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Full Length Article

Transcriptome Analysis of Salt Stress Response in Roots of Halophyte *Zoysia macrostachya*

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Abstract

Zoysia macrostachya Franch. et Sav. is a halophyte with very strong tolerance to salinity, which can serve as an alternative turfgrass for landscaping in saline-alkali land and provide the salt-tolerance genes for turfgrass breeding. To further illustrate the salt-tolerance mechanisms in this species at molecular level, the roots transcriptome of *Z. macrostachya* was investigated under salt stress using the Illumina sequencing platform. Altogether 47,325 unigenes were assembled, among which, 32,542 (68.76%) were annotated, and 87.61% clean reads were mapped to the unigenes. Specifically, 14,558 unigenes were shown to be the differentially expressed genes (DEGs) following exposure to 710 mM NaCl stress compared with control, including 7972 up-regulated and 6586 down-regulated DEGs. Among these DEGs, 24 were associated with the reactive oxygen species (ROS) scavenging system, 61 were found to be related to K^+ and Na^+ transportation, and 16 were related to the metabolism of osmotic adjustment substances. Additionally, 2327 DEGs that encoded the transcription factors (TFs) were also identified. The expression profiles for 10 DEGs examined through quantitative real-time PCR conformed to the individual alterations of transcript abundance verified through RNA-Seq. Taken together, results of transcriptome analysis in this study provided useful insights for salt-tolerance molecular mechanisms of *Z. macrostachya*. Furthermore, these DEGs under salt stress provided important clues for future salt-tolerance genes cloning of *Z. macrostachya*.

Keywords: Analysis; Roots; Salt stress; Transcriptome assemble; *Zoysia macrostachya*

Introduction

Soil salinization is not only a leading cause of the deteriorating ecological environment, but also a major abiotic factor affecting crop yield around the world (Zhu 2001). According to related statistics, there is 4.0×10^8 hm^2 saline-alkali land in the world, of which, an area of 3.6×10^7 hm^2 is located in China (Zhang *et al*. 2007). The cultivation of new salt-tolerance plant varieties is an effective way to utilize the saline-alkali land. Meanwhile, investigating the salt-tolerance mechanisms in plant and identifying the salt-tolerance genes can lay a solid foundation for cultivating the new salt-tolerance plant varieties.

Research on the salt-tolerance mechanisms of plants had been carried out over the past many decades. Selective ion absorption and compartmentalization play key roles in maintaining ion homeostasis in cytoplasm. For instances, salt overly sensitive (SOS) and Na^+/H^+ antiporter (NHX) can keep lower Na^+ content in cytoplasm (Zhu 2003), whereas the K^+ transporters with high affinity (HKTs) can improve K^+ and limit Na^+ transportation from root to leaf (Tang *et al*. 2015). Salt stress can induce ROS production, including hydrogen peroxide (H_2O_2), superoxide radicals ($\bullet O_2^-$) and hydroxyl radicals (•OH), eventually causing oxidative damage to cytomembrane (Miller *et al*. 2008). For alleviating the ROS-induced peroxidation damage, two kinds of ROS scavenging systems have evolved in plant, including the non-enzymatic antioxidants and the enzymatic antioxidants. The non-enzymatic antioxidants include glutathione (GSH) and ascorbic acid (AsA) etc.; whereas the enzymatic antioxidants consist of peroxidase (POD), catalase (CAT) and superoxide dismutase (SOD) etc., and they play crucial roles in altering the ROS homeostasis (Deinlein *et al*. 2014). Salt stress leads to imbalanced osmotic regulation; as a result, some osmolytes, such as free proline, sugar and betaine, are synthesized in cells to regulate the osmotic balance in plants (Ingram and Bartels 1996; Ashraf and Foolad 2007). In addition, the expression of salt-tolerance genes in plants are regulated by transcription factors (TFs), and many of which, including DREB, MYB, AP2/ERF and NAC families etc, exert vital parts in plant tolerance to salt stress (Deinlein *et al*. 2014).

Halophyte is a plant that grows regularly and completes the life cycles under the single salt concentration of >70 $mmol\cdot L^{-1}$ (Flowers and Colmer 2008). *Z. macrostachya*, the perennial warm-season turfgrass native

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to China, Japan as well as Korean Peninsula, and mainly grows in the coastal wetlands of Shandong, Jiangsu and Zhejiang provinces in China. *Z. macrostachya* can rapidly spread through rhizomes and stolons to form the dense turf with the deep root system, which can thereby be used as the soil-conserving, dike-protecting and sand-fixing turf. Moreover, *Z. macrostachya* is an euhalophyte, which exhibits tolerance to salinity and may be potentially used in landscaping of saline-alkali land. According to our previous research, *Z. macrostachya* tolerated 355 mM NaCl stress (Hu and Zhang 2010) and further research found that the roots of *Z. macrostachya* limited Na^+ absorption while improved K^+ absorption and transportation from roots to leaves, accumulated free proline and soluble sugars and enhanced the POD activity under salt stress, and all of these improved the salt-tolerance of *Z. macrostachya* (Hu and Zhang 2009, 2010; Hu *et al*. 2016). Nonetheless, the above studies are limited to physiological indexes, and the molecular mechanisms of salt-tolerance in this species remain unclear so far.

RNA-seq has emerged as a powerful tool to analyze genes expressional changes, which reflects the molecular mechanisms of plant response to salt stress, and has been utilized for investigating the molecular mechanisms of salt-tolerance in many halophytes, such as *Iris lactea var. Chinensis* (Gu *et al*. 2018), *Prunellae Spica* (Liu *et al*. 2020) and *Rhizophora mucronata* (Meera and Augustine 2020). To date, changes in global genes expression of *Z. macrostachya* under salt stress are still unknown, which limits the understanding towards the molecular mechanisms of salt-tolerance in this species. In this study, the Illumina HiSeq XTen sequencing platform was used to generate a roots reference transcriptome dataset and to explore DEGs with aims to improve our comprehensive understanding of the mechanisms of salt-tolerance at transcriptome level and to identify the DEGs involved in the salt-tolerance of *Z. macrostachya.*

Materials and Methods

Plant material, salt stress and RNA extraction

Zoysia macrostachya samples were collected from coastal wetland located 45.0 km east of Yancheng of Jiangsu province, China. Samples were brought back to the laboratory and planted in six PVC tubes (35 cm in length and 15 cm in diameter) filled with river sand, respectively. The plants were grown in a growth chamber with a 12 h light/12 h dark cycle, 30/20℃ day/night temperature, 800 μmol $m^{-2}·s^{-1}$ light intensity and a relative humidity of 80%. The plants were watered at intervals of three days, and irrigated weekly with 200 mL 1/2 Hoagland's nutrient solution during growth. After 30 d of growth, the plants were pulled out from the tubes and river sand were washed by water. Then, the roots of *Z. macrostachya* were soaked in 710 mM NaCl solution (treatment) and distilled water (control), respectively. Three biological replications were set for each treatment, three treatments were marked as $treatment_1$, $treatment_2$, $treatment_3$; and three controls were marked as $control_1$, $control_2$, $control_3$. After 8 h, 3 cm root tip were harvested and stored in liquid nitrogen for extracting RNA. Total RNA was extracted from three treatment and three control samples, respectively, using a mirVana miRNA Isolation Kit (Ambion) in accordance with the manufacturer's protocol.

Preparation and sequencing of cDNA library

The integrity of RNA was assessed by the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Afterwards, all samples with the RNA Integrity Number (RIN) of ≥7 were used for constructing cDNA libraries. Six cDNA libraries were constructed by the TruSeq Stranded mRNA LTSample Prep Kit (Illumina, San Diego, CA, USA) in accordance with the manufacturer's protocol. The above libraries were sequenced using the Illumina HiSeq XTen system to generate the 125/150 bp reads with paired-end.

Quality control and *de novo* assembly

Original data (raw reads) were processed by Trimmomatic (Bolger *et al*. 2014). Reads that contained ploy-N and were of low quality were eliminated to obtain clean reads. Later, the adaptor and sequences of low quality were removed, clean reads were assembled into expression sequence tag clusters (contigs), then *de novo* assembled into transcript through Trinity (version: trinityrnaseq_r20131110) according to the paired-end approach (Grabherr *et al*. 2011). Subsequently, transcript with the greatest length was selected as the unigene based on the sequence length and similarity.

Function annotations

Unigene functions were annotated through aligning them with SwissProt protein, NCBI non-redundant protein (NR), Clusters of orthologous groups for eukaryotic complete genomes (KOG) and Pfam databases using Blastx (Altschul *et al*. 1990), with the cut-off E-value of 10^{-5}. Typically, those proteins with the greatest hits to the above unigenes were utilized for assigning the functional annotations. According to SwissProt annotations, the gene ontology (GO) analysis was performed based on mapping relation between SwissProt and GO terms. All unigenes were mapped to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database for annotating the underlying metabolic pathways (Kanehisa *et al*. 2008).

Unigenes quantification, analysis of DEGs, cluster analysis, GO and KEGG enrichment analyses

FPKM value (Trapnell *et al*. 2010), together with the read counts for every unigene, were computed through eXpress

(Roberts and Lior 2013) and bowtie2 (Langmead and Salzberg 2012). Afterwards, DEGs were identified through nbinom Test and DESeq (Anders and Huber 2012) functions estimate Size Factors. Unigenes with P-value of ≤0.05 and $\log_2$foldChange of ≥1 were selected as thresholds of significant DEGs. Later, DEGs were performed hierarchical cluster analysis for exploring the expression profiles of transcripts. Then, the DEGs were carried out for GO enrichment and KEGG pathway analyses using the R software based on the hypergeometric distribution.

Annotation of TFs

The Hidden Markov Model (HMM) motif sequences of TFs were obtained based on Plant Transcription Factor Database (TFDB), which contained 58 TF families of green plant (Jin *et al*. 2014). Specifically, one unigene with ≥90% sequence homology was annotated as the putative TF (E-value of ≤$1E^{-10}$). Thereafter, DEGs, together with the candidate TFs in reference transcriptome, were clustered according to the TF families.

Quantitative real-time PCR (qRT-PCR)

For further validating the results of transcriptomic analyses, 10 DEGs including 1 POD-related, 2 SOD-related, 2 KEA-related, 2 KUP-related, 1 KOC-related, 1 Proline-related and 1 Betaine-related DEGs were selected for qRT-PCR analysis. *Z. macrostachya* were in exposure of 710 mM NaCl solution and distilled water for 8 h, respectively, then the root RNA were extracted according to the method mentioned above. Reverse transcription reactions were performed using SuperScript III reverse transcriptase (Invitrogen, Grand Island, NY, USA) following the manufacturer's instructions. Primers (Table 1) for qRT-PCR were designed using the Premier v5.0 software (Premier Biosoft, Palo Alto, CA, USA) with β-actin genes as the internal controls. The qRT-PCR was carried out by the Two-color Real-time PCR Detection System (Bio-Rad, USA) following amplification protocol: 3 min at 95°C, and then 3 s at 95°C and 30 s at 60°C for 45 cycles. All reactions were performed in triplicate, the relative expression levels of the selected unigenes normalized to β-actin was calculated using the $2^{-\triangle\triangle Ct}$ method (Livak and Schmittgen 2001).

Results

Sequencing results and assembly

A mean of 49.49 million raw reads were produced from controls, whereas 49.44 million raw reads were produced from 710 mM NaCl treatments (Table 2). Over 93.99% of all raw reads possessed the Phred-like quality score of Q30 level (error probability=1‰). An average of 48.23 million (controls) and 48.19 million (treatments) clean reads were obtained, and the valid base ratio and GC content were above

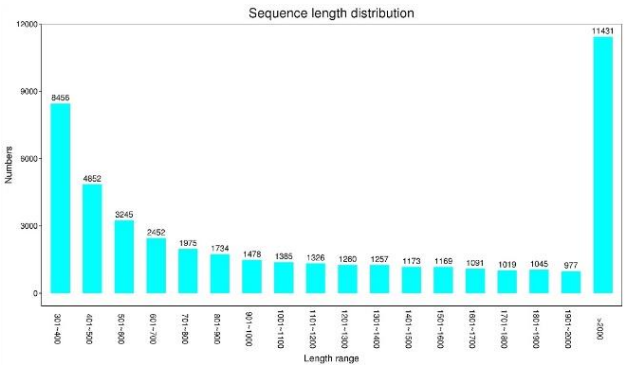

Fig. 1: Unigene length distribution

95.28 and 51.05%, respectively, indicating high quality of sequencing and cDNA library establishment. A total of 4,7325 unigenes with mean length of 1397 bp and the N50 of 2169 bp were obtained. As shown in Fig. 1, 24,192 unigenes had the length of 301–1000 bp, 10,317 had the length of 1001–2000 bp, and 11,431 had the length of >2000 bp.

Clean reads of each sample were mapped to *Z. macrostachya* unigenes to confirm the quality of sequencing for those six samples (Table 3). As observed, the average total mapped reads proportion was 87.61% (range, 86.98–88.44%), and the multi-position matched reads accounted for approximately 21.65%, and 64.89–67.06% reads were uniquely matched unigenes in all the six samples. An average of 81.43% reads was mapped in pairs.

Functional annotations of unigenes

Unigenes were annotated by aligning to the publicly accessible databases (Table 4). Estimated number of 32,033 (67.69%) unigenes were aligned in the NR database, 24,155 (51.04%) in the Swissprot database, 11,601 (24.51%) in the KEGG database, 17,906 (26.78%) in the KOG database, 22,309 (47.14%) in the GO database, and 51(0.11%) in the Pfam database. Altogether 32,542 unigenes (68.76%) were annotated against at least one of the following databases, including NR, SwissProt, KEGG, Pfam, GO and KOG.

According to the homology of sequences, 22,309 unigenes were clustered to 3 major GO classifications, including biological process, cellular component and molecular function (Fig. 2). These unigenes were subdivided into 57 GO terms. Among biological process classifications, the term of "biological regulation", "cellular process", "metabolic process", "regulation of biological process" and "responses to stimulus" were the dominant clusters, whereas only a few unigenes belonged to the "biological adhesion", "cell killing", "locomotion" and "rhythmic process" term. With regard to the cellular component classification, the term of "cell", "cell part" and "organelle" had the bigger unigenes proportions. As to the molecular function, most unigenes were classified into the "binding", "catalytic activity" and "transporter activity"

Table 1: The qRT-PCR primers for detecting the accuracy of transcriptome data

Unigene number	Unigene name	Annotated as	Forward primers (5'-3')	Reverse primers (5'-3')
1	TRINITY_DN11437_c0_g1_i1_2	POD	GCAAGTCAAGCACCTCAACCT	TGATTGGAATGGTCTGCTGGGA
2	TRINITY_DN10853_c0_g1_i2_1	SOD	GACTGAATCTCTACGCCTGT	GACGACGTATGGCACCAGAG
3	TRINITY_DN19735_c0_g1_i6_2	SOD	AGCTGCTCCATTGCCATTCCT	CCGAACCTCCTGTAAGTCAACC
4	TRINITY_DN19255_c0_g2_i4_1	KEA	TAGTAAGGGAGAATCTTTGCAAG	ATGGCAGATCCGTGACAAGTC
5	TRINITY_DN22016_c0_g1_i24_2	KEA	TCACCGCCATCCCAGTCATC	CCCAAATACACGCACTCCTG
6	TRINITY_DN16474_c0_g3_i4_2	KUP	CTTGTCGACCAACATTCACACC	CGAGTAAGCAACGGTCCA
7	TRINITY_DN16569_c0_g2_i2_1	KUP	ACTGGACGAGGAGCAACACT	TCTCACTCACTCCTCCAGAGC
8	TRINITY_DN16911_c0_g2_i5_2	KOC	TCCCAAACCAGCTTCACCGATT	CACCAGAGTATGCCTCGACAT
9	TRINITY_DN20583_c0_g1_i5_1	Free proline	TTACAACGGTTCGCAACTGT	TGCGTGGACTACTCAGAGACC
10	TRINITY_DN15030_c0_g1_i1_1	Glycine betaine	TCTCCTCATCCTCACCTCAT	ATTGCCAGTTCCTAGTGTTCC

Table 2: Summary of the sequence analysis

Sample	Raw reads	Raw bases	Clean reads	Clean bases	Valid bases	Q30	GC
$Control_1$	49536428	7430464200	48215536	7090647459	95.43%	94.42%	51.16%
$Control_2$	49533550	7430032500	48398632	7115121942	95.76%	94.68%	51.84%
$Control_3$	49424632	7413694800	48083930	7063618562	95.28%	93.99%	51.40%
$Treatment_1$	49686582	7452987300	48368100	7120357990	95.54%	94.22%	51.59%
$Treatment_2$	49108932	7366339800	47923128	7058090438	95.82%	94.58%	51.05%
$Treatment_3$	49536672	7430500800	48291630	7112106699	95.72%	94.38%	51.26%

Table 3: Mapping of clean reads to unigenes

Terms\Samples	$Control_1$	$Control_2$	$Control_3$	$Treatment_1$	$Treatment_2$	$Treatment_3$
Total reads	48215536 (100.00%)	48398632 (100.00%)	48083930 (100.00%)	48368100 (100.00%)	47923128 (100.00%)	48291630 (100.00%)
Total mapped reads	42448603 (88.04%)	42802853 (88.44%)	42159835 (87.68%)	42069655 (86.98%)	41718189 (87.05%)	42224960 (87.44%)
Multiple mapped	10306193 (21.38%)	10344313 (21.37%)	10207699 (21.23%)	10490280 (21.69%)	10622363 (22.17%)	10640511 (22.03%)
Uniquely mapped	32142410 (66.66%)	32458540 (67.06%)	31952136 (66.45%)	31579375 (65.29%)	31095826 (64.89%)	31584449 (65.40%)
Reads mapped in proper pairs	39451806 (81.82%)	39954228 (82.55%)	39326280 (81.79%)	38891334 (80.41%)	38763888 (80.89%)	39160458 (81.09%)

Table 4: Blast analysis of non-redundant unigenes against public databases

Databases for annotation	Unigene numbers	Percentages	300≤ Length<1000nt	Length≥1000nt
Annotated in NR	32033	67.69 %	10665	21368
Annotated in Swissprot	24155	51.04 %	6615	17540
Annotated in KEGG	11601	24.51 %	3571	8030
Annotated in KOG	17906	37.84 %	4774	13132
Annotated in GO	22309	47.14 %	6231	16078
Annotated in Pfam	51	0.11 %	41	10
Annotated in at least one database	32542	68.76%	21547	12995

term. Interestingly, 225 unigenes were categorized into the "antioxidant activity" term, while 894 as the "transporter activity" term.

The KOG database was searched to identify unigenes to predict and classify their functions. Based on the sequence homology, 17,906 sequences showed a KOG classification (Fig. 3). Among the 25 KOG clusters, the cluster of "general function prediction only" (3674, 20.52%) was the largest group, followed by the "posttranslational modification, protein turnover, chaperones" (2180, 12.17%), the "signal transduction mechanisms" (1821, 10.17%) and the "translation, ribosomal structure and biogenesis" (1266, 7.07%). By contrast, the clusters of the "cell motility" (3, 0.17‰), the "nuclear structure" (51, 0.28%) and the "extracellular structures" (52, 0.29%) were smaller groups.

A total of 11,601 annotated unigenes were mapped to the reference canonical pathways in the KEGG database (Fig. 4). The most represented pathways were "metabolism" (5711 unigenes), "genetic information processing" (2822 unigenes), "cellular processes" (1430 unigenes) and "environmental information processing" (1298 unigenes). In addition, "translation" (1335 unigenes), "signal transduction" (1216 unigenes) and "carbohydrate metabolism" (1049 unigenes) were the pathways most closely related to the mapped unigenes, which suggested the presence of active pathways in Z. *macrostachya*.

Recognition and annotation of DEGs

A total of 14,558 DEGs were recognized, among which, 7972 were up-regulated and 6586 were down-regulated

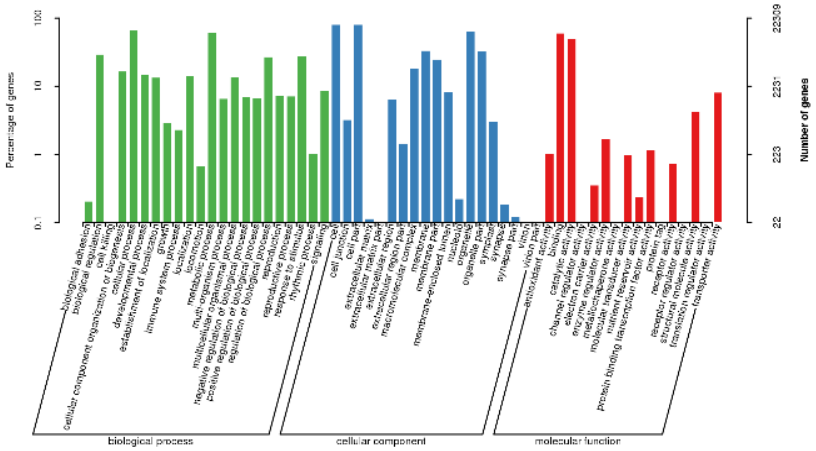

Fig. 2: GO categorization of unigenes

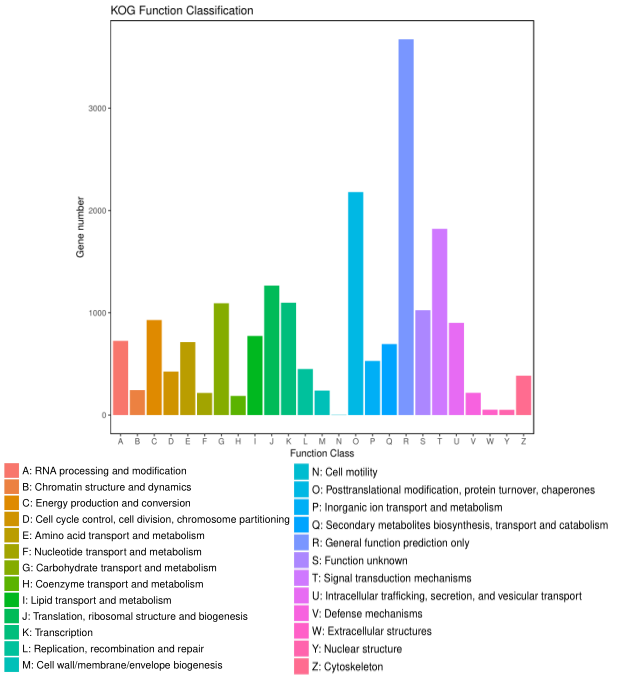

Fig. 3: KOG classification of unigenes

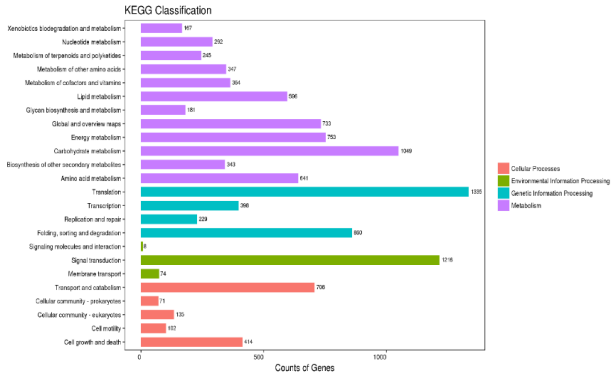

Fig. 4: KEGG categorization of unigenes

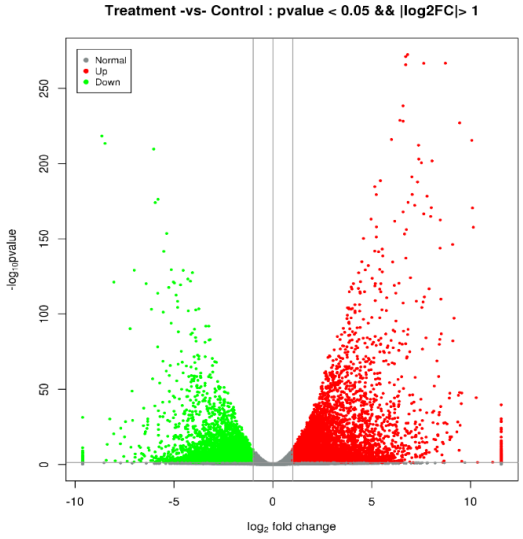

Fig. 5: Volcano plots of DEGs

(Fig. 5). According to our findings, 7424 DEGs were enriched into 64 GO terms. With regard to biological process, the term of "cellular process", "metabolic process", "biological regulation", "regulation of biological process" and "response to stimulus" had the greater unigenes proportions. As to cellular component, the "membrane", the "cell part" and the "cell" term were the dominant clusters. The "binding", "catalytic activity" and "transporter activity" term contained more unigenes in the molecular function category (Fig. 6).

In addition, DEGs were performed for KEGG analysis. The results suggested that, 2093 DEGs were assigned with the KEGG ID, which were classified into 197 pathways (Fig. 7). Typically, the most representative pathways were the "environmental information processing-signal transduction" (370 unigenes), the

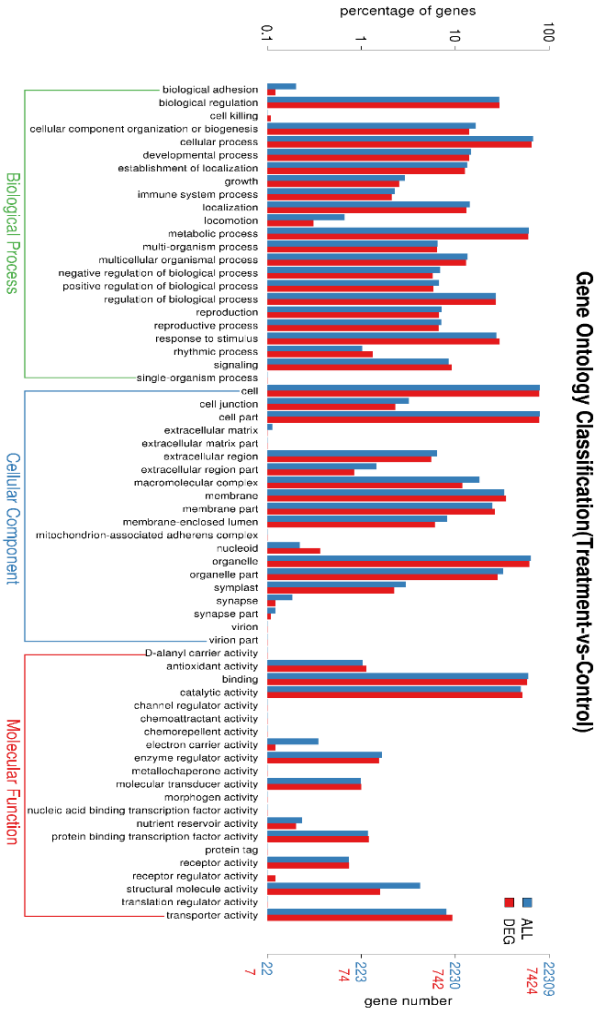

Fig. 6: GO categorization of DEGs in *Z. macrostachya*. under salt stress

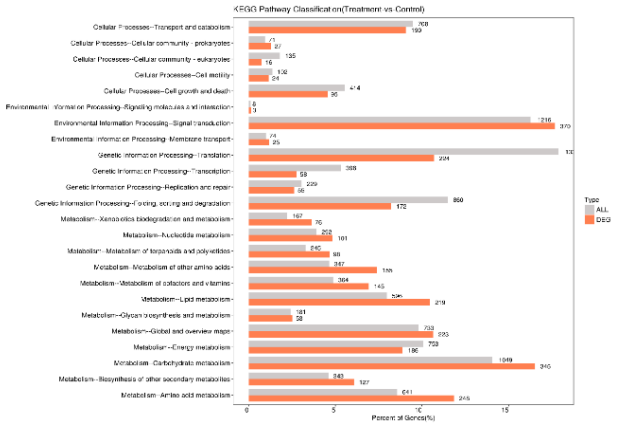

Fig. 7: KEGG categorization of DEGs in *Z. macrostachya* under salt stress

"metabolism-amino acid metabolism" (248 unigenes), the "genetic information processing-translation" (224 unigenes), the "metabolism-global and overview maps" (223 unigenes) and the "metabolism-lipid metabolism" (219 unigenes).

DEGs associated with ROS scavenging

A total of 24 DEGs were identified to encode ROS scavenging-associated enzymes (Table 5). Among which, 12 encoded PODs and constituted the largest group. The expression of TRINITY_DN11379_c0_g1_i1_2, TRINITY_DN11437_c0_g1_i1_2 and TRINITY_DN12963_c0_g1_i2_2 increased under salt stress, with high transcript levels. Four genes encoded SODs, the expression of one gene increased, while that of the other three genes decreased. Two genes encoded CATs, among which, TRINITY_DN21281_c0_g4_i5_2 showed higher transcript abundance, but TRINITY_DN21281_c0_g1_i8_2 was down-regulated under salt stress. Additionally, five genes were found to encode Ascorbate peroxidase (APX) and one gene encoded glutathione peroxidase (GPX). The encoded APX gene TRINITY_DN19584_c0_g2_i1_2 was up-regulated by nearly 26 times (FC 26.80), with the highest transcript levels.

DEGs involved in ion transportation

Fifty-two DEGs were identified to be related to the regulation of K^+ transportation (Table 6), which accounted for the greatest proportion of identified genes. 37 out of them were up-regulated, while 15 were down-regulated under salt stress. Eleven of them were involved in K^+ efflux antiporter (KEA), twenty were related to K^+ transmembrane transporter (KUP), three were associated with K^+ channel (AKT), nine were involved in the outward rectifying K^+ channel (KOC) and nine were related to cyclic nucleotide-gated channel (CNGC). Three genes were identified to encode the plasma membrane P-ATPases (PM-H^+-ATPases), while five encoded the vacuolar V-ATPases (V-H^+-ATPases), and these unigenes were up-regulated. Two unigenes that encoded Na^+/H^+ exchanger (NHX) were also identified, but their expressions levels were down-regulated.

DEGs associated with osmotic adjustment

In this study, 12 DEGs were identified to involve in free proline metabolism, eight out of them were up-regulated, while four were down-regulated under salt stress (Table 7). Four DEGs were identified to involve in Glycine betaine metabolism, 3 out of them were up-regulated, only TRINITY_DN15030_c0_g1_i1_1 was down-regulated under salt stress (Table 7).

DEGs related to TFs

A total of 2,327 DEGs were annotated to the TFs database, which belonged to 57 families (Fig. 8). 604 out of them were annotated to the BHLH family, which accounted for

Table 5: DEGs related to the ROS scavenging system

Gene ID	Log_2FC	P-value	Gene ID	Log_2FC	P-value
POD			SOD		
TRINITY_DN10724_c0_g1_i2_1	-1.61	1.46E-02	TRINITY_DN10853_c0_g1_i2_1	-2.67	1.13E-11
TRINITY_DN11379_c0_g1_i1_2	4.18	2.69E-11	TRINITY_DN19735_c0_g1_i6_2	1.97	3.98E-04
TRINITY_DN11437_c0_g1_i1_2	5.43	9.90E-03	TRINITY_DN699_c0_g1_i1_2	-1.13	5.85E-06
TRINITY_DN11915_c0_g1_i1_1	-2.11	1.28E-25	TRINITY_DN7158_c0_g1_i2_1	-2.22	1.73E-11
TRINITY_DN12563_c0_g1_i1_1	-3.11	2.07E-16	APX		
TRINITY_DN12830_c0_g1_i3_1	1.20	4.15E-07	TRINITY_DN18445_c0_g1_i11_1	-4.10	1.56E-08
TRINITY_DN12963_c0_g1_i2_2	4.89	5.57E-14	TRINITY_DN18597_c0_g2_i1_2	1.56	1.30E-02
TRINITY_DN13326_c0_g1_i1_2	1.42	2.82E-02	TRINITY_DN19584_c0_g1_i3_2	1.99	1.39E-29
TRINITY_DN13354_c0_g1_i1_1	-3.66	5.97E-12	TRINITY_DN19584_c0_g2_i1_2	4.74	1.61E-03
TRINITY_DN14879_c0_g3_i1_1	-2.59	1.10E-15	TRINITY_DN9766_c0_g1_i2_2	-2.91	7.99E-46
TRINITY_DN14924_c0_g1_i1_1	-2.67	2.84E-02	GPX		
TRINITY_DN15006_c1_g1_i1_1	2.78	7.16E-11	TRINITY_DN13470_c0_g1_i1_1	1.21	9.32E-05
CAT					
TRINITY_DN21281_c0_g1_i8_2	-2.45	5.95E-44			
TRINITY_DN21281_c0_g4_i5_2	2.28	3.62E-32			

Table 6: DEGs related to K^+ and Na^+ ion transport

Gene ID	Log_2FC	P-value	Gene ID	Log_2FC	P-value
KEA			KOC		
TRINITY_DN14177_c0_g1_i4_2	-1.65	9.15E-22	TRINITY_DN14314_c0_g1_i7_1	1.52	1.39E-21
TRINITY_DN14664_c0_g1_i3_2	1.82	2.80E-07	TRINITY_DN16211_c0_g1_i2_2	2.52	5.34E-06
TRINITY_DN15615_c0_g1_i2_2	-1.10	2.96E-04	TRINITY_DN16911_c0_g2_i5_2	7.38	1.02E-203
TRINITY_DN17765_c1_g1_i9_1	3.65	5.91E-08	TRINITY_DN19502_c0_g3_i1_2	1.21	5.76E-06
TRINITY_DN17765_c1_g2_i5_1	-1.06	3.66E-02	TRINITY_DN20260_c0_g1_i20_2	2.08	3.86E-32
TRINITY_DN19255_c0_g1_i3_1	-3.11	3.36E-06	TRINITY_DN20505_c0_g1_i1_1	3.24	4.61E-54
TRINITY_DN19255_c0_g2_i4_1	-3.14	7.72E-05	TRINITY_DN20585_c1_g2_i17_2	1.17	2.61E-05
TRINITY_DN21559_c1_g1_i5_2	2.23	1.58E-18	TRINITY_DN22190_c2_g1_i9_2	3.48	6.03E-49
TRINITY_DN21559_c1_g5_i3_2	1.85	1.67E-05	TRINITY_DN22190_c2_g2_i1_2	3.45	3.48E-15
TRINITY_DN22016_c0_g1_i24_2	3.69	2.89E-12	CNGC		
TRINITY_DN22016_c0_g2_i3_2	1.12	1.64E-04	TRINITY_DN12577_c0_g1_i1_2	2.10	6.61E-23
KUP			TRINITY_DN19156_c0_g1_i14_2	1.99	1.11E-05
TRINITY_DN15916_c0_g1_i8_1	1.80	5.39E-04	TRINITY_DN19236_c0_g1_i7_2	2.09	1.67E-37
TRINITY_DN16437_c0_g1_i1_2	1.74	1.27E-07	TRINITY_DN20366_c0_g3_i7_1	-1.15	1.52E-04
TRINITY_DN16474_c0_g2_i1_2	2.08	6.62E-05	TRINITY_DN20693_c0_g1_i17_1	-2.42	3.16E-08
TRINITY_DN16474_c0_g3_i4_2	4.16	9.84E-103	TRINITY_DN21311_c0_g1_i4_2	1.53	7.62E-09
TRINITY_DN16569_c0_g2_i2_1	-2.69	1.11E-08	TRINITY_DN21317_c0_g1_i1_2	1.34	1.39E-09
TRINITY_DN16999_c0_g1_i4_1	2.05	8.74E-26	TRINITY_DN21317_c0_g2_i2_2	1.27	1.29E-07
TRINITY_DN18621_c0_g1_i6_2	1.78	4.17E-09	TRINITY_DN22290_c0_g1_i1_2	1.58	1.26E-06
TRINITY_DN18799_c0_g1_i2_2	-1.65	6.76E-07	P-ATPase		
TRINITY_DN19410_c0_g1_i7_1	1.17	1.81E-02	TRINITY_DN20505_c0_g2_i2_1	4.50	1.70E-135
TRINITY_DN19574_c0_g1_i1_2	2.07	3.17E-14	TRINITY_DN20585_c1_g2_i17_2	1.17	2.61E-05
TRINITY_DN19850_c0_g1_i9_2	2.38	3.45E-23	TRINITY_DN22190_c2_g1_i9_2	3.48	6.03E-49
TRINITY_DN19858_c0_g1_i37_2	2.97	4.48E-65	V-ATPase		
TRINITY_DN19995_c2_g1_i15_1	-1.30	2.63E-06	TRINITY_DN13353_c0_g1_i2_2	5.91	2.81E-35
TRINITY_DN21041_c0_g1_i3_2	1.94	5.25E-31	TRINITY_DN15633_c0_g1_i2_2	1.64	2.75E-09
TRINITY_DN22105_c0_g1_i4_2	3.27	1.64E-54	TRINITY_DN14314_c0_g1_i7_1	1.52	1.39E-21
TRINITY_DN24434_c0_g1_i1_1	-2.95	1.47E-04	TRINITY_DN20260_c0_g1_i20_2	2.08	3.86E-32
TRINITY_DN4618_c0_g1_i1_1	-3.05	6.68E-35	TRINITY_DN20505_c0_g1_i1_1	3.24	4.61E-54
TRINITY_DN5960_c0_g1_i1_1	1.95	4.30E-20	NHX		
TRINITY_DN7740_c0_g1_i1_1	-3.82	6.23E-05	TRINITY_DN14878_c0_g1_i1_2	-1.02	1.16E-03
TRINITY_DN7928_c0_g1_i2_1	-2.28	1.36E-10	TRINITY_DN9153_c0_g1_i2_1	-2.51	2.12E-04
AKT					
TRINITY_DN13806_c0_g1_i1_1	-2.04	3.70E-08			
TRINITY_DN13806_c0_g2_i4_1	-2.04	1.73E-02			
TRINITY_DN24232_c0_g1_i1_1	1.71	2.59E-09			

the largest group, followed by NAC (470 DEGs), MYB-related (402 DEGs) and ERF (356 DEGs) family. The LSD (5 DFGs), LFY (5 DEGs) and SAP (1 DEGs) family had the least corresponding unigenes.

Validation of DEGs relative expression by qRT-PCR

The qRT-PCR results showed the expression patterns of 10 selected DEGs in good agreement with the relative expression results at transcriptome level (Fig. 9). This indicated that transcriptome data of *Z. macrostachya* roots obtained in this experiment were accurate.

Discussion

In this study, the roots informative transcriptome dataset for *Z. macrostachya* after NaCl treatment revealed that 68.76% of the 4,7325 unigenes were annotated by BLAST analysis,

Table 7: DEGs related to osmotic adjustment substances

Gene ID	Log_2FC	P-value
Free proline		
TRINITY_DN12426_c0_g2_i1_1	-2.54	2.81E-02
TRINITY_DN16249_c0_g1_i5_2	5.09	7.53E-51
TRINITY_DN16737_c0_g1_i1_2	4.69	3.33E-17
TRINITY_DN20583_c0_g1_i5_1	6.72	7.84E-272
TRINITY_DN22133_c0_g1_i14_2	3.78	3.74E-60
TRINITY_DN22200_c0_g1_i19_2	7.87	0.00E+00
TRINITY_DN20075_c0_g1_i5_1	2.19	8.55E-36
TRINITY_DN20857_c1_g4_i6_1	2.40	1.83E-47
TRINITY_DN19370_c0_g1_i3_1	-2.85	1.21E-08
TRINITY_DN20069_c0_g1_i5_2	1.74	8.26E-22
TRINITY_DN15955_c0_g1_i5_1	-1.31	5.68E-03
TRINITY_DN19808_c0_g2_i7_1	-2.45	7.36E-10
Glycine betaine		
TRINITY_DN12892_c0_g1_i2_1	2.29	1.74E-22
TRINITY_DN15030_c0_g1_i1_1	-2.86	5.60E-05
TRINITY_DN18859_c0_g1_i5_2	1.82	9.97E-04
TRINITY_DN12184_c0_g1_i3_2	2.14	9.46E-19

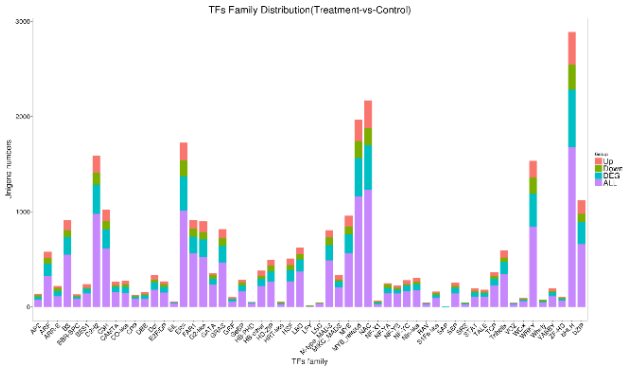

Fig. 8: Distribution of TFs family

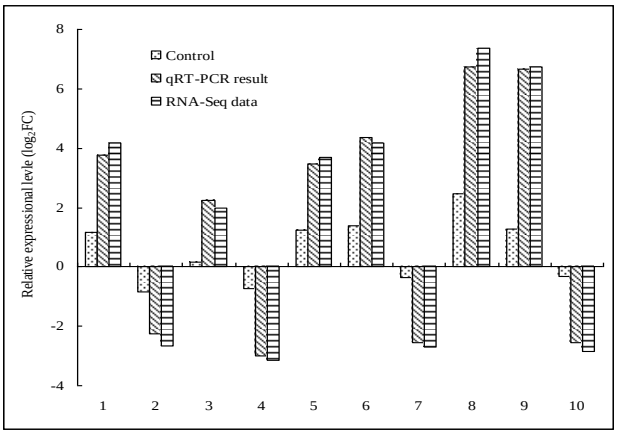

Fig. 9: Expression pattern validation of selected genes by qRT-PCR

which suggested that the sequences of the *Z. macrostachya* unigenes generated in the present study were assembled and annotated correctly. A total of 14,558 DEGs were identified from *Z. macrostachya* roots under salt stress, these unigenes provided a comprehensive understanding towards the genes transcription profiles of *Z. macrostachya*, and laid a solid foundation for further study of salt-tolerance mechanisms and identification of new genes in this species.

ROS accumulation induce cytoplasmic membrane damage (Xiong *et al*. 2020). Study has shown that salt-tolerance of plant was related to scavenging capacity of antioxidative proteins to some extent (Zhang *et al*. 2012; Bose *et al*. 2014). SOD is the first-line of defense in resistance to oxidative injury, which can dismutate $\bullet O_2^-$ into O_2 and H_2O_2 (Qu *et al*. 2010), CAT and POD can dismutate H_2O_2 into H_2O and O_2 (Xu *et al*. 2013), whereas, APX exerts a vital part in the catalysis of H_2O_2 conversion to H_2O, and H_2O_2 can also be reduced by GPX in the GPX pathway (Gu *et al*. 2018). In this study, these DEGs encodesd enzymatic antioxidants might be the vital factors involved in ROS elimination in *Z. macrostachya*.

Ions transport is a crucial factor in plant response to salt stress. To deal with salt stress, plants have evolved specific mechanisms for coordinating the Na^+ discharge and K^+ uptake processes (Guo *et al*. 2016; Lv *et al*. 2018). KUP, KEA and AKT constitute the K^+ uptake system in plant, PM-H^+-ATPase and V-H^+-ATPase can generate the driving force to extrude Na^+ out of cell and compartmentalize Na^+ to vacuole, and these are regarded as the mechanisms that a plant employs to restore the homeostasis of ions in cell (Chinnusamy *et al*. 2006). In our study, 37 up-regulated DEGs involved in K^+ uptake, 7 up-regulated DEGs involved in Na^+ extrusion was identified, respectively, indicating these DEGs played critical roles in reestablishing the cellular K^+ and Na^+ homeostasis. In addition, NHX were probably responsible for Na^+ sequestration, but 2 DEGs encoding NHX were down-regulated, revealing that NHXs were not likely involved in Na^+ discharge.

It is well-known that plants synthesize some osmolytes, such as free proline and Glycine betaine, so as to alleviate the negative effects of toxic ions and osmotic imbalance (Ingram and Bartels 1996; Ashraf and Foolad 2007). In this study, 11 DEGs involved in free proline and Glycine betaine metabolism were identified, their expression were up-regulated, it suggested that these DEGs exerted vital parts in regulating osmotic balance in Z. *macrostachya*.

The TFs can modulate the downstream genes expression, which play important roles in plant response to abiotic stresses (Capella e*t al*. 2015). Specifically, some TFs families have been identified from halophytes through RNA-Seq, such as *Iris lactea var. chinensis* (Gu *et al*. 2018), *Reaumuria trigyna* (Wang *et al*. 2014) and *Suaeda fruticosa* (Diray-Arce *et al*. 2015). In our study, some DEGs were annotated to the BHLH, NAC, MYB-relate or ERF TFs family. It is reported that the expression of TabHLH13 isolated from wheat was rapidly up-regulated after salt stress (Kim and Kim 2006), while that of bHLH92 in *Arabidopsis thaliana* was triggered under drought and high salinity (Jiang and Deyholos 2006; Liu *et al*. 2014), indicating that the BHLH TFs family exert vital parts in the salt-tolerance

of plant. Previous studies showed that, the over-expression of NAC TF GhATAF1 improved the salt-tolerance of cotton (He *et al*. 2016); besides, the ERFs TFs family can modulate the responses to abiotic stress and ethylene, as well as disease resistance, which were achieved through targeting the downstream promoter GCC-box (Ohme-Takagi and Shinshi 1995; Stockinger *et al*. 1997). By contrast, the expression of MYB-related TF AtMYBL in *A. thaliana* is activated by salt stress (Zhang *et al*. 2011). Thus, the TFs identified in our study laid the basis for more intensive studies.

Conclusion

A total of 4,7325 unigenes were assembled, and 68.76% of the 4,7325 unigenes were annotated by BLAST analysis. Altogether 14,558 DEGs were identified between salt stress and control samples, many DEGs were identified to encode enzymes related to the ROS scavenging system, K^+ and Na^+ transport proteins, osmotic adjustment substances and TFs, which potentially played vital roles in salt stress response in *Z. macrostachya*. Our findings provide useful insights for salt-tolerance molecular mechanisms of *Z. macrostachya* and provide basis for future cloning of salt-tolerance genes in this species.

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Author Contributions

HG Hu and ZM Zhang designed the experiments; HG Hu, ZM Zhang and SN Min performed the experiments; HG Hu and ZM Zhang analyzed RNA-Seq data; HG Hu, ZM Zhang and SN Min wrote the manuscript.

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