

Comparative Transcriptomics Reveals Osmotic and Ionic Stress Key Genes Contributing To Salinity Tolerance Differential in Two Pak Choi Cultivars

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Abstract

Background: Pak choi is an important leafy vegetable crop. Salinity is among the most harmful agents that negatively influence pak choi yield. However, the mechanism of salinity tolerance in pak choi has not been well understood. In this study, the root transcriptomics of two cultivars differing in salinity tolerant, Shanghaijimaocai (S, salinity tolerant) and Te'aiqing (T, salinity sensitive), were investigated under 0 and 100 mM NaCl treatments.

Results: Using de novo assembly, 214,952 assembled unigenes were generated. Totals of 6765, 2454, 2451 and 5798 differentially expressed unigenes (DEUs) were identified in comparison of S₁₀₀/S₀, T₁₀₀/T₀, S₀/T₀ and S₁₀₀/T₁₀₀, respectively. Shanghaijimaocai is more sensitive to NaCl stress than Te'aiqing in terms of root transcriptomics. Based on GO and KEGG pathway analysis, several osmotic and ionic stress-related genes including *MP3K18*, *PYL8*, *PP2C15/16/49*, *ARF2*, *bHLH112*, *bZIP43*, *COL5*, *CDF1/3*, *ERF25/60*, *HSFA6*, *MYBS3/59/92/CCA1/PHL5*, *POD21*, *GOLS7*, *CIPK4/7/12*, *NHX7*, *SLAH1* and *ALMT13*, showed higher expression in Shanghaijimaocai than in Te'aiqing. These genes, therefore, might be contributed to the difference in salinity tolerance. Moreover, the physiological shift of peroxidase activity was in accordance with dynamic transcript profile of the relevant unigenes.

Conclusions: We determined digital expression profile and discovered a broad survey of unigenes associated with the difference of salinity tolerance between Shanghaijimaocai and Te'aiqing. These findings would be useful for further functional analysis as potential targets to improve resistance to salinity stress via genetic engineering.

Background

Salinity is among the most harmful agents that negatively influence cultivated land fertility, crop productivity and quality [1]. In saline land, salinity ions (e.g. Na⁺ and Cl⁻) accumulation limit most crops growth and reduce water and nutrient uptake, resulting in nutrient imbalances, ion toxicity and osmotic stress [2-4]. Salinity on arable land problem is caused by the natural and anthropogenic activities [2, 3]. More than 20% of the world's arable land is affected by salinity and these values increase daily [5]. Under the pressure of increasing population, various strategies have been applied to improve and utilize salinized land to alleviate the crisis [2, 3, 5]. Among them, screening or breeding salinity tolerant crop varieties is a promising approach for maintaining global food production in agriculture [3, 6]. Hence, understanding the processes regulating the salinity tolerance in crop plants is necessary.

Generally, a high salinity concentration causes ion toxicity, hyperosmotic stress, and secondary stresses such as oxidative damage in plants [2, 3]. Stress regulatory molecular mechanisms in plants already been analyzed by researching many of the genes related to abiotic stress tolerance [1]. Increasing evidence has revealed that the various signal transduction pathways are involved in osmotic, ionic and reactive oxygen species (ROS) homeostasis [4, 7]; these include Ca²⁺ dependent calcium-dependent protein kinase (CDPK) pathway [8] and salt overly sensitive (SOS) pathway [9, 10], as well as mitogen-activated protein

kinase (MAPK) cascade pathway [7, 8], abscisic acid (ABA) pathway and ROS signaling pathway [11, 12]. In fact, the activated Ca^{2+} , MAPK and ABA signaling cascades further activate transcription factors (TFs), such as ZATs, bHLHs and MYBs [4, 13-15], which can cause changes in the expression of various osmotic stress-responsive genes, such as (P5CSs) [16, 17], and ionic stress-responsive genes, such as Na^+/H^+ exchanger (*NHXs*) [15, 18], which ultimately contribute to plant salinity tolerance. In addition, salinity stress tolerance mechanisms also include a series of ROS scavenging enzyme genes [4], compatible solute synthesis genes [4, 19, 20] and ion transporters[1, 4], which enhance plant adaptations to salinity stress. Some studies have shown that overexpression of the stress-associated genes encoding the ROS scavenging protein *TaPRX-2A* [21], compatible solute *KvP5CS1* [22], ion transporter *AtNHX1* [23] and *SpSOS1/NHX7*[10] results in enhanced tolerance to salinity stress in transgenic plants, respectively.

Pak choi (*Brassica rapa* L. ssp. *chinensis*) is an important leafy vegetable crop that is moderately sensitive to salinity [24]. Pak choi exhibits a wide variation in tolerance to soil salinity levels among cultivars [24, 25]. The previous studies in pak choi were focused on salinity-induced morphological, biochemical and physiological changes [24-26]. However, the molecular mechanisms underlying salinity stress tolerance in pak choi has not been well understood. Moreover, the root is the main site of plant sensing of salinity stress, and thus the root is an important aspect of salinity stress affecting plant growth and productivity [27]. Here, there are few transcriptome studies on the roots of pak choi in response to salinity stress.

Taken together, we hypothesize that salinity-induced expression of ions transport, signal transduction, antioxidative defense and osmotic regulation related genes may be responsible for the enhanced tolerance to salinity in pak choi. To test this hypothesis, a comparative transcriptome analysis was first performed on the roots of salinity tolerant and salinity sensitive cultivars of pak choi under control and NaCl-treated conditions. The main object of this paper are: (i) to reveal the changes in gene expression of two pak choi cultivars in NaCl-treated roots; (ii) to identify the key genes related to pak choi salinity stress tolerance; and (iii) to elucidate the gene regulatory network that is responsible for the cultivar differences of pak choi. The results presented here would be useful for the understanding molecular mechanism of salinity tolerance as well as for the breeding new varieties of salinity tolerance in pak choi.

Methods

Plant materials and salinity treatments

On the basis of our previous study (data unpublished), two pak choi cultivars, Shanghaijimaocai (S, salinity tolerant) and Te'aiqing (T, salinity-sensitive), were used in the present study. Seeds of the two cultivars were surface sterilized in 0.1% mercuric chloride for 8 min and fully rinsed with distilled water, then directly planted into pots (13 cm × 12 cm) with washed sand. They were cultivated in a growth chamber with 16 h light at 25 °C and 8 h darkness at 16 °C. One week after sowing, the seedlings with

uniform size were transferred to polyethylene pot (four seedlings of each cultivar per pot). The seedlings were fertilized with 2.8 L of Hoagland nutrient solution (pH 5.8) for one week. Then, the seedlings were treated with 2.8 L Hoagland nutrient solution with 0, 100 and 200 mM NaCl solution, respectively. The solution was replaced every two days. After two weeks of treatment, the roots and leaves samples were selected for morphological and physiological analysis. Moreover, other roots were selected for RNA-seq and RT-qPCR analysis, and those roots were immediately frozen in liquid nitrogen and then stored at -80 °C. Four plants (per pot) from each treatment were pooled collecting as a replicate. Two biological replicates were analyzed for RNA-seq, and three replicates for RT-qPCR validation.

Measurement of plant growth and physiological characteristics

After 14 days of NaCl treatment, the seedlings from 0, 100 and 200 mM NaCl in each cultivar were washed thoroughly with distilled water, blotted dry in filtered paper. Seedlings for each treatment (three replicates) were dissected into roots and leaves, and the fresh weight of leaves (LFW) and roots (RFW) were weighed using precision scales. Then, the dry weight of leaves (LDW) and roots (RDW) were measured after drying in an oven-dried at 105 °C for 1h, and then at 80 °C to a constant weight. In addition, the leaves samples (three replicates for each treatment) from 0 and 100 mM NaCl in each cultivar were used for determining the physiological characteristics. Based on above, the salinity tolerance coefficient (STC) of each index measured was calculated using Eq. (1).

$$STC_{ij} = \frac{X_{ij-treated}}{X_{ij-control}}$$

In the Eq. (1), STC_{ij} represents the salinity tolerance coefficient of the index (j) for the cultivar (i); $X_{ij-control}$ and $X_{ij-treated}$ represent the values of the index for the cultivar evaluated under NaCl-free and NaCl treatments, respectively. Finally, Na^+ and K^+ contents were measured at Shandong Institute of Agricultural Sustainable Development (Jinan, Shandong, China) using the flame photometer (FP-640, Shanghai, China), and subsequently, the shoots K^+/Na^+ ratios were calculated. The enzymes activities of APX, CAT, POD and SOD were conducted using Comin Biochemical Test Kits (APX-2-W, CAT-2-Y, POD-2-Y, SOD-2-Y, respectively; Suzhou, Jiangsu, China; <http://www.cominbio.com>) according to the manufacturer's instructions.

RNA extraction, sequencing and de novo Assembly

Total RNA were extracted separately from the root samples using Trizol reagent (Invitrogen, USA) and purified using the RNeasy Plant Mini Kit (Qiagen) following the manufacturer's protocol. RNA quality was determined using Agilent 2100 Bioanalyzer (Thermo Fisher Scientific, MA, USA). Eight cDNA libraries named S_{0_1} , S_{0_2} , S_{100_1} , S_{100_2} , T_{0_1} , T_{0_2} , T_{100_1} and T_{100_2} from two replicates RNA samples from control and 100 mM NaCl treatment were constructed and sequenced on the BGISEQ-500 platform (BGI, Shenzhen, China). The resulting de novo assembly was performed according to the methods described by Xu et al. [57]. Briefly, the clean reads were generated by removing adaptor reads and low quality reads

from the raw reads. Then, the clean reads from all the eight samples were assembled using Trinity paired-end assembly method [58] with default settings for all parameters, and mapped to transcripts. The transcripts which would be further clustered and eliminated into non-redundant unigenes using TIGR Gene Indices clustering tools (TGICL) [59]. For the study of multi-samples, the previous step using the TGICL for down-stream analysis of the unigenes was repeated. The processed unigenes were referred to as "All-unigenes." The All-unigenes were divided into two classes. One is clusters, several unigenes with over 70% similarity among them (prefix is "CL", followed by the gene family ID), and the other unigenes were singlettons (prefix is "Unigene").

Functional annotation and classification

The assembled unigene sequences were functional annotated against seven databases, including NCBI non redundant protein (Nr, <ftp://ftp.ncbi.nlm.nih.gov/blast/db>), Gene Ontology (GO, <http://geneontology.org>), Clusters of Orthologous Groups (COGs, <https://www.ncbi.nlm.nih.gov/COG/>), Swiss-Prot protein and the Kyoto Encyclopedia of Genes and Genomes (KEGG, <http://www.genome.jp/kegg>), Protein family (Pfam, <http://pfam.xfam.org>) and Non-redundant nucleotide sequence databases (Nt, <ftp://ftp.ncbi.nlm.nih.gov/blast/db>), by Blast (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) searches with an E -value $\leq 10^{-5}$. And then, GO annotation and functional classifications of the unigenes was performed based on the best hits from Nr annotation using BLAST2GO program (v2.5.0, <https://www.blast2go.com>).

Identification and functional enrichment analysis of DEGs

All clean reads were mapped to the unigene sequences using Bowtie2 program (v2.2.5, <http://bowtiebio.sourceforge.net/bowtie2/index.shtml>). Expected count tables for each gene were obtained by RSEM (v1.2.8, <http://deweylab.biostat.wisc.edu/rsem/rsem-calculate-expression.html>). Gene expression levels of each library were normalized to FPKM (fragments per kilobase of transcript per million mapped reads). Based on the negative binomial distribution, read counts were used to determine the differential expression analysis among four groups (S_0 , S_{100} , T_0 and T_{100} , two biological replicates per group) by the DEseq 2 package as described by Love et al.[60]. The gene with $|\log_2 \text{fold-change}| \geq 2$ and $P_{adj} \text{-value} \leq 0.05$ was considered as differentially expressed between two groups. The identified DEGs were subsequently carried into GO and KEGG pathway enrichment analysis with Phyper in R package by a corrected P -value ≤ 0.05 .

RT-qPCR analysis

Total RNA was extracted from three biological replicates of roots samples of both pak choi cultivars exposed to 0 and 100 mM NaCl treatments, respectively. Then the first strand cDNA synthesis was performed using Prime Script[®] RT reagent Kit (Takara, Dalian, China). RT-qPCR was conducted using a SYBR Premix EX TaqKit (Takara) in a 20 μ l reaction mixture on an ABI7300 (Applied Biosystems, Foster City, CA, USA) according to the method described by Yu et al.[61]. The primers of all selected genes were

designed using Beacon Designer 7.0 software (Premier Biosoft International, USA) (Additional file 2: Table S9). The equation ratio=2^{-ΔΔCT} was applied to calculate the relative expression level of the selected genes using *Actin* gene as an internal control [62]. Data statistical analysis with IBM SPSS Statistics Version 25 software (Armonk, NY, USA) was performed using Duncan's multiple range test at the *P*<0.05 level of significance.

Results

Physiology assay

Salinity tolerance coefficient worked well in screening the cultivars for salinity-tolerance based on salinity tolerant indices. It well showed the results of the comparison between the control and salinity-treated cultivars for each index. When pak choi seedlings were cultivated under NaCl conditions, the LFW, LDW, RFW and RDW in two cultivars showed considerable variability in salinity tolerance coefficient (Additional file 1: Figure S1). Compared with the values under normal conditions, the values of LFW, LDW, RFW and RDW in Shanghaijimaocai were higher under 100 mM NaCl treatment (STC>1), while in Te'aiqing, they were lower (STC<1). Furthermore, the values of LFW, LDW, RFW and RDW in two cultivars were lower under 200 mM NaCl treatment (STC<1). Therefore, 100 mM NaCl was used for physiological and transcriptome analyses in the present study.

Since K⁺ and Na⁺ uptake and sequestration are considered as among the key components differentiating between sensitive and tolerant genotypes, the K⁺ and Na⁺ concentrations in leaves of two pak choi cultivars were measured (Fig. 1). The Na⁺ concentrations in two cultivars were no difference in control. The control leaves K⁺ concentration showed higher quantity in Te'aiqing than in Shanghaijimaocai, whereas, salinity-stressed leaves K⁺ and Na⁺ concentrations showed significantly higher quantity in Shanghaijimaocai than in Te'aiqing. Moreover, the cultivar Shanghaijimaocai has higher K⁺/Na⁺ value than the cultivar Te'aiqing under NaCl treatment. These findings indicate that Shanghaijimaocai differed in the properties of Na⁺ (K⁺) translocation and accumulation from Te'aiqing.

To investigate whether the cellular antioxidant defence system was activated, the activities of key antioxidant enzymes including superoxidase dismutase (SOD), peroxidase (POD), catalase (CAT) and ascorbate peroxidase (APX) were tested. In Shanghaijimaocai, under NaCl stress, the SOD (Fig. 2a) and APX (Fig. 2d) activity obviously decreased compared with the control, whereas POD (Fig. 2b) and CAT (Fig. 2c) activities significantly increased. In Te'aiqing, NaCl stress induced a significant increase in POD activity compared with the control, while the SOD, CAT and APX activities were unchanged (Fig. 2).

Sequencing, de novo transcriptome assembly and annotation

To obtain an overview of pak choi transcriptome in roots, and identify candidate genes involved in response to NaCl exposure, eight cDNA libraries prepared from two replicates per treatment were sequenced using a BGISEQ-500 platform. Approximately 46.63–49.08 million raw reads were produced

for the eight libraries through RNA sequencing (Table 1). After removing low quality reads and reads containing adapter sequences, a total of 87.64, 88.25, 87.13 and 85.44 million clean pair-end reads remained for S₀, S₁₀₀, T₀ and T₁₀₀, respectively. The clean reads from each library were assembled and then merged into 214,952 all-unigenes as the reference transcripts of pak choi (Table 2). Of all-unigenes, the total length was 267,453,003 bp, mean length was 1244 bp, the N50 was 1888 bp, which were longer than that obtained in pak choi [28, 29], implying a good assembled quality of the transcriptome in the present study. The length distribution of all unigenes were as shown in Additional file 1: Figure S2, the length of assembled unigenes were mostly ranged from 200 to 500 nt accounted for 32.23%, 500 to 2000 nt accounted for 48.43% and 41,582 unigenes (19.34%) with length > 2000 nt. These results suggested that the quality of this assembled unigenes was credible.

Due to the lack of a reference genome for *B. rapa* L. ssp. *Chinensis*, BLASTx \times E-value $\leq 10^{-5}$ -searches were carried out to perform functional annotations with assembled unigenes against seven public databases, including NCBI non redundant protein (NR), Gene Ontology (GO), Clusters of Orthologous Groups (COG), Kyoto Encyclopedia of Genes and Genomes (KEGG), Protein family (Pfam), nucleotide databases (NT) and SwissProt public protein databases (Additional file 1: Figure S3). Out of the 214,952 unigenes, 186,301 (86.67%) unigenes were matched with the public databases, suggesting that this project has captured a majority of the pak choi transcriptome. Among these annotated unigenes, 171,134 (79.61%) unigenes annotated in the NR database, 133,774 (62.23%) unigenes annotated in the KEGG database, 142,027 (66.07%) unigenes annotated in the COG database, 118,909 (55.32%) unigenes annotated in the GO database, and 129157 (60.09%) Unigenes annotated in the SwissProt database (Additional file 1: Figure S3). Furthermore, the CDSs of 141,274 (75.83%) unigenes were predicted based on the SwissProt and Pfam public protein databases (Table 3). Of these CDSs, 3189 (0.02%) were longer than 3000 bp, 100,248 (70.96%) ranged from 500bp to 3000 bp, and 37,837 (26.78%) were shorter than 500 bp (Additional file 1: Figure S4).

Identification of differentially-expressed unigenes (DEUs)

Pairwise comparison analysis for each gene was performed between Shanghaijimaocai and Te'aiqing (S₀/T₀ and S₁₀₀/T₁₀₀) or between control and NaCl-treated samples in each cultivar (T₁₀₀/T₀ and S₁₀₀/S₀). DEUs were identified by the threshold of $|\log_2 \text{fold-change}| \geq 2$ and $P_{\text{adj}}\text{-value} \leq 0.05$. A total of 12,836 unigenes were differentially regulated in the four comparisons (Additional file 2: Table S1). Under control condition, 5798 DEUs were identified between two cultivars, while this value reached 2451 DEUs under NaCl-treated condition (Fig. 3a). Of which, only 354 unigenes were common ones (Fig. 3b). Compared with the control, 6765 DEUs including 2298 up-regulated and 4467 down-regulated unigenes were differentially regulated in Shanghaijimaocai in NaCl exposure. Whereas, only 2454 NaCl-treated DEUs were identified in Te'aiqing, including 1195 up-regulated and 1259 down-regulated unigenes. Among them, only 242 unigenes were common salinity-responsive genes in two cultivars (Fig. 3b).

Gene ontology (GO) analysis of DEUs

Based on GO functional annotation, a total of 8457 (65.89%) DEUs including 4314(S_{100}/S_0), 1693 (T_{100}/T_0), 3698 (S_0/T_0) and 1666 (S_{100}/T_{100}) DEUs, were assigned into 54 Go terms consisting of 25 biological process, 16 cellular component and 13 molecular function subcategories (Additional file 1: Figure S5a-d). Among these GO terms, the top five abundant categories of biological process ('cellular process', 'metabolic process', 'biological regulation', 'response to stimulus' and 'regulation of biological process'), cellular component ('cell', 'cell part', 'membrane', 'membrane part' and 'organelle') and molecular function ('binding', 'catalytic activity', 'transporter activity', 'structural molecule activity' and 'transcription regulator activity'), were similar in the four comparisons.

Furthermore, the results of significantly enriched GO terms (P_{adj} -value ≤ 0.05) are listed in Additional file 2: Table S2. For salinity-responsive DEUs, the enriched GO terms for DEUs of Shanghaijimaocai were assigned into 65 enriched GO terms, including 27 biological process ('translation', 'oxylipin biosynthetic process', 'response to oxidative stress', etc.), 8 cellular component ('ribosome', 'extracellular region' and 'mucus layer', etc.), and 30 molecular function ('structural constituent of ribosome', 'zinc ion transmembrane transporter activity' and 'chitin binding', etc.); meanwhile, the DEUs in T_{100}/T_0 were assigned into 45 enriched GO terms, including 23 biological process ('L-proline biosynthetic process', 'response to abscisic acid' and 'lipid transport', etc.), 3 cellular component ('chloroplast inner membrane', 'integral component of membrane' and 'vacuole'), and 19 molecular function ('glutamate 5-kinase activity', 'glutamate-5-semialdehyde dehydrogenase activity' and 'transaminase activity', etc.) subcategories (Additional file 2: Table S2). For DEUs between Shanghaijimaocai and Te'aiqing, the DEUs in S_0/T_0 were assigned into 42 enriched GO terms, including 14 biological process ('translation', 'microtubule-based process' and 'cyanide metabolic process', etc.), 10 cellular component ('ribosome', 'cytosolic small ribosomal subunit' and 'lysosome', etc.), and 18 molecular function ('structural constituent of ribosome', 'chitin binding' and 'cellulose binding', etc.); the DEUs in S_{100}/T_{100} were assigned into 21 enriched GO terms, including 12 biological process ('phosphorelay signal transduction system', 'circadian rhythm', 'cellular ion homeostasis', etc.) and 9 molecular function ('protein serine/threonine phosphatase activity', 'UDP-glucose 4-epimerase activity' and 'voltage-gated anion channel activity', etc.) subcategories (Additional file 2: Table S2).

KEGG metabolic pathway analysis of DEUs

Based on Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology-Based Annotation System, a total of 2700 (S_{100}/S_0), 1086 (T_{100}/T_0), 2428 (S_0/T_0) and 1118 (S_{100}/T_{100}) DEUs were respectively assigned to 127, 125, 129 and 126 pathways (Additional file 2: Table S3). The four comparisons differed from each other in metabolic pathways of DEUs (Fig. 4). For the salinity-responsive DEUs, the top five pathways in Shanghaijimaocai were ribosome, nitrogen metabolism, linoleic acid metabolism, alpha-Linolenic acid metabolism and carbon fixation in photosynthetic organisms; (Fig. 4a); glucosinolate biosynthesis, Ubiquinone and other terpenoid-quinone biosynthesis, Isoquinoline alkaloid biosynthesis, Phenylalanine, tyrosine and tryptophan biosynthesis and Carotenoid biosynthesis were the top five

pathways in Te'aiqing (Fig. 4b). Among these pathways, they were all significantly enriched pathways (P_{adj} -value ≤ 0.05)

For DEUs between Shanghaijimaocai and Te'aiqing, ribosome, photosynthesis, oxidative phosphorylation, carbon fixation in photosynthetic organisms and phagosome were the top five pathways under control condition, and only the ribosome and photosynthesis was the predominantly enriched pathway (Fig. 4c). Under salinity-treated condition, the top five pathways were circadian rhythm-plant, glucosinolate biosynthesis, 2-Oxocarboxylic acid metabolism, amino sugar and nucleotide sugar metabolism and C5-Branched dibasic acid metabolism; among these pathways, they were all significantly enriched pathways (Fig. 4d).

DEUs related to signal intermediates

Stress sensing and signal transduction are an important process for plants to combat both osmotic and ionic stress. In the present study, a total of 212 DEUs involved in SOS, ABA, MAPK and CDPK signaling pathway were identified, including calcineurin B-like proteins (CBLs), CBL-interacting serine/threonine-protein kinases (CIPKs), serine/threonine-protein kinases (SRK2s), abscisic acid receptor pyrabactin resistance like proteins (PYLs), protein phosphatase 2Cs (PP2Cs), MAPKs/MPKs, MAPK kinase kinases (MAP3Ks), and CDPKs/CPKs (Additional file 2: Table S4). Among them, 96 were salinity-responsive DEUs, 59 were DEUs between Shanghaijimaocai and Te'aiqing, while the remaining 57 were salinity-responsive DEUs, which also expressed differentially between two cultivars (Additional file 2: Table S4).

Furthermore, a significant distinction in the expression profile was also observed among different gene families. All unigenes belonging to *PYL* gene family was notably up-regulated by NaCl treatment in both Shanghaijimaocai and Te'aiqing (Table 4). In contrast, most unigenes belonging to *CBL* and *CPK/CRK* families were obviously down-regulated by NaCl treatment in both Shanghaijimaocai and Te'aiqing. *MPK* family in Shanghaijimaocai and *CIPK* in Te'aiqing showed mostly down-regulation after NaCl treatment (Table 4), while *CIPK* in Shanghaijimaocai showed mostly upregulation. Under NaCl exposure, most unigenes belonging to *PYLs*, *CIPKs*, *PP2Cs* and *SRK/KINs* showed higher expression in Shanghaijimaocai than that in Te'aiqing, while the majority of DEUs, such as *CPK/CRK*, *MAP3K* and *MPKs* were lower in Shanghaijimaocai than in Te'aiqing (Table 4). Moreover, the up-regulated expression of signal intermediates in S_{100}/S_0 and S_{100}/T_{100} were also analyzed, including *CIPKs* (Unigene6888, CL13846.Contig6, CL13846.Contig1), *PYL8* (CL16764.Contig5, CL16764.Contig3), and *MAP3K* (CL9384.Contig1) (Table 5). The *PP2Cs* (CL18346.Contig10, CL1555.Contig17, Unigene22896) downregulation in S_{100}/S_0 and S_{100}/T_{100} were also found (Table 5).

Identification of DEUs associated with TFs

The transcriptome data identified 547 DEUs mapped to 21 different TF families, such as APETALA2/ethylene responsive factors (*AP2/ERFs*), basic leucine zippers (*bZIPs*), basic helix-loop-helix (*bHLHs*), zinc finger proteins CONSTANS-LIKE (*C2C2-CO-likes*), ethylene-responsive factors (*ERFs*), golden2-likes (*G2-likes*), myeloblastosis proteins (*MYBs*), no apical meristem domain-containing proteins

(*NACs*), nuclear transcription factor Y subunits (*NF-Ys*), *WRKYs* and zinc finger proteins (*C2H2s*, *C3Hs* and *Dofs*) (Additional file 2: Table S5). Among them, 284 were salinity-responsive DEUs, 154 were DEUs between Shanghaijimaocai and Te'aiqing, while the remaining 103 were not only salinity-responsive but also differentially expressed between the two cultivars (Additional file 2: Table S5).

Meanwhile, most unigenes homologous to *C3Hs*, *Dofs*, *G2-likes*, *MYBs*, and *NF-Ys* were notably up-regulated by NaCl treatment in both Shanghaijimaocai and Te'aiqing (Table 4). Besides the aforementioned five TF families, five families (*ARRs*, *B3s*, *bZIPs*, *C2C2-Cos* and *MADSs*) in Shanghaijimaocai and two families (*NACs* and *WRKYs*) in Te'aiqing showed mostly up-regulation after NaCl treatment (Table 4). In contrast, six TF families (*AP2/ERFs*, *bHLHs*, *GRASs*, *HSFs*, *Tifys* and *WRKYs*) in Shanghaijimaocai and *AP2/ERFs* in Te'aiqing showed mostly down-regulation after NaCl treatment (Table 4). Under NaCl exposure, most unigenes homologous to *AP2/ERFs*, *ARFs*, *bHLHs*, *bZIPs*, *C2C2-CO-likes*, *Dofs*, *HSFs* and *MYBs* showed higher expression in Shanghaijimaocai than that in Te'aiqing, while the majority of DEUs, such as *ARRs*, *B3s*, *C3Hs* and *NACs* were lower in Shanghaijimaocai than in Te'aiqing (Table 4). Moreover, the expression abundance of up-regulated TFs in S_{100}/S_0 and S_{100}/T_{100} were also analyzed, including eight *MYBs*, three *Dofs*, *AP2/ERFs* and *C2C2-CO-likes* which contained two unigenes each, and *ARF*, *ARR*, *bHLH*, *bZIP* and *HSF* contained one unigene each (Table 5).

DEUs involved in ions transport

According to GO functional annotation, 91 DEUs were identified to highly similar with transporters that may be involved in the translocation of Na^+ , Cl^- and K^+ in plants, including potassium transporter 9 (*POT9*), potassium channels (*AKTs*, *KATs*), K^+ efflux antiporter 6 (*KEA6*), cyclic nucleotide-gated ion channels (*CNGCs*), sodium/hydrogen exchangers (*NHXs*), sodium/proton antiporter1 (*NHD1*), cation/ $\text{H}(+)$ antiporters (*CHXs*), plasma membrane (PM) H^+ -ATPases (*AHAs*, *PMAs*, *HA1*), vacuolar V-type proton H^+ -ATPases (*VHAs*), pyrophosphate-energized vacuolar membrane proton pumps (*AVPs*), aquaporins (*NIPs*, *PIP*s, *TIPs*, *SIP1-2*), Aluminum-activated malate transporters (*ALMTs*), Chloride channel protein CLC-b (*CLC-B*), mechanosensitive ion channel proteins (*MSLs*) and S-type anion channel SLAHs (*SLAHs*) (Additional file 2: Table S6). These transporters were divided into seven categories (I-VII) based on their expression patterns (Additional file 2: Table S6). The 12 unigenes in the first category and the five unigenes in the second category were specifically up-regulated in Shanghaijimaocai and Te'aiqing roots by NaCl treatment, respectively. 15 unigenes in the third category and four unigenes in the fourth category were specifically down-regulated by NaCl treatment in Shanghaijimaocai and Te'aiqing roots, respectively. The expression of 14 unigenes in the fifth category was higher in Shanghaijimaocai roots than that in Te'aiqing. The sixth category had 9 unigenes expressed lower in Shanghaijimaocai than that in Te'aiqing. Categories I to IV were salinity-responsive DEUs whereas categories V and VI were DEUs between two cultivars. The 32 unigenes in category VII were salinity-responsive DEUs between Shanghaijimaocai and Te'aiqing (Additional file 2: Table S6). Among of them, the expression of *PIP2-1* (Unigene18298) gene was higher in Shanghaijimaocai than in Te'aiqing, while it did not change under NaCl treatment in both cultivars (Additional file 2: Table S6). More importantly, three major transporter genes *NHX7* (CL850.Contig14), *ALMT13* (CL1489.Contig2) and *SLAH1* (CL10603.Contig2) were significantly up-

regulated by NaCl treatment in Shanghaijimaocai, and their expressions were higher in Shanghaijimaocai than in Te'aiqing under NaCl treatment (Table 5).

Reverse transcription-quantitative PCR (RT-qPCR) validation

To verify the differential expression patterns of DEUs identified in our transcriptome data, a total of ten candidate unigenes were selected for RT-qPCR analysis. The results indicated that the expression patterns of nine out of ten DEUs showed good agreement with the RNA-seq-based gene expression patterns, and only one gene (*MYBS3*) was not well consistent with the results of sequencing (Fig. 5). For example, the NaCl-responsive up-regulated DEUs also indicated relatively high expression level in Shanghaijimaocai under NaCl treatment in RT-qPCR analysis (Fig. 5). For DEUs between Shanghaijimaocai and Te'aiqing, the expression levels of *NHX7*, *SLAH1*, *ALMT13*, *MYB59*, *CDF3*, *ERF60*, *PYL8*, *CIPK7* and *POD21* genes were higher in Shanghaijimaocai than that in Te'aiqing under NaCl treatment (Fig. 5). These results suggested that the data detected by RNA-Seq analysis of this study provide reliable inference.

Discussion

Comparison of gene expression in roots between Shanghaijimaocai and Te'aiqing

RNA sequencing (RNA-seq) analysis has been widely utilized to reveal the mechanism of salinity tolerance in some plant species [30-32]. To elucidate the mechanisms involved in cultivar differences in salinity tolerance in pak choi, root gene expression profiles of Shanghaijimaocai and Te'aiqing were first analyzed under control and NaCl-treated conditions by RNA-Seq. Totally, 6765, 2454, 5798 and 2451 unigenes exhibited significant differential expression in comparison of S_{100}/S_0 , T_{100}/T_0 , S_0/T_0 and S_{100}/T_{100} , respectively (Fig. 3). Among them, NaCl-induced 2454 unigenes in the roots of Te'aiqing, whereas 6765 unigenes were identified between 0 and 100 mM NaCl treatments in Shanghaijimaocai, which was 2.8-fold higher than that of Te'aiqing (Fig. 3a), thereby suggesting that Shanghaijimaocai is more sensitive to salinity stress than Te'aiqing. Furthermore, between the two cultivars, 67.6% (NaCl-free) and 57.0% (NaCl-treated) DEUs showed higher expression in Shanghaijimaocai (Fig. 3a). Moreover, only 242 unigenes were common salinity-responsive genes in two cultivars (Fig. 3b). These results suggested that the two cultivars differed in the molecular mechanisms of roots adaption to salinity stress tolerance.

Potential DEUs may determine differences between Shanghaijimaocai and Te'aiqing in salinity-tolerance

Osmotic stress-related DEUs

High osmotic pressure on plant roots is one of the problems plants face when growing in salinized soils. The first response of plants to salinity is signal perception, and relay of this information via signal transduction pathways that eventually re-establish cellular osmotic and ionic homeostasis [1, 4, 7]. $\text{Ca}^{2+}/\text{CDPK}$ signaling pathways have been shown to be involved in maintaining osmotic homeostasis [4, 7]. In the current study, 52 DEUs were identified to be involved in CDPK signaling pathways (Additional file

2: Table S4). Among of these unigenes, the majority of unigenes encoding CDPKs (e.g. *CPK14*, *CPK28* and *CPK32*) were down-regulated by NaCl treatment in Shanghaijimaocai (85.2%) and Te'aiqing (88.2%), respectively (Table 4). Moreover, most unigenes (71.4%) encoding CDPKs showed lower expressions in Shanghaijimaocai than in Te'aiqing under NaCl treatment (Table 4). Thus, the CDPK may not be the key pathway to help fight against salinity stress in the two pak choi cultivars.

MAPK cascades and phosphatidic acid are also involved in the regulation of osmotic, ionic, and ROS homeostasis under salinity stress [4, 7, 14]. In the present study, 27 MAPK unigenes were found to expression altered in four comparisons (Additional file 2: Table S4). Of these, more importantly, one transcript encoding *MAP3K18* was up-regulated by NaCl treatment in Shanghaijimaocai, but in Te'aiqing, it was unchanged, and it showed higher in Shanghaijimaocai than in Te'aiqing under NaCl treatment (Table 5). The overexpression of the *AtMAP3K18* enhanced the resistance to drought in transgenic Arabidopsis plants [33]. Thus, NaCl induced up-regulation of *MAP3K18* may contribute to salinity tolerance in Shanghaijimaocai.

ABA signaling pathway is also activated by osmotic stress [4, 34]. It contains ABA receptor PYL/PYR/RCAR, negative regulator type PP2Cs, and positive regulator SnRK2 (sucrose nonfermenting 1-related protein kinase 2) [7, 35]. The SnRK2, a family of plant-specific serine/threonine kinases, acts downstream components such as TFs (e.g. bHLHs and MYBs), in response to salinity or osmotic stress [7, 16, 17]. Furthermore, the PP2C/ABI binds to SOS2, which also represses SOS2 activity [7, 36]. In the present study, 12 *PYL/PYR* unigenes, 64 *PP2C* unigenes and 9 *SRK2* unigenes were found to expression altered in four comparisons (Additional file 2: Table S4). Interestingly, four unigenes homologous to *PYL/PYR* genes (*PYL4*, *PYL8* and *PYR1*) were found to be up-regulated by NaCl treatment in Shanghaijimaocai, while in Te'aiqing all *PYL/PYR* differential expression unigenes were down-regulated (Table 4). Moreover, NaCl-induced upregulation *PYL8* (CL16764.Contig3 and CL16764.Contig5) in Shanghaijimaocai showed higher expressions than in Te'aiqing under NaCl treatment (Table 5). Overexpression of *AtPYL8* enhances the resistance to drought and osmotic stress by positively regulating ABA signaling in transgenic Arabidopsis [37]. The *PP2C15* (CL18346.Contig10), *PP2C16* (Unigene22896) and *PP2C49* (CL1555.Contig17) were found to be down-regulated by NaCl treatment in Shanghaijimaocai, while in Te'aiqing, they were unchanged, as well as they showed lower expressions in Shanghaijimaocai than in Te'aiqing under NaCl treatment (Table 5). These results indicated that the *PYL8*, *PP2C15*, *PP2C16* and *PP2C49* genes may be the critical factors in determining the contrasting salinity tolerance in two cultivars.

TFs, which are crucial components in osmotic stress mediated signaling pathways, are generally phosphorylated by protein kinases and then activate the expression of numerous downstream salinity stress-responsive genes [1, 4, 13, 16, 17]. In this study, we identified 547 DEUs mapped to 21 TF families, among which 284 were salinity-responsive DEUs, 154 were DEUs between Shanghaijimaocai and Te'aiqing, while the remaining 103 were not only salinity-responsive but also differentially expressed between the two cultivars (Additional file 2: Table S5). These results indicated that the two pak choi cultivars differed in the expression of TFs that may contribute to the difference in salinity tolerance.

Interestingly, we found that 20 DEUs mapped to 15 TFs including *ARF2*, *ARR2*, *bHLH112*, *bZIP43*, *CCA1*, *CDF1*, *CDF3*, *COL5*, *ERF25*, *ERF60*, *HSFA7*, *MYBS3*, *MYB59*, *MYB92* and *PHL5* were up-regulated in S_{100}/S_0 and S_{100}/T_{100} (Table 5). The TFs such as *ARF2* [38], *ARRs* [39], *bHLH112* [40], *bZIPs* [41], *CDF1/3* [42], *COLs* [43], *ERFs* [44], *HSFA7* [45] and *MYBs* [11, 46] are believed to participate in the abiotic stress responses and tolerances in many plant species. Thereby, we proposed that these TFs, induced under salinity were likely to play a crucial role in salinity tolerance of Shanghaijimaocai.

Under osmotic stress, ROS as toxic products, which result in oxidative damage and cell death [1]. Antioxidant defence system can alleviate oxidative damage in response to salinity/oxidative stress [1, 4, 21]. Our physiological work showed that the antioxidative enzymes, POD (Fig. 2b) and CAT (Fig. 2c) activities significantly increased by NaCl in Shanghaijimaocai, while in Te'aiqing, only POD activity was increased by NaCl (Fig. 2). Moreover, the POD, CAT and APX activities were obviously higher in Shanghaijimaocai than in Te'aiqing under 100 mM NaCl treatment. Meanwhile, our transcriptome analysis also showed that 70 antioxidative enzymes-related DEUs including *APXs*, *CATs*, *PODs*, *SODs*, *GPXs* and *GRs*, non-enzymatic antioxidants-related DEUs including *GSTs* and *GSHs* (Additional file 2: Table S7) were identified in pak choi. Of these, the majority of *PODs* were up regulated by NaCl treatment in both Shanghaijimaocai and Te'aiqing. It was well consistent with our physiological data (Fig. 2b). Importantly, we also found that the all *POD* unigenes showed higher in Shanghaijimaocai than in Te'aiqing (Additional file 2: Table S7). Of these, *POD21* (Unigene3772) gene was up regulated by NaCl in Shanghaijimaocai, while in Te'aiqing, it was unchanged (Table 5), suggesting that *POD21* may contribute to the cultivar difference in pak choi salinity tolerance.

Accumulation of compatible solutes/osmolytes such as glycine betaine, proline, polyamines, trehalose and other soluble sugar in cytosol or chloroplast, is an effective strategy to alleviate osmotic stress imposed by salinity [1, 4]. Totally, 77 DEUs related to osmolytes synthesis, including phosphoethanolamine N-methyltransferases (*PEAMTs*), Delta1-pyrroline-5-carboxylate synthetases (*P5CSs*), proline dehydrogenase 1 (*POX1*), trehalose-phosphate phosphatases (*TPPs*), trehalose-phosphate synthases (*TPSs*), inositol 3-phosphate synthase isozyme 3 (*IPS3*), hexokinases (*HXKs*), galactinol synthases (*GOLSs*), arginine decarboxylases (*ADCs*), ornithine decarboxylases (*ODCs*), and spermidine synthase 1 (*SPDS1*), were identified in four comparisons (Additional file 2: Table S8). Compared with control, most unigenes belonging to *P5CSs* and *GOLSs* were up-regulated in both Shanghaijimaocai and Te'aiqing after NaCl treatment. Overexpression of *KvP5CS1* [22] and *TsGOLS2* [47] increase salinity tolerance in transgenic plants, respectively. These results indicated that *P5CSs* and *GOLSs* may be involved in the NaCl-response in roots of both Shanghaijimaocai and Te'aiqing. Besides, one unigene of *GOLS7* (Unigene6912), whose expression was unchanged by NaCl in Te'aiqing, was up-regulated in both S_{100}/S_0 and S_{100}/T_{100} (Table 5). Whether *GOLS7* is the key factor regulating the cultivar difference in salinity-tolerance need to be further investigated.

Ionic stress-related DEUs

Ionic stress (also called “ionic imbalance” or “ion toxicity”) is another of the problems plants face when growing in salinized soils. Ionic stress (mainly Na^+) triggers an increase in cytosolic Ca^{2+} , and thereafter, Ca^{2+} -binding proteins further activate downstream signaling pathways[4, 7]. The Ca^{2+} -SOS signaling is well known as the central salinity excretion system that helps plants against Na^+ accumulation [48]. The genes for SOS signaling pathway (*CBLs-CIPKs* as *SOS3-SOS2*) have shown to provide enhanced resistance to salinity via positive regulation of the plasma membrane-localized Na^+/H^+ antiporter *SOS1/NHX7* [7, 49, 50]. We found that the majority of CBL unigenes were down-regulated by NaCl treatment in both Shanghaijimaocai (71.4%) and Te'aiqing (100%) (Table 4; Additional file 2: Table S4). The *CBLs* were not DEUs as judged by our criteria between the two cultivars under NaCl treatment (Table 4). This result suggested that *CBLs* may not be involved in the difference of salinity tolerance between the two cultivars. Most unigenes (62.5%) belonging to *CIPK* genes (*CIPK3, CIPK4, CIPK7, CIPK10, CIPK11, CIPK12, CIPK 21* and *CIPK25*) were found to be up-regulated by NaCl treatment in Shanghaijimaocai, while in Te'aiqing only one *CIPK1* transcript was up-regulated (Table 4). Moreover, *CIPK4* (Unigene6888), *CIPK7*(CL13846.Contig1, CL13846.Contig6) and *CIPK12* (CL16987.Contig7) showed higher expressions in Shanghaijimaocai than in Te'aiqing (Table 5). Thus, we speculate that three *CIPKs* (*CIPK4, CIPK7* and *CIPK12*) might contribute to the difference of salinity tolerance between two pak choi cultivars.

The CBL-CIPK modules can also interact with and regulate the activity of many ion transporters [9, 10]. Previous studies have reported that a number of transporters can control Na^+ , Cl^- and K^+ transport, which are essential for ion homeostasis to improve salinity tolerance in plants [50, 51]. Based on the physiological data (Fig. 1), we reasoned that differential root uptake and translocation capacity of K^+ and Na^+ may be main factors determining the contrasting of salinity tolerance in Shanghaijimaocai and Te'aiqing. Within the pak choi transcriptome, we identified 128 transporter-encoding DEUs, among which 65 (including 24 up and 41 down-regulated) were salinity-responsive DEUs in Shanghaijimaocai, and only 31 (including 16 up and 15 down-regulated) were salinity-responsive DEUs in Te'aiqing (Additional file 2: Table S6). Some transporters including NHXs, ALMTs, MSLs and SLAHs have been verified to involve in the cellular Na^+ and Cl^- exclusion or sequestration in plants, and thus enhanced the tolerance to salinity [52-54]. In this study, 33 DEUs that showed similarity to *NHXs* (6 unigenes), *ALMTs* (4 unigenes), *MSLs* (11 unigenes) and *SLAHs* (12 unigenes) (Additional file 2: Table S6). Of them, most *ALMT13* (100%), *MSLs* (75%), *SLAHs* (100%) and *NHXs* (60%) unigenes showed higher expressions in Shanghaijimaocai than in Te'aiqing. We speculate that the salinity tolerance mechanism of Shanghaijimaocai may be the inherent characteristics. Moreover, *NHX7* (CL850.Contig14), *ALMT13* (CL1489.Contig2) and *SLAH1* (CL10603.Contig2), whose expressions were not affected by NaCl in Te'aiqing, were significantly up-regulated by NaCl treatment in Shanghaijimaocai, and their expressions were higher in Shanghaijimaocai than in Te'aiqing under NaCl treatment (Table 5). *NHX7, ALMT13* and *SLAH1* of pak choi were homologous with *AtNHX7, AtALMT13* and *AtSLAH1* in *Arabidopsis*, respectively. Overexpression of the plasma membrane-localized transporter genes *AtSOS1/AtNHX7*[10, 52], (mediating Na^+ efflux), and *AtSLAH1* [54], (mediating Cl^- extrusion), resulted in increased salinity tolerance in transgenic plants. These results indicated that *NHX7* and *SLAH1* could contribute to elevate salinity tolerance in

Shanghaijimaocai. Besides *SLAC1*, *ALMT* transporters localize to the plasma and/or vacuolar membrane and might also contribute to Cl⁻ exclusion or sequestration [53], indicating that *ALMT13* may be responsible for the Cl⁻ detoxification, and consequently, increasing salinity tolerance in Shanghaijimaocai.

The cellular balance between K⁺ and Na⁺ is among the key salinity-tolerance mechanisms during salinity stress [55, 56]. In this study, 11 DEUs (homologous with *KEA6*, *AKT1* and *POT9*) were identified as K⁺ transporter or K⁺ channel protein (Additional file 2: Table S6). Of them, two *AKT1* were down-regulated by NaCl treatment in Shanghaijimaocai (Unigene19806) and Te'aiqing (CL4.Contig8), respectively. Three *POT9* were not affected by NaCl treatment in Shanghaijimaocai roots, as well as they were lower expression in Shanghaijimaocai than in Te'aiqing roots under NaCl treatment. These results indicated that *AKT1* and *POT9* may not involve in the difference of salinity-tolerance between the two cultivars. Two *KEA6* (CL6150.Contig22, CL6150.Contig12) and one *KAT1* (CL2130.Contig1) were specifically up-regulated by NaCl treatment in Shanghaijimaocai, but unchanged between the two cultivars exposed to NaCl. This result indicated that *KEA6* and *KAT1* may be related to NaCl-response in roots of Shanghaijimaocai, but not be involved in the difference of salinity-tolerance between the two cultivars.

Proton pumps including plasma membrane H⁺-ATPase, vacuolar membrane H⁺-ATPase and H⁺-pyrophosphatase, play an essential role in cellular Na⁺ extrusion or sequestration into vacuoles [10, 23]. Here, 28 DEUs that showed similarity to various H⁺ pumps (e.g. *AHA1*, *AHA3*, *AHA6*, *HA1*, *PMA1*, *AVP1*, *VHA-a3*, *VHA-b1/3*, *VHA-c1/2*, *VHA-e1/2/3* and *VHA-g1*) (Additional file 2: Table S5). Of them, *AHA3* (Unigene33507), *AHA6* (CL9664.Contig2), *VHA-a3* (Unigene41408) and *VHA-b1* (Unigene16791) were notably up-regulated by NaCl treatment in Shanghaijimaocai, but in Te'aiqing, except for *VHA-a3* (down-regulated), they were unchanged. Moreover, they were not DEUs as judged by our criteria between the two cultivars under NaCl treatment. These results indicated that *AHA3*, *AHA6*, *VHA-a3* and *VHA-b1* may be related to NaCl-response in roots of Shanghaijimaocai, but not are the key factors regulating the cultivar difference in salinity tolerance. Intriguingly, we found that the majority of DEUs, such as *HA1* (100%), *AVPs* (100%) and *VHAs* (67%) were higher in Shanghaijimaocai than in Te'aiqing under NaCl-free condition. Moreover, all these H⁺ pumps were suppressed or unchanged by NaCl in Shanghaijimaocai, while they were induced or unchanged in Te'aiqing. It seems that the inherent higher expression of these H⁺ pumps in roots of Shanghaijimaocai may contribute to its higher salinity tolerance.

Conclusions

Our study is the first systematic report of root transcriptome changes between two contrasting pak choi cultivars in response to NaCl stress. The sequence was assembled into 214,952 unigenes. Next, a total of 6765, 2454, 2451 and 5798 DEUs were identified during S₁₀₀/S₀, T₁₀₀/T₀, S₀/T₀ and S₁₀₀/T₁₀₀ comparison, respectively. The two cultivars differed in the molecular mechanisms in response to NaCl stress. Shanghaijimaocai is more sensitive to NaCl stress than Te'aiqing in terms of root transcriptomics. Moreover, many candidate functional genes including four protein phosphorylation cascades (*CIPK4/7/12*, *MP3K18*), four ABA signaling genes (*PYL8*, *PP2C15/16/49*), 14 TF genes (*ARF2*, *bHLH112*,

bZIP43, *COL5*, *CDF1/3*, *ERF25/60*, *HSFA6* and *MYBS3/59/92/CCA1/PHL5*, three ion transporter genes (*NHX7*, *SLAH1* and *ALMT13*), one antioxidant enzyme gene (*POD21*) and one osmoprotectant-related gene (*GOLS7*), which are involved in salinity tolerance mechanism, were found to be up-regulated by NaCl treatment in Shanghaijimaocai, as well as they showed higher expressions in Shanghaijimaocai than in Te'aiqing under NaCl stress. Thereby, these genes might be involved in cultivar difference in salinity tolerance of pak choi (Fig. 6), and these should be studied further. Our findings could provide information for further investigations of key genes and molecular mechanisms of salinity tolerance in pak choi.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

All the authors have signed the consent form.

Competing interests

The authors declare that they have no competing interests for this research.

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Authors' contributions

YR designed the experiments. DX, SC, WY, SW, JM, WX and MR performed the pak choi cultivation and sample collection. YR, DX, ST, SC, WY, SW, JM, WX and MR performed the experiments. DX and YR wrote the manuscript draft. YR, MR and ST edited and revised the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1 Overview of raw and clean reads in two pak choi cultivars exposed to 0 or 100 mM NaCl for two weeks

Sample	ID	Total raw reads (M)	Total clean reads (M)	Total clean bases (Gb)	Clean reads q20 (%)	clean reads q30 (%)	clean reads in raw reads (%)
Shanghaijimaocai	S _{0_1}	49.08	43.78	6.57	98.18	91.56	89.21
	S _{0_2}	48.7	43.9	6.58	98.24	91.8	90.13
	S _{100_1}	49.08	43.98	6.6	98.34	92.13	89.6
	S _{100_2}	49.08	44.27	6.64	98.19	91.62	90.2
Te'aiqing	T _{0_1}	49.08	43.63	6.54	98.28	91.91	88.89
	T _{0_2}	49.08	43.5	6.52	98.27	91.86	88.63
	T _{100_1}	46.63	41.42	6.21	98.24	91.78	88.82
	T _{100_2}	49.08	44.02	6.6	98.22	91.7	89.69

Table 2 Results of assembled samples

Sample	ID	Total unigene Number	Total Length (bp)	Mean Length (bp)	N50 (bp)	GC (%)
Shanghaijimaocai	S _{0_1}	92,591	98,585,640	1064	1627	43.11
	S _{0_2}	83,232	93,088,560	1118	1650	43.11
	S _{100_1}	88,503	95,785,900	1082	1580	43.49
	S _{100_2}	97,772	104,740,410	1071	1568	44.03
Te'aiqing	T _{0_1}	88,100	101,769,004	1155	1626	42.87
	T _{0_2}	81,670	98,575,905	1207	1671	43.03
	T _{100_1}	76,629	89,957,106	1173	1630	43.23
	T _{100_2}	81,688	98,224,732	1202	1687	43.11
All-Unigene		214,952	267,453,003	1244	1888	43.16

Note: GC, is short for guanine-cytosine

Table 3 Results of assembled samples

Total number	Total length	N50	N90	Max length	Min length	GC (%)
141,274	140,597,907	1260	492	15,234	297	46.38

Note: GC, is short for guanine-cytosine

Table 4 Statistics analysis of gene expression for signal intermediates and regulatory proteins in roots of two pak choi cultivars.

Gene family	S ₁₀₀ /S ₀ (Unigene)		T ₁₀₀ /T ₀ (Unigene)		S ₀ /T ₀ (Unigene)		S ₁₀₀ /T ₁₀₀ (Unigene)	
	Up	Down	Up	Down	Up	Down	Up	Down
Signal intermediates								
CBL	4	10	0	2	3	0	0	0
CIPK	10	6	2	4	3	4	8	2
SRK/KIN	2	2	2	1	4	1	3	0
PYL	4	0	2	0	1	1	8	1
PP2C	15	14	11	10	7	6	16	11
CPK/CRK	4	23	2	15	10	4	4	10
MAP3K	2	3	0	0	1	1	1	3
MPK	1	10	3	4	6	1	0	1
Transcription factors								
AP2/ERF	12	28	3	7	9	7	12	3
ARF	4	3	1	1	2	6	3	1
ARR	5	2	1	1	1	1	1	3
B3	2	0	0	1	4	3	1	4
bHLH	8	17	4	5	4	3	10	2
bZIP	3	1	1	2	6	3	7	2
C2C2-CO-like	6	0	0	0	1	0	4	0
C2H2	1	3	0	1	2	0	2	1
C3H	4	1	3	2	5	3	0	2
Dof	9	2	1	0	1	1	6	0
G2-like	8	0	3	0	4	0	1	2
GATA	2	1	2	2	1	2	3	2
GRAS	2	6	2	2	2	3	2	1
HSF	3	6	0	3	1	3	2	0
MADS	5	0	1	1	1	5	0	0
MYB	53	15	22	10	19	13	11	3
NAC	9	9	6	4	7	7	1	3

NF-Y	10	2	3	0	4	1	1	1
Tify	2	22	1	0	8	7	2	1
Trihelix	2	1	1	1	0	0	2	1
WRKY	2	21	9	7	10	2	3	4

Table 5 Critical DEUs involved in the salt tolerance of pak choi in this study

Gene ID	Description	Abbr.	log ₂ FoldChange			
			S ₁₀₀ /S ₀	T ₁₀₀ /T ₀	S ₀ /T ₀	S ₁₀₀ /T ₁₀₀
Signal intermediates						
CL16764.Contig5_All	Abscisic acid receptor PYL8	PYL8	2.5*	-2.6	-0.9	4.4*
CL16764.Contig3_All	Abscisic acid receptor PYL8	PYL8	2.3*	-1.8	-0.7	3.4*
CL16987.Contig7_All	CBL-interacting serine/threonine-protein kinase 12	CIPK12	2.1*	1.9	1.2	1.3*
Unigene6888_All	CBL-interacting serine/threonine-protein kinase 4	CIPK4	3.1*	-0.1	-0.4	2.8*
CL13846.Contig6_All	CBL-interacting serine/threonine-protein kinase 7	CIPK7	3.0*	-1.1	-1.0	3.1*
CL13846.Contig1_All	CBL-interacting serine/threonine-protein kinase 7	CIPK7	2.5*	0.9	0.8	2.3*
CL9384.Contig1_All	Mitogen-activated protein kinase kinase kinase 18	MAP3K18	2.0*	-0.6	0.4	2.9*
CL18346.Contig10_All	probable protein phosphatase 2C 15	PP2C15	-7.1*	-1.0	-0.1	-5.6*
CL1555.Contig17_All	probable protein phosphatase 2C 49	PP2C49	-4.6*	0.5	0.4	-4.0*
Unigene22896_All	Protein phosphatase 2C 16	PP2C16	-3.9*	0.9	0.4	-4.3*
Transcription factors						
CL9433.Contig5_All	ethylene-responsive transcription factor ERF060-like	ERF60	2.1*	0.8	0.7	2.0*
CL7472.Contig3_All	ethylene-responsive transcription factor ERF025-like	ERF25	4.7*	-0.5	-1.0	3.8*
CL1771.Contig2_All	auxin response factor 2	ARF2	4.7*	-0.3	-0.6	3.8*
Unigene6026_All	Two-component response regulator ARR2	ARR2	4.5*	#N/A	#N/A	3.6*

CL493.Contig43_All	Transcription factor bHLH112	bHLH112	4.0*	-2.0	-2.2	3.7*
CL960.Contig2_All	Basic leucine zipper 43	bZIP43	2.3*	-0.2	0.6	3.2*
CL15144.Contig3_All	zinc finger protein CONSTANS-LIKE 5	C2C2-CO-like/COL5	2.3*	1.2	3.2*	4.7*
CL15144.Contig2_All	zinc finger protein CONSTANS-LIKE 5	COL5	6.6*	0.0	-0.8	5.4*
CL14111.Contig2_All	Cyclic dof factor 1	Dof/CDF1	3.2*	0.4	1.2	3.6*
CL13584.Contig1_All	cyclic dof factor 3	CDF3	4.4*	0.6	0.9	4.7*
CL13584.Contig2_All	cyclic dof factor 3	CDF3	4.0*	1.2	0.6	3.1*
CL9101.Contig2_All	Heat stress transcription factor A-7a	HSFA7	2.3*	-0.7	0.8	3.9*
Unigene34163_All	Myb family transcription factor PHL5	MYB/PHL5	2.6*	-3.5*	-1.7	4.4*
CL18014.Contig11_All	Transcription factor MYB92	MYB92	2.3*	-4.6*	-1.8	5.6*
CL8798.Contig1_All	CCA1	MYB/CCA1	2.4*	1.4	2.5	3.4*
CL8798.Contig2_All	CCA1	MYB/CCA1	3.0*	0.9	1.6	3.2*
Unigene13784_All	Transcription factor MYBS3	MYBS3	4.2*	1.2	0.6	3.0*
CL4419.Contig2_All	Transcription factor MYBS3	MYBS3	3.0*	0.7	0.4	2.7*
CL16149.Contig1_All	transcription factor MYB59-like isoform X3	MYB59	3.8*	-0.6	-1.3	2.9*
CL4419.Contig5_All	transcription factor MYBS3-like	MYBS3	7.2*	-0.2	-3.4	3.6*
<i>Na⁺ and Cl⁻ transporters</i>						
CL850.Contig14_All	Sodium/hydrogen exchanger 7	NHX7	6.3*	-0.9	-1.8	4.3*
CL10603.Contig2_All	S-type anion channel SLAH1-like	SLAH1	2.8*	-1.4	-1.0	3.1*
CL1489.Contig2_All	Aluminum-activated malate transporter 13	ALMT13	4.1*	0.8	0.1	2.8*

Antioxidant						
Unigene3772_All	peroxidase 21-like	POD21	2.3*	-1.8	-0.5	3.7*
Osmotic adjustment substance						
Unigene6912_All	Galactinol synthase 7	GOLS7	3.0*	-0.2	0.0	3.2*

* indicates the genes significant differential expression between two groups.

Figures

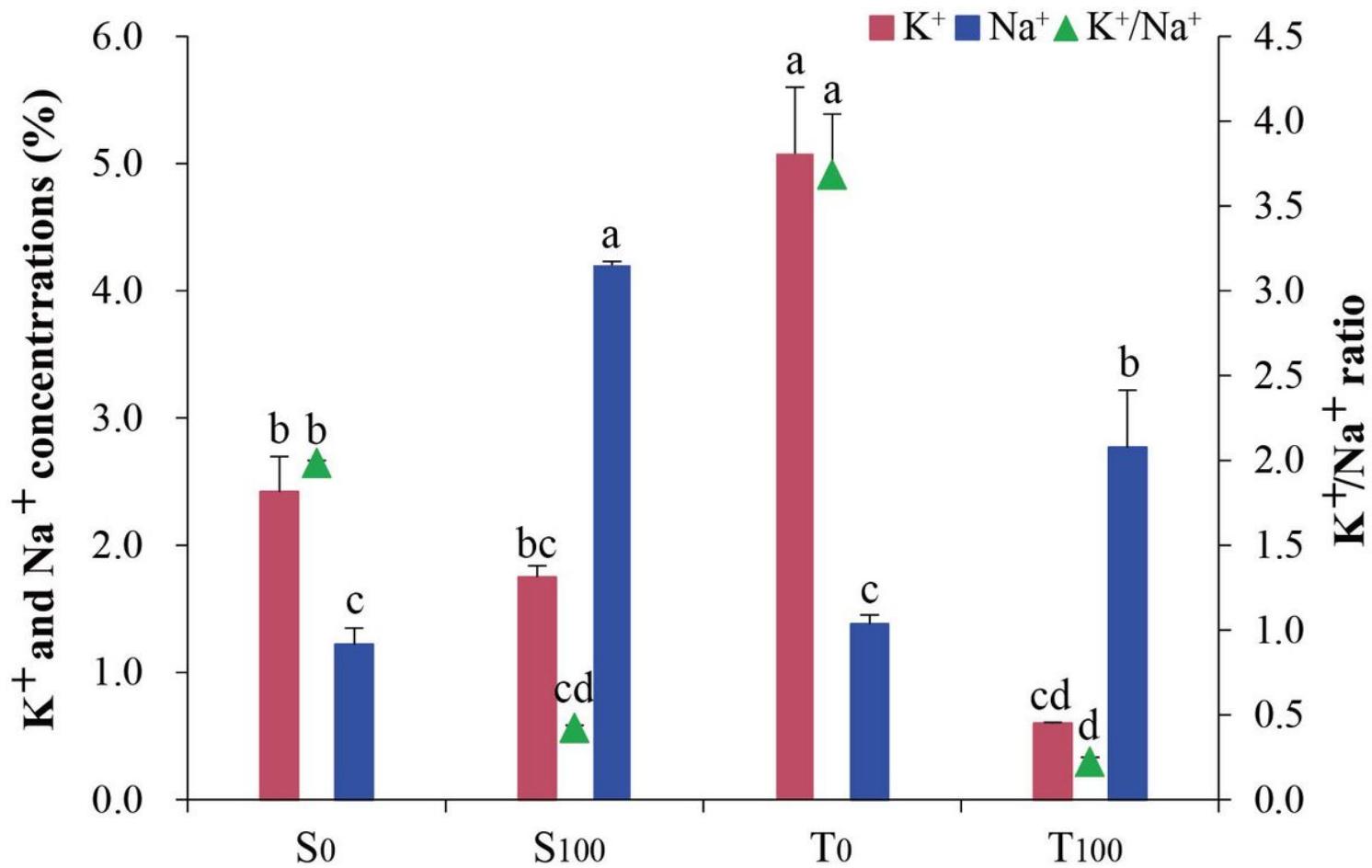


Figure 1

Comparison of the amount of Na⁺, K⁺ accumulations (in %) and the K⁺/Na⁺ ratio in the leaves of the two pak choi cultivars under control and 100 mM NaCl treatments. The results are the means and SDs of three replicates. Different letters above the bars indicate significant difference at the 0.05 level according to Duncan's multiple range test.

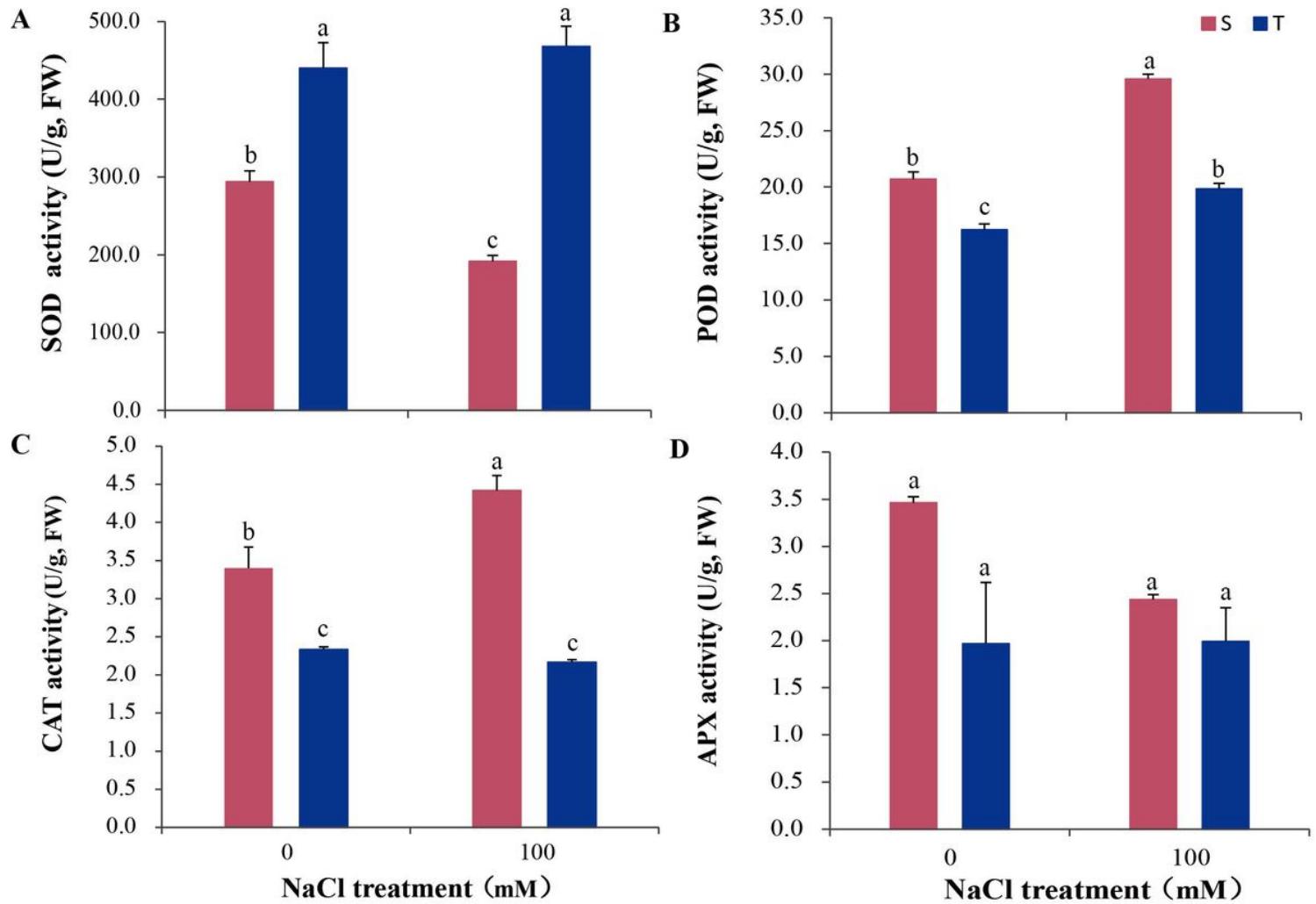


Figure 2

Analyses of dynamic physiological responses under different NaCl treatments. A. SOD activity. B. POD activity. C. CAT activity. D. APX activity. The results are the means and SDs of three replicates. Different letters above the bars indicate significant difference at the 0.05 level according to Duncan's multiple range test.

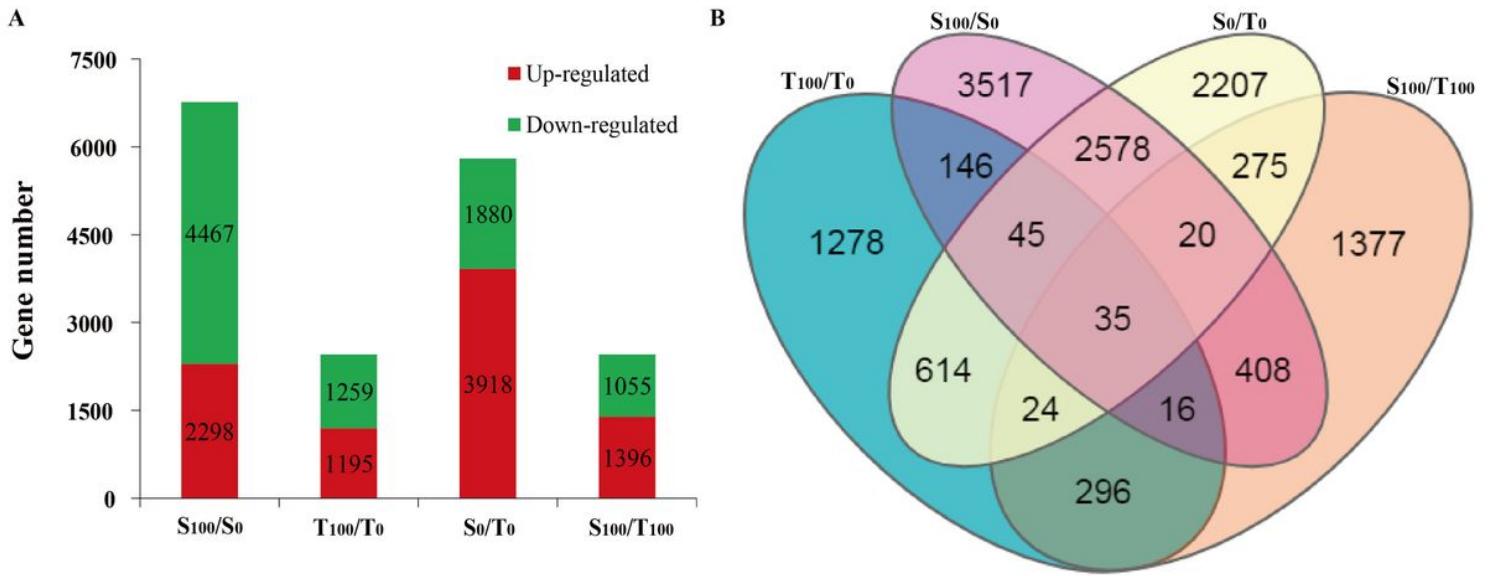


Figure 3

Analysis of DEGs following salt exposure in two pak choi cultivars. (a) The total number of up-regulated and down-regulated genes. (b) Venn diagrams of DEGs.

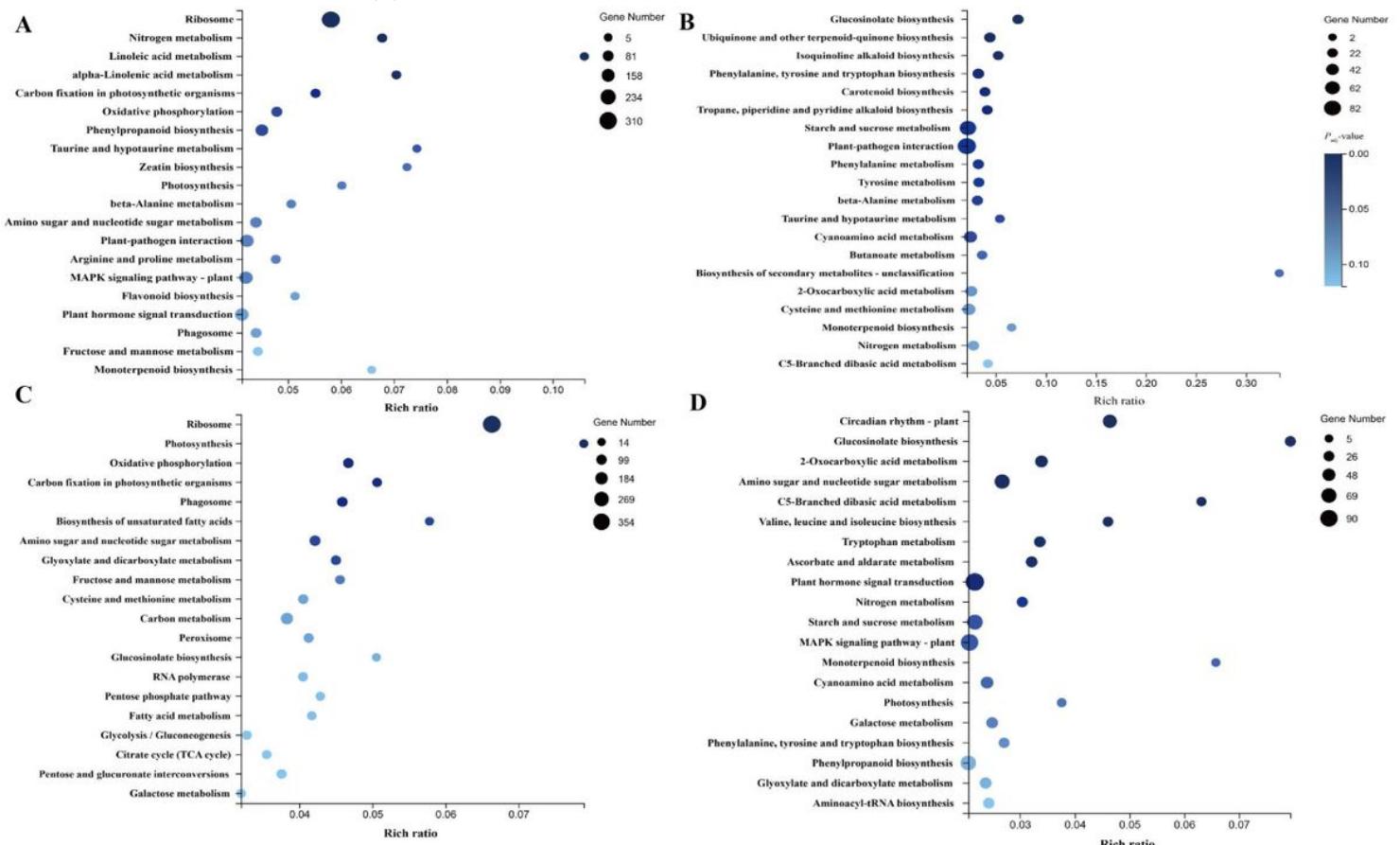


Figure 4

KEGG pathway enrichment analysis based on the differentially expressed genes in four comparisons. (a) S100/S0, (b) T100/T0, (c) S0/T0 and (d) S100/T100.

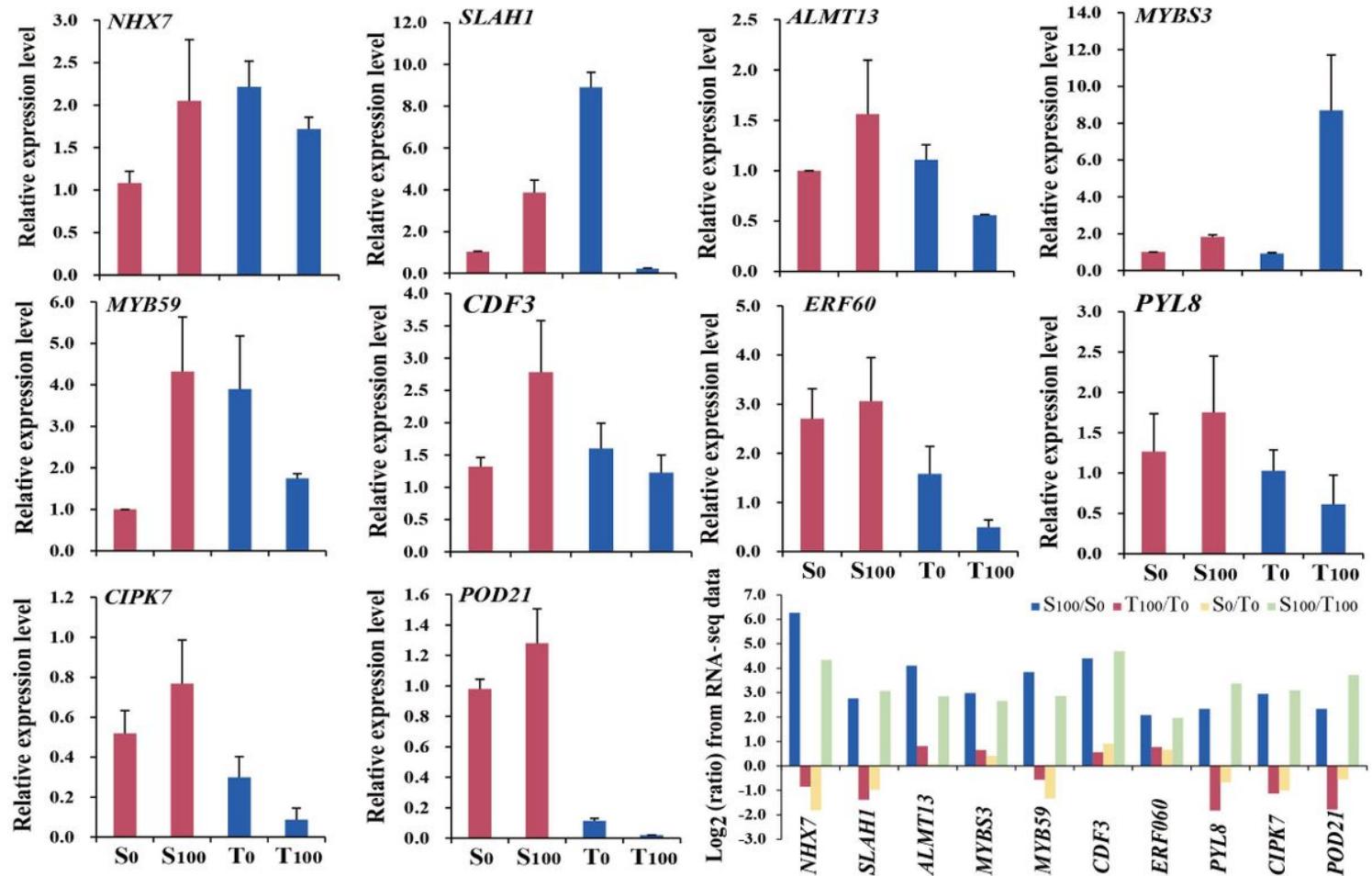


Figure 5

RT-qPCR analyses of 10 candidate DEGs under control and 100 mM NaCl treatments in roots of two pak choi cultivars. Each bar represents the mean \pm SD of triplicate assays. Different letters above the bars indicate significant difference at the 0.05 level according to Duncan's multiple range test.

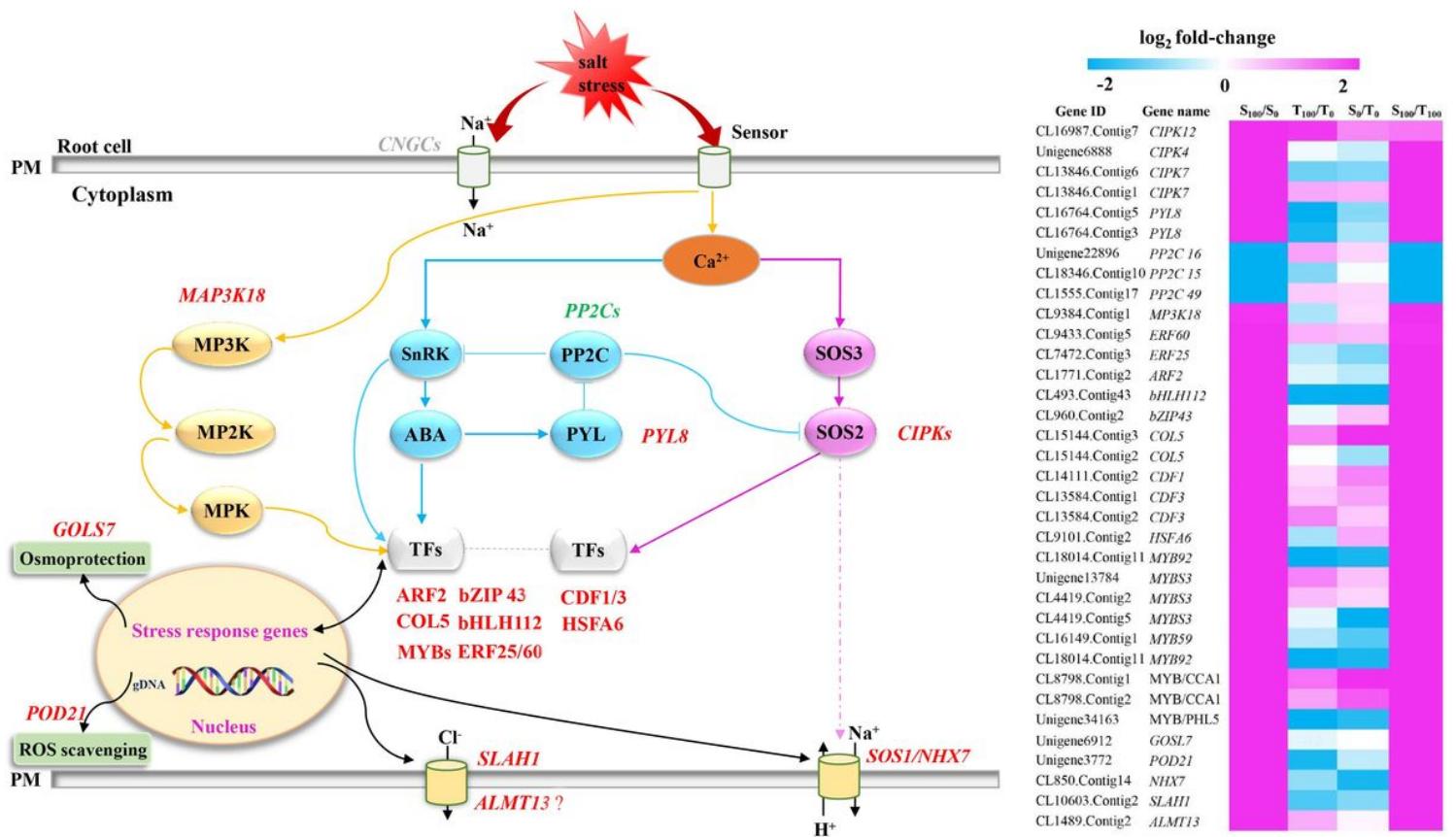


Figure 6

The putative model of the mechanism of salt-tolerance in Shanghaijimaocai compared with Te'aiqing. The grid represents the log₂ fold-change value of each gene in pairwise comparison. DEGs highlighted with red or green colour indicate that they were up or down-regulated by NaCl treatment and showed significantly higher or lower expression in Shanghaijimaocai compared with Te'aiqing.

Supplementary Files

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