimilation efficiency of *P. cornutum* under salt stress. Although these physiological traits in salt tolerance of *P. cornutum* have been documented, the corresponding molecular foundation remains elusive and should be investigated to identify the prominent genes involved. Furthermore, to date, studies on the molecular mechanisms involved in the ability of higher plants to tolerate Cl^--toxicity continue to lag behind and are mainly focused on the model plant *A. thaliana* and Cl^--sensitive crops such as soybean, citrus and grape [21]. Thus, understanding on the molecular basis in *P. cornutum* coping with Cl^--toxicity would help to further elucidate Cl^- tolerance in higher plants.

RNA sequencing (RNA-Seq) is a rapid and cost-effective method for both mapping and quantifying the transcriptome in eukaryotes and is widely used to analyze the gene expression in plants under specific developmental stages or physiological conditions, providing substantial insights into the molecular processes involved in plants' response to abiotic stresses [9, 22]. In the present work, we generated a transcriptomic dataset for studying the molecular mechanisms of salt tolerance in *P. cornutum* using Illumina high-throughput sequencing technology. Then, we analyzed the differentially expressed functional genes related to ROS scavenging, ion (especially Cl^-) transport and photosynthesis, as well as transcription factor genes under 50 mM NaCl treatment by using a tag-based digital gene expression (DGE) profiling technique.

Results

Transcriptome sequencing, *de novo* assembly and functional annotation

Transcriptome sequencing generated more than 119 and 118 million raw reads from the roots and shoots of *P. cornutum*, respectively (Additional file 1: Table S1). After exclusion of the low-quality reads, including

empty reads, adapter reads and reads with unknown nucleotide “N” or only one copy number, 117 and 116 million clean reads with GC percentage of 45% and 46% were obtained from the roots and shoots, respectively (Additional file 1: Table S1). The total clean reads/total raw reads percentages were more than 98% for both roots and shoots. Only a small number of reads was excluded by filtering, indicating that the data were highly reliable.

The *de novo* assembly of total clean reads generated 64,978 unigenes from shoots and 80,307 unigenes from roots, with an average mean length of 1091 and 997 bp, respectively (Table 1). After further assembly and redundancy elimination of these unigenes using the CAP Assembler, 72,068 gap-free unigenes with an average length of 1243 bp were obtained (Table 1). As shown in Additional file 1: Fig. S1, the length of 34,108 unigenes (more than 50%) was greater than 1000 bp.

In total, 63,396 unigenes were matched to known homologs from other plant species by being aligned against the protein databases including non-redundant protein (NR) database, non-redundant nucleotide (NT) database, Swiss-Prot, Kyoto Encyclopedia of Genes and Genomes (KEGG), Clusters of Orthologous Groups (COG), Gene Ontology (GO), accounting for 88% of the total unigenes (Additional file 1: Table S2). Specifically, 57,695, 60,628 39,893, 35,136, 24,608 and 53,435 unigenes had functional annotations in the above six databases, which accounted for 80%, 84%, 55%, 49%, 34% and 74% of the total unigenes, respectively (Additional file 1: Table S2).

Identification of DEGs in the roots and shoots of *P. cornutum* treated with 50 mM NaCl

The differentially expressed genes (DEGs) in roots and shoots were then identified. 3577 DEGs in roots and 3315 DEGs in shoots were upregulated exclusively after salt treatment for 6 h (Fig. 1). After salt treatment for 24 h, other 3621 and 1614 upregulated DEGs in roots and shoots were observed, respectively (Fig. 1). It was noteworthy that 443 DEGs in roots and 358 DEGs in shoots were upregulated after salt treatment for both 6 and 24 h. As for the downregulated DEGs, 2625 DEGs in roots and 2719 DEGs in shoots were observed exclusively after salt treatment for 6 h, and 7873 DEGs in roots and 2237 DEGs in shoots were observed exclusively after salt treatment for 24 h (Fig. 1); meanwhile, there were 711 and 696 downregulated DEGs in roots and shoots after salt treatment for both 6 and 24 h, respectively (Fig. 1).

DEGs related to ion transport

The absorption and accumulation of inorganic osmoticums such as Cl^-, Na^+, SO_4^{2-} and PO_4^{3-}, as well as maintaining homeostasis of the inorganic macronutrients K^+ and NO_3^-, are primary physiological mechanisms in the salt tolerance of *P. cornutum* [19, 20]. Therefore, we firstly analyzed the influences of

NaCl treatment on the transcript level of **DEGs related to** ion transport in the roots and shoots of *P. cornutum*.

After NaCl treatment for 6 and 24 h, 49 and 41 DEGs associated with Cl^-, NO_3^-, Na^+, K^+, Ca^{2+}, SO_4^{2-}, PO_4^{3-}, Zn^{2+}, Cu^{2+}, NH_4^+, Mn^{2+}, Mg^{2+}, Fe^{2+}, BO_3^{3-} and H^+ transport were respectively upregulated in roots, and 18 and 31 DEGs were respectively downregulated in roots (Fig. 2A and B). Among the upregulated DEGs, a large proportion was Cl^- and/or NO_3^- transporter-encoding genes, including slow-type anion channel-associated homolog 1 gene (*SLAH1*), chloride channel genes (*CLCs*) and nitrate transporter 1/peptide transporter genes (*NPFs*). It was noteworthy that the transcript levels of *SLAH1*, *CLCg*, *NPF6.4* were upregulated in roots after NaCl treatment for both 6 and 24 h (Table 2, Additional file 1: Table S3 and S4), indicating that these genes might be closely involved in Cl^- absorption and accumulation, as well as NO_3^- homeostasis of *P. cornutum* under saline conditions. Furthermore, it was observed that *SLAH1* was completely unexpressed in roots under the control condition (the RPKM value of *SLAH1* was 0.01 under the control condition), while its expression level was increased by over 8-fold after salt treatment for both 6 and 24 h (Additional file 1: Table S3 and S4), indicative of a vital role of *SLAH1* in salt response of *P. cornutum*. Among the downregulated DEGs related to Cl^- and/or NO_3^- transport, the transcript abundance of cation-chloride co-transporter 1 gene (*CCC1*), which encodes a Cl^- transporter facilitating the retrieval of Cl^- from xylem sap into roots [23], was substantially decreased in roots under salt treatment for 24 h (Additional file 1: Table S4). Na^+/H^+ antiporters (NHXs) are widely recognized to play important roles in Na^+ and/or K^+ homeostasis in plants under saline conditions [24-26]. Our results showed that the transcript abundance of plasma membrane-located Na^+/H^+ antiporters-encoding gene *SOS1* (also named *NHX7*) in roots was upregulated after salt treatment for 6 h but downregulated after salt treatment for 24 h (Fig. 2A and B, Additional file 1: Table S3 and S4), indicating that *SOS1* in roots might regulate salt tolerance in *P. cornutum* during relatively short-term salt treatment. The transcript abundance of the tonoplast-located Na^+/H^+ antiporters-encoding gene *NHX1* was upregulated in roots after 24 h of salt treatment, and the golgi-located Na^+/H^+ antiporters-encoding genes *NHX5* and *NHX6* were upregulated after both 6 and 24 h of salt treatment (Fig. 2A and B, Additional file 1: Table S3 and S4); therefore, these three genes very likely confer Na^+/K^+ homeostasis in roots of *P. cornutum* under saline conditions. Furthermore, several K^+ transporter-encoding genes that also contribute to Na^+/K^+ homeostasis, including members of K^+ transport gene family (e.g. *KT2*, *KT3*, *KUP2*, *HAK3*, *KEA1*, *KEA4*, *KEA5* and *KEA6*), were upregulated under salt treatment for either 6 or 24 h (Fig. 2A and B, Additional file 1: Table S3 and S4). The cyclic nucleotide-gated channels (CNGCs) are important plasma membrane-located cation channels mainly permeable to K^+, Na^+ and Ca^{2+}; the glutamate receptors (GLRs) as glutamate receptors can provide Glutamate to activate plasma membrane-located nonspecific cation channels in root cells and, therefore, trigger large and rapid rises in the concentrations of cytosolic cations, especially Ca^{2+} [27-29]. Our results also characterized many upregulated *CNGC* and *GLR* genes in roots of *P. cornutum* under salt treatment (Fig. 2A and B), indicating that *P. cornutum* is also likely to maintain root cationic homeostasis under saline conditions by increasing the expression of these *CNGC* and *GLR* genes in roots to control K^+,

Na^+ and Ca^{2+} transport across the cell membrane. Many upregulated DEGs related to the transport of other inorganic ions, including SO_4^{2-} (e.g., *Sultr1.2*, *Sultr2.1*, *Sultr3.1*), PO_4^{3-} (e.g., *PhT1.3*, *PhT2.1*, *PhT4.3*, *PHO1*), Zn^{2+} (e.g., *ZnT1*, *ZnT8*, *ZnT12*), Cu^{2+} (e.g., *CTR2*, *CTR5*, *CCH*, *PAA1*), Mn^{2+} (e.g., *PDR2*), Fe^{2+} (e.g., *IRT1*), Mg^{2+} (e.g., *MgT*), NH_4^+ (e.g., *AMT1.1*, *AMT1.2*, *AMT2*), and BO_3^{3-} (e.g., *BOR3*, *BOR4*), were also identified in roots of *P. cornutum* under either 6 or 24 h salt treatment (Fig. 2A and B, Additional file 1: Table S3 and S4). Additionally, as the transport of inorganic ions across cell membranes generally couples H^+ as a proton motive force [30], the upregulated plasma membrane H+ ATPase gene (*P-H^+ ATPase*) and vacuolar H+ ATPase gene (*V-H^+ ATPase*) in roots under salt treatment identified by our data (Fig. 2A and B) likely play essential roles in ion transport across the root cell plasma membrane and tonoplast, in addition to maintaining the cellular membrane electrochemical gradient.

After salt treatment for 6 and 24 h, 46 and 30 DEGs in shoots associated with inorganic ion transport were respectively upregulated, and 23 and 26 DEGs in shoots were respectively downregulated (Fig. 2C and D). In contrast to the observation in roots, the transcript level of *CCC1* in shoots was considerably upregulated under both 6 and 24 h of salt treatment (Fig. 2C and D, Additional file 1: Table S5 and S6), which might be beneficial for the exclusion of more Cl^- from shoot vascular tissues to facilitate Cl^- uptake by mesophyll cells. Cl^- can only be largely accumulated in plant shoots by compartmentalization into vacuoles of mesophyll cells [31, 32]. This process in higher plants is dominated by the tonoplast-located chloride channel CLCg [33]. Our results showed that the transcript abundance of *CLCg* in shoots was upregulated under salt treatment for both 6 and 24 h (Table 3). Hence, *CLCg* should play a key role in the Cl^--accumulating characteristic of *P. cornutum* under saline conditions. In addition to *CLCg*, *CLCb* that encodes tonoplast NO_3^- transporter [34], and several *NPFs* such as *NPF6.3* and *NPF6.4* that encode NO_3^- and/or Cl^- transporters [35], were also upregulated under salt treatment (Additional file 1: Table S5 and S6), suggesting that these genes might also be essential for Cl^- accumulation and NO_3^- homeostasis in shoots of *P. cornutum* under saline conditions. Certain DEGs found in roots encoding Na^+/H^+ antiporters such as *SOS1* and *NHX1*; K^+ transporters such as *KT2*, *KUP2*, *KEA1*, *KEA4*, *KEA6*; cation channels such as *CNGC1* and *CNGC6*; and glutamate receptors such as *GLR3.3*, were upregulated in shoots as well after salt treatment for either 6 or 24 h (Additional file 1: Table S5 and S6), indicating that these genes might also regulate Na^+/K^+ homeostasis in shoots of *P. cornutum* under saline conditions. Furthermore, several DEGs related to Na^+ or K^+ transport, including the high-affinity K+ transporter gene (*HKT1*), stelar K+ outward rectifying channel gene (*SKOR*) and guard cell outward-rectifying K^+ channel gene (*GORK*), were only found in shoots but not in roots after salt treatment (Fig. 2). HKT1-type proteins facilitate Na^+ absorption and transport in the roots of model plants *A. thaliana* [36, 37]. But, in *P. cornutum*, *HKT1* is likely to be distinctively responsive to salt stress in shoots. As an outward rectifying K^+ channel, GORK has been proven to participate in stomatal movement [38], therefore, the observed upregulation of *GORK* in shoots after salt treatment (Fig. 2D) are likely essential for stomatal movement and, consequently, affect salt tolerance in *P. cornutum*. Many DEGs related to the transport of other inorganic nutrients or osmoticums, such as SO_4^{2-}, Zn^{2+}, Cu^{2+}, Mn^{2+}, Fe^{2+}, Mg^{2+}, and NH_4^+, were identified in shoots after salt

treatment for either 6 or 24 h (Fig. 2C and D), indicating that these genes might also regulate shoot osmotic adjustment of *P. cornutum* under saline conditions. Among the DEGs encoding V-H^+ ATPase, P-H^+ ATPase and plasma membrane Ca2+ ATPase (P-Ca^{2+} ATPase) in shoots, *V-H^+ ATPase c2*, *V-H^+ ATPase e1* and *V-H^+ ATPase h* were not expressed under the control condition but were highly expressed under salt treatment (Additional file 1: Table S5 and S6), suggesting that these three genes might play vital roles in vacuole compartmentation of inorganic osmoticums for facilitating shoot osmotic adjustment.

DEGs related to ROS-scavenging system

The ROS-scavenging system in higher plants mainly consists of the ascorbate-glutathione (AsA-GSH) cycle, glutathione peroxidase (GPX) pathway, catalase (CAT) pathway and peroxiredoxin/thioredoxin (PrxR/Trx) pathway [9]. After salt treatment for 6 and 24 h, a total of 19 and 15 DEGs categorized in the abovementioned ROS-scavenging pathways in roots of *P. cornutum* were respectively upregulated and 5 and 29 DEGs were respectively downregulated (Fig. 3A and B). Among the upregulated DEGs in roots after 6 h of salt treatment, most were glutathione S-transferase genes (*GSTs*) that are involved in both the ASA-GSH and GPX pathways and thioredoxin genes (*Trxs*) that are involved in the PrxR/Trx pathway (Fig. 3A). Hence, the ASA-GSH, GPX and PrxR/Trx pathways might be the major components of the root ROS-scavenging system of *P. cornutum* in adaption to salinity. After 24 h of salt treatment, although many DEGs associated with ROS scavenging were downregulated, the transcript abundance of *GST-U10* (no expression under the control condition) and *TrxH8* was still obviously upregulated (Fig. 3B, Additional file 1: Table S8), indicating that these two genes might play vital roles in ROS scavenging in roots of *P. cornutum* under saline conditions. Furthermore, after salt treatment for 24 h, 2 upregulated *PODs*, 1 upregulated *CAT* and 1 upregulated *SOD* emerged (Fig. 3B). These genes probably facilitate root ROS scavenging of *P. cornutum* during relatively long-term salt treatment.

Following 6 h of NaCl treatment, the expression levels of 3 *GST* genes and 2 *GPX* genes in the GPX pathway were upregulated in *P. cornutum* shoots (Fig. 3C). In particular, *GST-F9* showed no expression under the control condition, but high expression detected in the shoots after 6 h of NaCl treatment (Additional file 1: Table S9). Despite the finding that fewer *POD* genes were upregulated than downregulated, one *POD* (*POD43*) was not expressed under the control condition, whereas its expression was substantially induced in the shoots after 6 h of NaCl treatment (Fig. 3C, Additional file 1: Table S9). Almost all the differentially expressed peroxisomal biogenesis factor genes (*PEXs*), *Trxs* and peroxiredoxin genes (*PrxRs*) in shoots after 6 h of salt treatment were upregulated (including 1 *PEX* and 2 *Trx*s showing no expression under the control condition but high expression under salt treatment) (Fig. 3C, Additional file 1: Table S9), indicating that the CAT and PrxR/Trx pathways should mainly function in the shoot ROS-scavenging system of *P. cornutum* under salt treatment. Following 24 h of salt treatment, only a few genes associated with ROS scavenging were differentially expressed in shoots, and most of them were downregulated (Fig. 3D). However, *PEX3-1a* showed no expression under the control condition

but upregulated expression under both 6 and 24 h of salt treatment (Additional file 1: Table S9 and S10), suggesting that *PEX3-1a* might play an essential role in ROS scavenging in shoots of *P. cornutum* under saline conditions.

DEGs related to photosynthesis

The maintenance of high carbon assimilation efficiency is an important strategy by which *P. cornutum* adapts to soil salinity [19]. The oxygenic photosynthesis of higher plants consists of photosynthetic electron transport with many components such as chlorophyll, photosystem II-light harvesting complex (PS II), photosystem I-light harvesting complex (PS I), cytochrome b_6f complex, ferredoxin, ATP synthase, and carbon fixation using various enzymes [39]. Thus, we analyzed the expression of DEGs related to the abovementioned processes in *P. cornutum* treated with 50 mM NaCl.

Under NaCl treatment for 6 h, the majority of DEGs related to the components of PS II complex and chlorophyll biosynthesis and all of the DEGs related to the components of PS I complex, cytochrome b_6f complex and ATP synthase in shoots of *P. cornutum* were upregulated (Fig. 4A), suggesting that the expression of these genes in shoots might be essential for light energy absorption and photosynthetic electron transport to generate more ATP for carbon fixation. Furthermore, it was observed that nearly 20 DEGs encoding various enzymes (mainly malate dehydrogenase and phosphoglycerate kinase) in carbon fixation process were upregulated (Fig. 4A, Additional file 1: Table S11), indicative of a vital role of these genes in maintaining high carbon assimilation efficiency in *P. cornutum* under salt stress.

Under NaCl treatment for 24 h, the numbers of upregulated DEGs in shoots related to the components of PS II complex, PS I complex, chlorophyll biosynthesis and ATP synthase were much lower than that under NaCl treatment for 6 h, but the expression of one cytochrome f-encoding gene, one ferredoxin-dependent glutamate synthase-encoding gene and one uroporphyrinogen III methyltransferase-encoding gene was still upregulated (Fig. 4B, Additional file 1: Table S12). These three genes might play a crucial role in light energy absorption and photosynthetic electron transport in *P. cornutum* under saline conditions. Furthermore, the majority of the upregulated DEGs involved in carbon fixation after 6 h of salt treatment were also upregulated after 24 h of salt treatment (Fig. 4, Additional file 1: Table S11 and S12).

DEGs related to transcription factors

Along with functional genes, regulatory genes also participate in the responses of plants to environmental stresses by regulating signal transduction or functional gene expression [40]. Transcription factors (TFs) are important and abundant regulatory genes in higher plants. The major classes of TFs include NAC (NAM/ATAF/CUC), AP2/ERF (APETALA2 and ethylene-responsive element binding proteins), bHLH (basic helix-loop-helix), MYB (myeloblastosis), WRKY (WRKY-domain), bZIP/HD-ZIP (basic region-

leucine zipper/homeodomain-leucine zipper), ZF (zinc finger) and HSP (heat shock protein), some of which have been validated to confer salt and drought tolerance in various plant species by transcriptional regulation of downstream target stress-responsive genes [9]. As *TFs* generally show rapid responses to abiotic stresses, we only analyzed the differentially expressed TF genes in roots and shoots after 6 h of salt treatment (the number of the differentially expressed TF genes after salt treatment for 24 h was much lower than that after salt treatment for 6 h in our results; data not shown).

After 6 h of NaCl treatment, more than 100 TF genes were differentially expressed in roots; approximately two-thirds were upregulated, among which the majority were *WRKYs*, *MYBs*, *ZFs*, or *bZIPs*/ *HD-ZIPs,* including *WRKY33*, *WRKY54*, and *MYB3*, CCCH-type *ZFs* and *bZIP24* (Fig. 5A, Additional file 1: Table S13), which have been validated to play important roles in salt tolerance and drought resistance in other plants [41-45]. Although less research have shown that *MADS-box* genes are closely related to the responses of plants to salinity, all 5 differentially expressed *MADS-box* genes in roots after salt treatment were considerably upregulated, with 3 members (*AGL16*, *AGL27* and *AGL29*) showing no expression under the control condition but high expression under salt treatment (Fig. 5A, Additional file 1: Table S13). Up to 6 upregulated AP2/ERF-encoding genes were also found in roots. Only a few NAC, bHLH and HSP-encoding genes were differentially expressed after salt treatment (Fig. 5A).

After 6 h of NaCl treatment, nearly 120 TF genes were differentially expressed in shoots (Fig. 5B). The number of upregulated WRKY and AP2/ERF-encoding genes in shoots was much lower than that in roots (Fig. 5), suggesting that *WRKYs* and *AP2/ERFs* might mainly regulate stress-responsible functional genes in roots to confer salt tolerance in *P. cornutum*. Similarly, the upregulated MADS-box-encoding genes in shoots were less abundant than in roots after salt treatment, but the expression of *AGL30* was upregulated more than 5-fold in both shoots and roots (Fig. 5, Additional file 1: Table S13 and S14), suggesting that *AGL30* is distinctively implicated in salt tolerance in *P. cornutum*. Many upregulated *MYBs* and *ZFs* respectively including 6 and 7 members that showed no expression under the control condition were also observed in shoots after salt treatment (Fig. 5B, Additional file 1: Table S14). Notably, the upregulated HSP-encoding genes were only found in shoots but not in roots (Fig. 5), suggesting that *HSPs* might mainly regulate stress-responsible functional genes in shoots of *P. cornutum* under saline conditions.

The validation of RNA sequencing results

The transcript abundance of 40 randomly selected DEGs (20 from roots and other 20 from shoots) was determined by using the Quantitative Real-time PCR (qRT-PCR) method to validate the RNA sequencing (RNA-seq) data. As shown in Additional file 1: Table. S15 and S16, the fold changes of the selected DEGs under salt treatment measured by qRT-PCR were highly consistent with the results obtained from RNA-seq data. Moreover, the correlation coefficient R^2 of the DEGs from roots between qRT-PCR and RNA-seq results under salt treatment for 6 and 24 h was 0.93 and 0.92 respectively (Fig. 6A and B); the correlation

coefficient R^2 of the DEGs from shoots between qRT-PCR and RNA-seq results under salt treatment for 6 and 24 h was 0.86 and 0.91 respectively (Fig. 6C and D), indicating that the RNA-Seq data in the present study were highly reliable.

Discussion

Ion transport plays a vital role in the response to salt stress in *P. cornutum*

Chloride (Cl^-) is the most common and abundant anionic salt in saline soils [1]. For most crops, especially legumes and perennial woody species, Cl^- toxicity is a major cause of yield reduction under saline conditions [21, 46]. However, it has been proven that the large absorption of Cl^- from external surroundings and translocation of Cl^- from roots into shoots for osmotic adjustment are important physiological mechanisms in salt tolerance of the xerophyte *P. cornutum,* which can adapt well to halomorphic arid soils [19, 20]. Therefore, *P. cornutum* must have evolved multiple molecular mechanisms of Cl^- transport to survive in these harsh conditions.

Until recently, the molecular mechanisms underpinning Cl^- transport in higher plants were poorly defined and the molecular identities of certain basic Cl^- transport processes remained elusive [21]. As a micronutrient, Cl^- is primarily absorbed at the epidermis cells of root hairs via several transporters and channels [31]. Decades ago, active Cl^- transporters such as $Cl^-/2H^+$ symporters and passive Cl^- influx/efflux channels at the plasma membrane of root epidermis were identified using electrophysiological approaches [47, 48], whereas to date, the corresponding candidate genes are still unknown. A previous work on *Zea mays* proposed a hypothesis that ZmNPF6.4 is likely a component of the plant root Cl^- uptake system, as ZmNPF6.4 is a plasma membrane-localized, proton-coupled, chloride-selective transporter in root epidermis cells [49]. However, the transcriptional responses of *NPF6.4* in plants to salt stress has not been investigated. In the present study, two transcripts of *NPF6.4*, *NPF6.4a* (CL7387.Contig1_All) and *NPF6.4b* (CL7387.Contig2_All), were found in *P. cornutum*, and the transcript abundance of *NPF6.4a* in roots was continuously induced by NaCl treatment for both 6 and 24 h (Table2, Additional file 1: Table S3 and S4), indicating that NPF6.4a might be a candidate protein that facilitates the uptake of Cl^- by roots in *P. cornutum* under saline conditions.

The overaccumulation of Cl^- in shoots generally triggers Cl^- toxicity in photosynthetic organs and ultimately restricts plant growth [1, 50]. The key rate-limiting gatekeeper step modulating Cl^- accumulation in the shoots has been shown to be the loading of Cl^- from the root stelar symplast into the xylem apoplast [21]. SLAH1 currently is the only known protein certainly mediating Cl^- loading into xylem under saline conditions [51, 52]. In the Cl^--sensitive plant *A. thaliana*, the expression of *AtSLAH1* in roots is substantially downregulated under salt stress [52], indicating that Cl^--sensitive species mainly avoid Cl^- overaccumulation by repressing the expression of *SLAH1* in roots. Conversely, in the roots of *P. cornutum*, *SLAH1* showed no expression under the normal condition but was highly expressed under salt treatment for both 6 and 24 h (Table 2, Additional file 1: Table S3 and S4), indicating that *P. cornutum* likely

accelerates its Cl^--accumulating characteristic by increasing the expression of *SLAH1* in roots. Therefore, the function of *SLAH1* in regulating salt tolerance between salt-sensitive and -tolerant plant species should be distinctly different. In *O. sativa*, the expression of *OsCCC1*, a gene involved in the Cl^- retrieval from xylem sap, was increased in roots under salt stresses, which contributes to reducing the Cl^- accumulation in shoots [23]. However, our results showed that the transcript abundance of *CCC1* in roots of *P. cornutum* was downregulated under salt treatment (Fig. 2B), which would be beneficial to decrease the amount of Cl^- withheld in roots and, ultimately, translocate more Cl^- into shoots. Therefore, *CCC1* might also be an important component in regulating Cl^- accumulation of *P. cornutum* under saline conditions.

AtCLCg is a unique protein mediating Cl^- compartmentalization into vacuoles of mesophyll cells in Arabidopsis [33]. In the typical Cl^--sensitive plant *Glycine max*, *GmCLC1*, a homolog of *AtCLCg*, is preferentially expressed at the tonoplast of root cells, and thus the main function of its encoded protein was withholding Cl^- in roots to decrease Cl^- accumulation in shoots [53]. In the present study, the transcript abundance of *P. cornutum CLCg* in shoots was induced under salt treatment at both 6 and 24 h (Table 3). Considering that *P. cornutum* is a typical Cl^--accumulating plant, the expression of *CLCg* in shoots under salt treatment might be essential for accumulating Cl^- in the vacuoles of the succulent shoot tissue to enhance osmotic adjustment capacity. Therefore, there should be a distinctly different responsive model of *CLCg* to salt stress between Cl^--sensitive and Cl^--tolerant species.

The uptake and storage of NO_3^- in plants under salt conditions is generally antagonized by the uptake of Cl^-, due to the competition between Cl^- and NO_3^- for the major binding sites of transmembrane channels or transporters [21, 48]. Our previous studies have shown that the capacity to maintain NO_3^- homeostasis in shoots is also a principal trait in the Cl^--accumulating capability of *P. cornutum* under saline conditions [19, 20]. The nitrate transporter NPF6.3 (also known as CHL1 or NRT1.1) has been identified as a key protein that mediate NO_3^- uptake by plant roots [54]. Our results obtained two transcripts for *NPF6.3*, *NPF6.3a* (CL8124.Contig1_All) and *NPF6.3b* (CL8124.Contig3_All) in *P. cornutum*, and the former was upregulated after 24 h of salt treatment, the latter was upregulated after 6 h of salt treatment in roots (Additional file 1: Table S3 and S4). These two NPF6.3 proteins might play an essential role in salt tolerance in *P. cornutum* by mediating NO_3^- uptake in roots. NPF7.3 (also known as NRT1.5) was proved to participate in the root-to-shoot transport of NO_3^- in plants [55]. In *A. thaliana*, the expression of *AtNPF7.3* in roots is downregulated in response to NaCl treatment [56]. However, the expression of *NPF7.3* in *P. cornutum* remained unchanged under salt treatment (since *NPF7.3* was not found among the DEGs) to render a constant NO_3^- transport from roots into shoots under salt treatment. Therefore, the superior long-distance transport ability of NO_3^- regulated by *NPF7.3* likely represents another important trait in the salt tolerance of *P. cornutum*.

The efficiency in maintaining Na^+ and K^+ homeostasis is crucial for salt tolerance in higher plants [1]. Numerous proteins involved in these processes have been identified and functionally characterized. For example, CNGC and Na^+/Ca^{2+} exchanger (NCX) mediate root Na^+ and/or K^+ uptake [57, 58], HKT1 and SOS1 mediate Na^+ long-distance transport from roots to shoots [25, 36], KT/KUP/HAK mediate K^+ transport in roots [59, 60], and tonoplast NHX mediates Na^+ and/or K^+ compartmentalization into vacuoles [24]. In the present study, we found that many genes encoding the abovementioned proteins were upregulated after salt treatment (Fig. 2). It has been proven that *Zygophyllum xanthoxylum*, another important xerophyte in northwestern China, could accumulate extremely high Na^+ concentrations in leaves as an important osmoticum to improve photosynthesis and hydration under salt and drought stresses [61, 62]. Subsequent studies have validated the vital roles of some Na^+ and/or K^+ transporters/channels in maintaining Na^+/K^+ homeostasis in *Z. xanthoxylum* [63, 64]. Therefore, the molecular mechanisms underlying Na^+ and K^+ transport in the xerophytic desert plants are much clearer than those involved in Cl^- transport. However, our results also provide new insights into Na^+/K^+ homeostasis in desert plants. For example, the transcript abundance of *HKT1* in *P. cornutum* was upregulated in shoots but remained stable in roots under salt treatment for both 6 and 24 h (Fig. 2, Table 3), indicating that *HKT1* is mainly responsive to salt in shoots of *P. cornutum*. To date, the molecular factors involved in Na^+ transport into mesophyll cells in plants are still unknown. As HKT1-type proteins locate at the plasma membrane and mediate Na^+ transport [36, 37], the expression of *HKT1* in shoots might contribute to Na^+ entry into shoots for using Na^+ as an osmoticum in *P. cornutum*. Furthermore, the transcript abundances of *NHX5* and *NHX6* were upregulated in roots and shoots of *P. cornutum* under salt treatment for both 6 and 24 h (Table 2 and 3). In *A. thaliana*, both NHX5 and NHX6 are Golgi and trans-Golgi network-located Na^+/H^+ antiporters that synergistically trigger plant salt tolerance by maintaining organelle pH and ion homeostasis [65]. However, the function of *NHX5* and *NHX6* in salt tolerance of desert plants has not been investigated. Our results suggested that both *NHX5* and *NHX6* might also two important candidates in the adaptation of desert plants to harsh environments.

The ROS-scavenging system are important for salt stress adaptation in *P. cornutum*

Plants undergo a series of oxygen metabolism reactions during growth, causing them to accumulate a certain amount of ROS in the cells. Under salt stress, the destruction of intracellular osmotic homeostasis can lead to dysfunction in ROS generation and scavenging mechanisms, resulting in the considerable accumulation of oxygen free radicals in the cells, which in turn exerts toxic effects on the cells [66]. It has been proven that under salt and drought stresses, *P. cornutum* exhibits a high ROS-scavenging capacity, with markedly increased SOD, CAT and POD activities [16-18].

SOD is the primary antioxidant enzyme in the ROS-scavenging system. It can convert superoxide anion radicals into free oxygen radicals and hydrogen peroxide. Subsequently, the large amounts of resulting free oxygen radicals and hydrogen peroxide are scavenged mainly through the ASA-GSH cycle, the GPX

pathway, the CAT pathway, and the PrxR/Trx pathway [67]. SODs in higher plants are classified by their metal cofactors into three known types: the Cu/Zn SOD, Mn SOD and Fe SOD, among which Cu/Zn SOD has the widest subcellular distribution (cytosolic fraction and chloroplast) [66]. The upregulation of *SODs* has been proven to substantially contribute to combating oxidative stress resulting from both biotic and abiotic stresses [68, 69]. In this study, the transcript abundance of *Cu-Zn SOD* was upregulated after salt treatment for 24 h in both roots and shoots of *P. cornutum* (Fig. 3, Additional file 1: Table S8 and S10). Hence, Cu-Zn SOD should be a core component in the ROS-scavenging system of *P. cornutum* confronted with salinity. CAT is indispensable for ROS detoxification during stress conditions; it has the highest efficiency for converting H_2O_2 in plant cells into H_2O and O_2 among all the antioxidant enzymes in plants [70]. In the present study, only the *CAT3* was induced in roots and shoots by salt treatment (Fig. 3, Additional file 1: Table S8 and S10), suggesting that under saline conditions, *P. cornutum* mainly increases its CAT activity by upregulating the expression of *CAT3* in roots and shoots. Among the upregulated DEGs involved in the four ROS-scavenging pathways in roots and shoots after salt treatment, most were *GSTs* and *Trxs* (Fig. 3). In the AsA-GSH cycle and GPX pathway, glutathione (GSH) acts as a sensor of redox to maintain lower levels of ROS; it must be first catalyzed by GSTs as an antioxidant, after which it can function in ROS scavenging [67]. Many studies have found that the overexpression of *GSTs* could substantially improve ROS-scavenging capacity in plants under salt stress [71, 72]. Therefore, the upregulated *GSTs* identified in the current study probably also play vital roles in ROS-scavenging system of *P. cornutum* under saline conditions (Fig. 3). The thioredoxin (Trx) proteins serve as redox transmitters within the cellular thiol/disulfide redox network, and the peroxiredoxin (PrxR) proteins act as thiol-dependent peroxidases with high affinity for peroxides, especially for H_2O_2, to protect protein thiols from oxidation. Thus, the PrxR/Trx pathways also function in the ROS-scavenging system in plants [73]. In the present study, many *Trx* genes in roots and shoots were upregulated after salt treatment (Fig. 3), suggesting that Trx may play essential roles in the ROS-scavenging system of *P. cornutum* under NaCl treatment.

High efficiency for stomatal movement and carbon fixation is essential for the adaption of *P. cornutum* to salt stress

Stomatal closure to reduce water loss through transpiration is an important strategy of *P. cornutum* adapting to salt stress, which in turn triggers stomatal limitation for photosynthesis; however, although the net photosynthetic rate of *P. cornutum* is significantly decreased, its dry biomass remains stable due to its high carbon assimilation capacity [19]. Therefore, the genes implicated in stomatal movement and carbon assimilation should play essential roles in salt tolerance in *P. cornutum*.

The aperture of the stomatal pore is regulated by changes in the osmotic potentials of the guard cells. These changes are mainly achieved by transporting K^+ and organic as well as inorganic anions across cellular membranes [74]. The outward K^+ channel GORK participates in K^+ efflux through the plasma membrane of guard cells and are consequently involved in stomatal movement [38]. In the present study,

the salt-induced expression of *GORK* in shoots of *P. cornutum* was observed (Fig. 2D), suggesting that this gene is closely related to stomatal closure in *P. cornutum* confronted with salt stress. In *A. thaliana*, several anion transporters also mediate NO_3^- and/or Cl^- transport across the plasma membrane or tonoplast of guard cells. For example, the slowly activated anion conductance (SLAC1) and aluminium-activated malate transporter 12 (ALMT12) mediate NO_3^- and/or Cl^- release or uptake from guard cells, while CLCc and ALMT9 mediate Cl^- compartmentalization into vacuoles of guard cells [75, 76]. In *P. cornutum*, the transcript abundance of these genes in shoots was unaltered after salt treatment, indicating that there should be other components involved in anion release or uptake from guard cells.

Photosynthetic electron transport is the primary step in the process of oxygenic photosynthesis, which converts sunlight into active chemical energy to provide ATP for the subsequent carbon assimilation [39]. In the present study, numerous upregulated genes associated with all the major components of photosynthetic electron transport, such as chlorophyll biosynthesis, the PS II complex, the PS I complex, the cytochrome b_6f complex, the ATP synthase and ferredoxin, were identified in the shoots of *P. cornutum* after salt treatment for either 6 or 24 h (Fig. 4), these photosynthetic electron transport-related genes in shoots of *P. cornutum* are probably involved in the enhancement of photosynthetic performance by activating a stronger capacity for light energy conversion and ATP generation. Furthermore, our results identified many upregulated genes that encode various enzymes directly involved in carbon assimilation, including phosphoenolpyruvate, malate dehydrogenase, alanine aminotransferase/transaminase, ribulose-1,5-diphosphate carboxylase, phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase-encoding genes (Fig 4, Additional file 1: Table S11 and S12). Hence, these genes likely play central roles in enhancing the carbon assimilation capacity of *P. cornutum* under saline conditions.

Transcription factors play important roles in regulating salt-responsive genes in *P. cornutum* under salt stress

The bZIP TFs play an important and integrative function in salt- and drought-signaling networks of higher plants [77]. Especially, bZIP24 has been identified as a key regulator in the salt tolerance of *A. thaliana* since it could stimulate the transcription of a wide range of stress-inducible functional genes such as *HKT1* and *SOS1* [78]. In the present study, the transcript level of *bZIP24* in roots of *P. cornutum* was upregulated after salt treatment (Additional file 1: Table S13), suggesting that *bZIP24* in roots might predominately regulate transcriptional networks in salt stress adaption of *P. cornutum*. Except for *bZIP24*, many other salt inducible *HD-ZIPs*, which were rarely reported in plants, were also identified in roots and shoots of *P. cornutum* (Additional file 1: Table S13 and S14), these *HD-ZIPs* might play specific roles in modulating the salt tolerance of *P. cornutum*. WRKY proteins regulate diverse plant processes including biotic and abiotic stresses adaption [79]. In *A. thaliana*, WRKY33 is indispensable for salt tolerance [41]. In our study, one *WRKY33* transcript *WRKY33a* was highly induced by salt in both roots and shoots of *P. cornutum*, and another transcript, *WRKY33b*, was induced by salt in roots (Additional file 1: Table S13 and S14). Therefore, WRKY33 likely also plays essential roles in salt signaling networks in *P. cornutum*.

WRKY11 and *WRKY20* has been proven to modulate salt adaption of various plant species [80, 81]. In *P. cornutum*, the *WRKY11* (CL2682.Contig2_All) and three transcripts for *WRKY20* (CL2519.Contig2_All, CL2519.Contig6_All and CL2519.Contig9_All) were all downregulated in roots and shoots of *P. cornutum* after 6 h of salt treatment (Additional file 1: Table S13 and S14), suggesting a specific role of *WRKY11* and *WRKY20* in regulating salt adaption in *P. cornutum*. The Cys2/His2-type ZF proteins have been proven to control and regulate WRKY functions, the ROS-signaling pathway and stomatal closure [82, 83]. In our results, two transcripts (CL7040.Contig1_All and CL7040.Contig2_All) for *Cys2/His2 2* were both induced by salt treatment in shoots (Additional file 1: Table S14), indicating that these two genes might also be important for salt tolerance in *P. cornutum*. In addition to Cys2/His2-type ZF proteins, certain members of the bHLH family, such as bHLH92, confer salt tolerance in plants also by controlling ROS-scavenging signaling as various PODs are their downstream targets [84]. In *P. cornutum*, the transcript abundance of *bHLH92* in shoots was upregulated after salt treatment (Additional file 1: Table S14). Thus, *bHLH92* probably participates in the enhancement of ROS-scavenging capacity in salt adaption of *P. cornutum.* The members of the MADS-box TF family were previously thought to be mainly involved in organ development such as floral emergence and root architecture [85, 86]. However, considering that some *MADS-box* genes showed almost no expression under the normal condition but were highly expressed in shoots or roots of *P. cornutum* under saline conditions (Additional file 1: Table S13 and S14), we can hypothesize that *MADS-boxs* might play specific roles in regulating salt tolerance in desert plant species. Additionally, our results identified some other salt-inducible TF genes in *P. cornutum,* such as *NAC62* and *MYB59* (Additional file 1: Table S13 and S14)*,* whose regulatory mechanisms in salt adaption in other plants have been less intensively investigated. These genes might represent novel regulators in salt tolerance in *P. cornutum*.

Conclusions

This study provides a first analysis of gene transcripts in *P. cornutum* under salt treatments. Candidate genes that probably confer salt tolerance of *P. cornutum* by facilitating Cl^- accumulation and NO_3^- hemostasis, as well as the transport of other inorganic osmoticums such as Na^+, K^+, SO_4^{2-} and PO_4^{3-} were identified. Meanwhile, many salt-responsive genes associated with the enhancement of ROS-scavenging capacity and carbon assimilation efficiency to improve the salt tolerance of *P. cornutum* were identified. Additionally, possible transcription factor genes with multiple functions in regulating the adaption of *P. cornutum* to salt stress were found. These results would promote the research on the molecular mechanisms of salt tolerance in the xerophytic species and lay a foundation for genetic improvement of stress-resistance of important forage and crop species in arid areas

Methods

Plant growth and treatments

All the species of the genus *Pugionium* have been identified by many researchers according to differences in silicle wings and the lobe shape of the cauline and radical leaves [13, 87, 88]. *P. cornutum* is the model species of this genus, this species is a wild vegetable in desert area of northwestern China, and it is free to collect this plant and its seeds [14, 15]. In the present study, seeds of *P. cornutum* were collected from plants grown in Mu Us Sandland in the Inner Mongolia Autonomous Region, China. Corresponding voucher specimen (No. Q.S. Yu, 6065) has been deposited in the Herbarium of School of Life Science, Lanzhou University [13]. We identified these collected seeds according to their typical features described by Yu et al. [13]. After removal of the bracts, seeds were disinfected with 75% alcohol for 30 s, sterilized with 5% NaClO for 10 min, and then rinsed 6 times with distilled water, soaked in distilled water for 1 day and then germinated at 28°C in the dark. After approximately 5 days, the seedlings were transplanted to plastic pots (one seedling/pot) filled with coarse-grained silica sand and irrigated with Hoagland solution containing 2 mM KNO_3, 0.5 mM KH_2PO_4, 0.5 mM $MgSO_4$, 0.5 mM $Ca(NO_3)_2$, 60 μM Fe-citrate, 50 μM H_3BO_3, 10 μM $MnCl_2$, 1.6 μM $ZnSO_4$, 0.6 μM $CuSO_4$, and 0.05 μM Na_2MoO_4 (pH was adjusted to 5.7 by using 1 M Tris). The solution was refreshed every 3 days. All seedlings were grown in a greenhouse, where the daily photoperiod was 16 h/8 h (light/dark), the temperature was 28°C/23°C (day/night), the light flux density was approximately 500 μmol m^{-2} s^{-1} and the relative humidity was approximately 60%.

After one month, uniform seedlings were randomly divided into two groups: control group (C) and salt treatment group (S). For salt treatment group, we used 50 mM NaCl since this concentration of NaCl did not affect the growth of *P. cornutum* [19, 20], while severely impaired the growth of the glycophytes such as Arabidopsis and even the salt-tolerant rice cultivar [89, 90]. In the control group, seedlings were irrigated with normal Hoagland solution; after 6 and 24 h, root (R) and shoot (S) samples were collected and labeled as C6R, C24R, C6S, and C24S, respectively. In the salt group, seedlings were treated with Hoagland solution containing 50 mM NaCl; after 6 and 24 h, root (R) and shoot (S) samples were collected and labeled as S6R, S24R, S6S, and S24S, respectively. Each sample was collected from 5-6 individual seedlings, and immediately frozen in liquid nitrogen for total RNA extraction.

Transcriptome sequencing

Total RNA was extracted from the four root samples (C6R, C24R, S6R, S24R) and four shoot samples (C6S, C24S, S6S, S24S) using the RNeasy Plant Mini Kit (Qiagen, Netherlands). Then, residual DNA was removed from the extracted RNA by treatment with DNase I at 37°C for 40 min. RNA integrity and purity were determined using a NanoDrop ND 1000 instrument (Thermo Scientific).

To obtain the transcriptome in roots and shoots of *P. cornutum*, the four RNA samples from roots were pooled together and the four RNA samples from shoots were pooled together. Poly (A) mRNA in the two pooled RNA samples was purified and then fragmented into small pieces as described by Qiu et al. [91]. The first-strand cDNA and second-strand cDNA of the mRNA fragments were synthesized for end-repair

and poly (A) addition and then linked to sequencing adapters according to Dang et al. [92]. Finally, we constructed two paired-end cDNA libraries (one for root and one for shoot) and sequenced the cDNA in each library on an Illumina HiSeq™ 2000 platform at the Beijing Genomics Institute (BGI, Shenzhen, China). The raw data has been deposited in the NCBI Sequence Read Archive (PRJNA512400).

The raw sequencing reads were then filtered to obtain high-quality clean reads as described by Qiu et al. [91], after that, transcriptome *de novo* assembly and unigene acquisition were performed [12, 92]. Finally, the obtained unigenes were clustered into the following two classes: (i) clusters: each cluster contained several unigenes sharing ≥ 70% sequence similarity with each other that were named with a "CL + cluster digital ID" as the prefix followed by the contig number (e.g., CL1. Contig 1, 2, 3…); (ii) singletons: each singleton represented one individual sequence that did not reach 70% similarity with any other sequence or fall into any assembly and was named with an "Unigene" as the prefix followed by the gene digital ID (e.g., Unigene 1, 2, 3…). To obtain their protein functional annotations, the all-unigene sequences were aligned against the protein databases, including Nr, Nt, Swiss-Prot, KEGG, COG and GO, using BLASTX with a significance threshold of *E*-value < 10^{-5}.

Differentially expressed genes (DEGs) analysis

The eight extracted total RNA from *P. cornutum* tissue samples were used to construct eight independent sequencing tag libraries (C6R, C24R, S6R, S24R, C6S, C24S, S6S and S24S) in parallel with a tag-based digital gene expression (DGE) system according to Ma et al. [12] and Xue et al. [93]. Then, each sequencing tag library was sequenced on an Illumina HiSeq™ 2000 platform at the Beijing Genomics Institute.

The obtained tags in each library were filtered as described by Ma et al. [12]. Then, the clean tags in each cDNA library were mapped to the all-unigenes data from the transcriptome sequencing using the short reads alignment program SOAPaligner/soap2 to determine the protein functional annotations of the genes. The transcript abundance of each gene was determined by the RPKM (reads per kilobase of exon model per million mapped read) method [94].

To identify genes responding to salt treatment, the $\log_2$ ratio of gene transcript abundance between the salt-treated tissue sequencing tag library and the corresponding untreated tissue sequencing tag library (S6R vs. C6R, S24R vs. C24R, S6S vs. C6S, S24S vs. C24S) was calculated. The statistical significance of the differential expression value for each gene was determined by evaluating the probability (P-value) of a gene between two tag libraries being transcribed equally using the formula proposed by Audic et al. [95]. The results of all statistical tests were corrected for multiple tests, and the false discovery rate (FDR) was used to determine the threshold P-value in multiple tests [12]. In this study, a gene with FDR < 0.001 and the absolute value of $\log_2$ ratio > 1 was termed as a differentially expressed gene (DEG).

The upregulated and downregulated DEGs related to ion transport, ROS-scavenging system, photosynthesis and transcriptional factors were then analyzed in roots and shoots respectively. To investigate the gene nucleotide sequence similarity between *P. cornutum* and the model plant *A. thaliana*, the upregulated DEGs under salt treatment with no expression (RPKM value = 0.01) under control condition were selected, then the nucleotide sequences of these DEGs were aligned with their homologous gene segments in *A. thaliana*.

Real-time quantitative PCR validation of DEG results

An independent real-time quantitative PCR (qPCR) experiment was conducted to validate the DEG results from RNA sequencing. *P. cornutum* seedlings were cultured and treated exactly the same as described above, then root and shoot samples were harvest for qRT-PCR validation. We randomly selected 20 DEGs from roots and 20 DEGs from shoots under both 6 and 24 h of salt treatments from our RNA sequencing data. Then, the expression changes of these DEGs under 50 mM NaCl treatment relative to the control condition were investigated using the qPCR method as described by Yuan et al. [64], where *ACTIN2* served as an internal reference. Subsequently, a correlation analysis of the obtained data between RNA sequencing and qPCR was performed as Guo et al. [96].

Abbreviations

RPKM: reads per kilobase of exon model per million mapped read; DEG: differentially expressed gene; FDR: false discovery rate; SLAH: slow-type anion channel-associated homolog; CCC1: cation-chloride co-transporter 1; CLC: chloride channel; NPF: nitrate transporter 1/peptide transporter; SOS1: plasma membrane Na+/H+ antiporter; NHX: tonoplast Na+/H+ antiporter; NCX: Na^{+}/Ca^{2+} exchanger; GLR: glutamate receptor; HKT: high-affinity K+ transporter; KT/HAK/KUP: K+ transporter; KEA: K^{+} efflux antiporter; SKOR: stelar K+ outward rectifying channel; CNGC: cyclic nucleotide-gated channel; V-CHX: vacuolar cation/H^{+} exchanger; GORK: guard cell outward-rectifying K^{+} channel; Sultr: SO42− transporter; PhT: PO43− transporter; ZnT: Zn2+ transporter; CTR: Cu2+ transporter; MnT: Mn^{2+} transporter; MGT: Mg2+ transporter; AMT: NH4+ transporter; IRT: iron transporter; BOR: $BO_3$3− transporter; V-H+ ATPase: vacuolar H+ ATPase; P-H+ ATPase: plasma membrane H+ ATPase; P-Ca2+ ATPase: plasma membrane Ca2+ ATPase; ROS: reactive oxygen species; GLR: glutaredoxin; APX: ascorbate peroxidase; GST: glutathione S-transferase; GPX: glutathione peroxidase; POD: peroxidases; CAT: catalase; PEX: peroxisomal biogenesis factor; Trx: thioredoxin; PrxR: peroxiredoxin; SOD: superoxide dismutase; HSP: heat shock transcription factor; ZF: zinc finger protein; MADS-box: MADS-box transcription factor; bHLH: basic helix-loop-helix protein; NAC: NAM/ATAF/CUC; AP2/ERF: APETALA2 and ethylene-responsive element binding proteins; bZIP/HD-ZIP: basic leucine zipper/ homeodomain-leucine zipper protein; MYB: MYB-domain protein; WRKY: WRKY-domain protein

Declarations

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

QM designed the study, Y-NC performed the experiment, Y-NC, F-ZW, C-HY, J-ZY, HG collected and analyzed the data, Y-NC wrote the manuscript, QM, J-LZ, S-MW revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1 Summary of *de novo* sequence assembly

Unigenes	Total number	Total length (bp)	Mean length (bp)
Shoot	64978	70865211	1091
Root	80307	80091654	997
All	72086	89575317	1243

Table 2 The upregulated DEGs related to ion transport in roots of *P. cornutum* after 50 mM NaCl treatment for both 6 and 24 h.

Gene ID	Gene name	Function
CL5647.Contig1_All	*SLAH1*	Cl^- transport from roots to shoots
CL2477.Contig4_All	*CLCg*	Cl^- vacuole compartmentalization
CL7387.Contig1_All	*NPF6.4*	Cl^- uptake at root epidermis
CL1096.Contig4_All	*NHX5*	Na^+/H^+ antiporter
CL1096.Contig10_All	*NHX6*	Na^+/H^+ antiporter
CL3604.Contig8_All	*GLR3.3*	Cation channel activator
CL3206.Contig3_All	*Sultr1.2*	SO_4^{2-} uptake
CL7785.Contig1_All	*Sultr3.3*	SO_4^{2-} transporter
CL564.Contig8_All	*PHT4.3*	PO_4^{3-} transporter
CL2740.Contig1_All	*PDR2*	Mn^{2+} transporter
CL666.Contig1_All	*BOR4*	BO_3^{3-} exporting from cytoplasm

Table 3 The upregulated DEGs related to ion transport in shoots of *P. cornutum* after 50 mM NaCl treatment for both 6 and 24 h.

Gene ID	Gene name	Function
CL4002.Contig2_All	*CCC1*	Cl^- retrieval from xylem sap
CL2577.Contig2_All	*CLCf*	Cl^- channel
CL2477.Contig4_All	*CLCg*	Cl^- vacuole compartmentalization
Unigene7084_All	*NPF5.2*	Cl^- and/or NO_3^- transporter
CL3282.Contig2_All	*NPF6.2*	Cl^- and/or NO_3^- transporter
CL6278.Contig1_All	*NPF8.1*	Cl^- and/or NO_3^- transporter
CL2487.Contig3_All	*NPF5.9*	Cl^- and/or NO_3^- transporter
CL1096.Contig4_All	*NHX5*	Na^+/H^+ antiporter
CL1096.Contig10_All	*NHX6*	Na^+/H^+ antiporter
CL3834.Contig2_All	*HKT1*	Na^+ and/or K^+ cellular efflux
CL1280.Contig5_All	*KUP5*	K^+ transporter
Unigene6900_All	*CNGC1*	Ca^{2+} root uptake
Unigene13264_All	*CTR5*	Cu^{2+} transporter
Unigene1617_All	*MGT10*	Mg^{2+} transporter

Additional File Legends

Additional file 1: **Figure S1** Length distribution of all assembled unigenes. **Table S1** Sequencing production statistics. **Table** S2 Summary of sequence annotation. **Table S3** Differentially expressed genes (DEGs) related to ion transport in roots of *P. cornutum* after 50 mM NaCl treatment for 6 h. **Table S4** Differentially expressed genes (DEGs) related to ion transport in roots of *P. cornutum* after 50 mM NaCl treatment for 24 h. **Table S5** Differentially expressed genes (DEGs) related to ion transport in shoots of *P. cornutum* after 50 mM NaCl treatment for 6 h. **Table S6** Differentially expressed genes (DEGs) related to ion transport in shoots of *P. cornutum* after 50 mM NaCl treatment for 24 h. **Table S7** Differentially expressed genes (DEGs) related to ROS-scavenging system in roots of *P. cornutum* after 50

mM NaCl treatment for 6 h. **Table S8** Differentially expressed genes (DEGs) related to ROS-scavenging system in roots of *P. cornutum* after 50 mM NaCl treatment for 24 h. **Table S9** Differentially expressed genes (DEGs) related to ROS-scavenging system in shoots of *P. cornutum* after 50 mM NaCl treatment for 6 h. **Table S10** Differentially expressed genes (DEGs) related to ROS-scavenging system in shoots of *P. cornutum* after 50 mM NaCl treatment for 24 h. **Table S11** Differentially expressed genes (DEGs) related to photosynthesis in shoots of *P. cornutum* after 50 mM NaCl treatment for 6 h. **Table S12** Differentially expressed genes (DEGs) related to photosynthesis in shoots of *P. cornutum* after 50 mM NaCl treatment for 24 h. **Table S13** Differentially expressed genes (DEGs) related to transcript factors in roots of *P. cornutum* after 50 mM NaCl treatment for 6 h. **Table S14** Differentially expressed genes (DEGs) related to transcript factors in shoots of *P. cornutum* after 50 mM NaCl treatment for 6 h. **Table S15** Expression pattern validation of 20 randomly selected genes in roots of *P. cornutum* under 50 mM NaCl treatment for 6 and 24 h by qRT-PCR method. **Table S16** Expression pattern validation of 20 randomly selected genes in shoots of *P. cornutum* under 50 mM NaCl treatment for 6 and 24 h by qRT-PCR method

Additional file 2: Sequence alignment of key upregulated DEGs from *P. cornutum* with their homologous genes in *A. thaliana*.

Figures

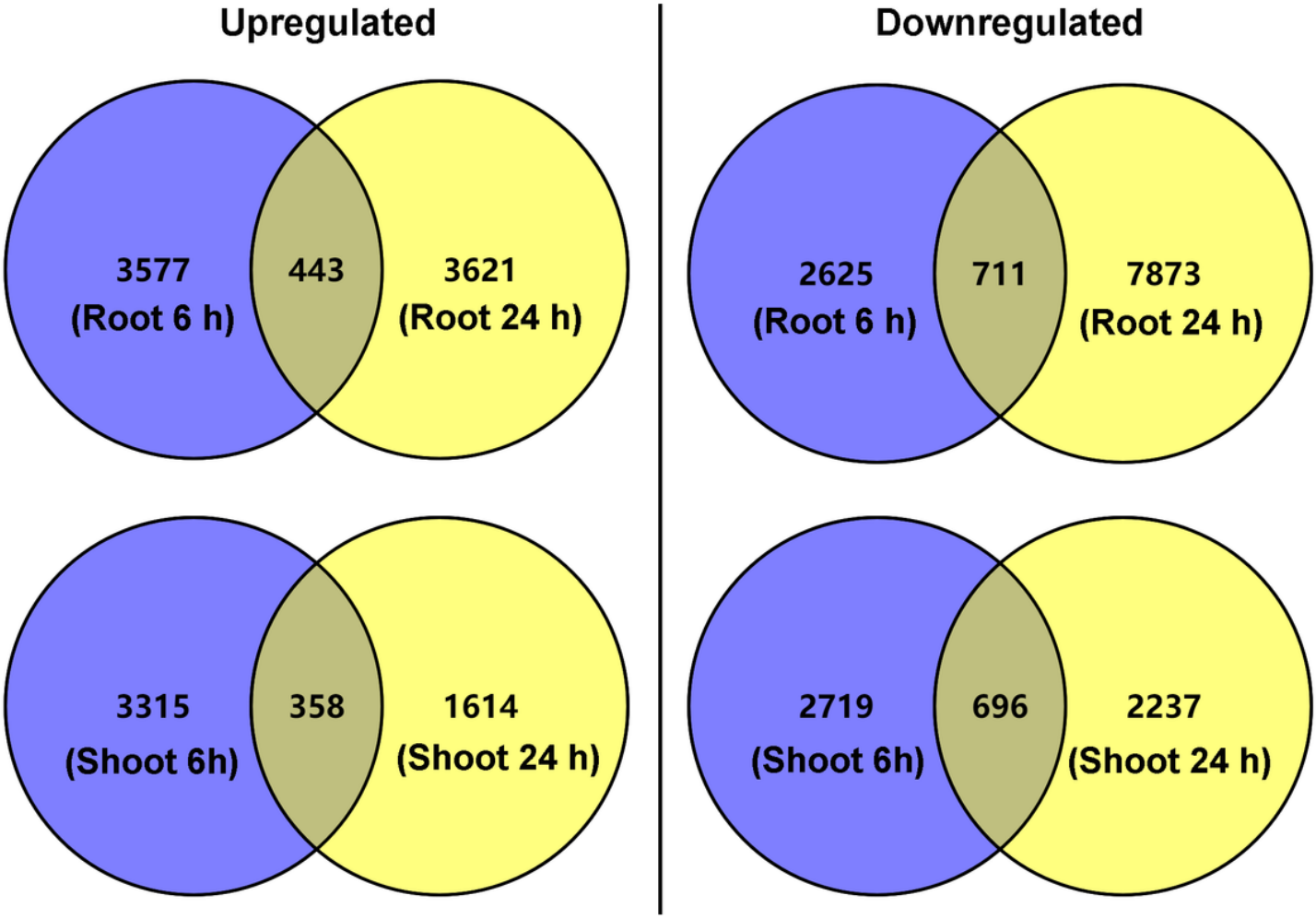

Figure 1

Venn diagrams showing the total number of differentially expressed genes (DEGs) in roots and shoots of P. cornutum under 50 mM NaCl treatment for 6 and 24 h. The blue circles represent the number of upregulated or downregulated DEGs in roots and shoots exclusively under 50 mM NaCl treatment for 6 h. The yellow circles represent the number of upregulated or downregulated DEGs in roots or shoots exclusively under 50 mM NaCl treatment for 24 h. The overlaps between blue circle and yellow circle represent the number of upregulated or downregulated DEGs in roots or shoots under 50 mM NaCl treatment for both 6 and 24 h.

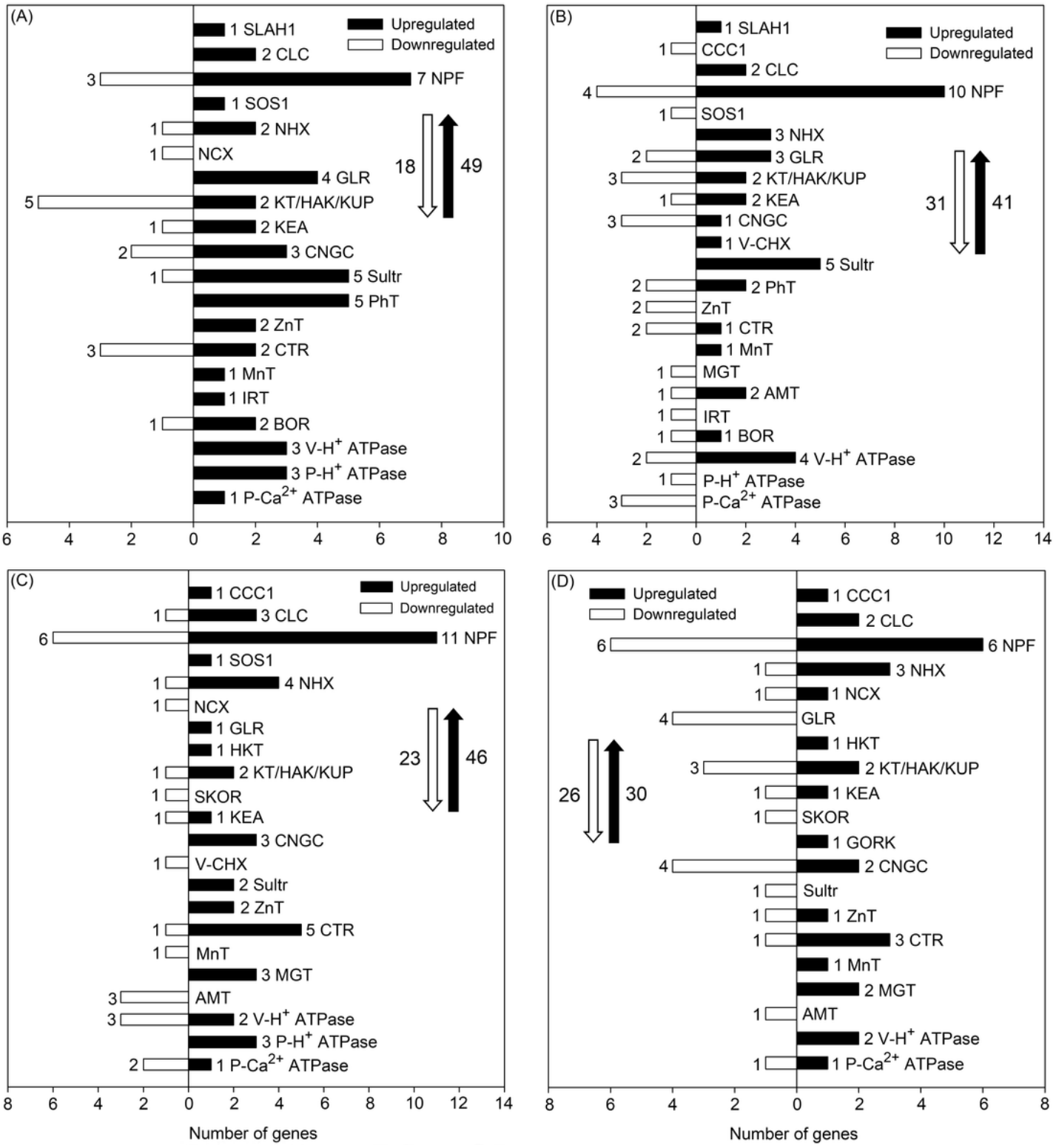

Figure 2

The number of DEGs related to ion transport under 50 mM NaCl treatment for 6 and 24 h in roots (A and B respectively) and in shoots (C and D respectively) of P. cornutum.

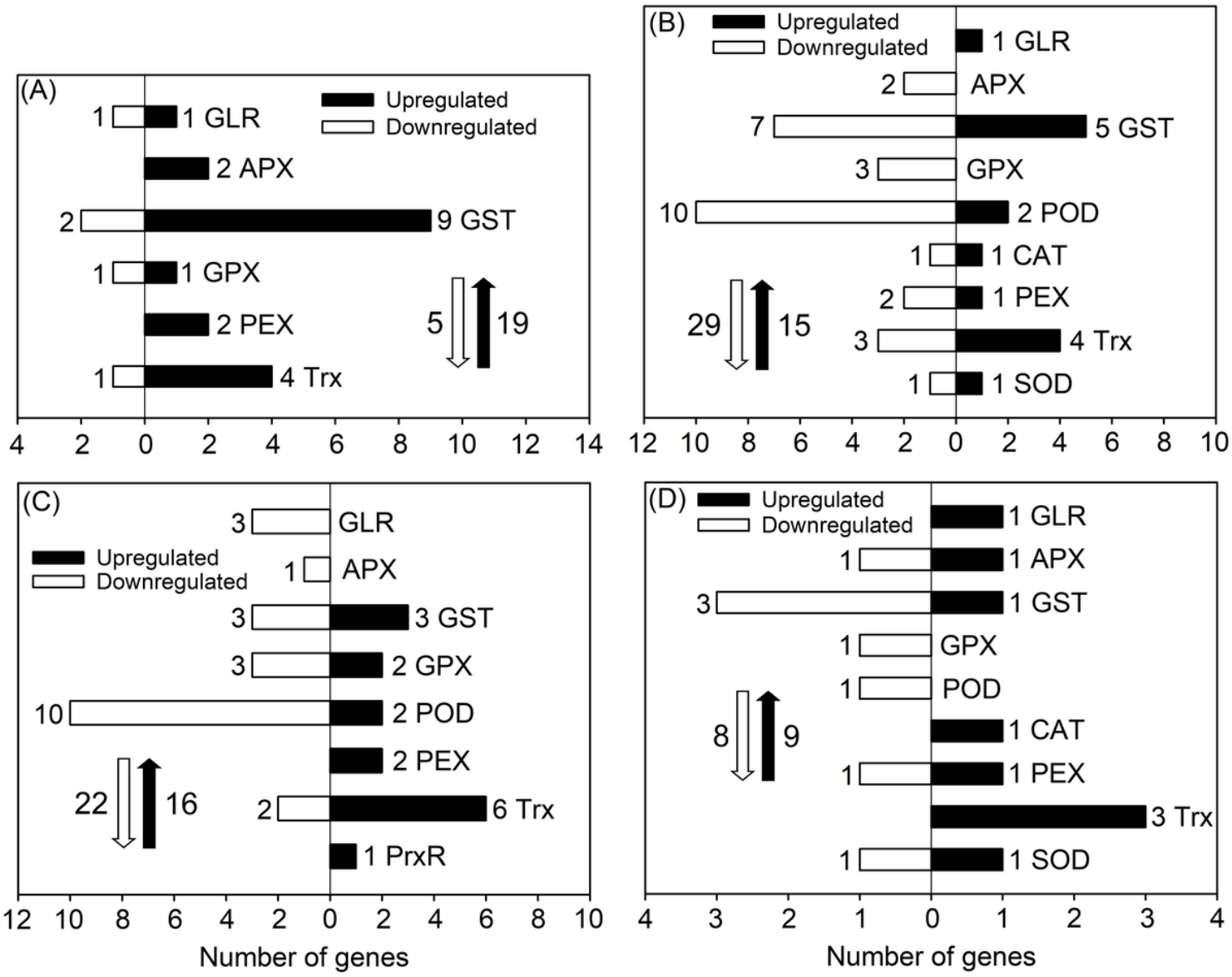

Figure 3

The number of DEGs related to ROS-scavenging system under 50 mM NaCl treatment for 6 and 24 h in roots (A and B respectively) and in shoots (C and D respectively) of P. cornutum. GLR, APX and GST are involved in ASA-GSH cycle, GST, GPX and POD are involved in GPX pathway, CAT and PEX are involved in CAT pathway, Trx and PrxR are involved in PrxR/Trx pathway.

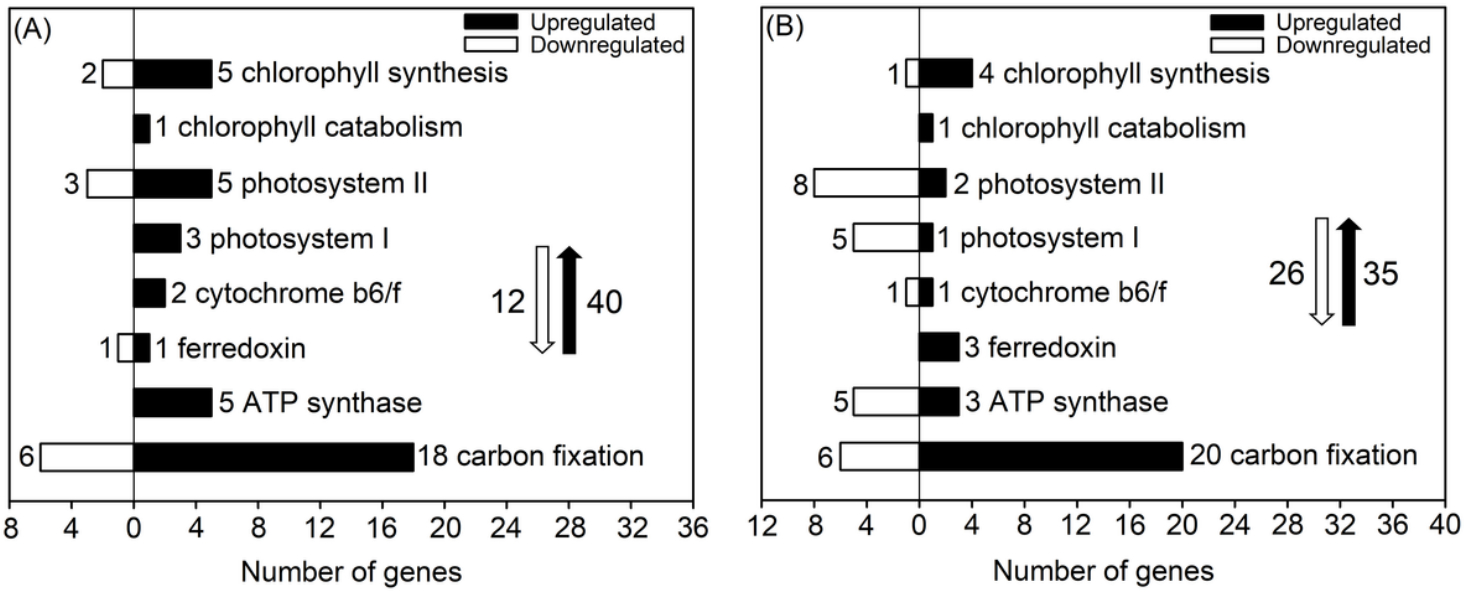

Figure 4

The number of DEGs related to photosynthesis in shoots of P. cornutum under 50 mM NaCl treatment for 6 h (A) and 24 h (B).

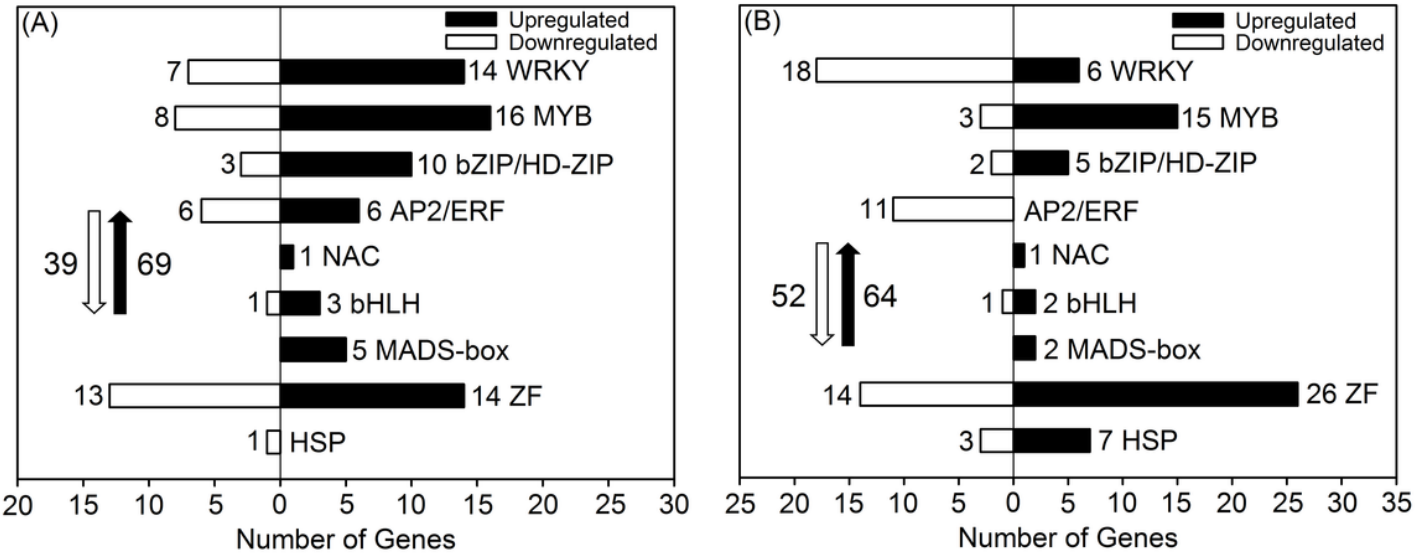

Figure 5

The number of DEGs related to transcription factors in roots (A) and shoots (B) of P. cornutum under 50 mM NaCl treatment for 6 h.

Supplementary Files

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- Additionalfile1.pdf

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